Characterisation and biofilm screening of the predominant bacteria isolated from whey protein concentrate 80

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Abstract The source of microbiological contamination of whey protein concentrate (WPC), a quality problem for the dairy industry, has not been thoroughly investigated. The objectives of this study were to identify the bacteria isolated from whey protein concentrate containing 80% protein (WPC 80) and determine their ability to form biofilms as a potential source of contamination in a whey processing line. Six *Bacillus* species including (percentage of isolates in brackets) *Bacillus licheniformis* (67%), *Bacillus cereus* (19%), *Bacillus thuringensis* (4%), *Bacillus subtilis* (4%), *Bacillus pumilus* (4%) and *Paenibacillus glucanolyticus* (2%) were identified based on BLAST databases in six different WPC80 batches. *B. licheniformis* was the predominant isolate. *B. licheniformis* are thermo-tolerant bacteria with the ability to form biofilm. This biofilm is a potential source of microbial contamination of product, resulting in microbial specification limits being exceeded. Spoilage of product may result from the metabolic products, such as enzymes, produced by these biofilms.

Keywords *Bacillus licheniformis* · *Bacillus cereus* · *Paenibacillus glucanolyticus* · Whey protein concentrate

1 Introduction

Whey is the liquid remaining after the production of cheese or the removal of fat and casein (80% of the proteins) from milk and contains predominantly Gram-positive

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organisms from the starter population used in cheese and casein manufacture (lactic acid bacteria) or thermo-resistant species such as spore-forming *Bacillus* species (Schreiber 2001). Some starter bacteria, such as *Streptococcus thermophilus* and thermo-resistant *Bacillus* species can tolerate heat treatment such as thermalisation, pasteurisation and evaporation used in the manufacture of whey protein concentrate (WPC). These bacteria are therefore likely to form biofilm on manufacturing plant surfaces where they can grow and then be released into the whey being processed. Whey contains about 50% of the nutrients present in milk, comprising milk sugar (lactose), serum proteins (whey proteins), minerals, a small amount of fat and most of the water soluble minor nutrients from milk such as vitamins (Smithers 2008; Zadow 1992). This whey is concentrated by ultrafiltration and evaporation and then spray dried to provide a product that can be used as an ingredient in many foods.

Whey, formerly a waste product, became interesting to the food processing industries due to its physical and chemical properties, especially its protein, and nutritional qualities. The major whey product is WPC (Zadow 1992). It can be defined as the product derived from milk whey containing 50, 65 or 80% native proteins (Luck et al. 2013) and called WPC 50, WPC 65 and WPC 80, respectively. The individual protein composition in ultrafiltered WPC is about 68% β -lactoglobulin, 21% α -lactalbumin and 10% serum proteins. This is approximately the same as present in whole milk (Delaney 1976). The amino acid composition of WPC and skim milk were compared, and the results showed WPC contains higher tryptophan and cysteine. This is due to the activity of starter bacteria in the manufacture of cheese whey (Macgibbon 2014). A schematic diagram of the WPC manufacturing process at one dairy plant in New Zealand is presented in Fig. 1.

The microflora in WPC varies depending on the type of whey with sweet whey showing a higher prevalence of thermophilic and mesophilic sporeformers compared with acid whey (Watterson et al. 2014). Aerobic sporeformers of particular concern in dairy products include the psychrotolerant *Paenibacillus* spp., mesophilic *Bacillus* spp.; for example, *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus pumilus* and thermophilic *Anoxybacillus flavithermus* and *Geobacillus* species in a group that is not well defined (Burgess et al. 2009, 2010; Watterson et al. 2014). Biofilm formation by some

Fig. 1 A generic whey protein concentrate manufacturing process highlighting temperatures throughout the process that will influence microbial growth

of these bacteria from the dairy industry, in particular the *A. flavithermus* and *Geobacillus* spp. in milk powder, has been reported and is believed to be the main cause of product contamination and possibly enzymes that lead to product spoilage (Scott et al. 2007). Sporeformers found in the dairy industry are the resistant forms of *Bacillus* and *Clostridium* species that withstand heat and chemical treatment that is used to control most bacteria in dairy manufacture. Generally, the microbial contaminants in products such as WPC are recorded as either thermophilic or mesophilic bacteria in routine testing. Dairy manufacturers have specifications for these groups of bacteria, but there is no requirement for further testing to identify the bacteria comprising these groups. The identification and characterisation of these bacteria could help in determining the effect on product quality and the risk of the manufacture of unacceptable product and the risk of product spoilage. This study aimed to identify the contaminants in six WPC 80 samples.

2 Materials and methods

2.1 Source of samples

Six WPC 80 samples from different manufacturing runs were obtained from one dairy manufacturing site in New Zealand. The samples in powdered form were received in 100-g foil-lined paper pouches used to store samples from the manufacturing process for testing. The WPC 80 samples are listed in Table 1. Six different WPC 80 samples provided due to reported high bacterial counts were analysed to obtain the total microbial load using the aerobic plate count technique (see below) as used in the dairy industry to monitor the quality of the product.

2.2 Isolation of bacteria

Ten grams of each WPC 80 sample were diluted in 90 g of sterile 1% peptone water (pH 7.0) and homogenised using a peristaltic blender for 1 min prior to serial tenfold dilution to 1×10^{-6} in 1% peptone. The pour plate technique was performed using milk plate count agar (MPCA) (Merck, BDH, Palmerston North, New Zealand) in duplicate

Table 1 Microbial load determined by plate counting on MPCA from WPC 80 samples incubated at 30 °C for 24 h

Sample	Mesophilic aerobic plate count (CFU.g ⁻¹)	Thermophilic aerobic plate count (CFU.g ⁻¹) ^a
A	5.5×10^4	$<10^2$
B	5.9×10^5	$<10^3$
C	1.0×10^4	$<10^3$
D	7.3×10^4	$<10^3$
E	1.2×10^5	$<10^2$
F	3.3×10^4	$<10^3$

^a Spreading colonies for the thermophilic aerobic plate count meant no accurate counts could be obtained for this test

to obtain numbers in the range of 30–300 colonies per plate. The agar plates were incubated aerobically at 30 °C for 24 h for mesophilic bacteria and at 55 °C for 18 h following an agar overlay to prevent spreading of thermophilic bacteria. Two to three single isolated colonies of different colony morphology were taken from the plates of each WPC 80 sample and restreaked onto MPCA to obtain pure cultures. Pure strains were stored at –80 °C on beads (Microbank, Pro-Lab Canada).

2.3 Phenotypic characterisation of isolates

The pure cultures grown on MPCA were screened using microscopic observation of Gram-stained cells. The motility tests were done by the hanging drop technique and observed under light microscopy. These were used as additional confirmatory tests for some of the isolates, selected on the basis of colony morphology as presumptive *B. licheniformis*. Each culture was inoculated into Trypticase Soy Broth (TSB) and incubated 30, 37 and 55 °C to observe growth at different temperatures based on optical density.

2.4 Identification by PCR

Gram positive bacilli with colonies indicative of *B. licheniformis* were further tested using species specific PCR targeting the gyrase B gene using one set of species specific primers Blich-F1 5'-AKACGGAAGTGACGGGAAC-3' and Blich-R1 5'-AGAAACTTTTCRAGCGCTT-3'(Huang et al. 2012). PCR amplification involved 20 µL Master mix (5 Prime MasterMix-100 Rxns GmbH, Germany) consisting of dNTPs, magnesium chloride and Taq DNA polymerase, 24 µL UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen), 1 µL forward primer, 1 µL reverse primer and 4 µL DNA template (unknown culture) to achieve a final volume of 50 µL. Amplification conditions were as follows; denaturing step at 94 °C for 7 min followed by 35 cycles with denaturation (49 °C for 60 s), annealing (55 °C for 60 s) and extension (72 °C for 60 s) followed by a final extension at 72 °C for 15 min. All PCR assays were carried out on the Techno thermal cycler (TC-400, Total Lab Systems, Auckland, New Zealand). The PCR products were visualised (E-Gel iBASE™, Invitrogen) using premade 2% agarose electrophoresis gel (E-Gel® EX with SYBR Glod II) and then visualised under UV Transilluminator, UVP, Inc (Chromator-View, San Gabriel, Californis, USA) and UVITEC (Cambridge, UK).

2.5 Partial 16S rDNA gene sequencing

For the colonies that were not indicative of *B. licheniformis* and for those isolates that did not produce a positive result with the species specific primers for the gyrase B gene of *B. licheniformis*, universal primers Bac27F 5'-AGAGTTTGATCCTGGCTCAG-3' and U1492R 5'-TACGGCTACCTTGTTACGACTT-3' were used to amplify a 1000 bp part of the 16S ribosomal deoxyribonucleic acid (rDNA) genome for sequencing, using the conditions in Flint et al. (1999b). Prior to sequencing, the PCR products were purified using a Zymo DNA Clean & Concentrator™-5, USA kit and sent to UV/VIS spectrophotometry to check the DNA concentration (1–2 µL) using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). Sequencing was done at the

Massey University Sequencing Unit using the BigDye terminator v3.1 cycle sequencing kit and the results were analysed using the BLAST GeneBank.

2.6 Biofilm screening

A microtitre plate assay (Oh et al. 2007) was used to determine the ability of isolates to attach (adhere to a surface) and grow (reproduce on a surface) and form biofilm. A sterile 96-well flat-bottomed polystyrene microplate (Falcon® 96, 35 3072, Becton, Dickinson & Company, USA) was filled with 230 μL of TSB. Three wells were filled with TSB only as negative controls. Overnight culture (20 μL) was added into the test wells, three wells per culture, and the plates were incubated aerobically for 24 h at 30, 37 and 55 °C. The contents of the plates were removed by inverting the plates, and then, the wells were washed three times with 300 μL of sterile distilled water. The remaining attached cells were fixed with 250 μL of methanol per well for 15 min. The liquid was discarded and the plates air-dried. The microplate wells were stained with 250 μL of 0.05% (w/v) crystal violet for 5 min. The excess stain was rinsed off by placing the microplate under running distilled water. After the microplates were air-dried, the dye bound to the adherent cells was resolubilized with 250 μL of 33% (v/v) glacial acetic acid per well. The optical density (O.D.) of each well was measured at 570 nm using an automatic 96-well microplate reader (BMG Labtech Spectrostar microplate reader, Bio-Tek Instruments, INC, Winooski, VT, USA). The biofilm formation of each *B. licheniformis* isolate (the predominant isolate) was tested using the microtitre plate assay with the strength of biofilm formation using the following range of O.D. values based on Oh et al. (2007). The cut-off O.D. (O.Dc) was defined as three SDs above the mean O.D. of the negative control. Strains were classified as follows: O.D. < O.Dc = no biofilm former, O.Dc < O.D. < (2 $\times$ O.Dc) = moderate biofilm former and O.D. > (2 $\times$ O.Dc) = strong biofilm former. Differences in the degree of biofilm formation were examined by the Friedman test, followed by the Wilcoxon signed-rank test. *P* values of <0.05 were considered significant. The O.Dc was determined as 0.5 for this study. The tests were done in triplicate at three different temperatures (30, 37 and 55 °C) to explore the influence of temperature on biofilm formation as the temperatures vary in different parts of the manufacturing process.

3 Results and discussion

3.1 Total aerobic plate count

The predominant bacteria in the WPC 80 samples were mesophilic bacteria (Table 2) with total mesophilic bacterial counts close to or exceeding the specification limits. The Australia New Zealand Food Safety Authority guideline for the mesophilic plate count for dried milk including whey powder is 5×10^4 CFU.g⁻¹ (acceptable level) and 2×10^5 CFU.g⁻¹ (maximum). The thermophilic bacterial counts were all < 10^3 CFU.g⁻¹. There is no guideline for thermophilic plate count.

Table 2 Bacteria species identified using species specific PCR for *B. licheniformis* and 16S rDNA sequencing from six WPC 80 samples

Bacteria species	Number of positive isolates
<i>Bacillus licheniformis</i>	33
<i>Bacillus cereus/Bacillus thuringensis</i>	11
<i>Bacillus subtilis</i>	2
<i>Bacillus pumilus</i>	2
<i>Paenibacillus glucanolyticus</i>	1
Total	49

3.2 Isolation of bacteria

The colony formations typical of *B. licheniformis* on MPCA were large opaque, adherent with irregular edges. These isolates were retrieved from MPCA and incubated at the appropriate temperature (30 °C). Although some of the isolates were observed to be facultative anaerobes, their growth in strict anaerobic conditions was not tested. Most of the facultative anaerobic and spore-forming bacteria produced pellicles after incubation at 30, 37 and 55 °C in TSB medium. Cells in pellicles are held together by an extracellular matrix consisting of exopolysaccharide and amyloid-like fibres largely composed of protein (Colodkin-Gal et al. 2012). This air-liquid interface might indicate that these bacteria could produce exopolysaccharide that generate biofilm formation.

Although the isolates were recovered at 30 °C (mesophiles), some grew at 55 °C (thermophilic). These results are similar to those of Lücking et al. (2013). Watterson et al. (2014) reported that sweet whey and non-fat dry milk showed higher prevalence of thermophilic and mesophilic sporeformers compared with acid whey and WPC 80. Some of the bacteria isolated in the present trial could grow at both 30 and 55 °C. Facultative thermophiles in dairy processing belong to the *Bacillus* genus and include species such as *Bacillus coagulans*, *B. licheniformis* and *B. pumilus* that are able to grow at both mesophilic and thermophilic temperatures (Burgess et al. 2013; Flint et al. 2011a, b).

The isolates obtained from the six WPC 80 samples were predominantly *Bacillus* species with rod-shaped purple-violet colour on Gram staining, as expected from colony morphology. All of the isolated bacteria were Gram positive, mostly sporeformers and motile based on microscopic observation.

3.3 PCR identification and 16S rDNA sequencing

The identification of suspect *B. licheniformis* isolates based on colony morphology was confirmed as expected using species specific PCR. *B. licheniformis* was the predominant isolate making up 33 of the 49 isolates (Table 2).

These aerobic-spore-forming bacteria are frequent contaminants isolated from milk or dairy processing environments (Alvarez-Ordóñez et al. 2014; Aouadhi et al. 2013; Brown 2000; Buehner et al. 2014; Burgess et al. 2013; Flint et al. 2001; Frank 1997; Lücking et al. 2013; Masiello et al. 2014) predominantly in milk powder (Reginensi et al. 2001; Scott et al. 2007; Yuan et al. 2012) but not commonly reported in whey

powder. The origin of *B. licheniformis* is believed to be from soil and the heat resistant spores survive pasteurisation of milk and the cheese manufacturing process, thus potentially contaminating the whey. Buehner et al. (2014) reported that *B. licheniformis* was the major contaminant in milk regardless of the seasons (winter or summer). *B. licheniformis* can contribute to product spoilage through the production of enzymes (Teh et al. 2012). The second most isolated *Bacillus* species were *Bacillus cereus*/*B. thuringensis* with 11 isolates. *B. cereus* is capable of producing emetic and diarrhoeal toxin that can cause food poisoning (Burgess et al. 2010; De Jonghe et al. 2010). The next most isolated species were *B. subtilis* and *B. pumilus*. These three *Bacillus* species are also common contaminants in dairy products (Frank 1997; Masiello et al. 2014). Certain mesophilic *Bacillus* spp. (e.g. *B. subtilis*, *B. pumilus* and *B. licheniformis*) can also grow at temperatures that are used to detect and enumerate thermophilic organisms (i.e., 55 °C). In one recent trial, WPC 80 contained thermophilic spore counts detected by direct plating (Watterson et al. 2014).

The present study identified one isolate as *Paenibacillus glucanolyticus* (Table 2). This is the first time that this bacterium has been isolated from WPC 80, and no records can be found of this bacterium being isolated in New Zealand. It was identified using 16S rDNA gene sequencing with confidence level 99% (BLAST) and 94% (Seqmatch). The *P. glucanolyticus* isolate was observed using scanning electron microscope (SEM) and the intercellular properties of the cells together with the spores were observed using transmission electron microscope (TEM). The cells were long >3.0 µm and thin <0.9 µm and produced oval terminal spores that markedly distended the sporangium. Interestingly, their spores portrayed a very unique shape, different from spores of *Bacillus* species.

This bacterium, formerly included in the genus *Bacillus*, is a facultative anaerobic, long, thin rod-shaped bacterium with terminal spore formation and may be isolated from various soils. Normal environments of *Paenibacillus* strains include soil, the rhizosphere of plants, water, diseased insect larvae and food products (Daane et al. 2002; Lorentz et al. 2006). Recently, Ferrand et al. (2013) published a case report on *P. glucanolyticus* infection in a 65-year-old patient with type 2 diabetes who developed a cardiac device-related endocarditis. This is believed to be the first ever report associating *P. glucanolyticus* with human infection.

3.4 Biofilm formation screening by *B. licheniformis* isolates

There is a lack of information on the biofilm-forming ability of *B. licheniformis* and the importance of biofilms of this bacterium in the contamination of dairy products.

Three *B. licheniformis* isolates (C55C01, E30C11 and F55C12) produced strong biofilm O.D._{570nm} ≥0.5 at 30 °C with an O.D. at 3.5 (the highest absorbance from microtitre plate reader) (Table 3, Fig. 2). Nine *B. licheniformis* isolates produced strong biofilms at 37 °C suggesting 37 °C is the optimum temperature for *B. licheniformis* to form biofilm. *B. licheniformis* is known as a thermo-tolerant bacterium and able to grow at temperatures up to 55 °C (Dhakal et al. 2013; Smithers 2008). However, in this study, most of the *B. licheniformis* isolated from six different whey samples did not produce biofilm at 55 °C. Biofilm formation at 55 °C was generally poor with only three isolates showing a tendency to form biofilm at this temperature. Moderate biofilm formation in TSB at 37 °C was produced by three *B. licheniformis* isolates. The majority of

Table 3 Biofilm strength of *B. licheniformis* measured using the microtitre plate assay in trypticase soy broth at three different temperatures (strength determined by the method of Oh et al. (2007))

Temperature (°C)	Biofilm formation (number of isolates in each category)		
	No biofilm	Moderate biofilm	Strong biofilm
30	30	0	3
37	21	3	9
55	30	2	1

B. licheniformis strains were unable to produce biofilm at any of the three temperatures (30, 37 and 55 °C) in the TSB medium. However, they may produce biofilm under the conditions of a dairy manufacturing plant on a protein-fouled surface.

3.5 General discussion

Microbial contamination of dairy products is controlled by heat treatment such as pasteurisation to kill pathogens and prevent spoilage. However, some thermophilic and mesophilic organisms as well as spore-forming bacteria can survive and contribute to the spoilage of the milk. These bacteria include *S. thermophilus* and spore-forming bacteria *Anoxybacillus*, *Geobacillus* and *Bacillus* species which can survive subsequent dairy manufacturing processes and contaminate whey (Flint et al. 1997, 1999a, b, 2000). Parkar et al. (2003) showed a variety of thermophilic bacilli strains including *Geobacillus*, *Anoxybacillus*, *B. licheniformis*, *B. coagulans* and *B. pumilus* were able to attach to both stainless steel and milk foulant in similar numbers. Dairy product contamination is believed to be enhanced by the growth of biofilms in the manufacturing plant. Biofilms may develop on any surface where the conditions are suitable for bacterial growth (Costerton 1995; Donlan 2002; Flint et al. 2001). The contribution of biofilms of these thermo-resistant bacteria to the contamination of whey products is unknown.

Product quality can be affected by these sporeformers in three different ways: by production of toxin or spoilage enzymes or by interfering in downstream processing steps (De Jonghe et al. 2010; Pasvolsky et al. 2014). Enzymes like protease and lipase do influence the quality of milk and milk products by altering the sensory qualities such as texture, taste, aroma and nutritional value (Chen et al. 2003; Teh et al. 2012, 2013). *Bacillus* and *Paenibacillus* produce a variety of proteases, lipases and phospholipases that impact the texture of dairy product and cause off-flavours (Lücking et al. 2013). The effect of the bacteria isolated in the present trial on the sensory quality of WPC 80 is unknown.

The ability of *B. licheniformis* to form biofilm has been demonstrated, and this is important as this is the most likely source of contamination of high numbers of *B. licheniformis* and their enzymes in product. Locating the zone in the manufacturing plant where these biofilms grow will be important for their control. We know that *B. licheniformis* cannot grow at 10 °C, the temperature used in the ultrafiltration plant; therefore, this cannot be a site of biofilm development for these bacteria. Other parts of the WPC manufacturing plant where conditions are likely to be appropriate for

Fig. 2 The O.D. scores of the dairy isolate biofilms at 570-nm absorbance using the microtitre plate assay at three different temperatures. The results represent the amount of crystal violet stain retained on the test plate indicating the presence of biofilm

B. licheniformis biofilm development are during clarification and evaporation, and this needs to be confirmed.

4 Conclusion

Mesophilic bacteria, in particular, *B. licheniformis* may predominate in WPC 80, contributing to aerobic plate counts close to or exceeding specification limits. These bacteria may produce biofilm under laboratory conditions with 37 °C being the favoured temperature compared with 30 and 55 °C. This may be a source of product contamination during the manufacture of WPC 80. The control of *B. licheniformis* during the manufacture of WPC 80 is important to maintain product quality.

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