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Using State-of-the-Art Analysis to Explain Atypical Cheese Defects

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Declassified Emmental aged 1.5 months with irregular eyes

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The quality of fermented foods like cheese is largely determined by the composition of the microbiome. This article draws on a case study to demonstrate the various analytical techniques used by Agroscope to investigate microbial defects in cheese.

Introduction

The Agroscope Cheese Microbiome research project aims to further our understanding of how the cheese microbiome influences product quality by studying both its desirable and undesirable effects. The project focuses on raw-milk cheese as its microbiome is expected to have higher biodiversity. During the project, new analytical methods will also be developed to help the Agroscope Cheese Consultancy shed light on complex case studies. In this context, one particular goal is to develop increasingly powerful and affordable molecular-biological methods which will provide deeper insights into the cheese microbiome.

The title image shows a declassified Emmental with irregular eye formation. Serious defects like this are unusual, even for experienced cheese consultants. Due to the very irregular formation of the eyes, the defects were assumed to be of microbial origin and caused by gas-producing spoilage microorganisms. Since commercial enterprises and regional advisory platforms have a limited range of analytical techniques at their disposal, Agroscope was called on to investigate the causes.

Investigating case studies like this also provides Agroscope with opportunities to share knowledge with practitioners in the form of educational and training events.

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Cheese varieties produced in Switzerland are highly valued by consumers due to their excellent and consistent quality. The Swiss cheese industry relies on independent quality assessments (known as 'taxations') to ensure that only cheese of impeccable quality is placed on the market. Defective cheeses are declassified, which causes significant economic losses for the cheese dairies concerned, especially if their entire production is affected for a prolonged period. In such situations, the sooner the causes can be identified, the sooner corrective measures can be taken to resolve the problem. A great deal of knowledge and experience is needed to shed light on rare cheese defects. Agroscope employs four cheese consultants who work closely with regional advisory platforms to provide support and guidance to cheese dairies throughout Switzerland. Thanks to the consultants' involvement in cheese research and culture development at Agroscope, cheese makers have access to a broad range of expertise to investigate complex cheese defects.

Analytical approaches to investigating microbiological cheese defects

Major advances have been made in the field of cheese analysis in recent years. Figure 1 gives an overview of the different methods used at Agroscope to investigate cheese defects. New molecular biological methods can now provide diagnostic clues to explain the microbiological and molecular causes of atypical cheese defects. For this reason, the cheese consultants at Agroscope are increasingly using molecular-biological methods alongside conventional microbiological tools. Limited opportunities to spot defects as they develop during cheese ripening using microbiological methods has led to the development of several chromatographic, enzymatic and biochemical analysis methods in recent decades which use selected metabolites to track fermentation behaviour as the cheese ages. The solutions currently available to explain atypical microbial cheese defects are outlined below.

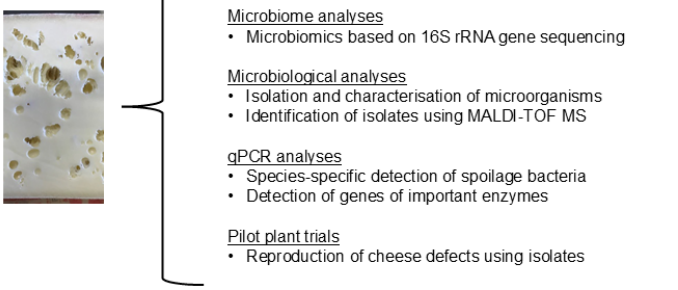

Figure 1: Solutions for investigating the cause of microbial cheese defects.

Metabolite analysis

Metabolite analysis is still the first step in investigating defective cheese samples. In most cases it can provide vital clues to explain the causes of quality defects. Volatile carboxylic acids and biogenic amines are among the most important metabolites in cheese. Analysis of the volatile carboxylic acid levels in cheese samples provides insights into the fermentation behaviour of the ripening cheese and permits the detection of undesirable types of fermentation (e.g. butyric acid fermentation) – many of which are also characterised by excess gas production.

The production of biogenic amines is also a common cause of undesirable gas formation in cheeses. In cheese, biogenic amines result from the breakdown of amino acids in a reaction that also releases CO_2. For this reason, the formation of these metabolites must always be considered in relation to the formation of cracks or defective eye formations. The formation of biogenic amines leads to a rapid rise in pH as the cheese ripens, accelerating the aging process and reducing the shelf-life of the cheese. Histamine and tyramine are among the most undesirable biogenic amines produced in cheese. Tyramine is problematic for people who are being treated with monoamine oxidase inhibitors (MAOs), as these drugs also inhibit the degradation of tyramine. Typical symptoms of tyramine poisoning are increased blood pressure combined with headaches, dizziness, blurred vision and nausea. Histamine produces an unpleasant burning sensation in the mouth. The ingestion of > 50 mg histamine per meal can cause health problems such as skin rashes and diarrhoea, even in healthy individuals.

In this case study, the first step was to analyse the levels of volatile carboxylic acids and biogenic amines in the defective cheese. The key findings are summarised in Table 1.

Table 1: Analysis results for the declassified Emmental.

Formic acid	5.5	mmol kg^{-1}
Acetic acid	30.5	mmol kg^{-1}
Propionic acid	8.9	mmol kg^{-1}
Total volatile carboxylic acids	45.7	mmol kg^{-1}
Cadaverine	853	mg kg^{-1}
Histamine	42	mg kg^{-1}
Tyramine	61	mg kg^{-1}
Total biogenic amines	956	mg kg^{-1}
Free amino acids (OPA)	186	mmol kg^{-1}

The low level of propionic acid indicates that propionic acid fermentation, still low at this early stage of ripening, accounts for only a fraction of the gas formation. Formic acid and acetic acid are produced during the breakdown of citrate by facultative heterofermentative lactic acid bacteria, which are added to Emmental as an adjunct culture. However, these volatile carboxylic acids can also be produced by enterobacteria during the mixed acid fermentation of lactose. This type of mixed acid fermentation releases CO_2 and hydrogen along with a range of organic compounds, which in contaminated cheese can result in defective eye formation even during pressing or in the first few days of aging, causing early blowing in extreme cases. Levels of volatile carboxylic acids in Emmental cheeses of the same age with normal eye formation were found to be similar, so there was no indication of mixed acid fermentation by enterobacteria.

In contrast, the determination of biogenic amines produced a surprising result. The cheese contained a remarkably high concentration of cadaverine (853 mg kg^{-1}), which can only be explained by the presence of spoilage microorganisms. The formation of cadaverine in cheese is mainly associated with the growth of enterobacteria, although it can also be linked to lactic acid bacteria (e.g. *Paucilactobacillus wasatchensis*). The production of Emmental at cooking temperatures of 52−54 °C and the slow cooling of the cheese curd after moulding normally results in a significant reduction of enterobacteria, which is why high levels of cadaverine are atypical for this variety of cheese. Further investigations were thus conducted to determine the microbiological cause of the high levels of cadaverine.

Analysis of the cheese microbiome

The term microbiome refers to the entire community of microorganisms within an ecosystem. Analysis of the cheese microbiome involves extracting genetic material directly from the cheese sample and sequencing it. The main difference between this technique and conventional microbiological analysis is that it does not require microorganisms to be cultured and is thus able to capture non-culturable microorganisms as well. A distinction is made between PCR-based sequencing methods (*amplicon-based sequencing*) and PCR-free methods (*shotgun metagenomic sequencing*).

Agroscope uses a technique based on sequencing the 16S rRNA gene to analyse the microbiome. Here, sections of the bacterial 16S rRNA gene in the extracted genetic material are amplified by PCR and then sequenced. 16S rRNA gene sequencing is particularly informative as this gene is common to all bacteria yet varies from one bacterial species to another. Thus, the bacterial species can be identified by determining the base sequence of this gene and comparing it with known sequences. This technique has been the standard method for identifying bacteria since 1994.

Unfortunately, however, the 16S rRNA gene sequence databases required for comparison often contain incorrect entries as they are not curated. So in some cases sequencing data can be ascribed to the wrong species of bacteria. A high-quality, curated database for microorganisms of relevance to the dairy industry has been compiled in recent years in partnership with INRAE (the French National Research Institute for Agriculture, Food and Environment) as part of Agroscope's Microbial Biodiversity research project (Meola et al., 2019). The entries in this DAIRYdb database provide more reliable results. A cheese microbiome analysis yields millions of sequence data. Molecular biological and computing knowledge is essential to interpret the results of this type of study. Figure 2 shows the steps involved in a cheese microbiome study at Agroscope.

In this case study, 10 good quality cheeses from different batches and 10 cheese samples from one batch of severely defective cheeses were investigated using the method described above. The species in the starter culture (*Lactobacillus delbrueckii* and *Streptococcus thermophilus)*, and the adjunct cultures (*Propionibacterium freudenreichii* and *Lactobacillus paracasei*) dominated the composition of the microbiome in all cheeses studied and together comprised over 99% of the microbiome in the cheese mass (Fig. 3). The relative abundance of *Lactobacillus delbrueckii* was lower in the defective cheese group than in the reference cheeses, while that of *Lactobacillus paracasei* was higher. The fundamental problem of using microbiomics with fermented foods is that individual species can dominate to such an extent that it becomes very difficult to detect smaller bacterial populations.

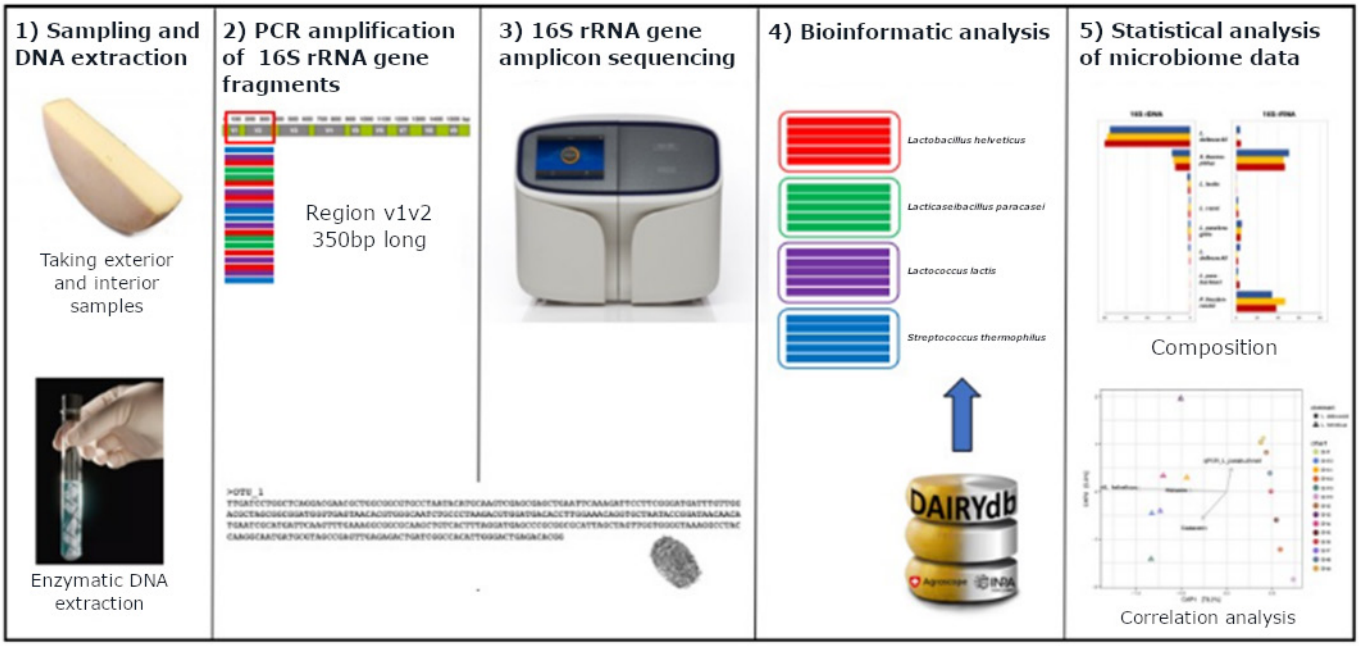

Figure 2: Steps involved in a 16S-based cheese microbiome analysis at Agroscope.

The results presented in Figure 3 show that the bacterial species of the added cultures dominate the cheese microbiome, while the naturally occurring (autochthonous) bacteria account for only a very small fraction. However, even marginal bacterial populations in the cheese microbiome can have a very significant effect on the sensory characteristics and quality of the cheese. This is particularly true of bacterial populations responsible for cheese spoilage, which – despite very low relative abundances of < 0.1% – can severely impair cheese quality.

Figure 4 shows the relative abundance of the minor components of the cheese microbiome in the analysed cheeses, which together make up less than 1%. The defective cheese samples were found to contain *Lentilactobacillus parabuchneri* along with other lactic acid bacteria such as *Lactiplantibacillus pentosus*, *Lactiplantibacillus plantarum* and *Loigolactobacillus coryniformis* (Fig. 4). Species belonging to the *Entercoccaceae* and *Enterobacteriaceae* family were also identified as minor components.

A correlation can be assumed to exist between the formation of the biogenic amines cadaverine (*Enterobacteriaceae*), tyramine (*Enterococcus* ssp.) and histamine (*Lentilactobacillus parabuchneri*) and the spoilage bacteria. Due to the very high similarity between the 16S rRNA gene sequences of closely related species, any species-specific statements made about the cause of the defective cheese quality would be more speculative than reliable. The growth of cheese spoilage bacteria in the defective cheese samples was traced back to an error when selecting the production recipe, which resulted in a too low cooking temperature (50 °C instead of 52−54 °C) and a too low moulding temperature (44 °C instead 52 °C). The much too low moulding temperature is thought to have resulted in incomplete lactic acid fermentation. Consequently, residual sugar was still present in the cheese after 24 hours, favouring the growth of undesirable cheese spoilage bacteria.

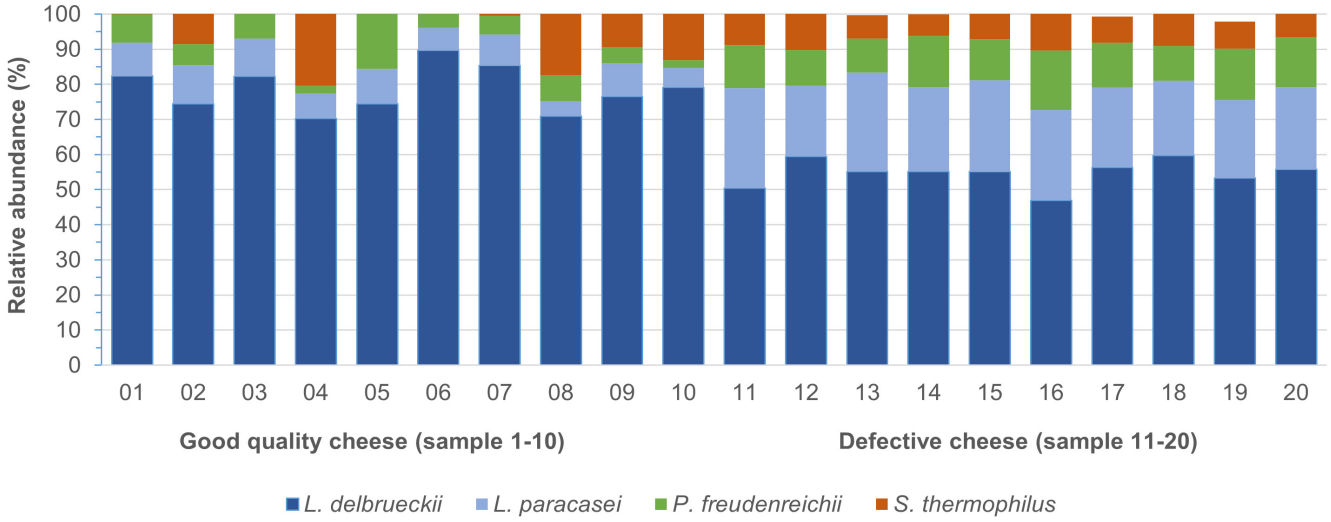

Figure 3: Relative abundance of the dominant species Lactobacillus delbrueckii, Streptococcus thermophilus, Propionibacterium freudenreichii *and* Lacticaseibacillus paracasei *in the analysed cheese samples.*

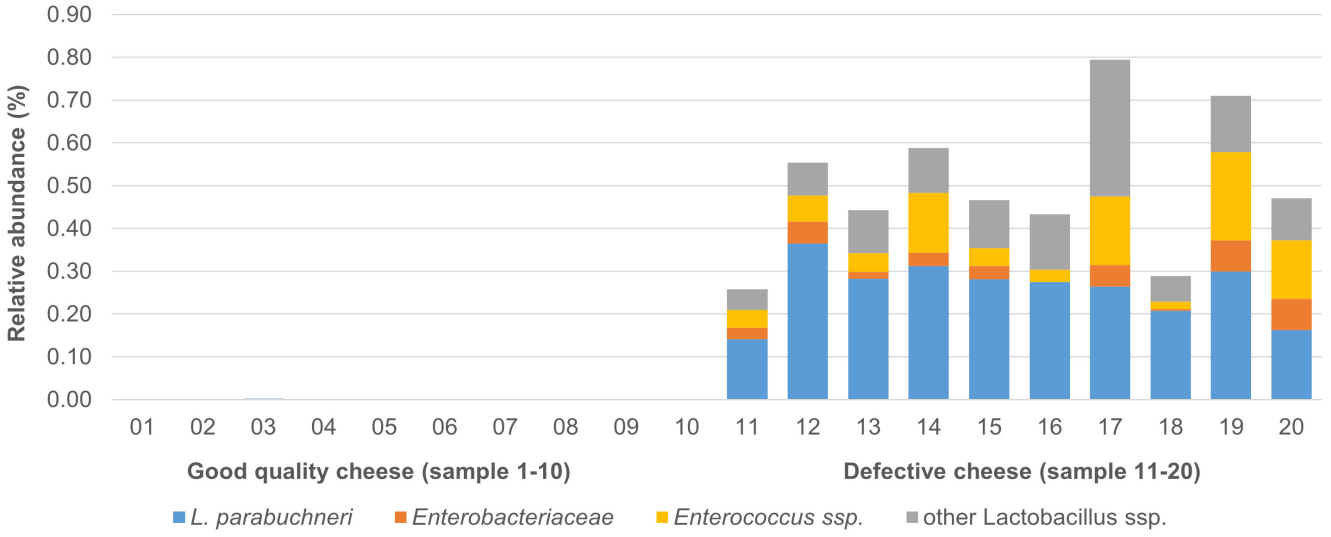

Figure 4: Relative abundance of minor components in the cheese microbiome.

In summary, it can be concluded that microbiome analysis provides worthwhile indications of the presence of undesirable microorganisms in cheese. However, the results of microbiome analysis did not permit statements to be made about the precise species of cheese spoilage bacteria in the defective, declassified Emmental cheese. For this reason, microbiological analyses were additionally undertaken.

Microbiological analyses

Biogenic amines are organic, basic compounds mainly formed by the decarboxylation of amino acids by microorganisms. For example, histamine is produced from histidine, tyramine from tyrosine and cadaverine from lysine. Different media are used in microbiology to isolate the bacteria which produce biogenic amines. These decarboxylase media all have a slightly acid pH and require the addition of a corresponding precursor amino acid for amine formation, and in addition, a pH indicator.

If a bacterium is able to decarboxylate the precursor amino acid, an amine is produced which increases the pH around the bacterial colony. The rise in pH is visually indicated by the colour change of the pH indicator.

Agar plates with lysine as the precursor amino acid were used in this case study due to the high level of cadaverine. A serial dilution series of a homogenate of the defective Emmental was spread out on these agar plates and subsequently incubated at 30 °C for 20 h, resulting in the growth of red colonies (Fig. 5). Since the red colour indicates a pH change alone rather than direct proof of amine formation, colonies were inoculated into a liquid medium containing the corresponding precursor amino acid in a second step. After further incubation at 30 °C for around 20 hours, the media supernatant was screened by thin-layer chromatography to determine the presence of biogenic amines (Fig. 6). This qualitative screening technique can determine the production of histamine, tyramine, tryptamine, cadaverine and putrescine in up to 16 samples at once within a few hours. This method confirmed that cadaverine was produced by the red colonies. Agroscope uses MALDI-TOF-MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry) to rapidly identify bacteria and yeasts (Fig. 7). With this method, colonies are transferred from agar plates to a metallic target plate to which a matrix solution is added (usually a cinnamic acid derivative). This type of target plate holds between 96 to 160 samples in individual wells. The target plate is then inserted in the MALDI-TOF-MS instrument. A laser is fired under high vacuum at the bacteria in the matrix solution. This releases and ionises the bacterial proteins, which are then accelerated in an electrical field passing through a flight tube. The mass of the released proteins can be determined from the time it takes for the molecules to pass though the flight tube.

Individual bacterial species have a characteristic total mass spectrum, like to a molecular fingerprint. The bacterial species can be identified by comparing the measured spectra with reference spectra. Thanks to this very rapid method of identifying bacteria, it took less than half a day to identify the red colonies as *Klebsiella pneumoniae*.

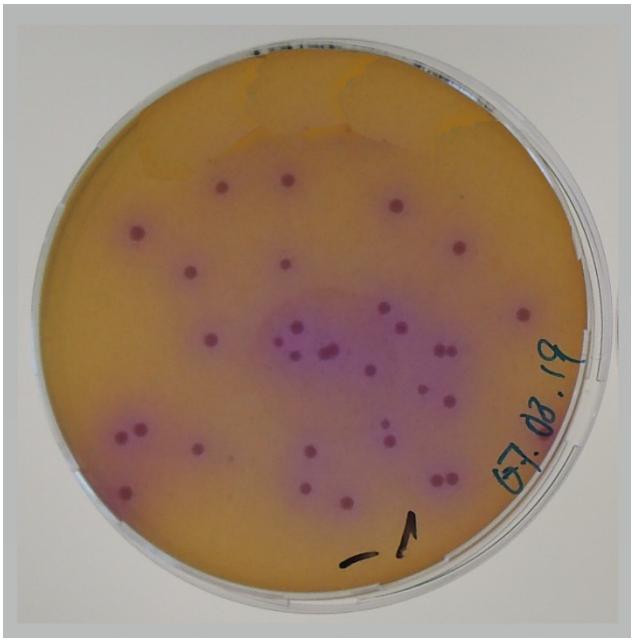

Figure 5: Agar plate with cadaverine-producing bacteria, which appear as red colonies on the specific medium.

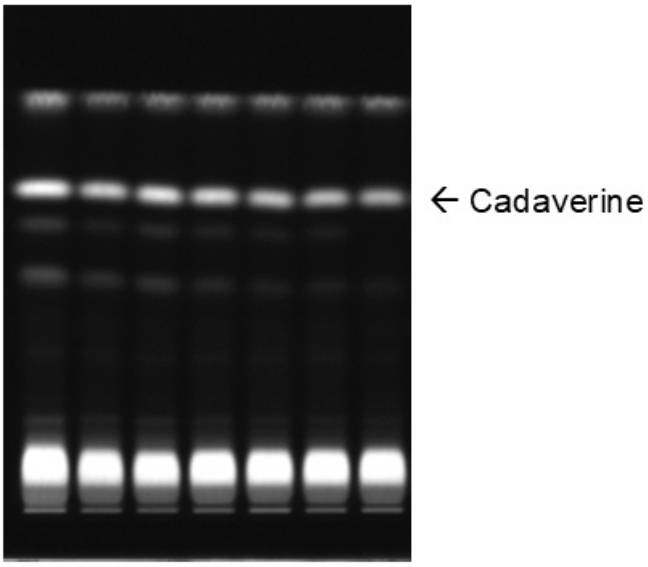

Figure 6: Thin-layer chromatography image confirming the production of cadaverine by the isolated bacterial colonies.

Occurrence of *Klebsiella pneumoniae* in cheese

Little is known about the occurrence of *Klebsiella pneumoniae* in cheese. An Italian study identified the species as a spoilage organism in commercially produced Mozzarella (Massa et al., 1992). Batches contaminated with *Klebsiella pneumoniae* showed high gas production, leading to the formation of holes in the cheese and causing the plastic pouches to swell. The Mozzarella cheese was made from pasteurised milk, so it was assumed that contamination occurred after the milk had been pretreated. To achieve the characteristic texture associated with Mozzarella, after production the cheese is stretched in a water bath at temperatures of up to 57 °C. The gas formation observed in the defective Mozzarella indicates that *Klebsiella*

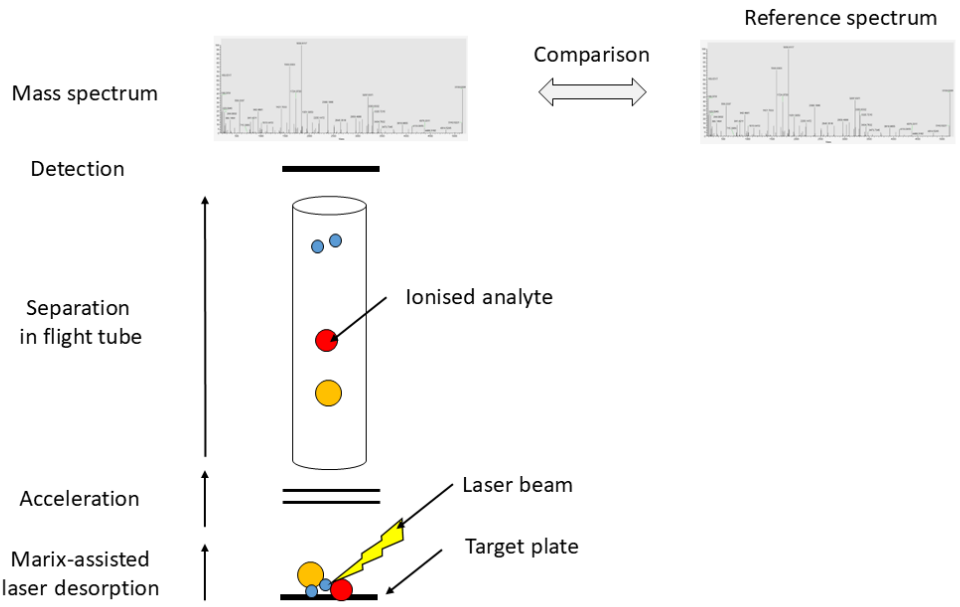

Figure 7: Schematic diagram of a MALDI-TOF-MS analysis.

pneumoniae can survive this type of heat treatment. Investigations of the thermal resistance of *Klebsiella pneumoniae* undertaken in the study showed that *Klebsiella pneumoniae* cultures with an initial bacterial count of 10^7 cfu ml^{-1} were inactivated after heat treatment at 63 °C for 15 min.
In this case study it could not be determined whether the processed raw milk was contaminated with *Klebsiella pneumoniae*. It is also conceivable that contamination with this spoilage bacteria did not occur until the moulding stage when the curds are filled into the moulds. The cross-sectional image shows that the defective eye formation was less pronounced in the middle of the cheese. This zonal difference suggests that *Klebsiella pneumoniae* was at least partially inactivated in the middle of the Emmental due to the slower cooling rate.

qPCR analysis

The availability of gene sequences for a very wide range of bacterial species in public databases is driving the advancement of species-specific detection systems. The development of a bioinformatics pipeline (Dreier et al., 2020) has enabled the number of species-specific detection systems to progressively grow in the last three years. Agroscope can now quantify over 30 bacterial species of relevance to the dairy industry using qPCR analysis (Tab. 2). This creates new opportunities for investigating field samples for cheese spoilage bacteria. The population densities of the species added with the cultures were also determined in this study (Fig. 8). The population density of *Lactobacillus delbrueckii* was found to be significantly lower in the defective cheeses (p-value = 0.001). The results of the qPCR analysis suggest that the growth of *Lactobacillus delbrueckii* in the defective cheese was inhibited by the low moulding temperature of 44 °C.

Table 2: Overview of species relevant to the dairy industry for which Agroscope has species-specific qPCR detection systems.

Species	EvaGreen qPCR	TaqMan qPCR
Clostridium butyricum	x	
Clostridium tyrobutyricum	x	x
Enterococcus durans	x	
Enterococcus faecalis	x	
Enterococcus faecium	x	
Lactobacillus acidophilus	x	x
Levilactobacillus brevis	x	
Lentilactobacillu buchneri	x	
Lacticaseibacillus casei	x	
Loigolactobacillus coryniformis	x	
Latilactobacillus curvatus	x	
Lactobacillus delbrueckii	x	x
Limosilactobacillus fermentum	x	x
Lactobacillus gasseri	x	
Lactobacillus helveticus		x
Lentilactobacillus parabuchneri	x	x
Lacticaseibacillus paracasei	x	x
Lactiplantibacillus pentosus	x	
Lactiplantibacillus plantarum	x	x
Lacticaseibacillus rhamnosus	x	
Latilactobacillus sakei	x	
Lactococcus lacremoris	x	
Lactococcus lactis	x	
Pediococcus acidilactici	x	
Pediococcus pentosaceus	x	
Propionibacterium freudenreichii		x
Acidipropionibacterium acidipropionici		x
Acidipropionibacterium jensenii		x
Acidipropionibacterium thoenii		x
Streptococcus salivarius	x	
Streptococcus thermophilus	x	x

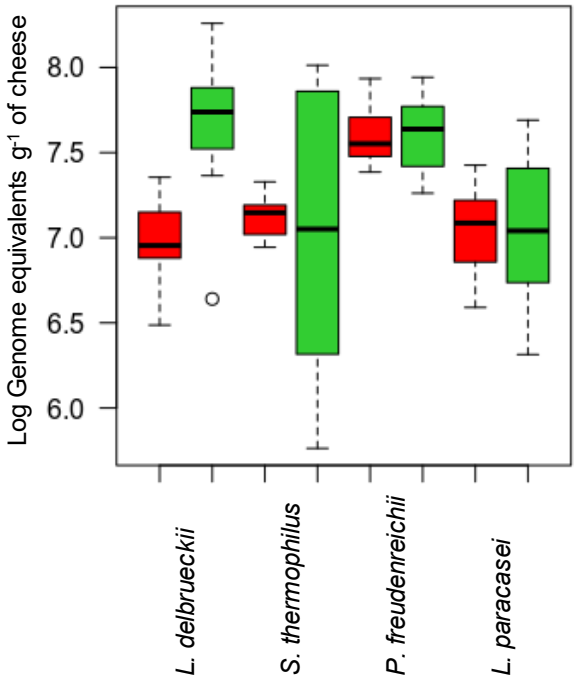

Figure 8: Detection of species from starter and adjunct cultures by qPCR in defective cheese samples (red; n = 10) and good quality cheeses (green; n = 10).

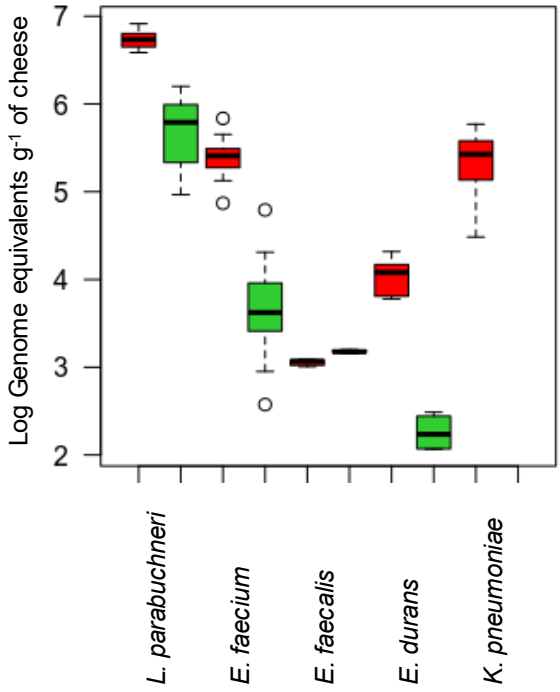

Figure 9: Species-specific detection of L. parabuchneri, E. faecium, E. faecalis, E. durans *and* K. pneumoniae *by qPCR in defective cheese samples (red; n = 10) and good quality cheeses (green; n = 10).*

Analysis of the microbiome clearly indicated the presence of spoilage bacteria in the defective cheeses (*Lentilactobacillus parabuchneri*, *Enterococcus* ssp. und *Enterobacteriaceae*; Fig. 4), although the relative abundance of the spoilage flora was very low (< 1 %). The results of the species-specific qPCR analysis of *Lentilactobacillus parabuchneri*, *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* and *Klebsiella pneumoniae* are shown in Figure 9. The qPCR results support the hypothesis that incomplete lactic acid fermentation and residual sugar led to the growth of cheese spoilage bacteria in the defective cheeses. The population density of the three species *Lentilactobacillus parabuchneri*, *Enterococcus faecium* and *Enterococcus durans* in the 10 defective cheeses was significantly higher than in the good-quality cheeses (p-value= 0.0002). *Klebsiella pneumoniae* contamination was detected only in the defective cheese, suggesting that this species is inactivated at the cooking and moulding temperatures normally used in the production of Emmental AOP. The detection of cheese spoilage bacteria by qPCR is very instructive since it is possible to assess from the population densities whether a causal relationship exists between the defect and the spoilage microorganisms. The defective cheese samples were found to contain histamine and tyramine in addition to very high concentrations of cadaverine. The production of these three biogenic amines is easily explained by the qPCR results. The population density of *Klebsiella pneumoniae* accounts for the high concentration of cadaverine in the defective cheese. The production of tyramine can be linked mainly to *Enterococcus faecium* due to the population densities found. The population density of *Lentilactobacillus parabuchneri* was an order of magnitude higher in the defective cheeses than in the good-quality cheeses. Various studies have shown that this species is very often present in cheeses highly contaminated with histamine (Berthoud et al., 2017).

The case study presented here shows the consistency of the results obtained with 16S metagenomics and qPCR and illustrates the complementarity of these two molecular biological methods. The limiting factor of qPCR analysis is that it can identify only cheese spoilage species for which a species-specific detection system exists. Furthermore, considerable analytical effort is required, since the numerous species in a cheese microbiome must currently be detected by serial qPCR analysis. Impressive advances in microfluidic technology have led to the development of a high-throughput qPCR system (Fluidigm®), which offers attractive new prospects, including for cheese diagnostics. This system enables up to 96 different qPCR analyses to be conducted in parallel, reducing analysis costs by around a factor of 100 compared with conventional qPCR analysis. Agroscope is currently researching the use of Fluidigm® technology to further advance cheese diagnostics.

Pilot plant trials

Investigating atypical cheese defects is a core element of the Agroscope Cheese Consultancy's work. A cheese defect can only be explained if it can be specifically reproduced. Pilot plant trials are an important tool when it comes to increasing our understanding of cheese spoilage bacteria. During the investigations, several isolates of cadaverine-producing *Klebsiella pneumoniae* were successfully isolated from the Emmental shown in the title image. The use of such isolates in pilot plant trials can yield valuable information about the thermal resistance and damage threshold of this species that is particularly relevant to the production of raw-milk cheese. For example, these trials can be used to explain whether the quality of

specific cheese varieties is impaired by the presence of this bacteria in the raw milk, and the bacterial count in the processing milk above which cheese defects can be expected. *Klebsiella pneumoniae* is a Class 2 organism according to the list of officially classified organisms published by the Federal Office for the Environment (FOEN). For reasons of biosafety, it was not possible to conduct corresponding trials at the Agroscope Liebefeld pilot plant using the isolates obtained. Agroscope's cheese research infrastructure must be adapted to comply with current safety requirements before cheese trials with isolates from Class 2 organisms can be resumed.

Summary and conclusions

This study set out to explain the reasons for the occurrence of an extremely defective eye formation in declassified Emmental using a case study. The results of the biogenic amines analysis showed that all the defective cheese contained spoilage bacteria that produce very high concentrations of cadaverine. It was assumed that this spoilage microorganisms could be responsible for the severely defective eye formation. Thus, efforts were made to identify the spoilage microorganisms by molecular biological and microbiological analysis. Analysis of the microbiome revealed that the defective cheese was contaminated with enterococci and *Lentilactobacillus parabuchneri*, the presence of which explains the low production of tyramine and histamine. *Enterobacteriaceae* were also detected, which could explain the defect due to their gas-producing potential and ability to produce cadaverine. Due to the extreme dominance of species introduced in the added cultures, the relative abundance of the detected undesirable bacteria was well below one per cent. The results of the microbiome analysis did not provide a conclusive explanation of the cause of the defect. Using MALDI-TOF MS, it was possible to identify cadaverine-producing isolates obtained from the declassified Emmental as *Klebsiella pneumoniae*. Relevant contaminations with *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus durans*, *Lentilactobacillus parabuchneri* and *Klebsiella pneumoniae* were detected in the defective cheese by qPCR analysis and thus a plausible explanation for the defect was found. Agroscope is currently conducting research to quantify desirable and undesirable species in cheese using high-throughput qPCR. Preliminary results with this complementary approach are very promising; the use of this cost-saving methodology would constitute a major advance in cheese analysis and cheese consultancy.

This study shows that, despite the availability of new methods of analysis, investigating atypical microbiological quality problems in cheese remains a complex and time-consuming task which can only be resolved with the aid of different, complementary techniques. Microbiome analysis can provide valuable information for rapidly resolving real-life case studies, but the results of these analyses must always be interpreted with caution and expertise, since this research method was not designed for diagnostic purposes.

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