Meat and meat products spoilage in the genomic era: analysis with metagenomics and culture-dependent methods to meet the suspects

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Corbion

Corbion is a producer of biobased ingredients. Corbion produces and sells amongst others organic acids like lactic acid and lactate salts to the food business. It is used to enhance taste, regulate acidity but for an important part also to enhance safety and increase shelf life of the products of our customers. A variety of products that are present in the supermarket contain our ingredients. Especially manufacturers of cooked meat like hotdogs and hams use our products to slow down spoilage and to increase protection against growth of the pathogen *Listeria monocytogenes*.

The importance of identification of spoilage organisms

At Corbion, we frequently determine the spoilage flora of different products from our customers, mostly meat products, to identify the main species that spoil a particular product group. We then determine the sensitivity of the main spoilers against several antimicrobials like lactic acid, acetic acid and others. With this information we can advise the best product and the usage level to our customers. We have a large database with the dose response curves of various spoilage organisms to various antimicrobials. We frequently update this database with new strains and new antimicrobials. The dose response data are derived from medium throughput growth experiments using bioscreen machines. The bioscreen can measure simultaneously 200 growth experiments in a single run.
We also subject the most important spoilers to different pH, temperature and water activity levels and together with the dose response curves we build predictive growth models according to the gamma concept. The gamma concept assumes that all different parameters (pH, temperature, etc) do not interact with each other and have each a separate effect on the growth rate [1]. Hence, the effects can be multiplied, leading to relatively simple and transparent mechanistic growth models.
Predictive models need a lot of data and effort and therefore, we must be sure that we do this only for the most important spoilers. Therefore, identification of the spoilage organisms plays an important role in Food Microbiology at Corbion.

Metagenomics vs classical isolation (figure 1)

Food is considered spoiled when the flora reaches a count of log 7 [2].

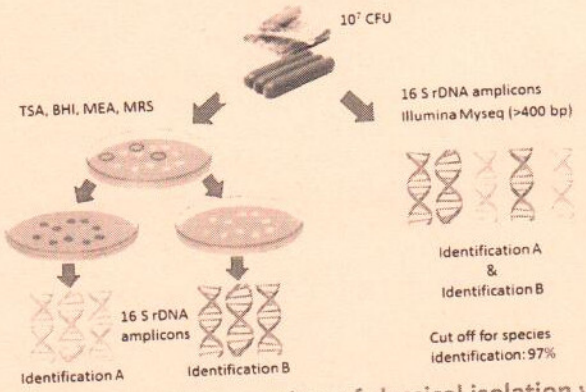

Figure 1: schematic overview of classical isolation vs. metagenomics

Traditionally, spoilage research is done by diluting a sample of spoiled food and isolating the organisms from the plates with the highest dilution that shows colonies. Thus, every colony on the plate is an important spoiler. However, spoiled food always contains several dominant species and to get a good

overview of the flora, many colonies need to be obtained in pure culture and subsequently identified (figure 1).
There is a bias introduced by the type of agar media that is used, so it is good practice to plate food samples on different media. Some organisms have trouble to grow on agar plates. Another bias is introduced when different colonies are picked for further isolation. Very small colonies can be as important as big colonies and one must carefully pick several different colony shapes, colors and sizes for further isolation and identification. At Corbion, identification of pure cultures is outsourced and a specialized company determines the identity based on a part of the 16 S rDNA sequence, and the homology to other sequences in the SILVA rRNA database. To isolate and identify several colonies is laborious and costly and the amount of bacteria isolated from a food sample is limited by the costs whereas a statistically sound method would require more isolates than we currently obtain per sample. However, due to the vast amount of spoiled vacuum packaged cooked meat samples analyzed at Corbion, we could derive a clear top 5 of spoiling bacteria (table 1 and next section).
It is no surprise that determining the spoilage flora with culture independent methods became more and more popular. Especially the development of metagenomics has clear advantages over other culture independent methods like DGGE. At Corbion we make use of 16 S rDNA amplicon sequencing methods of companies like Baseclear and Quality Partner. There are several other companies offering this service in Europe. In other regions of the world, this service is more difficult to find. In our case, the V1-V3 region of the 16S rDNA of a food sample is amplified and the amplicons are sequenced with the Illumina Myseq. This yields between 3000 and a million of reads depending on the depth of sequencing (and price!). One can imagine, due to the relative high amount of sequences, that the use of metagenomics thus gives a much better overview of the flora when compared to classical isolation and identification where cost limitation do not allow more than ~10 organisms isolated per sample. Metagenomics makes statistical and quantitative analysis of multiple samples also a lot easier [7].

Table 1: frequency of the most dominant spoilers in > 50 analysed spoiled VP cooked meat samples

	Absolute count	Share
Lactobacillus sakei	48	23%
Leuconostoc mesenteroides	35	17%
Leuconostoc carnosum	24	11%
Lactobacillus plantarum	23	11%
Lactobacillus curvatus	22	10%
Total share		**72%**

Studying the spoilage of cooked vacuum packaged (VP) meat.

Although cooked meat products like frankfurters and ham are subjected to a heating step of around 72 °C, it is not the spore forming bacteria that frequently spoil these meat products. It is well established that Lactic Acid Bacteria (LAB) are important spoilers of cooked meat and organoleptic defects and pH drop correlate strongly with the numbers of lactic acid bacteria [2, 3]. Beside color, taste or odour defects, growth of LAB can result in gas formation and slime production [4]. A level of 10^7 LAB per gram in cooked meat can be regarded as spoiled [2]. LAB like *Lactobacillus sakei* and *Leuconostoc mesentroides*, but also *Weisella* and *Carnobacterium* species have been found in spoiled cooked meat [5, 6]. However, studies up to now don't give a clear picture of the most dominant species and if there is variation in dominant species between regions worldwide.
From 2007-2014, more than 200 organisms were isolated from more than 50 cooked meat products that were past the sell by date. The samples were from 11 different countries, from all the regions in the world (NA, SA, EMEA, Asia). The strains were isolated from plates with the highest dilution of the sample. The identity of the strains was determined by a 500 bp 16 S RNA sequence analysis.

The most frequently found organism was *Lactobacillus sakei*, representing more than 20% of all isolates (Table 1). Also *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *Leuconostoc carnosum* were frequently found (11-17% each). *Lactobacillus curvatus* was the last in the top 5, at 10%.
The top 5 species accounted for 70% of all isolated organisms and the top 5 dominated the cooked meat spoilage flora in all regions (Figure 2).
Other species were isolated. None of them exceeded 4% abundance. Only 3 out of more than 50 cooked meat products did not contain any of the top 5 species (but a close related *Lactobacillus paraplantarum* was present in these cases)

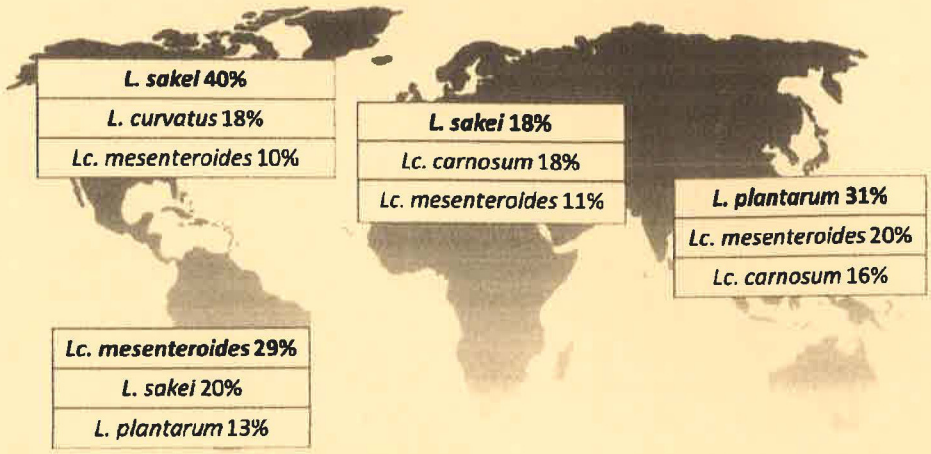

Figure 2 most dominant species in VP cooked meat per region in the world

One can imagine that isolation and identification of over 200 strains is costly, especially when taking the costs of working hours into account. Therefore, metagenomics were started to be used at Corbion from 2013 for spoilage analysis. During the first experiments a method was used, yielding over 1 million reads per sample after amplification of the V3-V5 region of the 16 S rDNA gene. Some of the samples were also analyzed by classical isolation of organisms, to verify the results from metagenomics. Among the samples there was a t=0 sample of an Asian vacuum packed hotdog (< 100 CFU/g), a spoiled sample of the same batch stored for 11 days at 10 °C (Asian supermarket conditions) as well as an spoiled sample that was intermittently stored at 30 °C and -18 °C (Asian wet market conditions). The analysis clearly showed that the initial microbial diversity was much higher at t=0 compared to the final spoiled sample (figure 3a and 3 b).

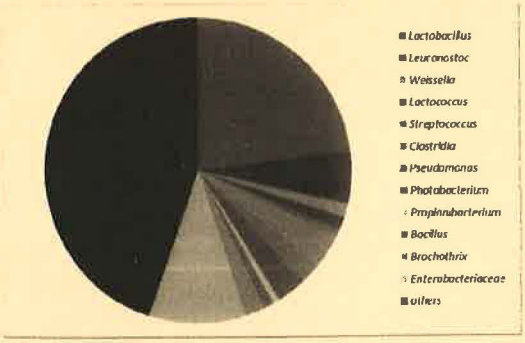

Figure 3a: Breakdown of the population to the genus level present in an unspoiled Asian hotdog

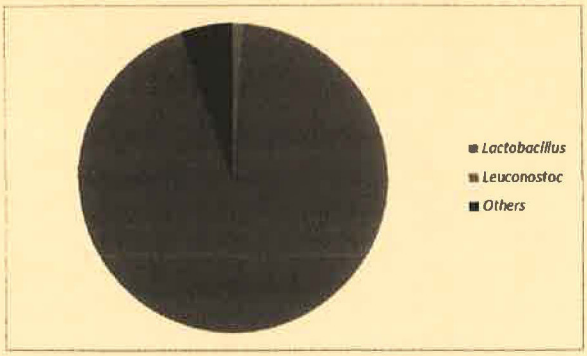

Figure 3b: Breakdown of the population to the genus level present in a spoiled hotdog from the same batch after incubation at 10 °C for 11 days.

Hotdogs incubated at constant temperature level (10°C) showed less diversity compared to hotdogs incubated at varying temperatures (daily shifts from -18 to +30°C). The metagenomics results coincided well with the isolations (Figure 4).

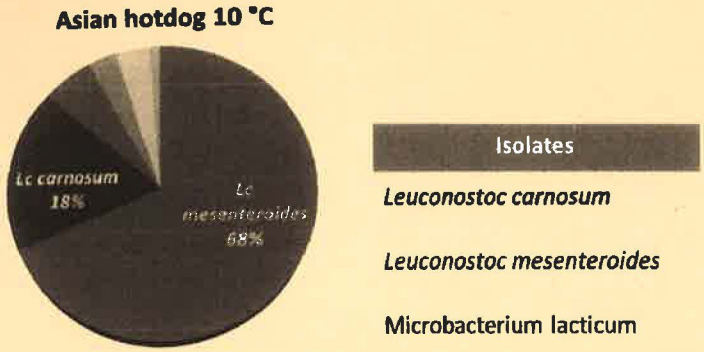

Figure 4: Breakdown to the species level of a spoiled Asian hotdog incubated at constant temperature, and the isolates obtained from the same batch.

Also other samples, like Dutch hotdogs, showed a spoilage flora that fits well with the top 5 dominant spoilers (Figure 5). Samples analyzed in duplicate showed nearly the same results.

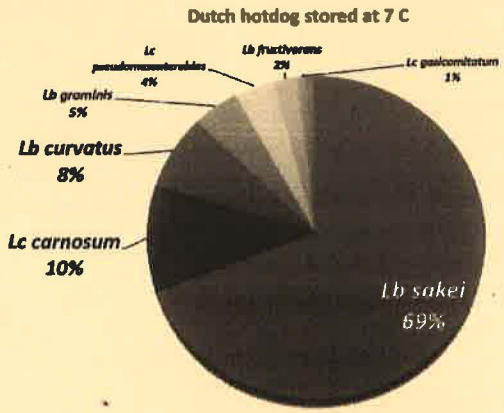

Figure 5: Breakdown to the species level of spoiled Dutch hotdogs, incubated at 7°C

Subsequent metagenomics analysis of other VP cooked meat samples affirmed the dominance of the top 5 spoilers.

Conclusions from spoilage analysis of VP cooked meat

A clear top 5 of cooked meat associated species could be determined, and consisted of *Leuconostoc* and *Lactobacilli* and this top 5 dominates products from all over the world. Metagenomics of (cooked) meat products was successful, reproducible and the results fit well with the top 5 dominant species obtained by isolations. Metagenomics greatly reduce costs related to spoilage research.

The spoilage of fresh meat.

Fresh meat is generally subject to fast spoilage. We have applied metagenomics to several spoiled samples of vacuum packaged fresh meat obtained from the supermarket and two separate batches of minced beef (total of 8 samples and both batches vacuum, air and MAP packaged). The V1-V3 region of the bacterial 16S rDNA gene was amplified, and high throughput sequencing was conducted with the Illumina Myseq technology. The method applied yielded 2000-3000 reads per sample were obtained. Percentages were calculated by dividing the amount of reads belonging to a operational taxonomic unit (OTU) by the total amount of reads of a sample.
Three vacuum packaged fresh rump (round) steak pieces slaughtered in different countries (Ireland, UK, Uruquay) and a piece of lamb steak, were invariably dominated by only *Lactococcus piscium* (56.8% ± 9.6)

and *Leuconostoc inhae* (15.9 ± 6.8), see figure 6. Both species were not found in spoiled cooked meat in previous research. Both species contributed to 70% or more of the total flora in each sample (figure 6).

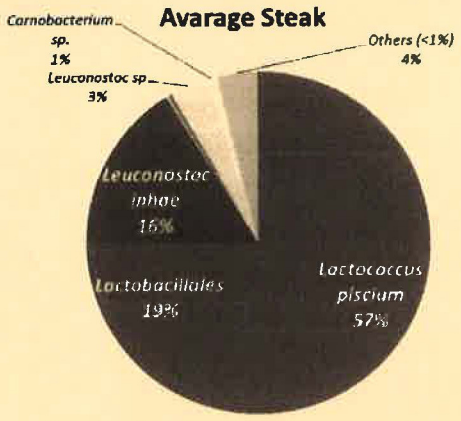

Figure 6: breakdown to the species level of average values from 4 different steaks

In the case of spoiled minced beef, the type of packaging seemed of some but limited importance (figure 7). Analysis was done on 8 different samples from 2 different batches. During the processing of the two batches, the meat was packaged in vacuum, air or MAP (80% N_2/20% CO_2 and 65% N_2/35% CO_2). The lack of real differences in the microbiome between the types of packaging may be because the minced meat samples showed high bacterial counts from the start.
Apart from four different species of LAB present above 10% in different samples (*Leuconostoc gelidum* and the closely related *Leuconostoc gasicomitatum*, *Lactobacillus algidus* and *Lactococcus piscium*), 6 out of 8 samples of minced beef contained at least 20% of Photobacteria. In 3 out of 8 samples, more than 70% of the reads belonged to Photobacteria, making Photobacteria an unexpected important spoiler of minced beef.

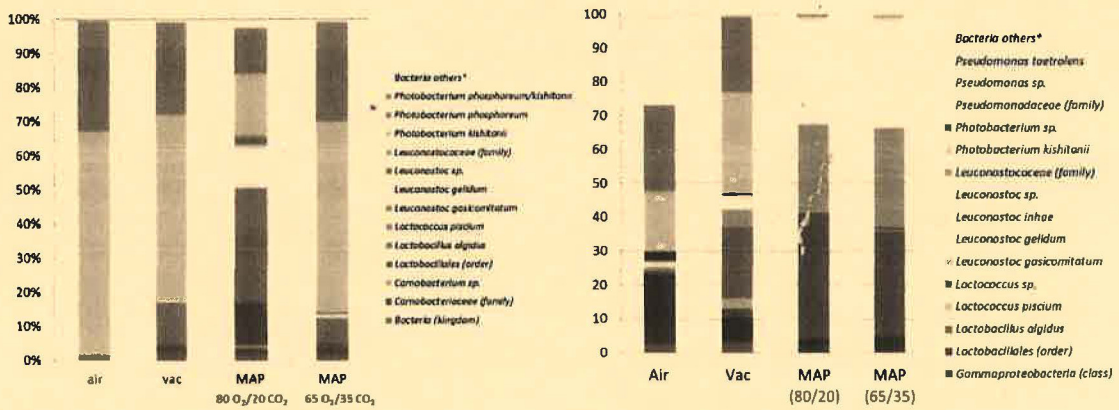

Figure 7: breakdown of the flora of spoiled minced beef in 4 different packages in two separate batches

The observation that *Photobacterium* species were present in large amounts was a surprise to us at the time that the experiments were done (late 2015). However, in 2011 already, *Photobacterium* was mentioned as an fresh meat spoiler when using PCR-DGGE to elucidate the spoilage flora of chilled beef [8]. Very recently, more and more papers show the presence of *Photobacterium* in fresh meat products and all the papers have in common that culture independent methods are used [7, 8, 9, 10, 11].

Conclusions from fresh meat spoilage research:

The lactic acid bacteria found in spoiled fresh meat are different from the flora of cooked meat spoilage and *Photobacterium* species are important spoilers as well.

Overall conclusion from meat spoilage research using different methods

Although classical isolation and identification gives a good overview of the spoilage flora, the costs and effort is considerable. Classical isolation is, naturally, the only way to obtain pure cultures for further characterization like obtaining dose response curves and data regarding growth at different pH, temperature and water activity values, and these are needed to build predictive growth models. Predictive modelling can only be automated to a certain level and will always need manual curation. Therefore the amount of different strains that can be used for modelling is limited, mainly due to the amount of hours that need to be spent on the data collection, processing and curation. There is no need for e.g. 200 different strains.

Metagenomics on the other hand allow for cheap and thorough analysis of the spoilage flora. The technique is independent of culturing and hence, does not have the biases that arise from culturing and picking. It is also clear that with metagenomics, species can be detected that were previously not found with culture dependent techniques. As a matter of fact, metagenomics has recently turned our view on fresh meat spoilage upside down by finding *Photobacterium* as a major spoiler.

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