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A RAPD based study revealing a previously unreported wide range of mesophilic and thermophilic spore formers associated with milk powders in China

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ABSTRACT

Aerobic spore forming bacteria are potential milk powder contaminants and are viewed as indicators of poor quality. A total of 738 bacteria, including both mesophilic and thermophilic, isolated from twenty-five powdered milk samples representative of three types of milk powders in China were analyzed based on the random amplified polymorphic DNA (RAPD) protocol to provide insight into species diversity. *Bacillus licheniformis* was found to be the most prevalent bacterium with greatest diversity (~43% of the total isolates) followed by *Geobacillus stearothermophilus* (~21% of the total isolates). *Anoxybacillus flavithermus* represented only 8.5% of the total profiles. Interestingly, actinomycetes represented a major group of the isolates with the predominance of *Laceyella sacchari* followed by *Thermoactinomyces vulgaris*, altogether comprising of 7.3% of the total isolates. Out of the nineteen separate bacterial species (except five unidentified groups) recovered and identified from milk powders, twelve proved to belong to novel or previously unreported species in milk powders. Assessment and characterization of the harmful effects caused by this particular micro-flora on the quality and safety of milk powders will be worth doing in the future.

1. Introduction

Milk is one of the most nutritionally complete foods containing proteins, carbohydrates, vitamins and minerals. The plethora of nutrients present in milk also makes it an ideal growth medium for a great variety of microorganisms including bacteria, yeasts and molds (Walker, 1988; Phillips and Griffiths, 1990). This is the reason why dairy products are routinely analyzed for microbial contaminants.

Spore-forming bacteria are present in many food processing environments and may pose a threat to food safety and quality. They have been frequently isolated from raw milk and throughout the milk processing continuum (Crielly et al., 1994; Postollec et al., 2012). Raw milk is usually considered as sterile and microbes are introduced into it from either the infected udder or from environmental sources during milk handling and processing (Phillips and Griffiths, 1990). Raw milk and the farm environment may contain spore counts up to 10^4 cfu/ml and are regarded as primary sources of milk contamination (Coorevits et al., 2008). Spores, being hardy in nature, retain their vitality even at elevated industrial processing temperatures and attach to stainless steel surfaces, where they germinate under ideal growth conditions and form biofilms. Due to the bio-transfer potential of a mature biofilm, spores and vegetative cells slough off and contaminate the liquid product flowing over it (Flint et al., 2001). Additionally, the higher number of spores in milk powders as compared to raw milk also reflects the concentration effect as milk solids are concentrated during the manufacture of milk powder (Hill and Smythe, 2012). The presence of high counts of these spore-formers lowers the value of the milk powder, and is regarded as representing a lack of good manufacturing practices and poor plant hygiene.

Aerobic spore-forming bacilli are major contaminants in the milk processing industries as the spores of these bacteria, present in raw milk, survive pasteurization and subsequent processing and thus become part of final product (Cook and Sandeman, 2000; Huck et al., 2007). Among the aerobic bacilli, mesophilic and thermophilic bacteria are of particular concern because of their highly thermoresistant spores (some surviving 125 °C for 30 min) and heat-stable spoilage enzymes (Lücking et al., 2013). Besides the spoilage potential, the role of *Bacillus cereus* as a potential food poisoning agent is well known. It causes two types of food poisoning, mainly described as emetic and diarrheal syndromes (Granum and Lund, 1997). Other *Bacillus* species have been considered of less importance in terms of food safety. However, various reports have confirmed the toxin production from the strains of *Bacillus licheniformis* (Salkinoja-Salonen et al., 1999), *Bacillus mojavensis*, *Bacillus fusiformis* (From et al., 2005), *Bacillus simplex*, *Bacillus firmus* (Taylor et al., 2005), *Bacillus circulans*, *Brevibacillus brevis*, *Bacillus lentus*, *Bacillus mycoides* (Beattie and Williams, 1999), *Bacillus subtilis*, *Bacillus*

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pumilus, *Bacillus megaterium* (Yoo et al., 2014) and various other aerobic bacilli (De Jonghe et al., 2010). The production of toxins has been reported from only mesophilic or facultative thermophiles at mesophilic conditions (Burgess et al., 2010). This suggests that bacilli other than *B. cereus* could also be food safety concerns. However, the presence of obligate thermophiles does not usually pose a health threat to consumers (Rückert et al., 2004).

It is well established that thermophilic and mesophilic bacteria are the major milk powder contaminants throughout the world. Ronimus et al. (2003) analyzed milk powders from New Zealand, to assess the types of thermophilic contaminants. The majority of the isolates were assigned to the groups representing *Geobacillus stearothermophilus*, *Anoxybacillus flavithermus*, *B. licheniformis* and *B. subtilis*. The comprehensive study which aimed to analyze and compare the isolates of milk powders from 18 different countries also reported the dominance of *A. flavithermus* followed by *B. licheniformis* (Rückert et al., 2004). In another study, commercial milk powders from Uruguay were analyzed for the presence of mesophilic and thermophilic sporeformers and *B. licheniformis* was found to be the most dominant milk powder contaminant followed by *A. flavithermus*. Interestingly, *G. stearothermophilus* was not found in any of the samples (Reginensi et al., 2011). In China, *B. licheniformis*, *A. flavithermus* and *G. stearothermophilus* were also reported as the prevalent microorganisms in Chinese milk powders (Yuan et al., 2012).

China is one of the world's largest milk producing countries with an expected milk production of 38 million tons in 2015 ([USDA/FAS] U.S. Department of Agriculture/Foreign Agriculture Services, 2015). The annual consumption of infant formulas, in China, is approximately 3000,000 t per year (Liu et al., 2012). Since 2010, China has established a very stringent criterion for aerobic plate counts in infant formulas, with an acceptable upper limit of 10^3 cfu/g (GB 10765, 2010). Any strategic approach aiming to remove spoilage and pathogenic microbes from susceptible foods requires a complete assessment of the nature and types of these contaminants. In China, the nature and diversity of mesophilic and thermophilic aerobic sporeformers have not been explored well. To the best of our knowledge, there is only one study that aimed to explore only thermophilic milk powder contaminants in Chinese milk powders and the prevalence of mesophilic bacteria remained unknown which represent a significant part of the microbial consortia associated with milk powders. Therefore, the objective of the work reported in this manuscript is to assess the over diversity of mesophilic and thermophilic bacteria associated with Chinese milk powders, based on random amplified polymorphic DNA (RAPD) to have better knowledge of current status of milk powder contaminants.

2. Materials and methods

2.1. Sample collection and selection of isolates

Twenty-five samples of milk powders representative of Chinese commercial markets that included infant formula powders (IFP), skimmed milk powders (SMP) and whole milk powders (WMP) were collected from different outlets, and wherever possible, product of companies which claimed that powders had been produced in China. While collecting the samples it was assured that the samples represent all the main milk powder manufacturing companies of China as well as various areas of production. All the powder packets were stored at room temperature until analysis and samples from the packages were taken aseptically and within the use by date.

Ten grams of each powdered milk sample were reconstituted in 90 ml of 0.1% sterile peptone water and agitated for 15–20 min in ice bath using sterile glass beads. Total thermophilic count (TTC) was determined by pour plating 1 ml aliquot of appropriate dilutions on Plate count Agar in triplicate followed by incubation at 55 °C (Ronimus et al., 2006) for 24 h. Similarly, mesophilic spore count (MSC) and thermophilic spore count (TSC) were determined after giving a pre-heating treatment (80 °C for 10 min in a water bath) to the reconstituted milk powders to kill the vegetative cells (Rückert et al., 2004) before plating on tryptic soya agar (TSA) supplemented with 0.2% potato starch followed by incubation at 37 °C and 55 °C for 24 h (Reginensi et al., 2011). Bacterial colony counts of each plate were determined after incubation. Isolates were randomly chosen from the highest dilution plates and sub-cultured onto TSA plates three times. For each sample, at least thirty different colonies were randomly chosen from the plates containing about 30 or more than 30 colonies. For some low count milk powders less than 30 colonies were selected and similarly for some high count milk powders more than 30 colonies were chosen. In case of a large number of isolates selected for a single sample, isolates were clustered based on morphology, Gram staining and catalase test. A sum of 738 isolates were selected and purified for genetic and DNA based analysis.

Table 1
Nucleotide sequences of primers used in this study.

Amplification target	Name of the primer	Primer sequence (5′ → 3′)	Reference
16S rRNA gene	rD1	AAGGAGGTGATCCAGCC	Weisburg et al. (1991)
	fD1	AGAGTTTGATCCTGGCTCAG	Weisburg et al. (1991)
Genome	OPR-13	GGACGACAAG	Ronimus et al. (1997)

2.2. DNA extraction and RAPD-PCR analysis

Total genomic DNA was extracted from 1.5 ml tryptic soya broth cultures using the DNA extraction kit following the manufacturer's instructions (Axygen, China). DNA was eluted with 100–200 μl of elution buffer and quantified by NanoDrop 2000 (Thermo Fisher Scientific, USA). The extracted DNA was stored at −20 °C until use.

RAPD assays were performed in a 25 μl reaction mixture, containing 10× Thermo buffer (Takara, Japan), 2.5 mM $MgCl_2$, 1 U Taq

Table 2
TSC, TTC and MSC of Chinese milk powders (cfu/g).

Sample no	Powder type[d]	Production region	TSC[a]	TTC[b]	MSC[c]
1	IFP	Shandong	7.4×10^3	1.1×10^4	1.4×10^4
2	IFP	Shandong	1.2×10^4	3.0×10^4	5.6×10^3
3	IFP	Inner Mongolia	2.6×10^2	1.1×10^3	4.5×10^3
4	IFP	Zhejiang	1.8×10^3	5.5×10^3	3.6×10^2
5	IFP	Shandong	5.0×10^3	2.0×10^4	3.0×10^4
6	IFP	Shanghai	1.2×10^3	5.5×10^3	1.5×10^3
7	WMP	Inner Mongolia	1.3×10^3	2.8×10^4	1.4×10^4
8	WMP	Heilongjiang	3.5×10^1	6.5×10^1	1.0×10^4
9	WMP	Heilongjiang	1.0×10^1	4.5×10^1	1.0×10^2
10	WMP	Shanxi	1.6×10^2	5.3×10^2	4.3×10^2
11	WMP	Shanxi	2.5×10^1	5.0×10^1	1.6×10^1
12	WMP	Inner Mongolia	3.2×10^2	8.1×10^2	2.3×10^2
13	SMP	Guangdong	2.5×10^2	3.3×10^3	3.3×10^2
14	SMP	Hebei	2.9×10^3	4.8×10^4	3.5×10^3
15	SMP	Shnaghai	2.0×10^4	4.3×10^4	1.1×10^3
16	SMP	Zhejiang	8.1×10^3	1.5×10^3	3.3×10^3
17	SMP	Zhejiang	1.9×10^2	3.8×10^2	6.0×10^2
18	SMP	Shanghai	1.4×10^2	5.8×10^2	1.2×10^2
19	SMP	Heilongjiang	1.8×10^2	2.3×10^2	2.0×10^2
20	IFP	Tianjin	1.0×10^2	1.6×10^2	9.0×10^2
21	IFP	Inner Mongolia	1.3×10^2	6.0×10^2	4.0×10^2
22	IFP	Inner Mongolia	8.5×10^1	1.9×10^2	4.8×10^2
23	IFP	Heilongjiang	7.3×10^1	1.0×10^2	3.5×10^2
24	IFP	Guangdong	4.0×10^2	5.0×10^2	2.1×10^2
25	IFP	Hebei	1.3×10^3	3.6×10^3	1.2×10^2

[a] TSC = thermophilic spore count.
[b] TTC = total thermophilic count.
[c] MSC = mesophilic spore count.
[d] IFP = Infant formula milk powders; SMP = Skimmed milk powders; WMP = Whole milk powders.