Using state-of-the-art DNA sequencing technology to reveal the bacteria present in Irish artisanal cheese

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Introduction

The production of cheese is believed to have commenced over 8000 years ago during the 'Agricultural Revolution'. Today, cheese is produced and traded throughout the world, with more than 1000 cheese varieties in existence, which can be classified in a number of different ways, such as according to texture (i.e., hard, semi-hard or soft), milk type (i.e., cow, goat or sheep's milk) or level of maturity (i.e., 'fresh'/'unripened' or 'ripened'). Special categories, such as 'blue-veined' and 'smeared' cheeses, also exist. Regardless of the variety of cheese, the production is reliant on the fermentation of milk by harmless microbes such as bacteria. These microbes may be added deliberately as starter cultures or be present naturally in the milk. The bacterial composition of each cheese product is unique, and these bacteria have a major influence on the taste, colour, odour and texture of a cheese. There have thus been considerable efforts made to characterise the microbial populations of cheeses by growing (culturing) these bacteria on agar plates, in an aerobic environment (where oxygen is present), in the laboratory.

Such culture-based approaches have highlighted the frequency with which lactic acid bacteria (L.A.B., i.e., bacteria which ferment lactose to lactic acid), such as *Lactococcus, Lactobacillus, Streptococcus, Enterococcus,* and *Leuconostoc*, are present in cheese. While other bacteria, such as *Propionibacterium*, *Staphylococcus*, *Corynebacterium*, *Brevibacterium*, as well as yeasts and moulds, frequently occur, there also exists a large spectrum of other microorganisms which occur less frequently (including disease-causing or 'pathogenic' bacteria) or are more difficult to detect. Significantly, it is now recognised that the aforementioned traditional, culture-based techniques, introduce biases as a consequences of the fact that some bacteria do not grow well (or at all!) in laboratory agars and broths. For these reasons, it is crucial to have tools that allow monitoring of bacterial populations without cultivation.

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Modern methods

The development of new DNA-based methods which allow scientist to study bacteria without culturing them in the laboratory, i.e., culture-independent approaches, has led to a revolution in microbiology. These techniques rely on the analysis of DNA extracted from bacteria present in a particular environment and, provided DNA can be extracted efficiently, an accurate picture of the bacteria present in any environment can be developed, regardless of whether the bacteria can be grown in a laboratory or not. Different bacteria can be distinguished on the basis of differences in the DNA sequence of a particular gene, called the 16s rRNA gene, which acts as a bacterial DNA fingerprint. Thus, following the extraction and DNA sequencing of the bacterial DNA from the food matrix, bioinformatic analysis (the application of statistics and computer science) can be employed to reveal all of the bacteria that are present. The availability of new and improved DNA sequencing technologies, known as Next-Generation (Next-Gen) Sequencing technologies, and, more specifically, access to the only Next-Gen sequencer of its type (454 Genome Sequencer FLX system) in Ireland, has facilitated the present detailed investigation of the bacterial composition of Irish artisanal cheeses.

Aim of research

The aim of my research was to apply, for the first time, state-of-the-art Next-Gen DNA sequencing technology to gain an in-depth insight into the microbial content of Irish artisanal cheese.

As mentioned above, this technology relies on culture-independent techniques. The first stage of this project was to develop a suitable DNA extraction protocol, followed by a sequencing of the 16S rRNA fingerprint of more than 100,000 bacteria. After developing a suitable DNA extraction protocol, a variety of handmade cheeses from artisanal cheese producers and farmers' markets throughout Ireland were obtained. In addition to analysing the bacterial composition of the cheeses, in many cases their associated rinds, which have been either naturally developed or smear-ripened, were also analysed.

DNA was extracted from62 cheeses and 11 cheese rind samples, 16s rRNA gene sequences were prepared for Next-Gen sequencing and bioinformatic analysis was then carried out to identify the bacteria present.

Outcome of study

The microbial composition of artisanal cheeses

Sixty two handmade cheeses manufactured from unpasteurised or pasteurised cows, goats or sheep's milk were obtained from artisanal cheese producers and farmers' markets throughout Ireland. This collection consisted of 18 soft cheeses, 31 semi-hard cheeses and 13 hard cheeses; 49 cheeses were produced from cows milk, 10 from goats milk and 3 from sheep's milk. Thirty four were produced from unpasteurised milk and 28 from pasteurised milk. Following Next-Gen sequencing, data analyses revealed that artisanal cheese have a diverse microflora, with the identification of 21 different bacterial genera (a classification level of bacteria). A difference in the composition of the microbes of different cheese types was noted, with the microbial diversity increasing from soft to semi-hard to hard cheese (Figure 1).

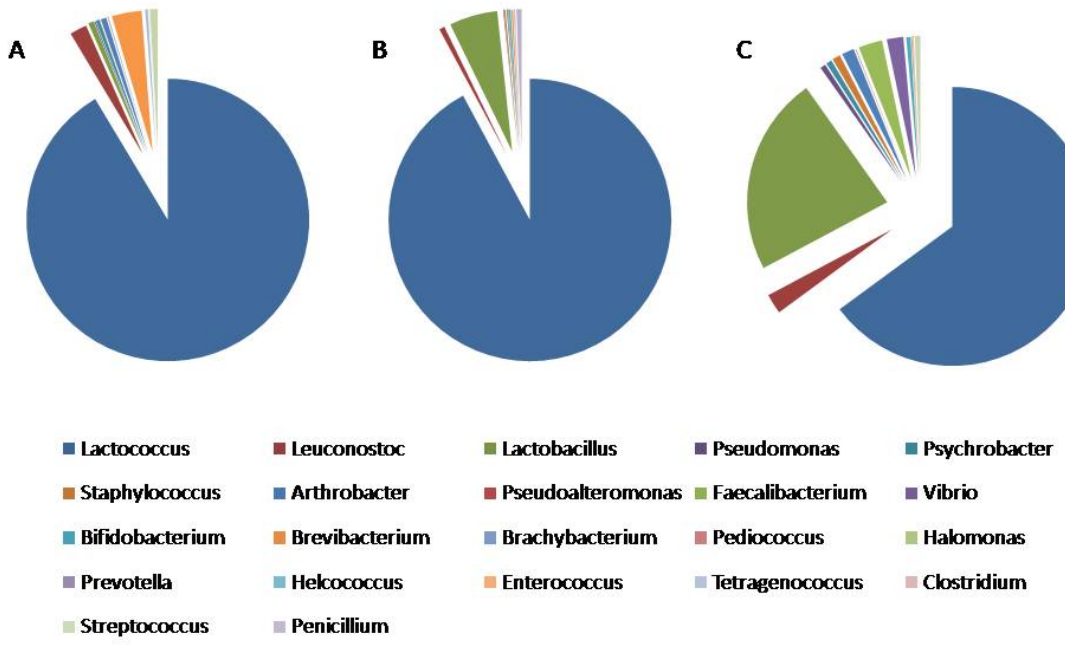

Figure 1: Microorganisms found in Irish artisanal cheeses using Next-Gen DNA sequencing technology. A = soft cheese; B = semi-hard cheese; C = hard cheese.

The dominant bacteria throughout was *Lactococcus,* a major milk microbe and a common cheese starter bacteria. When different cheese types (soft, semi-hard and hard) were compared, a total of eight genera were found in common. In addition to *Lactococcus*, these

were *Lactobacillus*, *Leuconostoc*, *Pseudomonas*, *Psychrobacter*, *Staphylococcus*, *Arthrobacter* and *Faecalibacterium*. *Vibrio* were found in soft cheese only, while *Helcococcus*, *Halomonas* and *Streptococcus* were found in the semi-hard cheeses only and *Enterococcus*, *Tetragenococcus* and *Clostridium* were found in hard cheeses only.

Three genera were shared between soft and semi-hard cheeses only. These were *Pseudoalteromonas*, *Pediococcus* and *Bifidobacterium*. Although *Pseudoalteromonas*, which is usually regarded as a marine bacterium, has been detected on the surface of smear-ripened cheese on one previous occasion, this is the first instance of its detection in cheese cores. *Brevibacterium* was the only genus shared between soft and hard cheeses, and *Prevotella* was the only genus common to semi-hard and hard cheeses.

Among these bacteria listed are three, i.e., *Prevotella*, *Faecalibacterium* and *Helcococcus*, that have never before been detected in cheese. These three types of bacteria are anaerobic, i.e., they do not grow or survive well in the presence of oxygen, which may explain why they had not previously been identified using culturing on plates under laboratory conditions. *Helcococcus* was detected in only one cheese, which was a semi-hard cheese made from unpasteurised, cows milk. This bacterium has been associated with clinical problems in humans but also in cows, sheep and horses and thus, in this instance, may reflect the sourcing of contaminated milk from an infected animal. *Prevotella* are associated with human infections, periodontal disease as well as with the rumen and hind gut of cattle and sheep. In this study, *Prevotella* was detected sporadically in semi-hard and hard cows and goats milk cheese at low levels, 0.02-0.38%.

Finally, despite not having been uncovered in cheese previously, we detected *Faecalibacterium* in soft, semi-hard and hard cheese samples made from unpasteurised and pasteurised cows milk. *Faecalibacterium* is a human gut bacterium with possible anti-inflammatory potential. Although detected at low levels (0.02-0.05%) it is notable that one of the main end-products of *Faecalibacterium* metabolism is a molecule called butyrate. While butyrate can contribute positively to cheese flavour development, in high levels this product can induce what is called the late-blowing defect in cheese, which results in undesirable cracks in the cheese, as well as abnormal aroma and flavour. The detection of these three anaerobes reveals that the microbiota of cheese is more diverse than previously appreciated, thus further highlighting the benefits of using Next-Gen sequencing to investigate these populations.

Interestingly, it was noted that cows milk cheese contained 21 different bacteria, whereas goats milk contained only 8 different bacteria. Of these eight, three, i.e., *Prevotella*, *Arthrobacter* and *Brevibacterium*, have not previously been detected in a goats milk cheese. Only two bacterial genera, *Lactococcus* and *Lactobacillus*, were detected in sheep's milk cheese.

It is also well known that pasteurisation of milk greatly influences the microbiota of resultant cheeses. However, previous studies have not employed Next-Gen sequencing tech-

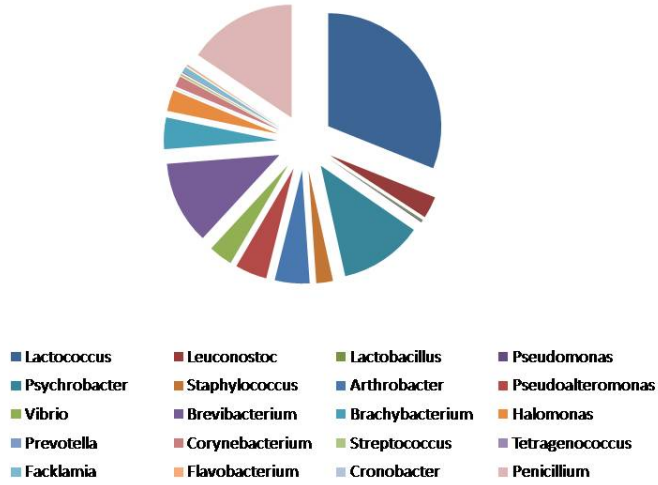

Figure 2: Microorganisms found in Irish artisanal cheeses rinds using Next-Gen DNA sequencing technology.

nologies to assess this influence in depth. Here, by comparing the bacterial genera present in artisanal cheeses manufactured from unpasteurised, relative to those made from pasteurised, milk, it is apparent that *Halomonas, Helcococcus, Streptococcus, Enterococcus* and *Tetragenococcus* were detected in raw milk cheeses only and that *Clostridium* and *Vibrio* were detected from pasteurised milk cheeses only.

The microbial composition of the rind of artisanal cheeses

As a consequence of its exposure to the external environment and, in some cases, steps taken during the manufacturing process, the bacteria present in the rind of a cheese will frequently differ dramatically from that of the rest of the cheese. We again used Next-Gen sequencing to analyse the microbiota of 11 of the artisanal cheeses rinds. These included smear/wash ripened rinds, naturally developed rinds, and one mould ripened rind. Analysis of sequence data revealed the presence of 19 different genera (Figure 2).

While some of these genera, including *Lactococcus, Leuconostoc and Lactobacillus*, were the same as those detected in the cheese core, a number were identified in cheese rinds only. These included *Corynebacterium*,*Facklamia*, *Flavobacterium* and *Cronobacter*.While *Lactococcus*remained the most common genus in cheese rinds, their proportions were significantly lower in the rind than in the core. Generally, smear/wash-ripened rinds had particularly low levels of lactococci (1.91-4.82%), while naturally developed rinds had levels of lactococci of up to 98%. It was also apparent that *Psychrobacter* and *Brevibacterium* represented a considerable proportion, ~10% on average, of the total population. Other

bacteria detected include*Leuconostoc, Lactobacillus, Pseudomonas, Psychrobacter, Pseudoalteromonas, Brachybacterium, Prevotella, Arthrobacter, Streptococcus* and *Tetragenococcus*.

While many of the genera listed above are common to cheese rinds, and indeed *Corynebacterium, Arthrobacter, Brevibacterium* and *Halomonas*, have previously been identified on the surface of Irish artisanal cheeses, we also detected two generain the rind that had not previously been detected, i.e.,*Prevotella* and *Facklamia*. The former has been referred to above and its detection in the cheese rind is surprising given its anaerobic nature. *Facklamia*, on the other hand, is an opportunistic human microbe and has also been detected in lactating cows with hematuria and urodynia and in raw cows milk (?, ?). To our knowledge, this is the first report of this genus in cheese. It is also interesting to note that *Vibrio* were only identified in rinds of the smear/washed variety and thus it may be that the washing process contributed to the presence of these salt-water-loving marine microbes.

Conclusion

We employed Next-Gen DNA sequencing to provide an in-depth understanding of the microbiota of Irish Farmhouse Cheeses. Here we highlighted the influence of this groundbreaking technology, identifying, for the first time, a number of bacteria previously undetected in cheese. These include the first recording of *Arthrobacter* and *Brachybacteria* in goats milk cheese. More significantly, a number of bacteria previously undetected in cheese were identified, including *Faecalibacterium, Prevotella, and Helcococcus* in cheese and *Facklamia* in cheese rind. The nature of the newly detected microbes suggests the presence of an underlying microbial population in cheese which has previously been overlooked. A thorough knowledge of the cheese microbiogy is very important for cheese manufacturers as many of these microbes impact on flavour and texture of the final cheese which in return impacts on the consumer opinion of a cheese. Therefore, to meet the cheese lovers' needs, it is important for the manufacturer to understand how and where the unique tastes and textures come from and how they can impart these into their cheeses. This work highlights the significant contribution that Next-Gen sequencing technology can make to our knowledge of food microbiology.

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