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The heat resistance and spoilage potential of aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders

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ABSTRACT

The propensity for aerobic bacilli and allied genera to produce highly heat-resistant spores and thermally stable spoilage enzymes are major bacteriological issues faced by the dairy industry. Most of the enzymes are able to survive any heat treatment applied during the manufacture of milk powders and have the potential to remain active in milk powders and other dairy products during storage, and may explain some of the sensory and functionality defects reported in dairy products. Despite many reports on the occurrence of spore-forming bacteria in dairy products, knowledge about food quality related properties of many aerobic sporeformers is still scarce. Therefore, the aim of this study was to determine thermal resistance and spoilage potential of a large pool of mesophilic and thermophilic sporeformers, representing 738 isolates and 31 different RAPD groups, recently isolated from Chinese milk powders. Spore formers producing highly heat resistant spores (surviving 125 °C for 30 min) included 2 thermophiles (*Geobacillus thermoleovorans* group and *Geobacillus stearothermophilus*) and one mesophilic species (*Brevibacillus brevis*). *Paenibacillus macerans* showed the highest proteolytic activity followed by members of the *Bacillus cereus* group, *Br. brevis*, *Bacillus subtilis*, *G. thermoleovorans* group and *Virgibacillus proomii*. The highest lipase producing strains belonged to *Bacillus licheniformis*. Phospholipase activity was only shown by members of the *B. cereus* group and *Brevibacillus parabrevis*. Ten strains showed positive β-galactosidase activity, while, 4 strains showed positive haemolytic activity. *B. licheniformis* strains, despite belonging to one RAPD group or sub-group showed markedly different phenotypic characters which support the previous findings of heterogeneity in RAPD-based *B. licheniformis* groups. The results of this study will broaden the knowledge about the spoilage potential and thermal resistance of many strains of dairy origin.

1. Introduction

Aerobic endospore-forming bacteria present major problems for the dairy industry because of their potential role in quality deterioration and reduced shelf life of dairy products. The longevity of these bacteria in the form of endospores (for simplicity hereafter termed as spores) in soil and their robust survival strategy in biofilms (Flint et al., 2001) make it difficult to completely eradicate them from dairy manufacture. Among the sporeformers, the bacteria belonging to the genus *Bacillus* and allied genera are of particular concern; spores of some bacilli do not only survive the pasteurization process but there is mounting direct or indirect evidence that some of them like *Bacillus sporothermodurans* are able to withstand the UHT (Ultra High Temperature, 138 °C–140 °C for 2–4 s) treatment of milk (Scheldeman et al., 2006; Cattani et al., 2013; Esteban et al., 2013). There are several other highly heat resistant bacilli with potential to sporadically contaminate the UHT process like *Geobacillus stearothermophilus*, *Brevibacillus brevis*, *Bacillus sphaericus* and *Paenibacillus lactis* (Scheldeman et al., 2006). Lücking et al. (2013) reported some sporeformers which could survive a heat treatment of 125 °C for 30 min. The survival and presence of spores in final product result in failure to comply with specifications for spore content.

Variation of heat resistance of spores among strains within the same species is a notable phenomenon and has previously been reported in *B. sporothermodurans* (Scheldeman et al., 2006), *Bacillus subtilis* (Oomes et al., 2007; Berendsen et al., 2015a) and many other bacilli (Lücking et al., 2013).

During the manufacture of dairy products, the preheater plate heat exchanger and the evaporator are the predominant sites of spore formation (Scott et al., 2007). Spores change into vegetative cells and result in the production of enzymes and the formation of biofilms. Biofilms, in

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additions, serve as important niches where bacteria can grow and produce thermally stable enzymes (Teh et al., 2012) and spores (Flint et al., 2001). These spores survive all the subsequent stages of milk powder manufacture process such as concentrate heating and spray-drying (Hill and Smythe, 2012). Most of the enzymes are also thermally stable and can survive pasteurization (Shamsuzzaman et al., 1986) as well as the UHT process (Koka and Weimer, 2001; Griffiths et al., 1981; Chen et al., 2004) and result in functional and flavour defects, instability problems and reduced shelf life of dairy products during storage (Cogan, 1977). For instance, lipases cause rancidity and produce fruity flavours (Fitz-Gerald and Deeth, 1983; Woods et al., 2001) while proteases produce defects described as rotten and bitter off-flavours by producing bitter peptides (Meer et al., 1991) and also result in age gelation in sterilised milk (Harwalkar, 1992). Phospholipases degrade the phospholipids of the milk fat globule membrane and as a result more lipolysis occurs by milk's natural lipases which leads to the production of bitter off flavours (Shah, 1994). β-Galactosidases catalyse the hydrolysis of β-1,4 galactosidic bonds in lactose and yield glucose and β-galactose. Haemolysins cause the lysis of red blood cells and thus indicate pathogenic potential rather than spoilage ability.

Chen et al. (2004) gave evidence that proteases and lipases produced by various *Bacillus* species can survive all the heat treatments applied during the manufacture of milk powder. Chopra and Mathur (1984) also demonstrated the remarkable heat resistance of proteases produced by *G. stearothermophilus* isolates.

Mesophilic (MP) and facultative thermophilic (TP) bacteria pose more threat to the quality of dairy products as most dairy products are stored at <37 °C. For instance, the production of extracellular slimy substances in pasteurised milk and cream by *Bacillus licheniformis* (Gilmour and Rowe, 1990), ropiness in raw and pasteurised milk and spoilage in UHT and canned milk caused by *B. subtilis* (Heyndrickx and Scheldeman, 2002) and acid production in canned and UHT milk by *Bacillus coagulans* (Gilmour and Rowe, 1990) are some of the examples. *Bacillus cereus* is a well-known pathogen that causes food poisoning by producing two types of toxins; emetic type and a diarrhoeal type (Granum, 2002) but also causes serious quality defects like 'bitty cream' and 'sweet curdling' in milk as a result of phospholipase and protease activities, respectively (Heyndrickx and Scheldeman, 2002). The importance of TP bacilli in the dairy industry and their role in deteriorating the quality of dairy products are reviewed by Burgess et al. (2010).

The dairy industry is facing major challenges not only due to the spoilage potential associated with psychrotrophic bacteria (Marchand et al., 2009; Stoeckel et al., 2016; von Neubeck et al., 2015; Caldera et al., 2016) but also with MP and TP species. *Bacillus* species produce more diverse protease enzymes with greater thermal stability as compared to the enzymes produced by *Pseudomonas* (Chen et al., 2003). De Jonghe et al. (2010) provided some useful insights into spoilage potential of aerobic spore formers, mainly comprising MP bacteria, recovered from raw milk. Additionally, quantitative assessment of lipases was based on titration which usually results in a poor recovery of short chain fatty acids thus compromising the results (Duncan and Christen, 1991). Lücking et al. (2013) provided some useful information on the spoilage potential of both MP and TP isolates based on qualitative screening on selective media rather than quantitative methods.

In our previous study, we found a wide range of MP and TP spore-forming bacteria in Chinese milk powders (Sadiq et al., 2016) that belonged to 31 different RAPD groups and 24 species (including some groups). The heat resistance of many of these isolates and their potential to affect the quality of milk powders is unknown. The objective of this study was to decipher the heat resistance and spoilage potential of the representative isolates of all the RAPD groups representing 738 bacterial isolates from milk powders and examine the inter and intra species diversity of these isolates that may influence dairy product quality.

2. Materials and methods

2.1. Materials

Chemical reagents, dimethylformamide (DMF) and acetonitrile were purchased from (Sinopharm Chemical Reagent Co., Ltd.). Phenylmethanesulphonyl fluoride (PMSF), 4-nitrophenyl butyrate, trinitrobenzene sulfonic acid (TNBS), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), isopropyl-β-D-thiogalactopyranoside (IPTG) and Clarifying Reagent were obtained from Sigma-Aldrich. Ethylenediamine-tetraacetic acid (EDTA) from Merck Millipore and bronopol, glycine, *p*-nitrophenol and sodium azide were purchased from Aladdin China. Rest of the chemicals were also of analytical grade. Full fat UHT milk purchased from the local market was used for the production of enzymes.

2.2. Selection of the isolates

All representative strains of 31 different RAPD groups as well as sub-groups, representing 738 different isolates recovered from Chinese milk powders (Sadiq et al., 2016), were studied. A total of 178 strains (see Table 1) were randomly selected (1–10 from each RAPD group or sub-group, depending on the number of previously recovered strains belonging to a particular group) to determine their enzymatic potential based on screening on different selective media. For the quantitative assessment of proteases and lipases, three strains (showing positive results) were further selected from each group (including sub-groups). In addition, some of the strains showing negative screening results were also confirmed by the quantitative method. In case of *B. licheniformis* groups and sub-groups, 5–6 strains were selected for quantitative assessment because of the diversity and heterogeneity of these isolates in Chinese milk powders. For heat resistance studies only 1–2 isolates from each group and sub-group were randomly selected for all isolates.

2.3. Spoilage potential of sporeformers

2.3.1. Screening assay for enzymatic potential

The spoilage potential of each strain was screened in terms of its ability to produce lipases, proteases, β-galactosidases, phospholipases and haemolysins following the methods described by De Jonghe et al. (2010) and Lücking et al. (2013). A single colony was streaked on respective agar plates to detect enzymatic activities. Following selective media were used: plate count agar (Sigma-Aldrich) supplemented with 4% skimmed milk powder (Sigma-Aldrich) for proteases, tributyrin agar for lipases, Columbia agar supplemented with 5% sheep blood for haemolytic activity, nutrient agar (NA, Difco) supplemented with 8% egg yolk emulsion (Sigma-Aldrich) for phospholipase activity and β-galactosidase activity was detected by using X-Gal agar (TSA plates spread with 35 μl X-gal and 20 μl IPTG).

2.3.2. Quantitative assessment of total proteolytic activity

The strains that were positive for protease activity on the agar plate were further chosen in order to quantify their total proteolytic enzymes following the method used by De Jonghe et al. (2010) with some changes. The strains were first grown in brain heart infusion broth (BHI; Oxoid) for 17–24 h (depending on the strain) on a shaking incubator (150 rpm) and the culture was diluted in UHT milk to the final concentration of 10^5 CFU/ml followed by the incubation for one week (37 °C for MP and 55 °C for TP) to allow protease production. After incubation, the samples were centrifuged for 10 min at 12,000 *g*. Then, 1 ml of the supernatant was diluted in 9 ml UHT milk followed by the addition of sodium azide and bronopol (final concentrations 0.01% and 0.025%, respectively) to inhibit bacterial growth. These samples were further incubated for 1 week at 37 °C (optimum temperature for proteolytic activity).