Meeting with Professor Mike Peck

Aim of this meeting/why we chose this approach:

Professor Mike Peck is a consultant microbiologist from the Quadram Institute. His research interests are primarily centred around *Clostridium botulinum*, and his contributions to *botulinum* research have resulted in social and economic impacts within the food industry.

Prof. Peck was visiting the University of Nottingham and giving a seminar titled "*Clostridium botulinum* and foodborne botulism". This was the perfect opportunity to gain professional advice on our project, so we created a PowerPoint presentation about it, followed by a Q&A session.

We felt that a Q&A was an ideal way to include Prof. Peck in our Human Practices work as he is knowledgeable and experienced with our target organism (and *Clostridia* in general). Since an academic expert on botulism was present in Nottingham, it would be a wasted opportunity if we didn't try to get as much information and feedback from him as possible, so we felt that a Q&A session would be the best way to go about this.

Challenge testing:

Prof. Peck spoke to us in detail about the process of challenge testing and the different groups of botulinum. There are 2 groups which cause foodborne botulism:

- Group I (proteolytic) is genetically the closest to *C. sporogenes.* It's mesophilic and primarily affects shelf-stable/canned foods. However, people know how to can foods safely and effectively nowadays, so no big innovations have been needed in can production/design.
- Group II (non-proteolytic). It's saccharolytic, grows at 3°C and is less heat-resistant than group I. This group is the main concern for the food industry as lots of chilled foods are produced now and can potentially harbour group 2 *botulinum* if incorrectly processed.

Although most work is done with group II botulinum, Prof. Peck said that using group I is adequate for proof of concept considering that the desirable endpoint of our project would be to have a cocktail of GM strains of *botulinum* anyway.

He also touched upon why retailers challenge test and how they go about it. They challenge test to improve the shelf life of their products and to determine what effect a reduction of preservatives has on it. It is usually carried out on ready meals which are vacuum packed (VP) and modified atmosphere packed (MAP). MAP products contain around 50% CO_2 and 50% N_2.

For a challenge test experiment, they would make an exponent with different dilutions and inoculate many products with spores of about 8 to 10 different strains. These products are then stored at the same temperature that is advised for it (8°C in the UK). It then undergoes a heat treatment and samples are extracted at different timepoints. The food is incubated

and tested for toxin production. This is done by putting the sample into a sterile plastic bag, adding a buffer then inserting the bag into a stomacher (a mixing device used in bacteriological sample preparation). The stomached samples are then centrifuged, and the *botulinum* toxins are tested for via an ELISA assay.

Other methods of testing:

Mike Peck also explained other methods which are also available or used in conjunction with challenge tests. Before the ELISA assay was used, mice bioassays were conducted in the UK. This involved injecting mice with food extracts from the inoculated samples and observing them for symptoms of botulism. Mice which show symptoms must be anaesthetised.

British food companies now dissociate themselves from mice testing and rely on the ELISA assays. However, in the US it is still common practice. Mike stated that thousands of mice are used in testing in the US. It takes many technicians to carry out the procedure and observe the mice. On top of this there are roughly 500 food samples to test, which takes weeks to extract the toxin from and another couple of weeks to carry out ELISA assays. The costs of doing this are also very significant.
He pointed out that when working with US companies, the Quadram Institute and any other British-based testing company must validate their ELISA assays using amounts of toxin which have been standardised by the mouse assay in the US – an ELISA test can detect 5 mouse lethal doses (MLD).

The FDA and CDC also use something known as the endopeptidase method. The food sample is added to a stomacher along with magnetic beads containing antibodies (Abs) for each toxin serotype. Each bead has Abs for all 9 serotypes on it. If toxin is present in the food sample, they will bind to the beads. A magnet is then put into the sample to extract the beads, which are then inserted into a microtitre plate. Botulinum toxins have endopeptidase activity – they cleave SNARE proteins at different places in neurons. This results in different sized peaks when analysed by mass spectrometry, and thereby determines which toxin serotype is present.

The endopeptidase method has similar costs to the ELISA method used in the UK. It is being slowly implemented as they try to move away from the mice assay as it is time-consuming, costly and requires a lot of manpower.

ELISA is useful in challenge testing as you know which strains/serotypes you used to inoculate the sample with, whereas the mouse assay is useful in botulism outbreaks as you can detect unknown strains/serotypes (Abs are raised against them in the mouse).

Project advice:

General pointers:

Prof. Peck also provided us with some pointers after we presented our project to him. He said that our project is the first step in improvement – avoiding extraction and reducing ELISA time would be ideal. Our method can do around 500 samples in a day, saving time and money due to an easier process of analysis. Mike also mentioned that we would need to validate our method and demonstrate that it is as sensitive as the mouse/ELISA test, and convince the US that our method is safe. The amount of toxin we need to detect would have to be in the picogram range to ensure it is equivalent to the current methods. In order to show that it's safe, we would need to run many challenge tests, plot growth curves and take viable cell counts.

Some foods form gas, so he suggested that perhaps a more complex reporter may be necessary instead of acetone e.g. something that's not found in food and is unique to our organism. However, for proof of concept acetone is fine. Our method would require some headspace in the packaging to measure acetone. He stated that the food testing industry dislike the use of needles for extracting samples from inoculated food, however for our acetone detection purposes it might be the way forward for us. In VP it is easy to increase the headspace, but it's harder to do so in MAP. We would also need a control set of food to observe natural/background acetone levels too.

Real food Media/broth to use:

As well as general pointers, he also suggested food we could use as media or broth in our experiments as real-world applications. Mike Peck mentioned that there was a group I *C. botulinum* outbreak in North America due to poor prevention methods in carrot juice in 2006. Botulinum proliferates in low acid conditions which have high water activity, and carrot juice was the perfect medium with these conditions. Nowadays, citric acids/fruits are added to lower the pH as an extra preventative measure.

For the purposes of our project and mutant strain, he suggested we should use carrot juice as it's easy to sample and was part of a case study. To prepare the juice we need to make sure the pH of it is above 6 and then sterilise it. Carrot juice can be used in exactly the same way as a microbiological broth medium.

When we do carrot juice experiments with our mutant strain, we need to ensure that there's no acetone present in the headspace of the tube/bottle in which we're testing.

Meat/chilled foods industry:

To provide us with some more clarity on what we'd learned from David Raine at Pro-Pak foods, Prof. Peck also touched upon current issues within the meat industry. For example, meat and chilled food companies are trying to reduce the heat treatment of their products in order to maintain freshness, nutritious value and increase shelf-life. Confirming what David Raine said too, they are under pressure to reduce the nitrite content of meat. This is because nitrites in meat can form nitrosamines when cooked at high temperatures. Nitrosamines are carcinogenic, and they are also being told to look at trying to reduce the

cooking temperature or lowering the pH of meat products to prevent nitrosamine formation too.

There has been a lot of work on chilled food products as the range and variety of products available is increasing, such as vegetarian substitutes for meat. Botulism prevention and control is becoming increasingly important for this sector.

Outbreaks which have occurred in the past:

Cases in the UK:

Prof. Peck also elaborated on a variety of outbreaks and why they occurred. The first botulism outbreak in the UK occurred in Scotland in 1922 due to the incorrect heat treatment of sandwiches. He stated that most UK outbreaks are small, but most of the ones which do occur are due to imported products which have been contaminated.

In Birmingham, 1978 canned salmon became contaminated with *C. botulinum* after processing. It had the correct heat treatment, but there was a puncture in the cans caused by the machinery. Because of this, as the cans were cooling, they were also drawing in the surrounding air. The raw salmon was then packaged into these cans and *botulinum* growth occurred.

Mike Peck pointed out that this was a highly unusual case as most outbreaks are not due to post-process contamination. A bigger problem in the UK is wound-borne botulism, and the biggest cause of it is from heroin drug abuse (caused by group I *botulinum*).

The worst food-borne botulism outbreak however, occurred in 1989 when a low sugar version of hazelnut yoghurt was produced. *C. botulinum* was able to proliferate in these yoghurt pots, and some exploded due to gas production by the organism. The ones which didn't explode were assumed to be fine and were sold as normal. This caused 27 cases of botulism and resulted in 1 death.

Cases in Europe and the US:

Prof. Peck recommended looking at outbreaks which occurred in other European countries as they have different eating habits than the UK. Cases/countries we should be looking at in particular are:

- Salting ham in homes/farms in France – 10 to 20 cases occur per year
- Vacuum packing fish in Germany
- 1998 canned mushrooms from Italy

We were also informed about infant botulism being the most common form of the illness in the US. Our adult gut flora is fully developed, so it can repel *botulinum* cells and spores. However, infants have an underdeveloped gut flora and are susceptible to botulism if they consume spores. People who are immunocompromised or receiving chemotherapy are also susceptible to botulism.

Q&A Session

We were told that the effects of nitrites in meat have little effect, but they do have anti-botulinum activity, known as the Perigo factor. Can you confirm this? (0:00)

The presence of nitrite does affect toxin formation as it has been observed in challenge tests with and without it. The theory is that when you heat nitrite, it releases oxygen free-radicals which destroy proteins (i.e. *botulinum* neurotoxins (BoNT)). However, there was a report done on bacon which showed that there wasn't enough nitrite activity to confirm what was going on.

How effective is retorting at killing *C. botulinum*? (1:00 min)

The process that's applied via retorting is called a "*bot* cook". It is said to reduce the number of spores of group I *C. botulinum* by a factor of 10^{12} (retorted at 121˚C for 3 mins). This doesn't kill all the spores, but it delivers a specified kill (specified reduction to the number of spores that's considered significant enough to prevent *botulinum* growth). *C. botulinum* outbreaks mainly occur when ineffective retorting takes place.

Are there any other methods aside from challenge testing used to test for BoNT? (2:20 mins)

Prof. Peck doesn't think there is for toxin testing. People use the endopeptidase/mass spectrometry method, ELISA or the mouse assay. There are also other endopeptidase methods used as well. However, the problem with this method is that other bacteria (including *C. sporogenes*) produce lots of proteases too. If these adhere to the magnetic beads, then they will produce false positives. The CDC is working hard to make sure the endopeptidase method doesn't bind non-specific proteases.

How much earlier is shelf life set before toxin production is observed in a challenge test? (3:35 mins)

A table of concentrations of toxin is produced, and a cut-off/limit would be applied. If there's more toxin than the limit it's positive, if there's less than the limit it's negative. So, the shelf-life is based around a detection limit and experience of using ELISA. The limit is about the equivalent of mouse lethal dose. Setting the shelf-life itself is a risk management decision made by food companies. They tend to set it a few days before the last negative result was observed.

Is the nitrite issue hurting the meat industry? (6:40 mins)

The meat industry is testing how much nitrite is necessary in their products. They are also looking at alternatives – sorbate, lysin and lactate. Increasing the shelf-life for the food industry in general is quite significant. Even a 2-day increase is important as it means that food can be on shelves for an extra weekend.

How do they extend shelf life? Do they change the ingredients? (8:55 mins)

They either add preservatives, increase the heat treatment or challenge test more precisely to see the maximum shelf life. The latter is the most common way.

Is there a set figure for the amount of toxin that needs to be detected, or is it as soon as toxin production begins? (11:00 mins)

ELISA has a detection limit is around 5 MLD50 (minimum mouse lethal doses). This is in the picogram range (roughly 10 pg), which is about the amount you can detect if you inject the inoculated food extract into a mouse. This is what people have accepted for decades, so we should use the same detection limit for our project too. We need to show that when we do a challenge test of wild-type *botulinum* in parallel with our mutant *botulinum,* if the wild-type is producing 5 MLD50, we can detect acetone in our one too. We also need to rationalise the concentration of acetone so that it's consistent with the concentration of BoNT. Lowering the detection limit isn't a concern as the food industry don't care about improving it (doesn't really help them).

Why do food companies want to reduce their heat treatment periods? (13:40 mins)

To increase the food quality – it will have better nutritional value and decrease energy costs.

What type of food industries approach you for challenge testing? (14:22 mins)

Usually bigger companies. A challenge test costs around £25k-100k.

What do smaller do instead of challenge testing? (15:38 mins)

They have to show due diligence and follow the FSA guidelines. The guidelines don't mention specific preservatives, but you must keep food below 3˚C. Almost all chilled foods have a shelf-life of less than 10 days at 10˚C. The guidelines also state that you must retort at 90˚C for 10 minutes, the pH should be below 5, a_w (water activity) of less than 0.97 and a salt concentration of more than 3.5% salt (the salt concentration of sea water). Alternatively, any other set of factors which work will suffice as well.

Why is *C. sporogenes* a better model for group I *botulinum*? (17:55 mins)

C. sporogenes is very closely related to group I. Group II is so different to group I that you can't really compare them. The average nucleotide identity between them was compared, and according to this they are separate species rather than strains.

Some *sporogenes* have been found to produce BoNT and some group I don't produce BoNT as they can lose the toxin plasmid. The advantage of working with *sporogenes* is that it's not called *botulinum*. Even if our mutant *botulinum* doesn't produce toxin, it's still classified as a *C. botulinum* so it still has to follow the same safety/security regulations i.e. a license will still be needed to work with it.

What is a good model for group II *botulinum*? (22:00 mins)

There isn't an equivalent model. We would have to make our project work with *sporogenes*, make group I mutants, then go straight to group II *botulinum* mutants. Group II degrades

carbohydrates, so it makes different end products e.g. acetobutyrate. Group I and *sporogenes* mainly degrade proteins (they are proteolytic) so their end products are the same as the end products of protein degradation.

What are the main differences between toxin types? (23:15 mins)

All *botulinum* toxins get inside nerve cells. These cells contain SNARE proteins which are involved in acetylcholine release across the synapse and nervous transmission. This causes muscle activation, but the presence of BoNT means that acetylcholine doesn't reach the muscles and causes them to become "floppy".

The main differences are where the toxins cleave the SNARE proteins. Type A, B, C, D, E, F, G, X, BoNT J (from *Enterococcus*). They bind to the nerve at a different place and cleave a different SNARE protein as well. 3 SNARE proteins, each cleave 1 of them, but in a different place. They essentially bind to protein at one place and cleave it in another.

All toxin types result in same symptoms. Some toxins last in nerve cells longer than others, such as type A which can last a few years – it continues to degrade the SNARE proteins.

What causes toxin production? (25:30 mins)

Prof. Peck was not entirely sure but suggested our project might be able to find it out. "Whatever activates *botR* in *botulinum*, will it activate botR in your system?" Some group II strains don't have *botR*. Toxin production is also tightly regulated but he's not sure how it's regulated this tightly. *botR* is only expressed for a couple of hours as well as it requires a lot of energy to make the toxin

A similar regulator to *botR* exists in *C. perfringens*. It was knocked out to observe its function but the knock-out was lethal, so it hasn't been determined whether or not it regulates toxin production. All we know is that *botR* is needed in its genome, and that it's lethal when knocked out (in *C. perfringens*).

There is something which *botR* responds to/turns it on, but it's not known. *botR* turns on other genes in the operon including itself.

Toxin production is in the late exponential phase/early stationary phase. Data from growth curves shows that at $5x10^7$ CFU per ml there's less than a mouse lethal dose per ml. By $5x10^8$ CFU per ml (10-fold increase), in stationary phase the amount of toxin produced is 1 million-fold.

Cell density is also a factor in toxin production, similar to quorum sensing.

Sometimes the toxin is released through cell lysis, sometimes it's released through other ways. Maybe through the flagella (which are hollow) or through hollins which are small flagella units. Toxin is not actively transported out of the cell; it just diffuses out through natural holes.

What use is the toxin to the bacteria? (29:22 mins)

The toxin has a poor use to the bacteria. Perhaps it creates an environment in which it can grow. If we consume *botulinum* spores it can't grow in us, but if we're dead it can. Therefore, if *botulinum* manages to kill us it can grow in us. However, it's not an avert pathogen – *C. difficile* is. *C. botulinum* would prefer soil, and its purpose isn't to cause botulism in us. Also, when *C. botulinum* is grown in the lab it can lose the toxin genes easily.

Avian botulism has big outbreaks in wild bird populations every year in the UK. The water can become very anaerobic in the late summer. *C. botulinum* grows in the sediment and becomes concentrated in clams and mussels, which the birds end up eating. They eventually die and decompose. This causes maggot growth which have toxin/spores in them too. Other birds come to eat these maggots, become intoxicated and die.

Avian botulism can also affect chickens, but that's mainly because of the way we keep them. If we keep them in a different way it may be preventable.

Are there preventative measures in place to prevent avian botulism? (32:10 mins)

In wild birds no, in chickens yes. It must be ensured that there's nothing in the feed. There is a problem in Northern Ireland where chickens are kept in big sheds. The chicks which die and decompose have botulinum spores. This gets spread on land e.g. cow pasture, and the cows inadvertently eat this and contract botulism. It isn't really a problem in the rest of the UK however, as cows and chicken sheds aren't kept in close proximity.

Are there a lot of botulism cases in general? (34:20 mins)

It's very rare compared to *campylobacter/salmonella,* but that's because people need to be very vigilant about *botulinum* – a large outbreak would have disastrous consequences in the UK.

The Quadram Institute has an advisory committee on the microbiological safety of food which advise the FSA. They decided to set up a new committee to look at *botulinum* again, which demonstrates the extreme vigilance that's currently in place.

Companies are also wanting to change things all the time, and unexpected things happen too. For example, loads of chilled canned crabmeat from Thailand appeared at the docks in Southampton. When it arrived in UK, food inspectors thought it was meant to be a shelf-stable product (it wasn't as it was meant to be a chilled product). They tested it and found there was lots of bacteria in it, so it got quarantined. They couldn't decide whether to allow it to be sold – the challenge was to decide whether a tin canned product should be allowed to be sold with an 18-month shelf life. In the end, a 10-day shelf life was put on it.

What happens now in the UK? E.g. companies like Unilever have shut down their *botulinum* lab, so what do they do now for risk analysis? (37:40 mins)

There is free software created by the Quadram Institute called Combase. You can put in the conditions of your product e.g. pH, temp, salt etc. and choose the bacterium of interest. The software will predict/model a growth curve for you. Food companies can use this software early on to initially inform them about the safety of their product, they wouldn't rely solely on it though.

They would also conduct a risk assessment and look at the FSA guidelines. Big companies will do 3rd party challenge tests.

Aside from the Quadram Institute there are also 2 other food research associations – Campden BRI and Leatherhead, which are funded exclusively by the food industry. They are capable of doing anything and everything that the food industry wants as they're quite broad in the services they provide, but not experts in any particular field. They also do work on *botulinum* too.

The cost of setting up a lab is expensive, especially a *botulinum* lab. The Quadram Institute tends to do more work for companies outside the UK.

How is *C. sporogenes* used as a surrogate for group I *botulinum*? (41:50 mins)

People are interested in high pressure and low heat treatment to ensure the safety of a food product. Companies will initially run tests with *sporogenes* as their spores are more heat resistant than group I *botulinum*. However, they're not necessarily a good model for everything e.g. making a shelf-stable ham product. It's all about the heat ability to kill the spores. The view now is that some *sporogenes* strains produce toxin, and some *botulinum* strains don't.

What strain of *sporogenes* should we use? (43:15 mins)

The strain recommended by FDA for heat-resistance testing is *C. sporogenes* PA3679. A group at the FDA in Chicago have about 6 strains. 3 of the strains were heat-resistant, and the other 3 were not, which resulted in them being identified as 2 separate classes – the work about these strains has been published.

Any other food to grow *sporogenes* on aside from carrot juice e.g. lasagne and mashed potato from Pro-Pak; can it be used in any way? (45:42 mins)

Lasagne and mash potatoes aren't very suitable for *sporogenes*. Carrot juice is perfect as it's runny and you can take a sample and plate it easily. We need to check that the pH hasn't been lowered beforehand though. The quantity of toxin found in the carrot juice was the highest ever recorded. Prof. Peck wasn't sure why so much toxin is produced in carrot juice medium.

If we use lasagne or mashed potatoes, it's not so easy to inoculate/sample. However, it's good for challenge testing.

If we have carrot juice in a tube with a headspace it's really good to work with. It's controllable as it's easy to sterilise so no other bacteria will be present in it. In lasagne there will be other bacteria, and if it's chilled *sporogenes* won't grow in it either. At room temperature it will be full of *Bacilli*. Heat treatment of 90˚C for 10 mins (which we found out from Pro-Pak) won't kill any *sporogenes* spores present in it, or more importantly it won't have any effect on *Bacillus* spores as well. It will start to swell at room temperature and make counting *sporogenes* quite difficult too.

We could macerate the lasagne/mash first in a food processor (homogenises it too), then add it to a tube, autoclave it then produce a growth curve. It will be easier to control as it'll be sterile as well. It may be worth a go to filter-sterilise carrot juice – we'll need to get a carrot juice with no bits in it, so it doesn't block the filter.

We could also buy canned food and try growing *sporogenes* in that to mimic post-process contamination. Something runny once again would be good, and we would need to homogenise it before we sterilise it to reduce sampling issues e.g. shake it before extraction.

Additionally, we could irradiate our lasagne before putting it into the food processor. There's a place in Swindon used by the Quadram Institute which irradiates food products. The only problem would be getting the lasagne there in a chilled condition.

Reasons for the questions we chose:

We learned about the Perigo factor in the nitrite issue from Dr Jones at Campden BRI, and we asked Professor Peck to confirm the effects of it as we thought that as an expert in botulism, he may have more information on the topic. Building on this, we then asked how the nitrite issue is affecting the meat industry and what they're doing about it.

After learning about challenge testing, we were also curious to see what other methods are also available to test for *botulinum* toxins - this information would be used to evaluate which test our device/project is most suitable for.

Another question that we felt was important to ask was whether there is a concentration threshold that needs to be met when conducting challenge tests/other assays for the toxin. This would allow us to ensure that our electronic nose device is sensitive enough to meet the current standards/thresholds for toxin detection.

We also asked what kinds of food companies approach the Quadram Institute for challenge testing, as it's important for us to understand who our market audience is. After asking this, we learned that it was mainly large companies who could afford to challenge test, so we then asked what smaller companies do instead. Engaging with smaller companies is something we aim to achieve with our project, as we believe it has the potential to make challenge testing cheaper and more accessible for them.

We then asked a series of questions relating to *C. sporogenes* and the causes of toxin production. Finding out the conditions in which toxin production occurs will allow us to mimic it in the lab so that our experiments and models are as accurate as possible. We also asked whether there were any other foods we could use as media to grow *C. sporogenes* on in order to see how we can make use of the food samples given to us from Pro-Pak Foods.

In addition to our questions that we had prepared earlier for Professor Peck, a lot of follow-up questions were also asked in relation to some of the answers that he had given. For example, we had questions regarding shelf life, the effectiveness of retorting, avian botulism and botulism in general. These questions were mainly asked for our own understanding and because of our inquisitive nature!

What we've learned from this meeting and Q&A:

- The mouse assay isn't really used in the UK as companies don't want to be associated with animal testing – it's still widely used in the US however
- Our project is the first step in improvement. It's a good proof of concept in improving the methods of challenge testing:
    - Has the potential to reduce ELISA time and costs
    - Avoiding extraction would be ideal too
- Americans are slowly moving away from the mouse assay and are starting to use magnetic beads with toxin Abs
- Although the endpoint of our project will produce safe strains of *C. botulinum*, they will still be subjected to the same rules and regulations as wild-type *botulinum* i.e. labs must still have *botulinum* licenses to work with our organisms
- Our method MUST be as sensitive and reliable as current methods for it to be viable
- We need to produce an acetone detection curve as well as a growth curve
- In order to measure presence of acetone in an industrial setting, we need to convince manufacturers to allow headspace in VP/MAP foods.
- Carrot juice broth is a good food media to use
- The most common type of botulism in the UK is wound botulism
- The most common in the US is infant botulism
- European countries tend to have more outbreaks of foodborne botulism than the UK as they have different eating habits
- The *C. sporogenes* PA3679 strain is recommended by the FDA to test heat-resistance

How we can integrate our findings:

- We could analyse the data of the FDA recommended *sporogenes* strain against the current strain we're using and see how they compare
- It may be easier for our electronic nose device to have a syringe/needle - this will make it easier to use within a challenge test.
- We could contact European food companies and see how they tackle botulism due to fact that they have more outbreaks than the UK
- We could grow *C. sporogenes* in canned foods to mimic post-process contamination
- We can also use carrot juice broth as a media to model our project in a "real food" environment as it is ideal for our purposes.
    - We could compare the shop bought one (containing fruit acids) against homemade carrot juice (without acid) and test the growth