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Biofilm formation by *B. licheniformis* isolated from whey protein concentrate 80 powder as a potential source of product contamination

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ABSTRACT

This study aimed to examine biofilm formation of *Bacillus licheniformis* isolated from whey protein concentrate 80 (WPC80) as a potential source of contamination in the manufacture of WPC.

Six WPC80 powder samples from one whey processing plant in New Zealand were used in this study. Six *Bacillus* species including (percentage of isolates in brackets) *B. licheniformis* (66%), *Bacillus cereus*/*Bacillus thuringiensis* (18%), *Bacillus subtilis* (4%), *Bacillus pumilus* (4%), *Paenibacillus glucanolyticus* (2%) and *Lactobacillus plantarum* (6%) were identified using colony morphologies, biochemical tests, species specific PCR and 16S ribosomal DNA gene sequencing and subsequent analysis using the BLAST and Seqmatch databases.

Preliminary screening for biofilm formation by the predominant contaminant, *B. licheniformis* using a microtitre plate assay with the bacteria grown in laboratory medium tryptic soy broth (TSB) at three different temperatures (30°C, 37°C and 55°C) showed most biofilm formation at 37°C with 9/33 isolates forming strong biofilm. In total 13/33 isolates formed strong biofilm at three different temperatures on the polystyrene microtitre plate surface.

Subsequent tests for biofilm formation on stainless steel (SS) showed an increased frequency of biofilm formation with 32/33 strains forming strong biofilm in TSB at 37°C. This demonstrates the limitation of the microtitre plate assay for screening for biofilm formation and suggests that biofilm growth of *B. licheniformis* favours a SS surface.

The attachment and biofilm formation was further investigated using SS coupons and reconstituted whey medium at different concentrations (1%, 5%, and 20%). The best medium for *B. licheniformis* isolates to form biofilm on SS at its best growth temperature (37°C) was 1% reconstituted WPC80. Interestingly, when 1% reconstituted WPC80 was supplemented with lactose and minerals (mainly calcium and magnesium)

to replicate the composition of Mozzarella cheese whey before ultrafiltration (UF), the *B. licheniformis* biofilm counts increased at least by one log.

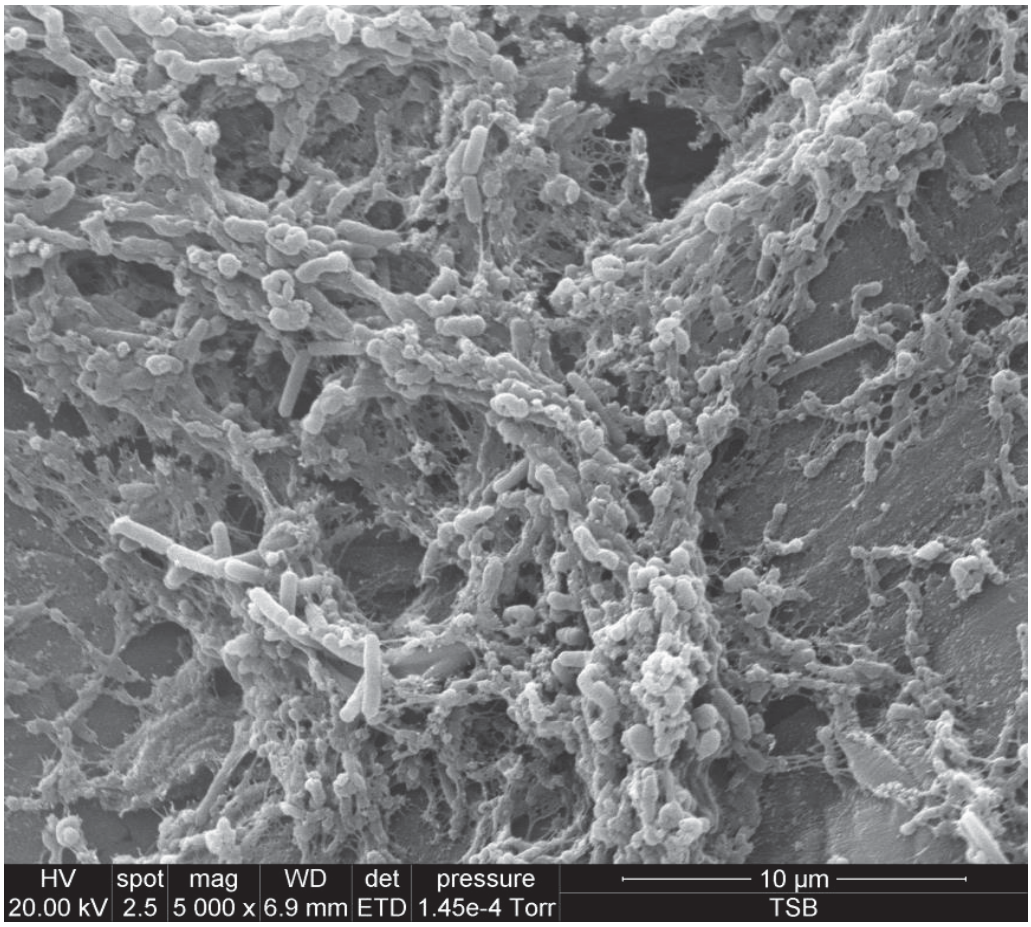
The production of protease enzyme, extracellular polymeric substances (EPS) and nitrate reduction by *B. licheniformis* showed the potential of *B. licheniformis* to influence the quality of dairy products. Biosurfactant production by *B. licheniformis* identified as lichenysin consisting of lipopeptide was detected and this may influence biofilm formation on SS. The inability of the *B. licheniformis* isolates to ferment lactose as their major carbon source was confirmed by lactose fermentation tests and shows that *B. licheniformis* is not ideally suited to a dairy environment. The *B. licheniformis* vegetative cells were found to be heat resistant with a $< \log_{10}$ reduction at the three temperatures tested; 72ºC, 75ºC and 80°C during 15 s, 30 s and 60 s heating intervals.

In order to thrive in a dairy system, synergistic interactions with other microflora were investigated as a possible mechanism to use lactose that has been broken down by other microflora. *Lactobacillus plantarum (L. plantarum),* another isolate from the WPC80 samples, has the ability to produce glucose and galactose from lactose. This was grown with each of two *B. licheniformis* isolates (E30C11 and F30C02) with different abilities to form biofilm. Interestingly this did not enhance the growth of *B. licheniformis* suggesting that another carbon source, most likely whey protein, must provide the energy source for this bacterium in a whey environment.

A review of the WPC80 processing plant showed the UF membranes had the largest surface area (3500 – 7500 m^2), providing most potential for biofilm growth. However, UF was run at 10°C, too low for the growth of *B. licheniformis* which has a minimum growth temperature of 20°C. The hypothesis that sections of the processing plant before the UF step are the sites for *B. licheniformis* biofilm growth was supported by analysing several samples from the raw whey balance tank, clarifier, thermaliser and separator where 7 *B. licheniformis* strains were isolated. This shows that *B. licheniformis* is present at several early stages of WPC processing, with the most likely areas for growth being the certain sections of the clarifier, thermaliser and the separator where temperatures are close to the best growth temperature for this bacterium (37°C).

Preventing *B. licheniformis* contamination of WPC needs to focus on adjusting the conditions in these sections of the processing plant to limit biofilm growth.

Keywords: dairy, *Bacillus* species, *L. plantarum*, lichenysin, stainless steel, membrane processing plant.



FRONTISPIECE Biofilm of *Bacillus licheniformis* embedded in extracellular polymeric substances on 304 grade stainless steel after 24 h incubation.

LIST OF PUBLICATIONS

This work has been published in part in the following publications:

1. Xuemei Tang, Steve Flint, Rod Bennett, John Brooks and **Siti Norbaizura Md Zain** (2015). Biofilm in the dairy industry. Chapter 8: Biofilm contamination of ultrafiltration and reverse osmosis plants. John Wiley & Sons. Oxford.

2. **Siti Norbaizura Md Zain,** Steve Flint, Rod Bennett and Tay Hong Soon (2016). Characterisation and biofilm screening of the predominant bacteria isolated from whey protein concentrate. *Dairy Science & Technology*, *96(3),* 285-295. doi:10.1007/s13594-015-0264-z

3. **Siti Norbaizura Md Zain,** Rod Bennett and Steve Flint (2017). The Potential Source of *B. licheniformis* Contamination During Whey Protein Concentrate 80 Manufacture. *Journal of Food Science*, *82(3),* 751-756. doi: 10.1111/1750-3841.13633

LIST OF PRESENTATIONS

This work has been presented in part in the following presentations:

Oral Presentations:

1. **Siti Norbaizura Md Zain.** *The role of biofilm development on ultrafiltration membranes in the contamination of whey products*. IFNHH Food Division Symposium, Massey University, Palmerston North, New Zealand, 15 November 2013.

2. **Siti Norbaizura Md Zain.** *Identification of bacteria isolated from whey powder.* IFNHH Food Division Symposium, Massey University, Palmerston North, New Zealand, 14 November 2014.

3. **Siti Norbaizura Md Zain,** Steve Flint, Rod Bennett and Tay Hong Soon. *Identification of bacteria isolated from whey powder.* New Zealand Microbiological Society Conference, Wellington, 18 - 21 November 2014.

4. **Siti Norbaizura Md Zain,** Steve Flint and Nur Hanizah Kamsani. *Biofilm formation of single and mixed strains of thermophilic bacteria isolated from whey powder.* Malaysian Society for Microbiology Conference, Terengganu, Malaysia, 6 - 8 December 2014.

5. **Siti Norbaizura Md Zain,** Steve Flint, Rod Bennett and Tay Hong Soon. *Biofilm formation by B. licheniformis isolated from whey protein concentrate 80 powder on two different media and surfaces*. New Zealand Microbiological Society Conference, Rotorua, 2 - 5 November 2015.

6. **Siti Norbaizura Md Zain,** Steve Flint and Rod Bennett. *Biofilm formation from a co-culture of Bacillus licheniformis and Lactobacillus plantarum on stainless steel in a whey environment.* New Zealand Microbiological Society Conference, Christchurch, 14 - 17 November 2016.

7. Steve Flint, **Siti Norbaizura Md Zain** and Rod Bennett. *The role of biofilms in the quality of dairy products in whey processing plants.* American Dairy Science Association Annual Meeting, Pittsburgh, Pennsylvania, 25 - 28 June 2017.

Poster Presentations:

1. Rod Bennett, **Siti Norbaizura Md Zain,** Steve Flint and Tay Hong Soon (2015). *Understanding microbial contamination during whey protein concentrate manufacture.* Seminar on Emerging Dairy Technologies, Munich, Germany, 16 - 18 September 2015.

2. **Siti Norbaizura Md Zain,** Mandy Kee and Steve Flint. *Isolation and identification of Paenibacillus species from New Zealand dairy product.* The New Zealand Institute of Food Science and Technology Conference, Rotorua, 4 - 7 July 2016.

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