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THE IMPORTANCE OF *GEOBACILLUS SPP.* AS GROUP OF BACTERIAL CONTAMINATES IN THE DAIRY INDUSTRY

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ABSTRACT

Infant formulae are modern artificial substitutes for human breast milk. Formulas are designed for infant consumption, and are usually based on either cow milk or soy milk. *Geobacillus* represents aerobic or facultative anaerobic, neutrophilic, obligatory thermophilic, endosporeforming bacteria. A total of ninety random samples of milk powder, Infant milk powder and milk-cereal based weaning food (30 samples each) were purchased from different shops and pharmacies in Assiut city. Samples were examined for total thermophillic count, total spore count at 37°C and 55°C. The incidences of *Geobacillus* spp. and other bacillus spp. were also determined. The mean value of total thermophillic colony count/g was 10×10^3; 13×10^3 and 12×10^3/ g of examined dried milk powder, infant milk formulae and milk-cereal based weaning food samples, respectively at 37°C. While at 55°C, it was 1×10^2 and 7.6×10^2/g in the same samples, respectively. Infant milk formulae were free. In addition, the mean of total spore count at 37 and 55°C were 22×10^2 and 10×10^3/g in milk powder samples, respectively and 11×10^3 and 59×10^2 in milk-cereal based weaning food samples and 13×10^3 in infant milk formulae at 37 °C. *Geobacillus stearothermophilus* could be isolated only from milk-cereal based weaning food in percentage of 16.7%. Some other *Bacillus spp.* could be detected at different temperatures with different percentages. Lipolytic and proteolytic activities of G. stearothermophilus were also evaluated.

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INTRODUCTION

Thermophilic bacilli, such as Anoxybacillus, Geobacillus and Bacillus are common contaminants growing within the processing lines of milk powder producing factories. Spores surviving processing (filling, temperature, sterilization of packaging material, etc.) can germinate, grow, and proliferate in the product (Scheldeman *et al.*, 2006). Moreover, these contaminants are used as indicator organisms for plant hygiene and specification limits based on their numbers have been implemented to ensure milk powder quality.

Recently, Nazina *et al.* (2001) reclassified some existing Bacillus species within group V into the new genus *Geobacillus* based on phenotypic and genotypic characteristics. So far, the most recently published and recognized species are *Geobacillus caldoxylosilyticus, G. debilis, G. gargensis, G. jurassicus, G. kaustophilus, G. lituanicus, G. pallidus, G. stearothermophilus, G. subterraneus, G. tepidamans, G. thermocatenulatus, G. thermodenitrificans, G. thermoglucosidasius, G. thermoleovorans, G. toebii, G. uzenensis and G. vulcani* (Nazina *et al.*, 2001, 2004; Banat *et al.*, 2004; Schaffer *et al.*, 2004; Zeigler *et al.*, 2005).

G. stearothermophilus spores (formerly *B. stearothermophilus*) are extremely heat resistant, up to 20 times more resistant than Clostridium *botulinum* spores (Watanabe *et al.*, 2003; Iciek *et al.*, 2008). *G. stearothermophilus* spores typically survive canning and sterilization procedures of food products (Denny, 1981 and Brackett, 2001) and may cause spoilage problems especially where foods must be stored at elevated temperatures for a long time. Growth of *G. stearothermophilus* spores results in flat sour spoilage because acid is produced but with little or no gas generated (Brackett, 2001). Due to its heat resistance, this microorganism is often used as a biological indicator for testing the efficacy of sterilization processes (Chopra and Mathur, 1984 and Phillips and Griffiths, 1990). Furthermore, the capacity of this bacterium to adhere to stainless steel and grow in biofilms appears to be a likely cause of contamination of manufactured dairy products (Flint *et al.*, 2001).

In the dairy industry, the thermophilic bacilli are usually enumerated using an aerobic plate count (APC) incubated at 55 °C. Those that have been isolated from dairy products at this incubation temperature can be divided into two groups: the obligate thermophiles and the facultative thermophiles (also known as thermotolerant microorganisms). The obligate thermophiles grow

only at elevated temperatures (approximately 40–68°C) and include Anoxybacillus flavithermus and Geobacillus spp. (Flint *et al.*, 2001; Ronimus *et al.*, 2003; Scott *et al.*, 2007). The facultative thermophiles belong to the Bacillus genus and tend to grow at both mesophilic and thermophilic temperatures, depending on the strain. Some examples of species include B. licheniformis, B. coagulans, B. pumilus, B. sporothermodurans and B. subtilis (Crielly *et al.*, 1994; Flint *et al.*, 2001; Ronimus *et al.*, 2003, Scheldeman *et al.*, 2005).

Feeherry et al. (1987) noted that many food products cannot withstand the heat treatment needed to inactivate thermophilic spores. Therefore, other measures such as the control of contamination of ingredients by thermophilic organisms, rapid cool below 4 °C after thermal processing, and controlled storage are required to prevent spoilage.

MATERIALS and METHODS

Collection, preparation and serial dilutions of samples:
A total of 90 random samples of milk powder, Infant milk powder and milk-cereal based weaning food (30 samples each) were purchased from different shops and pharmacies in Assiut city, Egypt. These samples were still valid for consumption as shelf life is at least to be more than one year from production time. Cartons and cans of samples were cleaned, thoroughly mixed and aseptically opened. Ten fold serial dilutions were carried out according to A.P.H.A. (1992).

Enumeration of total thermophiles:
1 ml of the sample and its decimal dilutions was plated using milk plate count agar (MPCA) and incubated at 37 and 55 °C for 48 h as described by Frank and Yousef (2004).

Enumeration of total Spores:
Firstly, the samples were heated at 80 °C for 20 min. to inactivate the vegetative cells and activate the spores, enabling them to germinate. The heat-treated sample is pour plated with MPCA supplemented with 0.2% starch and is incubated at 37°C and 55°C for 48 h. The starch is added as an aid for spore germination (Murphy *et al.*, 1999; McGuiggan *et al.*, 2002 and Coorevits *et al.*, 2008).

Isolation of Geobacillus spp.:
Tryptic soy agar with 0.2% soluble potato starch was chosen as the isolation medium and incubated at 37°C and 55°C for 48 h (Ronimus *et al.*, 2003).

Identification of isolates:
Identification of bacterial isolates was performed based on their morphological, physiological, and biochemical characteristics, as described in Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986).

Lipolytic and protylytic activity:
To evaluate the proteolytic activity, the strains were plated on milk agar. The milk agar was prepared with plate count agar supplemented with 1% skimmed milk powder (Beerens and Luquet, 1990). The plates were incubated at 30°C for 48 hours. The positive results were indicated by transparent halo zone around the colonies.

For the evaluation of lipolytic activity, the strains were plated on tributyrin agar, prepared with plate count agar supplemented with 1% tributyrin (Beerens and Luquet, 1990). The plates were incubated at 30°C for 48 hours. The positive results were indicated by transparent halo zone around the colonies.

RESULTS

Table 1: Statistical analytical results of Total Thermophilic Count at 37°C and 55 °C.

Examined samples	No. of the examined samples	At 37°C					At 55 °C				
		Positive samples		Count/ g			Positive samples		Count /g		
		No.	%	Min.	Max.	Mean	No.	%	Min.	Max.	Mean ±St Error
Milk powder	30	21	70	1×10^2	38×10^3	10×10^3	2	6.7	1×10^2	1×10^2	1×10^2
Infant milk powder	30	19	63.3	2×10^2	79×10^3	13×10^3	0	0	0	0	0
Milk- cereal based weaning food	30	25	83.3	4×10^3	33×10^3	12×10^3	13	43.3	2×10^2	1×10^3	7.6×10^2

Table 2: Statistical analytical results of Total Spore Count at 37°C and 55 °C.

Examined samples	No. of the examined samples	At 37°C					At 55 °C				
		Positive samples		Count/ g			Positive samples		Count /g		
		No.	%	Min.	Max.	Mean	No.	%	Min.	Max.	Mean
Milk powder	30	25	83.3	1×10^2	15×10^3	22×10^2	4	13.3	1×10^2	38×10^3	10×10^3
Infant milk powder	30	10	33.3	20	44×10^3	13×10^3	0	0	0	0	0
Milk- cereal based weaning food	30	18	60	1×10^3	81×10^3	11×10^3	13	43.3	1×10^3	19×10^3	59×10^2

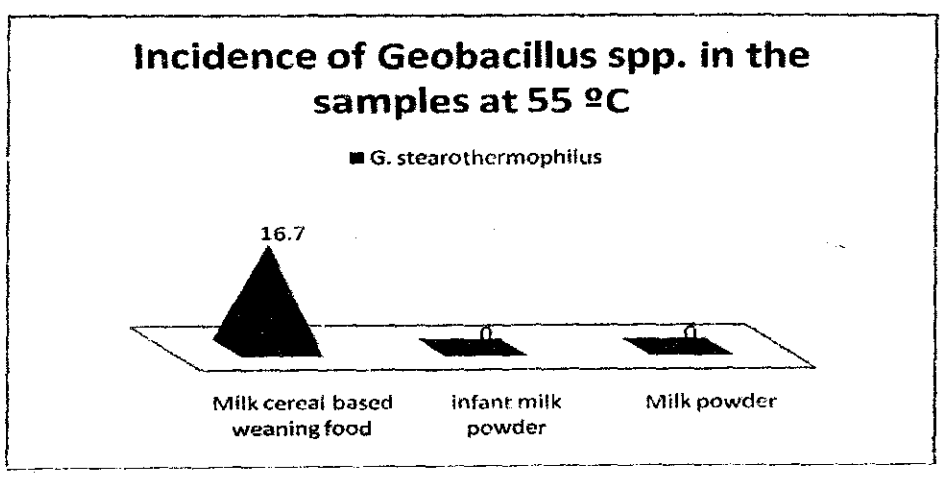

Figure 1: Incidence of Geobacillus spp. in the examined samples at 55 °C.

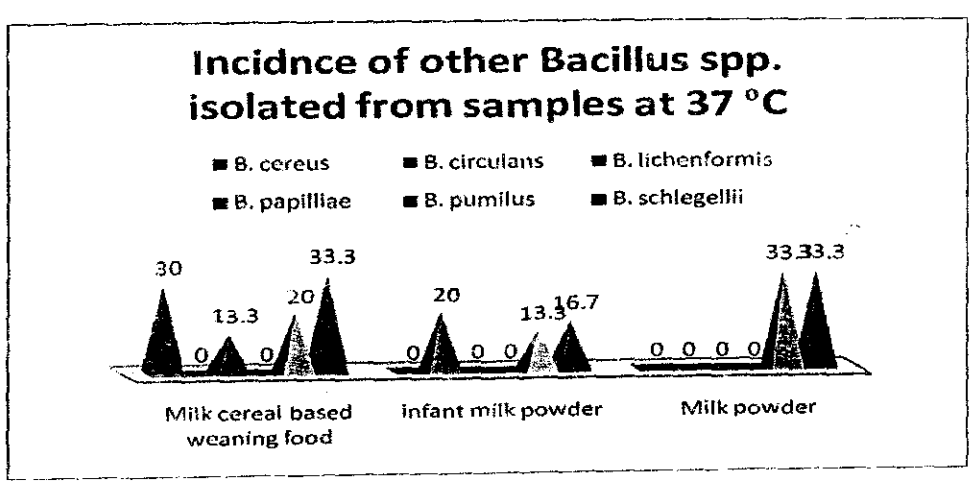

Figure 2: Incidence of other *Bacillus spp.* in the examined samples at 37 °C.

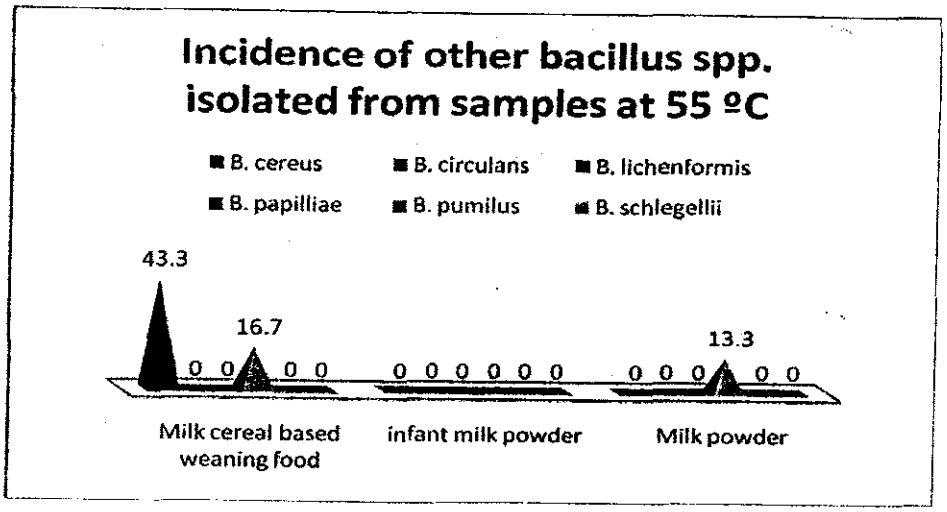

Figure 3: Incidence of other *Bacillus spp.* in the examined samples at 55 °C.

DISCUSSION

Although it is universally accepted that these thermophilic bacilli do not constitute a health risk to the consumer, they have been deemed to be an indicator of plant hygiene during processing, and specification limits have been implemented based on numbers of thermophilic bacilli in products (Murphy *et al.*, 1999; Kwee *et al.*, 1986; Ronimus *et al.*, 2003). Recently, the thermophilic bacilli could not only cause spoilage due to their production of acids and thermostable enzyme excretion, but also cause food-borne illness (Burgess *et al.*, 2010).

In the present study, the mean count of total thermophillic count at 37°C in the examined samples were 10×10^3, $13\times10^3$and 12×10^3/g and found to be 1×10^2, 0 and 7.6×10^2 /g at 55°C in examined samples of milk powder, infant milk formulae and milk-cereal based weaning food samples, respectively (Table 1). The percentage of total thermophilic counts at 37°C were 70, 63.3 and 83.3 %, while at 55 °C were 6.7, 0 and 43.3in the examined samples respectively. Dong *et al. (2012)* could isolate *B. licheniformis* in percent of 27.8% in the examined samples of milk powder and it considered higher than that obtained in this study. Thermophilic bacilli had been isolated from milk powder plants in New Zealand and also from milk powders collected from 18 countries around the world, and the dominant thermophilic bacilli in milk powder samples were relatively consistent (Ronimus *et al.*, 2003; Rueckert *et al.*, 2004; Scott *et al.*, 2007).

From Table (2), total spore count were detected in 83.3, 33.3 and 60 % in examined samples of milk powder, infant milk formulae and milk-cereal based weaning food samples, respectively at 37°C. At 55°C, total spore count failed to be detected in the examined samples of infant milk formulae, while the mean total spore count reached to 10×10^3 and 59×10^2 /g in the examined samples of milk powder and milk-cereal based weaning food, respectively.

Spores are resistant to heat, mechanical disruption and a wide variety of chemicals, making it very hard to destroy them in dairy manufacturing processes (Jones *et al.*, 2005 and Scheldeman *et al.*, 2006).

Typically, milk powder is produced continuously over an 18–24 h processing period during which the number of thermophilic bacilli in the product often mirrors that of a typical bacterial growth curve. Thus, with increased processing time, the number of thermophiles increases until specification limits are reached and the process run is terminated to prevent product downgrading. Many factors, such as the microbiological quality of the raw milk or the thermal operating conditions in the plant (low-, medium or high heat treatments) or the plant hygiene can all affect the growth of thermophiles in the processing line, making it difficult to predict a processing time that ensures thermophile numbers at the end of the run are below specified limits. In practice, actual numbers of contaminants in milk powders are determined retrospectively by plate counting, providing results commonly after 16–48 h. Due to these microbiological constraints, it is common practice to terminate processing runs by initiating CIP (cleaning in place) with the expectation that the numbers of thermophilic bacilli will be below their specification limits (Stadhouders *et al.*, 1982; Murphy *et al.*, 1999; Kwee *et al.*, 1986; Ronimus *et al.*, 2003).

It's of interesting observation, that *G. stearothermophilus* could be isolated only from milk-cereal based weaning food in percentage of 16.7 % (5 samples) and failed to be detected in other examined samples (Fig.1). Dong *et al.* (2012) could isolate *G. stearothermophilus* in higher percentage from milk powder (12.4%) *Geobacillus* strains have been isolated from temperate areas, as well as hot

environments, such as hot springs, oilfields, deep sea sediments, sugar refineries and dairy factories (Tai *et al.*, 2004).

Other *Bacillus spp.*, could be found in the examined samples of milk powder where *B. cereus* and *B. circulans* in percentage of 33.3 and 33.3 % at 37°C, respectively. While, *B. lichenformis* could be isolated in percentage of 13.3% at 55 °C. At 37°C, *B. cereus, B. circulans* and *B. pumilus* could be identified in 16.7, 13.3 and 20% of isolates of infant milk formulae, respectively. Milk-cereal based weaning food samples found to be contaminated with *B. cereus, B. circulans, B. papilliae* and *B. schlegellii* at 37 °C and contaminated with *B. lichenformis* and *B. schlegellii* in percentage of 16.7 and 43.3%, respectively at 55 °C (Figures 2 and 3).

Another study in New Zealand milk powder plants revealed that seven strains of thermophilic bacilli were able to grow at 55 °C or above and recognized as the major contaminants growing in the processing lines (Ronimus *et al.*, 2003). These are *G. stearothermophilus, Anoxybacillus flavithermus, B. licheniformis* and *B. subtilis*. Furthermore, a second investigation on milk powders from 18 different countries demonstrated that *A. flavithermus. B. Licheniformis* and *G. stearothermophilus* represent world-wide sources of contamination being near-ubiquitously present in milk powders, and are thus of economic importance in processing (Rueckert *et al.*, 2004).

Strains of obligate and facultative thermophiles are capable of producing acids, as well as a variety of heat-stable enzymes, including proteinases and lipases, which could result in the spoilage of dairy products (Gundogan and Arik, 2004; Murugan and Villi, 2009). In the present study, all *G. stearothermophilus* isolates have lipolytic and proteolytic activities. The real potential for the obligate thermophiles to spoil dairy products is thought to be low, as dairy products are generally stored at temperatures below 37 °C, temperatures at which obligate thermophiles will not grow. However, *G. stearothermophilus* has been associated with 'flat-sour' spoilage in a variety of canned food products, including evaporated milk (Kalogridou-Vassiliadou, 1992). In the case of facultative thermophiles, some strains of *B. licheniformis* are also capable of producing a slimy extracellular substance that can affect the quality of pasteurised milk and cream (Gilmour and Rowe, 1990).

In fact, the levels of thermophiles in raw milk are usually very low (e.g. b10 cfu/mL) (Hill and Smythe, 1994; McGuiggan *et al.*, 2002). On rare occasions when higher levels do occur (e.g. N100 cfu/mL), the predominant thermophilic species in this raw milk tend to be *B. licheniformis and B. coagulans*. However, when the final product from raw milk of this quality is processed, the predominant thermophilic species tend to be *A. flavithermus and Geobacillus spp* (Burgess *et al.*, 2010).

CONCLUSION

These contaminants are used as indicator organisms for plant hygiene and specification limits based on their numbers have been implemented to ensure milk powder quality. The attention of industry and researcher should be focused on dairy products stored at room temperature, since the occurrence of strains producers of proteases was highest at 30°C. Taking into account that these products have a long shelf life, these enzymes may act on proteins, altering their sensory characteristics. Consumers' demands increasingly aim at high-quality, minimally processed, nutritious and fresh-like products. Traditional thermal processing methods cause loss of desirable properties related to texture, flavor, color, and nutrient value. However, the most serious commercial problems with product sterility are caused by thermally resistant spores. In an attempt to provide alternatives to solve these problems,

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أهمية أجناس الجيوباسيلس كمجموعة من الملوثات البكتيرية في صناعة الألبان

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تعد كل من الألبان الجافة والأغذية الجافة المحتوية علي اللبن من الأغذية واسعة التدوال لدي الكبار والأطفال، وتلوثها بميكروب الجيوباسيليس من الأمور التي تستوجب الاهتمام والدراسة لما يسببه هذا الميكروب من فساد لمنتجات الألبان وذلك لقدرته علي إفراز الإنزيمات والأحماض التي تسبب تحلل البروتينات والدهون في اللبن. لذلك تضمنت هذه الدراسة فحص عدد ٩٠ عينة عشوائية من أغذية الأطفال اللبنية الجافة بواقع ٣٠ عينة من لبن البودرة، ٣٠ عينة من اللبن الجاف للأطفال و٣٠ عينة من أغذية الفطام الجافة المحتوية علي خلاصة الدبوب. وكانت صالحة للاستهلاك حيث تمتد فترة صلاحيتها لمدة لا تقل عن عام من تاريخ الإنتاج وتم جمع هذه العينات من العديد من المحال التجارية والصيدليات في مدينة أسيوط لمعرفة مدي تلوثها بالميكروبات المختلفة. وتم فحص العينات عند درجة حرارة ٣٧ و ٥٥ °C حيث ان ميكروب الجيوباسيليس من مجموعة البكتيرية التي تنمو في درجات حرارة عالية. ودلت النتائج أن متوسط العدد الكلي للميكروبات (عند ٣٧°C) كان 10×10^3 في اللبن البودرة بينما وصل الي 13×10^3 و 12×10^3 في لبن الأطفال وأغذية الأطفال الجافة المحتوية علي اللبن، علي التوالي. بينما كان متوسط العدد الكلي للميكروبات المحبة للدرجة الحرارة العالية عند درجة حرارة ٥٥°C 1×10^2 و 7.6×10^2 في اللبن البودرة وأغذية الأطفال الجافة المحتوية علي اللبن ، علي التوالي. بينما لم تتواجد في لبن الأطفال وكانت متوسطات الحويصلات عند درجة حرارة ٣٧°C علي التوالي هي 22×10^2 ، 13×10^3و 11×10^3 وبينما عند درجة حرارة ٥٥ °C كانت متوسطات الحويصلات 10×10^3 و 59×10^2 علي التوالي في اللبن البودرة وأغذية الأطفال الجافة المحتوية علي اللبن ولم تتواجد في لبن الأطفال أيضا. أما الجيوباسيليس ستريوسيرموفيليس فقد تم عزلها فقط من أغذية الأطفال الجافة المحتوية علي اللبن بنسبة 16.7% من العترات المعزولة عند درجة حرارة ٥٥°C. كما تم عزل بعض الأجناس الاخري من الباسيليس بنسب مختلفة وعند درجات حرارة مختلفة. وقد أثبتت النتائج قدرة الجيوبايليس علي تحليل البروتينات والدهون.