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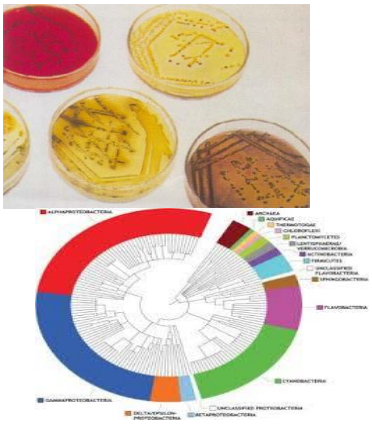
Systems microbiology applied to the reduction and control of bacterial transmission in the powdered infant formula production environment



Key external stakeholders:

Infant formula producers; research community

Practical implications for stakeholders:

The integration of flow cytometry and 16S sequencing data provide additional information over a single method on the different bacterial species in a processing facility.

Main results:

- In a food processing facility with different zoned areas (low, medium and high care, the greatest number of cells (regardless of their physiological state) was detected in the low care zone, followed by the medium and high care zones, but the number of viable cells per cm^2 in medium care was nearly three times greater than that detected in both low and high care zones.
- In the dry zone, plate counting under-estimated the number of viable cells, while in the medium care, a lower number of cells were detected by flow cytometry (FCM), compared to the plate count.
- Twenty out of 30 genera which were predominantly present in the low care zone were mainly associated with soil and the general environment, which included species belonging to *Pseudomonas*, *Spirosoma,* and *Sphingomonas* genera. On the other hand, those predominantly present in the wet care zone such as *Acinetobacter*, *Chryseobacterium,* and *Paucibacter* are mainly associated with water and sewage, aswell as soil and other general environment sources. In contrast, the greatest number of human and milk-associated genera such as *Streptococcus*, *Lactococcus*, *Corynebacterium*, *Lactobacillus,* and *Kocuria* were found in the high care zone.
- A closer look at the number of cells of the different species of the top three genera could be used as a good indicator of the possible transition of the cells between different zones
- No pathogens were detected

Opportunity / Benefit:

The results showed that the physical segregation of a production facility into different care zones has a positive impact on reducing the microbial load within the facility. However, better control measures such as stricter monitoring of staff and personal hygiene policies might be necessary to achieve a significant reduction in the human-associated microorganisms in high care.

Collaborating Institutions:

Teagasc, UCD.

Teagasc project team: Dr. Kieran Jordan (PI), Dr. Amir Anvarian

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1. Project background:

Currently, culture-based methods such as agar plates are the most commonly used technique for assessing the microbiological status of food processing environments. In using agar plates, it is only possible to determine the presence of bacteria that the investigator is looking for (using selective agars) and the method gives little information on the physiological status of the cells, except that they are alive if they grow. Some additional information can also be obtained by using different nutrient- content agars and assessing the number of stressed cells. With advances in microbiological techniques, there are a number of different methodologies that can be applied to samples to gain more information about the bacterial population of a processing environment and the physiological state of the bacteria present. Two of these methodologies are 16S rDNA sequencing and FCM, the former giving information about the type of bacteria present and the latter providing information on the physiological state of the bacteria. Combining the results of these two methods can give valuable information about the microbiological status of a food processing environment. Though most micro-organisms are cultured using traditional culture-based methods, under stress conditions, many microorganisms, some yet unidentified, are unable to grow on conventional growth media due to lack of effective culture techniques and/or induction of the so-called viable but non-culturable (VBNC) state. This has made normal culture techniques ineffective in describing the entire microbiome of complex environments. This problem could be overcome by using 16S rDNA sequencing, which is a culture-independent next generation sequencing method and has been successfully used for describing the composition of the microbiome in depth. The 16S rDNA gene encodes for 16S ribosomal RNA which is universally present in prokaryotic microorganisms. The variations within the 16S rDNA sequences facilitate identification of bacteria at the species level and, therefore, has made 16S rDNA gene sequencing an ideal tool for bacterial taxonomic studies. Compared to other molecular microbiology techniques, 16S rDNA sequencing is not only cheaper in price, but also the interpretation of the resulting data is easier and faster. With some online analysis platforms, such as Illumina Basespace or software such as QIIME and MOTHUR will not differentiate live from dead cells.

Flow cytometry is a powerful and rapid technique for simultaneous quantification and multi-parameter analysis of the microbial populations at single cell level. Cells are focused and aligned one behind the other in a narrow stream with a diameter close to the diameter of the cells so that single cells can be introduced to the light beam (generally laser).When cells are subjected to the light, they scatter light in all directions, although it is generally detected in two directions: forward scatter (FSC), along the axis of the light source; and side scatter (SSC), perpendicular to the light beam. Data from FSC and SSC scatters are generally used to characterize the morphological state of the cells, as rough indicators of the cell size and granularity, respectively. In addition, the light absorbed by the cells can result in emission of fluorescence (either due to presence of naturally fluorescent compounds or staining with various fluorophores), the intensity of which could be detected by FCM (Shapiro, 2003). Consequently, staining the cells with various fluorophores or fluorescence-conjugated antibodies can be used for understanding a wide range of physiological parameters of the cell (e.g., viability, metabolic and respiratory activities, internal pH, etc.) as well as detection of specific microorganisms at an analysis rate of up to 10,000 cells per second. Furthermore, comparing the viability results obtained with FCM with those of the plate counting can be used to determine the number of VBNC cells in a sample.

2. Questions addressed by the project:

- What protocols need to be established to use FCM and 16S rDNA sequencing to investigate the environmental microbiome in a food processing facility.
- Are agar plate counts representative of the microbiome of a food processing facility.
- Will FCM combined with 16S sequencing give a better understanding of the food processing facility microbiome, than a single method.

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3. The experimental studies:

The production facility consisted of three care zones of low, medium and high care. From each zone, twenty swab samples were collected, representing the critical production, storage and packaging sites within each production zone. Sponges pre-moistened with neutralizing buffer were used for environmental swabbing. Each hydrated sponge was used for swiping a single sampling zone of 50 × 50 cm. In total, the twenty samples taken represented 5 m^2 of that zone and were placed into five bags, each consisting of four sponges. To each bag, 40 mL of phosphate buffered saline (PBS) was added not earlier than 30 min post-sampling. The purpose of the delay was to prevent the dilution of the neutralizing buffer, allowing the effective neutralization of the possible chlorine and quaternary ammonium compound residues in the sample. The bags were sealed with the tabs provided, kept on ice, transported to the laboratory and processed within 24 h.

The sponges were transferred into a sterile beaker, representing three composite samples, one from each zone. Clarification of the suspension and separation of cells from debris were achieved by three-step centrifugation followed by a single-step filtration.

For FCM studies, the swabs were washed in 200 mL PBS. The cells were dispersed in fresh PBS and analyzed with a flow cytometer for membrane integrity, metabolic activity, respiratory activity and Gram characteristics of the microbiome using various fluorophores. The samples were also plated on agar plates to determine the number of culturable cells. For 16S rDNA sequencing studies, the cells were harvested by centrifugation only. Genomic DNA was extracted using a chloroform-based method and used for 16S rDNA sequencing studies, using the Illumina MiSeq platform.

4. Main results:

- The greatest number of cells (regardless of their physiological state) was detected in the low care zone, followed by the medium and high care zones ($p < 0.0001$). This was expected considering the implementation of stricter levels of hygiene and working practices in the latter two zones. However, the reduction in the total cell count, did not necessarily translate to a concurrent reduction in the number of viable cells as determined by FCM. By knowing the total cell count and the percentage of viable cells (based on the exclusion of PI viability dye), it was possible to calculate the density of viable cells per cm^2 of the sampling zone. The number of viable cells per cm^2 in medium care was nearly 3 times greater than that detected in both low and high care zones. This was probably due to greater level of humidity, hence greater access of microorganisms to available water in this zone. The lack of significance between the FCM viability results obtained for the two dry zones (low and high care) could be considered as further evidence of the primary role of humidity in improved viability of the cells in the wet medium care zone.
- In both dry zones, compared to the FCM, the plate counting significantly under-estimated the number of viable cells, while in the medium care, significantly lower number of cells were detected by FCM, compared to the plate counting. The under-estimation of viable cells by plate counting due to the presence of stressed and starved viable but non-culturable (VBNC) cells is well-documented and was, therefore, expected. On the other hand, the under-estimation of viable cells by FCM in medium care may be due to the presence of ultramicrobacteria and ultramicrocells (e.g., bacteria of the genera *Flavobacterium, Bacteroides*, and *Chryseobacterium*) with sizes smaller than the detection limit of the flow cytometer used in this study (< 0.5 µm). This could have resulted in the cells being considered as background noise. This could also mean that the number of FCM viable cells, hence VBNC cells in dry care zones of low and high could have been significantly higher than determined in this study. Moreover, the possible presence of very high concentration of surfactants, detergents and washing solutions in that zone, could have rendered some of the cells un-stainable, and hence not detectable based on the proposed protocol.
- The mean DNA content in low, medium and high care zones was 278, 168, and 53 pg/cm^2, respectively. Considering the vast difference between the DNA content of different bacterial species, it was not possible to establish a direct correlation between the DNA content of the sample and the cell count. Nonetheless, the greatest DNA content was found in the low care sample, followed by medium and high care zone samples, which closely resembled the results for cell counts obtained using from the FCM total count. In addition, the mean total RNA content for samples of low, medium and high care zones was 2, 29, and 1 pg/cm^2, respectively. By making the presumption that the presence of RNA in the cell is an indicator of protein synthesis, hence a degree of cellular vitality, the RNA content of the cells was in agreement with both the FCM viable count and plate counts.

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- Class A pathogens includes *Cronobacter* spp. and *Salmonella enterica* for which clear evidence of disease causality exists. No Class A microorganisms were detected in either of the three care zones. On the other hand, Class B (i.e., causality plausible, but not yet demonstrated) and Class C organisms (i.e., causality less plausible, or not yet demonstrated) were detected at both genus and species level in all three zones.

5. Opportunity/Benefit:

The results showed that the physical segregation of a production facility into different care zones has a positive impact on reducing the microbial load within the facility. However, the reduction in total cell count did not lead to a reduction in either the total viable count or the human associated pathogenic bacterial species. Therefore, better control measures such as stricter monitoring of staff and personal hygiene policies might be necessary to achieve a significant reduction in the human-associated microorganisms in high care.

- **Dissemination:**

Main publications:

- Anvarian AHP, Cao Y, Srikumar S, Fanning S, Jordan K. 2016. Flow cytometric and 16S sequencing methodologies for monitoring the physiological status of the microbiome in powdered infant formula production. *Frontiers in Microbiology*, Volume 7 Article 968.
- Cao Y, Fanning S, Proos S, Jordan K, Srikumar S. 2017. A review on the applications of next generation sequencing technologies as applied to food-related microbiome studies. *Frontiers in Microbiology*, Volume 8 Article 1829.

Popular publications:

- Anvarian AHP, Cao Y, Srikumar S, Fanning S, Jordan K. 2016. Development of a protocol for monitoring the physiological status of the microbiome in powdered infant formula production units using flow cytometric and metagenomic technique. *Proceedings of FoodMicro*, Dublin, July 2016.

6. Compiled by: Dr. Kieran Jordan

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