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Tyndallization does not Suppress *Bacillus megaterium* and May Explain Part of Potato Peel Colic

M Witty*

Math and Science Department, School of Pure and Applied Sciences, Florida SouthWestern State College,
8099 College Parkway, Fort Myers, Florida 33919, U. S. A.
*Corresponding author: mwitty@fsw.edu

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Abstract There is a recent cooking fad of leaving potato peel intact in food and eating it. Some bacteria from potato epidermis can survive cooking and this paper describes two examples of extremely thermoduric organisms which have been isolated from potato peel after it had been cooked rigorously and repeatedly in the process of Tyndallization. These bacteria have been identified by rDNA analysis of samples taken during Tyndallization. One of them, *Bacillus megaterium*, has a reputation for causing food poisoning through production of emetic toxins similar to cereulide and may account for some gastric features of Potato Peel Colic. The other, *Aeromicrobium flava* is only poorly characterized in previous literature.

Keywords: *potato, tuber, skin, peel, epidermis, colic, Bacillus megaterium, endophyte, rDNA, cereulide*

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1. Introduction

One of the most important sources of human nutrition is potato tubers [1], though they have some drawbacks, including occasional gastrointestinal irritation and colic [2]. This is especially true for the skin/epidermis which has conventionally been removed by peeling and rejected from the kitchen. Regrettably, after the 1940s, potato epidermis was popularized and accepted as the most nutritious part of tubers [3,4]. This is unfortunate because potato tuber epidermis is a very harmful part of the plant. Its pathology is usually entirely attributed to alkaloids [5] especially in greened tubers. There are hazards in addition to alkaloids in potato tuber epidermis, though these are much less well defined. They include heat resistant toxins from microbial endophytes which may be the cause of colics and irritable bowel by ingestion, even when potato epidermis is cooked [2,5,6].

Cooking potatoes often consists of heating until the tuber is softened. A common way to cook tubers is to peel and boil them in water for twenty to twenty five minutes which destroys most microbial vegetative cells, such as pathogens of the oral fecal route of infection. Some cells, including spores and especially endospores resist this regimen and their suppression requires more care. Even more rigorous than ordinary cooking at 100°C is the process of Tyndallization [7], which consists of repeated cycles of boiling followed by time at room temperature. Endospores are stimulated to germinate and make themselves vulnerable to subsequent cycles of boiling, a sterilization process consistent with cooking unlike other methods for suppressing bacteria which include rigorous chemical treatments [8]. Some species of soil bacteria from potato tuber epidermis are capable of resisting even this rigorous treatment and these include species capable of pathology as shown in this paper.

The best method for analysis of microbes in environmental or complex samples is Metabarcoding or analysis for rDNA (ribosomal DNA). It can determine entire microbial communities in a sample, including recalcitrant species which cannot be cultivated. This paper presents an example of the normal microbial components of potato epidermis and thermoduric species which can survive cooking.

Some significant species of bacteria were identified including examples of human pathogens which are present because of the use of animal dung [9,10] or even human wastewater as fertilizer [11]. We report examples of bacterial endophytes from potato skins, emphasizing organisms capable of causing colics and bowel irritation even after cooking. We hope that people will avoid eating potato epidermis in the future, even if it is cooked, because of the potential it has to cause ill health.

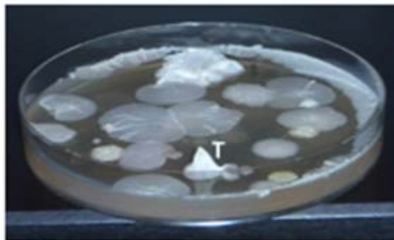
2. Materials and Methods

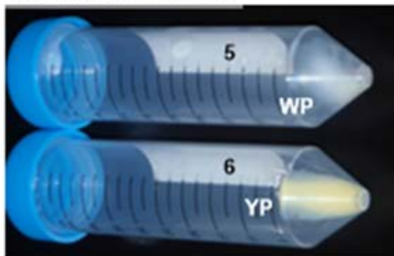
New Potato Tubers were purchased from Publix Supermarkets (Lakeland, Fl). Potato tuber epidermis was obtained in a way similar to material served as potato skins and chips i.e. by peeling 1mm of external tissue which retained residual soil, epidermis and buds.

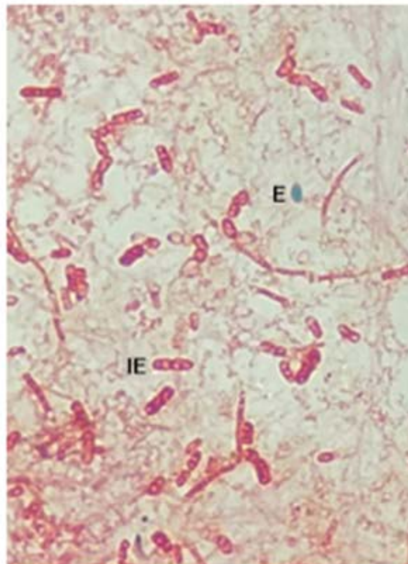
The raw potato peel was added to a sterile aliquot of Nutrient Broth (635ml) in a 1l Duran bottle until a little

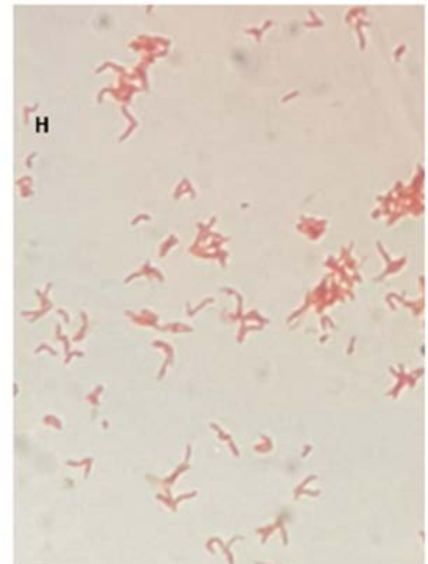
peel began to float beyond the surface of the agar (174.71g) and the new volume of broth and peel was approximately 800ml. Before treatment (cycle 0), the broth and peel mixture were swirled and then a 1ml sample spread on a 9cm Nutrient Agar plate, incubated at 25°C for two days and bacterial colonies counted. Then the method of repeated boiling (Tyndallization) was used to suppress microbes in the peel material as follows: the peel and broth material was brought to boiling point on a hot plate then left to cool to 25°C and incubated overnigh**t**. Then another 1ml sample was taken as before and spread on Nutrient Agar and also incubated at 25°C for at least two days. This was repeated daily seven times to achieve the **first** Tyndallization and generate the data used to compose Figure 1a and graph 1. The times to increase temperature from 25°C to 100°C (ramping times) for every cycle were, of course, variable. However, 55 minutes to increase temperature to 100°C and 65 minutes to cool to the point the material could be easily handled and pipetted are typical times. This process induced resistant spores to germinate and then they were easily destroyed by subsequent heating cycles.

The classical microbiological methods of enrichment culture and spore staining have been supplemented by the molecular method of rDNA analysis. To generate additional samples suitable for DNA analysis, material (1ml) from all steps of a **second** similar Tyndallization was transferred to 50ml aliquots of sterile Nutrient Broth in 250ml flasks. These were incubated at 25°C, swirling daily. Only the flasks with samples from cycles 0, 5 and 6 showed growth. The suspension of cell material from these three flasks was transferred to 50ml Falcon tubes, centrifuged at 4000g for 15 minutes, supernatant discarded (see Figure 1b) and the cells dried (50°C oven) to prepare material for molecular analysis. Bacterial diversity was determined by DNA sequence analysis using the Illumina sequencing platform [12] optimized for prokaryote rDNA targets (Scott Dowd, personal communication). Analysis of rDNA content is a method very well suited to this kind of study because it allows for detection of the vast majority of bacteria that cannot be cultivated. The Primers used were bac799F 5'-ACCMGGATTAGATACCCKG-3' and illbac1193R 5'-CRTCCMCACCTTCCTC-3'. DNA sequences with relative abundance values above 0.1 were used to draw graphs using the Excel program. Literature databases were searched to determine if the microbes were known to produce substances that are toxic, allergenic or irritant, especially heat resistant substances. One drop of the broth and peel mixture from this Tyndallization, steps 5 and 6, were also used to make heat fixed smears and these were stained with Malachite Green over boiling water for ten minutes and then counterstained with safranin to reveal mother cells and endospores (Figure 1c and Figure 1d).







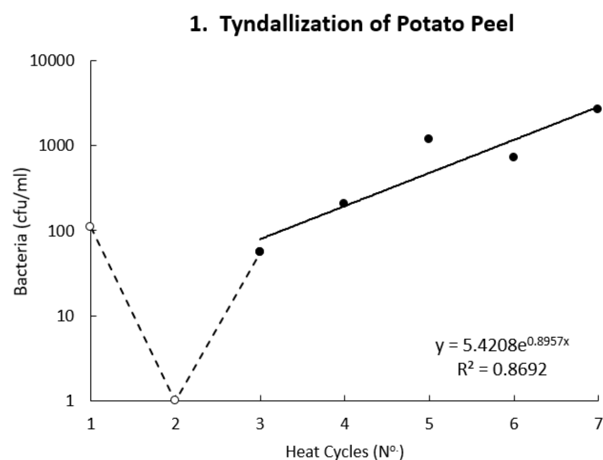


Caption: A; Representative colonies after step five of Tyndallization, grown on Nutrient Agar at 25°C for four days. Photographed at an angle to emphasize one of many tented colonies seen. B; Bacteria after five and six steps of Tyndallization. The bacteria obtained after five steps had loose fluid slime associated with them. C; light micrograph of the large step five bacteria, oil immersion microscopy. Only occasional endospores were seen, as in this field of view, though cytoplasmic inclusions which might be immature endospores were abundant. D; light micrograph of small step six bacteria, oil immersion microscopy. Unexpectedly, no endospores were seen. 5; 50ml Falcon tube with bacteria which survived five Tyndallization steps. 6; 50ml Falcon tube with bacteria which survived six Tyndallization steps. E; endospore. H; unavoidable halo from diffraction, not poor focus. IE; Inclusions, probably immature endospores. L; loose and watery part of pellet material which flowed easily when handled for photography c.f. firm yellow pellet. T; tented colony. WP; loose white pellet. YP; firm yellow pellet. Cameron L. Olivera assisted with microphotography.

Figure 1. Bacteria Enriched from Potato Peel

3. Results and Discussion

Individual bacterial colonies were seen by enrichment from potato epidermis in the **first** Tyndallization followed by growth on agar. Only a very few colony forms were seen (for example see Figure 1a). The colonies initially decreased in number but then increased logarithmically (Graph 1). The two samples of enriched bacteria which survived five or more cycles in the **second** Tyndallization produced enough material for detailed analysis, growing in separate 250ml flasks. These were examined microscopically and one sample was found to be enriched for endospore producers, though staining was imperfect and only occasionally was Malachite Green staining intense (Figure 1c). Many cytoplasmic inclusions were seen which we assume to be poorly stained or immature endospores. The other sample showed bacteria with no endospores, only small vegetative cells which diffracted strongly (Figure 1d). These results encouraged us to assume only a simple and easy to understand population of bacteria was present and to use a more precise method which could identify bacterial species and allow looking up the characteristics of these microbes in literature databases i.e. rDNA analysis.

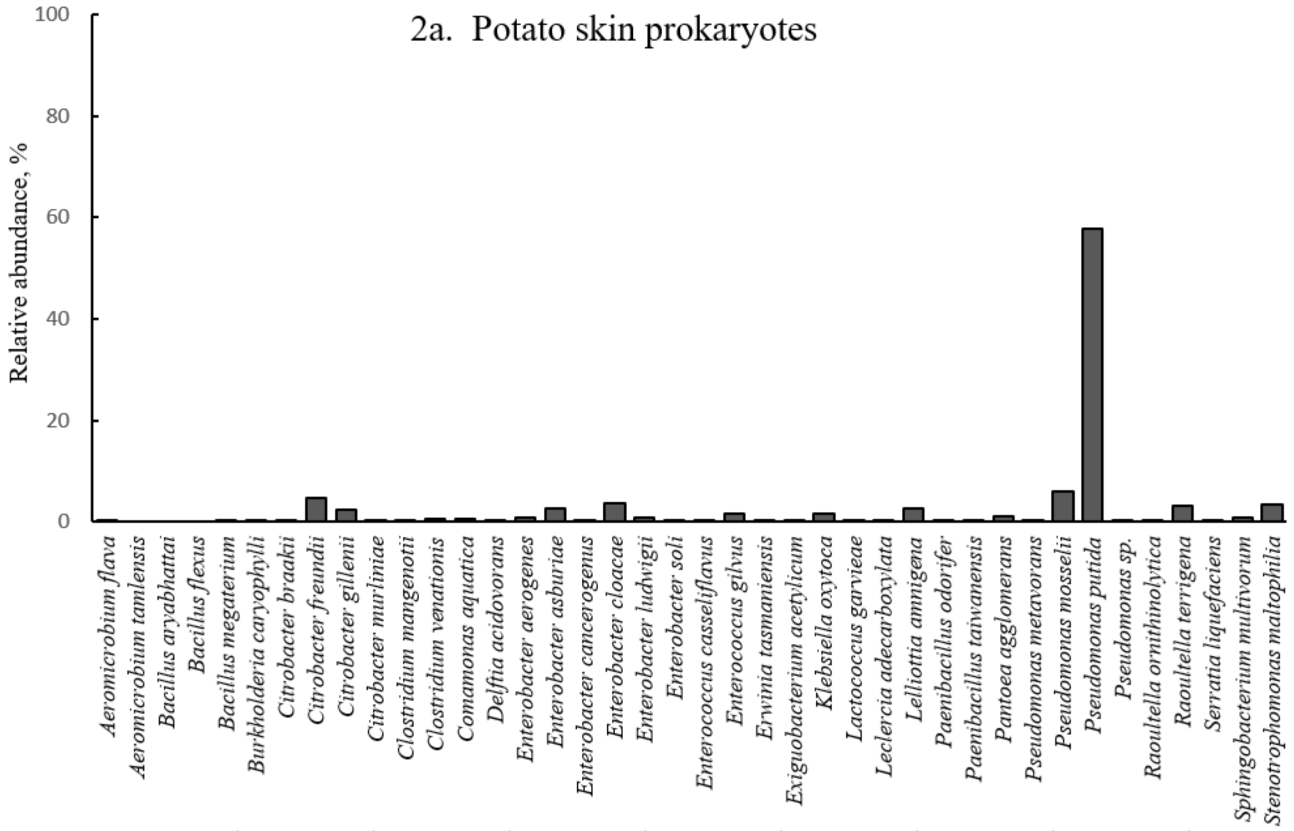


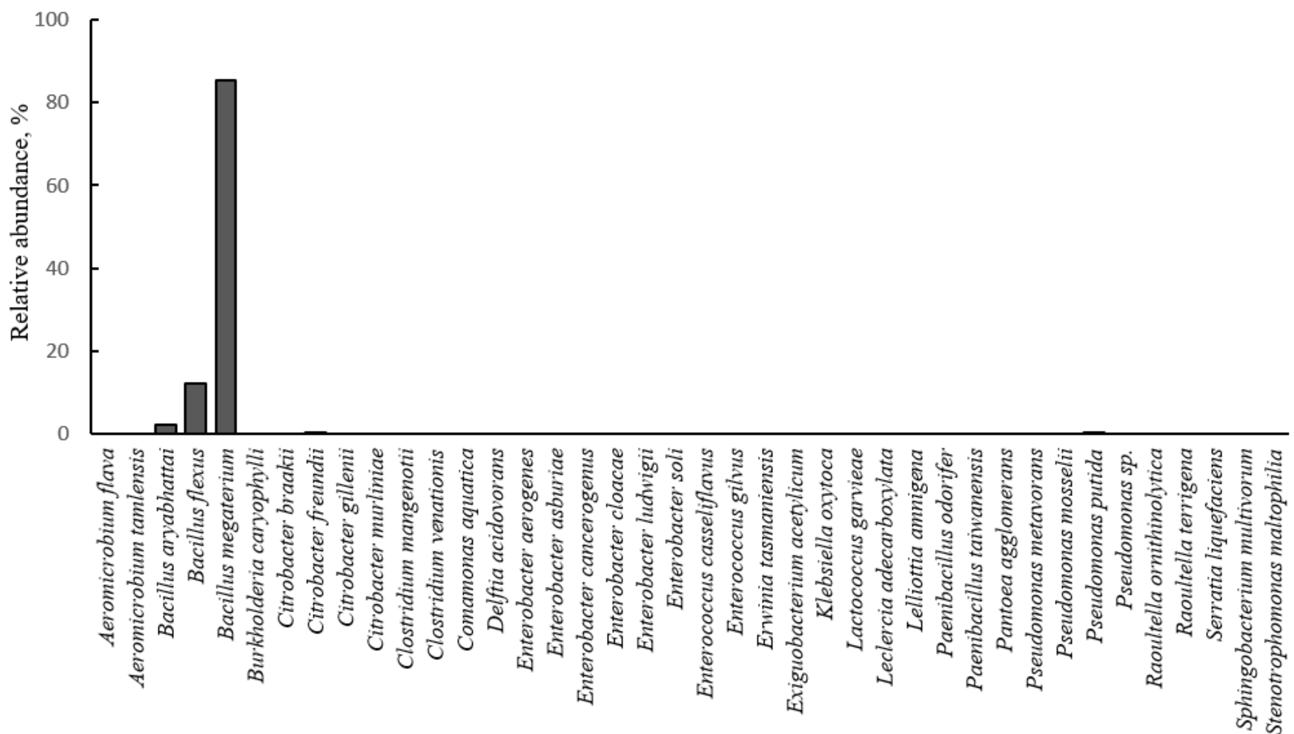
Graph 1. Dotted lines show that Tyndallization does not achieve sterility, using this regimen. Solid line: heat resistant bacteria proliferate and are detected by enrichment of some distinctive (by microscopy) spore formers and some surprising forms

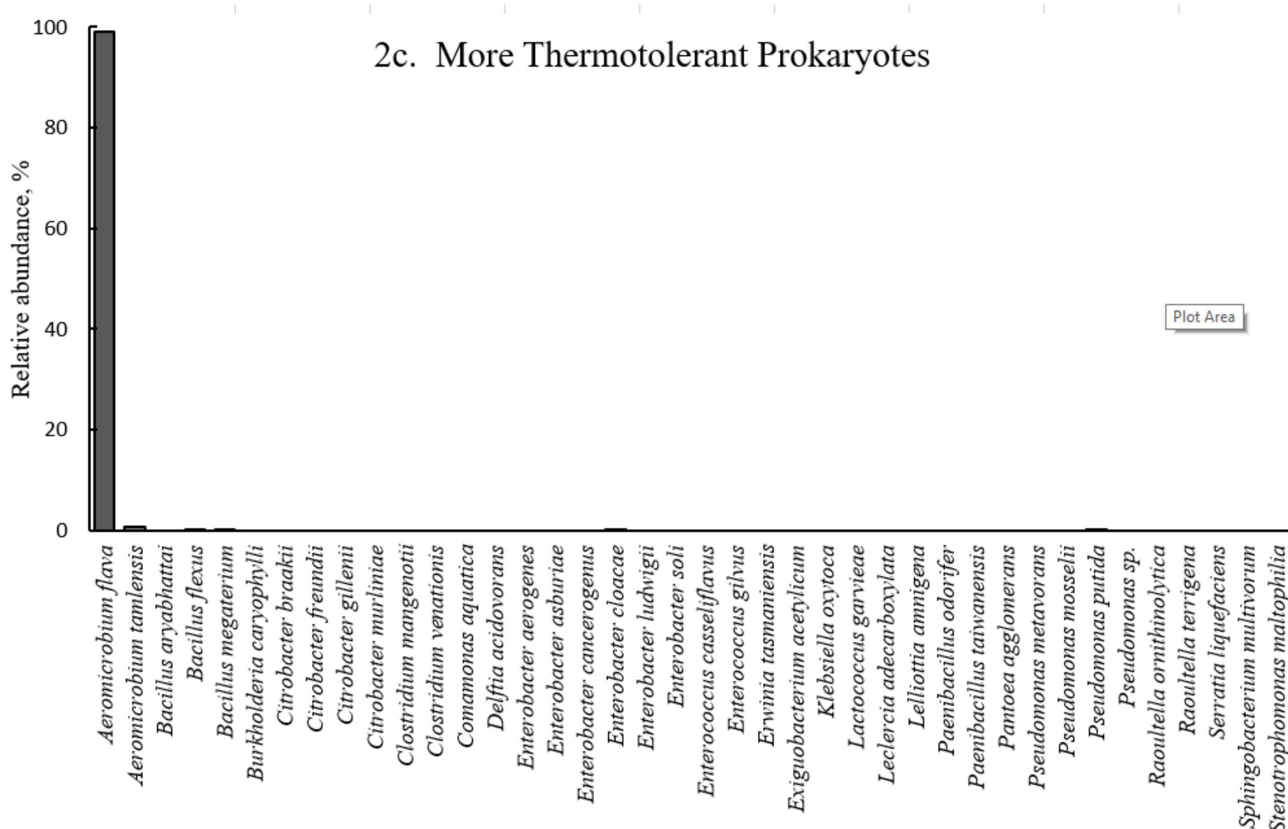
Table 1. Most abundant prokaryotes. Ribosomal RNA gene analysis was used to detect microbes and their abundance at three stages of Tyndallization. Relative abundance (numbers shown above) was used to rank species and those which ranked ≥ 0.1 were selected for inclusion in this table. Species were then reordered alphabetically. Only key references are shown.

Species	Not heated	Tyndallized (5 cycles)	Tyndallized (6 cycles)	Notes	Key references
Aeromicrobium flava	0.0	0.0	99.2	Not thermophilic, only thermotolerant	[21]
Aeromicrobium tamlensis	0.0	0.0	0.7	Not thermophilic, only thermotolerant	[22]
Bacillus aryabhattai	0.0	2.4	0.0	Endospores	
Bacillus flexus	0.0	12.4	0.0	Endospores. Not a pathogen	[23]
Bacillus megaterium	0.1	85.2	0.1	Heat stable toxins similar to cereulide	[15]
Burkholderia caryophylli	0.4	0.0	0.0	Plant pathogen	
Citrobacter braakii	0.4	0.0	0.0	Enterobacteriaceae	
Citrobacter freundii	4.8	0.0	0.0	Enterobacteriaceae	
Citrobacter gillenii	2.3	0.0	0.0	Enterobacteriaceae	
Citrobacter murliniae	0.5	0.0	0.0	Enterobacteriaceae	
Clostridium mangenotii	0.4	0.0	0.0	Closest relative to *Clostridium difficile*	
Clostridium venationis	0.6	0.0	0.0	Endospores	
Comamonas aquatica	0.5	0.0	0.0	Opportunist	
Delftia acidovorans	0.1	0.0	0.0	Opportunist	
Enterobacter aerogenes	1.0	0.0	0.0	Enterobacteriaceae	
Enterobacter asburiae	2.8	0.0	0.0	Enterobacteriaceae	
Enterobacter cancerogenus	0.2	0.0	0.0	Enterobacteriaceae	
Enterobacter cloacae	3.7	0.0	0.0	Enterobacteriaceae	
Enterobacter ludwigii	0.9	0.0	0.0	Enterobacteriaceae	
Enterobacter soli	0.3	0.0	0.0	Enterobacteriaceae	
Enterococcus casseliflavus	0.4	0.0	0.0	Enterobacteriaceae	
Enterococcus gilvus	1.6	0.0	0.0	Enterobacteriaceae	
Erwinia tasmaniensis	0.2	0.0	0.0	Soil saprophyte	
Exiguobacterium acetylicum	0.2	0.0	0.0		
Klebsiella oxytoca	1.6	0.0	0.0	Enterobacteriaceae	
Lactococcus garvieae	0.3	0.0	0.0		
Leclercia adecarboxylata	0.4	0.0	0.0	Opportunist	
Lelliottia amnigena	2.6	0.0	0.0	Enterobacteriaceae	
Paenibacillus odorifer	0.2	0.0	0.0	Endospores	
Paenibacillus taiwanensis	0.2	0.0	0.0	Endospores	
Pantoea agglomerans	1.1	0.0	0.0	Soil, ubiquitous	
Pseudomonas metavorans	0.3	0.0	0.0		
Pseudomonas mosselii	6.0	0.0	0.0		
Pseudomonas putida	57.7	0.0	0.0	Soil saprophyte	
Pseudomonas sp.	0.2	0.0	0.0		
Raoultella ornithinolytica	0.3	0.0	0.0	Enterobacteriaceae, pathogen	
Raoultella terrigena	3.1	0.0	0.0	Enterobacteriaceae	
Serratia liquefaciens	0.2	0.0	0.0	Opportunist	
Sphingobacterium multivorum	0.8	0.0	0.0	Rare opportunist	
Stenotrophomonas maltophilia	3.5	0.0	0.0	Rare opportunist	

The method of Tyndallization was used because it is similar to the domestic cooking method of boiling (≈100°C), though more rigorous and involves heating with ramping times of about 120 minutes, followed by time for bacteria to reveal their heat resistant nature by subsequent growth. Surprisingly, bacteria were able to survive repeated boiling, as shown by grown on agar after Tyndallization for 7 cycles of boiling. To obtain qualitative results the step of growth on agar was omitted and populations of bacteria during Tyndallization were analyzed to detect rDNA and determine species identity (Table 1).







Graph 2. Relative abundance of bacteria from A; potato peel, B; potato peel following five cycles of Tyndallization and C; potato peel following six cycles of Tyndallization

In order to emphasize the more abundant species in potato epidermis the results of nucleotide sequencing were ranked by abundance. This produced a very lengthy ranked list of prokaryotes so only species with relative abundance greater than 0.1, were retained and then Table 1 was alphabetized again. Literature searches showed that many of the species listed were significant to tubers used as food and in cooking because they were associated with research that showed heat resistant pathological features and also species that produce endospores. To save space only references to the most significant papers are cited. Graph 2 shows that the endospore producing bacteria were predominantly *Bacillus megaterium*. The small heat resistant cells which showed no sign of endospores in microscopy were identified as predominantly *Aeromicrobium flava* in rDNA analysis. These results support out aim of elucidating causes of Potato Peel Colic by revealing that, in addition to irritating substances accumulating of tubers in soil, some bacteria are capable of surviving rigorous cooking processes and remain a threat to human health if cooked peel is consumed or stored after cooking.

4. Conclusions

Soil is one of the most diverse environments known and contains many bacteria that are neutral, but some which are harmful to human health. This is why food grown in soil should be cleaned and peeled before cooking, to avoid encountering harmful bacteria (Table 1 columns one and two). However, this has been neglected in recent decades, especially for potatoes. Foolishly, potato tuber epidermis tissue has even been preferred for it's supposed superior vitamin content [13] at the expense of food safety. This is despite grave concerns regarding the alkaloid content of this tissue [14]. However, alkaloids are not the only danger and eating this material is fraught with multiple risks regarding colics. In addition to irritation from alkaloids, potato skins may cause colic which originates from resistant irritants of microbial origin that survive cooking, as described previously [2]. Even more serious is the possibility of live pathogens in potato skin/epidermis surviving the cooking process and producing toxins in food which can accumulate after the cooking process through bacterial growth.

The results of a first series of Tyndallization cycles showed only a small number of colony forms and this suggested repeating the process but to produce samples that could be analyzed for microbial diversity using rDNA analysis. Surprisingly, it was possible to produce two distinct groups of enriched bacterial that were very different in appearance (Figure 1b) and also showed mostly one cell type each. One was predominantly the endospore forming bacterium *Bacillus megaterium*, conspicuously heat resistant as expected, and also a species capable of causing food poisoning [15,16,17]. The other was more surprisingly not an endospore former; *Aeromicrobium flava* which is also not associated with food poisoning. This shows that potato epidermis contains microbes which can cause uncomfortable colics (reviewed in [2]) by the additional method of resisting rigorous cooking. Usually this problem is solved by peeling in addition to boiling for 20 to 25 minutes. This is not successful if the peeling part of food sanitation is left out. As can be seen in Table 1 dung bacteria are killed off but sinister species remain living in the cooked peel.

Bacillus megaterium is capable of human pathology (for example [18]). Significantly for food products, some strains of *B. megaterium* are also capable of producing toxins and contain genes for pathogenic factors (summarized in [16]). Particularly interestingly for this work, *B. megaterium* makes toxins similar to cereulide [15]. Cereulide is a heat resistant cyclic peptide toxin best known in *Bacillus cereus* [19]. It is very disturbing that something similar to cereulide is produced by some *B. megaterium* and that we have shown that this species survives rigorous cooking conditions like Tyndallization. There is also an interesting correlation in this: cereulide is emetic [19] and the symptoms of potato skin colic also relate to the stomach (unpublished observation from eating cooked potato skins).

Even though potatoes grown in the USA do not use solid human waste as a fertilizer, several fecal species were seen from the *Enterobacter* and *Enterococcus* genera. These may be destroyed easily by normal cooking, but many species previously implicated in producing heat stable mycotoxins and allergens have been seen by us previously [2] and also, this study shows that some suspicious bacteria that can survive rigorous cooking. It is easy to conclude that food safety may be improved if we do the sensible thing and discard the epidermal layer of tubers rather than expose ourselves to the danger of eating it. Obviously, even limited cooking kills almost all vegetative cells associated with food poisoning from fecal bacteria [20]. *Clostridium* was detected on potato peel at Tyndallization step 0 (Table 1) and this is relevant because they are endospore formers. However, they were not bacteria which grew during Tyndallization. I assume this was because they were poisoned by oxygen.

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