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Food microbiology

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Vertical Transmission

Dena Lyras
President of ASM

This will be my last communication to you as President of the ASM, after serving in this role for the past 4 years.

I never expected that I would hold the role of President of our Society and, in truth, I was nervous about taking on such an important task. What I can say now, at the end of my tenure, is that it has been a deeply fulfilling time, which has allowed me to connect with so many of you – I am very lucky to have so many new friends and colleagues in our community of microbiologists. More than that, it has been a tremendous privilege to serve you as the ASM President. I hope that I can use the knowledge I have gained to continue to serve the Society in other ways in the future.

One of the most wonderful aspects of my presidency has been the time I have spent working with our ASM Executive. These people are profoundly committed to the Society and our members, and it has been an honour to spend time with them. I would like to take this opportunity to thank Roy Robins-Browne, Kate Seib, Anthony Baker, Cheryl Power, Rebecca LeBard, our President Elect, Mark Schembri, and Priscilla Johanesen, who attends every executive meeting in her role as Chair of the Professional Development Committee; you are such a talented and passionate group of people and I am privileged to now call you friends.

I would also like to extend my thanks to our State Branch Chairs and the Chairs of our Standing Committees, and all of the members who sit on the committees under these portfolios. Thank you also to the members who take the time to represent the Society on National and State Advisory Committees and Boards. The members of the Microbiology Australia Editorial Board also deserve a very big thank you for the wonderful job they do getting interesting, readable issues together, and for giving our members an opportunity to showcase their science. I am also grateful for the hard work of our Scientific Advisors, especially during COVID-19 restrictions when, together with Kate Seib, they managed to deliver online content that kept us all connected and engaged.

A special thank you to Kara Taglieri at ASN events for many things, but mostly for helping me to solve problems along the way and tolerating my many, many questions.

Finally, I would like to thank all of our ASM members for your support. The ASM is you, our members. Please consider taking an active role in the Society at the state or national level, it will bring you so much more than you can imagine. I wish our new President, Mark Schembri, and our Society, the very best for the future, and I know that the Society is in good, caring hands. I look forward to seeing many of you at our upcoming meeting in Sydney, face-to-face for the first time in 3 years. Safe travels to all of you.

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Microbiology Australia

Food microbiology: current and future topics of investigation

Prudence Bramwell

This edition of *Microbiology Australia* is dedicated to food microbiology. The field of food microbiology is very important as microorganisms can be used in a positive way to make and enhance food products, or, from a negative perspective, microorganisms cause both food spoilage, with subsequent food wastage, and foodborne disease, with potentially life-threatening consequences. The multidisciplinary field of food microbiology is of great interest due to the possibility of studying food microorganisms in the context of food safety and hygiene, classic and new food preservation technologies, traditional and novel food fermentations, functional foods and probiotics, rapid automated methods of analysis, epidemiological and molecular investigations[1] and the future use of microbes as a sustainable food source and for recycling.[2] Therefore, this edition of *Microbiology Australia* focuses on articles related to food safety, advances in microbial food analysis, and novel microbes for new fermented food products that are relevant for the future.

An important topic in this edition relates to omics and its role in food microbiology. Omics (such as genomics, transcriptomics, proteomics, and metabolomics) generates data that can extend the range of information from a single system and increase detection, tracking and adaptation of foodborne microbes in processing environments.[3] The articles by both Gray and Pillidge *et al.* discuss the use of omics to better understand microbial communities in food systems. Gray reviews the use of omics to study leafy vegetables from farm to fork. Leafy green vegetables are well known as a source of foodborne illness such as *E. coli* 0157 infections.[4] Pillidge *et al.* describe the use of metagenomic analysis to study the stages in the production of cheese and the changes in cheese microbiota during fermentation and cheese ripening. Both articles discuss historical culture-based methods compared with high-throughput sequencing methods to obtain whole genome sequencing (WGS) data to develop an understanding of metagenomics, including microbes that might be missed using culture-dependent methods and how the microbiota changes during processing.

In addition to cheese, bread is another classic fermented food that has been made for thousands of years.[5] However, dietary preferences are changing due to the increased demand for gluten free and low fermentable oligo-, di-, monosaccharides and polyols (FODMAP) bread. Wittwer and Howell describe research into diverse breadmaking yeast that may cater for these demands using novel yeasts from potentially non-food environments in sourdough production to develop interesting flavour properties and as a support to the lactic acid bacteria (LAB) degradation of gluten and breakdown of fructans.

Another area of fermentation research is in plant-based products. According to the Bloomberg Intelligence Report, plant-based food sales are expected to increase fivefold by 2030.[6] Fermented plant-based foods are important as a potential replacement for fermented dairy products as they are naturally lactose-free and have a good source of bioactive compounds. However, there is a need for new strains of LAB that are more suited to fermentation of plants than dairy products and that could produce acceptable flavour, texture and nutritional value if proved safe. Huang *et al.* describes Citizen Science as a way to help identify new species of LAB that may be best suited to plant fermentation.

Laboratory analysis of suspected contaminated food is essential to maintain food quality, safeguard consumer safety and ensure emerging pathogens are identified.[7] In late 2021 public health investigations across Australia detected cases of gastroenteritis caused by *Vibrio parahaemolyticus*, linked to eating raw oysters from South Australia.[8] *Vibrio parahaemolyticus* is a bacterium found in marine and coastal waters where oysters normally grow. Outbreaks in Australia have been rare. However, in the last few years *Vibrio* infections have been sporadically reported and are now a notifiable infection in Tasmania.[9] The article by Hedges addresses the possible issue of *Vibrio* spp. as an emerging issue for Australia and discusses advances in molecular methods of analysis using PCR and gene sequencing.

As stated earlier, WGS is increasingly becoming a routine tool to detect and track foodborne outbreaks within hours to days.[10] The article by Bramwell *et al.* discusses the changes in analytical laboratories as traditional methods of detection of foodborne pathogens and spoilage microbes are replaced by rapid automated molecular technologies, such as WGS, as they become more accessible and affordable.[10] However, it explains the reasons why there is still a place for more traditional culture-based methods, in particular for food microbiology analysis in complex food matrices and processed food.

A key limitation when testing food is time-to-detection.[7] Therefore, another area of food microbiology related to rapid analytical technology is rapid *in situ* screening assays for foodborne pathogens, known as Point of Management (POM) assays, similar to 'Point of Care' diagnostics in the medical field. Most raw poultry contains *Campylobacter*.[11]

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Templeton *et al.* describes their work on a POM assay to detect *Campylobacter* in poultry production that uses a dipstick for molecular screening of *Campylobacter* DNA. They describe the advantages and disadvantages of this technology and how it could be extrapolated for other relevant pathogens in the poultry industry, such as *Salmonella*.

In recent years, novel processing and treatment methods such as high-pressure processing, ultrasound, cold plasma, and pulsed electric field have been developed to minimise the risk of unwanted microorganisms being present in food.[12] These new processing techniques have less impact on the organoleptic and nutritional qualities of food by preserving the characteristic properties.[13] Seididamyeh and Sultanbawa describe the use of photodynamic inactivation as an emerging technology on microbial safety in foods. They describe how this treatment can be used to inactivate both pathogenic and spoilage microbes in food, showing promise for the future in minimising nutritional and sensorial changes in a cost-effective and environmentally friendly way.

Another issue related to food processing is the production of biofilms in food processing facilities. Biofilms have been shown to be a source of transmission of pathogens from surfaces and equipment, where they adapt to, and successfully colonise, niche environments.[1] Omics can broaden the scope of knowledge gained studying biofilms, including identification and remediation of strains that persist in the processing plant environment.[3] Biofilms are addressed in Dykes' article, including difficulties in studying pathogen biofilms in food processing facilities *in situ* and issues related to the way biofilms are currently investigated.

In conclusion, the above knowledge can be used in practice to ensure there is a constant, plentiful and safe food supply for the future rapidly growing human population. And as our future is reliant on food availability and good nutrition,[14] experts in all food microbiology fields are essential for improving global health and wellbeing.

Biography

Prudence Bramwell is an Honorary Associate Professor at RMIT University. She has over 30 years' experience in food microbiology. Prior to her 25 years at RMIT as an academic educator in the fields of food microbiology and food safety, she has held positions in Microbiology at both the University of Melbourne and University of Sydney and at the Australian Government Analytical Laboratories (the latter now amalgamated into the National Measurement Institute) in the field of microbiological analysis. While at AGAL she was a registered NATA auditor of Biological Laboratories. While at RMIT she held certificates as an advanced food safety auditor and associate quality auditor with Exemplar Global. Her research interests are in methods for the isolation and identification of foodborne microbes.

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Omic applications to understand food system microbiomes

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ABSTRACT

Understanding the microbial communities associated with food systems has traditionally used culture-based techniques that can provide a snapshot of the microorganisms present. However, this approach requires multiple media types and only allows for the identification of a limited number of culturable species. Culture-independent methods such as sequencing and omic techniques provide a deeper understanding of the microbial community, how they interact and function together across the entire food system. This review provides a brief introduction to omic techniques used in microbiome studies and touches on microbiome research that has been undertaken across the farm to fork continuum focusing on leafy vegetables where possible.

Keywords: food systems, metagenomics, metaproteomics, metatranscriptomics, microbiomes, omic techniques.

Leafy vegetables are a part of a healthy and balanced diet providing many nutritional qualities including vitamins, minerals, fibre and phytochemical compounds.[1,2] Production and sale of leafy vegetables has increased due to humans consuming leafy vegetables as part of a healthier diet. Between 2019 and 2020, the Australian retail supply of leafy salad vegetables increased by 9%.[3] As production has increased, so too has foodborne illnesses associated with leafy vegetables, with some major outbreaks linked to lettuce, spinach or ready to eat salads due to their raw and ready to eat nature.[4–6] As a result, important strategies, guidelines and policies have been developed both within Australia and overseas.[7,8]

Traditionally, food safety regulatory measures have focused on the identification of foodborne pathogens at various locations within processing facilities and on the final product prior to sale. While it is important to track foodborne pathogens, they occur at low prevalence and low numbers within a broader microbial community that are often present in complex environments such as food production and processing environments as well as food products. This leads to challenges in detecting pathogens in food systems, determining how they enter and survive throughout the food chain and how best to control them.

Understanding the microbial composition and potential impact these communities have on pathogens and spoilage organisms could provide new ways of improving the safety and shelf life of foods. A microbiome is a microbial community and includes the environmental components (chemical, physical and biological) of the ecological niche in which the community exist.[9] The microbiome can be composed of bacteria, fungi, viruses, algae, or small protists, which is known as the microbiota, in a mutualistic or competitive manner. Within food systems, microbiome research can occur within the farm arena to understand the microbial interactions with the crop or animal system and the production of a healthy commodity; within the processing environment to ultimately prevent cross-contamination of products by pathogenic species and reduce spoilage; and within the human gut to understand the dietary effects and impact on the native microbiota of the commodity on human health.

Food microbiome research has historically been conducted using culture-based methods through the identification of pathogens of interest as well as determining total viable counts of bacterial and fungal species. With the development of molecular and sequencing methods and the reduction of costs associated with sequencing, microbiome research nowadays is typically performed using omic techniques like *16S* rDNA amplicon sequencing and whole genome sequencing, metagenomics, metabarcoding, metatranscriptomics, metaproteomics and metabolomics and provides a greater depth of information. These techniques can be used singularly or in combination to help overcome any limitations individual techniques may have as well as providing an understanding of community

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structure, networks and interactions.[10] Utilising omic techniques allows the analysis of the taxonomic composition of all microbial members (understanding who is there), analysis of the metabolic potential (what they can do) and the microbial functioning (what are they doing).[9] The majority of food system microbiome research has focused on understanding the soil microbiome for improving plant/crop growth, with a small amount of research concentrating on microbial communities within the red meat and poultry industry, with limited microbiome research in the horticultural farm to fork space.

A summary of some of the microbiome research and potential applications that have occurred within food systems are highlighted below. Jackson *et al.*[11] utilised culture-dependent and culture-independent methods to assess the bacterial composition of leafy salad vegetables. Culture-dependent methods identified bacterial colonies from six phyla in comparison to culture-independent methods which identified 11 different phylas. While most of the dominant taxa identified in the leafy salad vegetable samples were characterised by both dependent and independent methods, pyrosequencing was able to identify two additional bacterial taxa, *Ralstonia* (endophytic) and *Actinobacter* (associated with the leaf surface). Although *Ralstonia* is capable of growth on trypticase soy agar, the colonies are typically small and may be missed in isolate sampling, therefore the identification of *Ralstonia* by pyrosequencing ensured its presence was detected. Culture-independent methods are able to identify low abundant taxa otherwise missed using culture-dependent methods. Determining the entire microbial community from leafy vegetable samples also allows for the identification of microbial species which may influence the survival of pathogenic or spoilage organisms.

Bacterial *16S* and fungal ITS amplicon sequencing was employed to distinguish the bacterial and fungal communities on spinach and swiss chard with consideration of leaf damage.[12] The fungal community at all stages (baseline – manual harvest and no wash step, machine harvest, washing, packaging) remained consistent for both spinach and swiss chard with *Ascomycetes* followed by *Basidomycota* phyla the most dominant. Whereas the bacterial diversity varied with specific species abundant at different harvesting and processing stages. Spinach and swiss chard communities were both dominated by the phylum *Moraxellaceae*; however, a significant reduction in its abundance occurred following the washing stage. The family *Pseudomadaceae* increased and was the most abundant family in the washing and packaging microbiota.[12] A *16S* rRNA analysis before and after sanitisation in a fresh produce processing facility found greater reduction in bacterial populations and shifts in microbiomes following effective sanitation.[13] The microbial populations on production floors were also found to be consistently reduced by sanitation in comparison to peripheral surfaces like doors and walls. Several species were identified at multiple sites throughout the facility suggesting these species comprised part of the core microbiota of the processing facility. Understanding the microbiota at various stages of processing and how these communities are influenced by the various hurdles and processes associated with food production provides important information on the microbiota the consumer is exposed to and the potential influence it may have on consumer health.

A meta-transcriptomic study performed by Jung *et al.*[14] on the interactions of lactic acid bacteria during a kimchi fermentation identified *Leuconostoc mesenteroides* was most active during early phase fermentation and *Lactobacillus sakei* and *Weissella koreensis* dominated the later fermentation phase. They identified genes typical of heterolactic acid fermentation from pathways relating to carbohydrate transport, hydrolysis and lactate fermentation. The identification of active populations, gene expression and interaction of community members at important stages of the fermentation process would not have been possible with the use of culture-based methods. Proteomics was employed to assess the inhibition potential of modified atmospheric conditions, 30% carbon dioxide (CO_2) and 70% oxygen (O_2), of five typical meat spoilage microorganisms on a simulated meat medium.[15] Proteomic analysis identified the five species were able to co-exist as a result of alternative species-specific metabolic pathways in which synergistic spoilage occurred. Three of the meat spoilage species utilised a variety of mechanisms to reduce oxidative stress, maintain intracellular pH, osmotic balance and oxygen levels and alteration of the fatty acid composition. The use of proteomics provided an insight into nutrient utilisation and adaptation to industry adopted modified atmospheres designed to reduce the growth of spoilage microorganisms and therefore spoilage in general. Identification of members of the core microbiota, particularly if they are spoilage or pathogenic microorganisms, provides valuable awareness of the species which may support the survival of undesirable microorganisms. Metagenomic analyses also provide greater insight into the effectiveness of cleaning and disinfection treatments and offers the ability for facilities to tailor their sanitation methods to target species of interest.

There is substantial research in the human gut microbiome arena demonstrating the value in understanding microbial community interactions. However, microbiome research across food systems and in particular non-fermentative and or leafy vegetables is lacking. The availability of sequencing and omic technologies has the power to rapidly expand our understanding of microbial community members and interactions in this space. Understanding how community members interact and move from one area of the food chain to another area may allow for the development of rapid screening techniques or the development of healthy state (ideal) microbiomes that may increase crop production, reduce contamination by spoilage and pathogenic microorganisms, increase product shelf-life and improve health benefits for consumers.

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Biography

Dr Jessica Gray is a post-doctoral fellow within the Microbiome for One System Health Future Science Platform at CSIRO within the Food Microbiology group. Jess's research focuses on understanding the various microbiomes across the farm to fork continuum.

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Cheese quality and authenticity: new technologies help solve an age-old problem

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ABSTRACT

Cheese represents a complex ecosystem of starter and non-starter bacteria, with populations changing over time as the cheese matures. Successive microbial communities, particularly in aged cheeses like cheddar, have a profound impact on the final cheese flavour and quality. Being able to accurately predict cheese ripening outcomes at an early stage, based on cost-effective analyses, would be of great benefit to cheesemakers. In the past, there has been a significant gap between microbiological and chemical information obtained from omics and its application to the cheese industry, but thanks to recent advances in omics analytical methods, computing programs and sensor technologies, this gap is narrowing.

Keywords: cheese authenticity, cheese quality, metabolomics, microbial profiling, multi-omics, proteomics, sensors.

Introduction

Cheese is one of the most widely consumed dairy products. Many different varieties of cheese that vary in texture, taste, and aroma are made and consumed around the world. The number one cheese produced in Australia is cheddar,[1] although other cheese types are gaining in popularity (Fig. 1). For cheddar and other low moisture cheeses that require ageing (ripening), manufacturers have a keen vested interest in getting the ripening process right. If ripening does not proceed correctly, the cheddar made in a daily production run could end up being sold off cheaply, perhaps as an ingredient for locally made processed cheese rather than being exported to an overseas customer at top dollar.

Cheesemaking begins with raw milk. After standardisation of the milk to a pre-determined protein–fat ratio and high-temperature short-time (HTST) pasteurisation, the milk is pumped into a cheese vat. The starter culture is then added, followed by addition of rennet, a mixture of milk coagulating enzymes traditionally obtained from the lining of the abomasum from young calves. Alternatively, the rennet can be plant- or microbial-derived, or it may be a highly purified form of bovine chymosin obtained through recombinant DNA technology.[2] Based on the action of the rennet and starter, milk coagulation occurs. Subsequent cheesemaking steps include cutting the curd and whey drainage, heating, salting and pressing.[3] In large-scale industrial cheddar cheese-making, strains of *Lactococcus lactis*, a species of lactic acid bacteria (LAB), usually comprise the starter culture. Starter cultures can be added either in freeze dried form direct to the vat, known as direct vat inoculation (DVI), or grown in a separate tank then added as bulk starter. It is not uncommon for cheesemakers to also add adjunct cultures at lower levels, usually non-starter lactic acid bacteria (NSLAB) comprising strains of *Lactobacillus helveticus, Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*),[4] or other species. These NSLAB grow slowly in the young cheese during ripening to eventually reach high numbers where they modulate cheese flavour and texture development.[5] Cheese ripening becomes more complicated, however, because each factory has its own distinctive resident microbiota that naturally 'inoculate' the cheese before ripening begins. These adventitious microbiota also contribute to the complex succession of microbes critical in determining the final cheese properties.[3]

How is cheese ripening monitored and controlled as it progresses? In most large cheddar-making factories, experienced cheese-graders take core samples at different ripening stages to assess and predict the final cheese flavour and texture. Some basic chemical and microbiological analyses may also be done. Alongside the skill and

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Fig. 1. Hard cheeses such as cheddar are ripened for relatively long periods of time, in some cases for up to 3 years. Accurately predicting ripening outcomes at an early stage would save cheesemakers money and lead to improved product quality for consumers. New advances in omics and sensor technologies will help cheese graders achieve this goal with greater reliability and precision. Furthermore, by applying integrative omics, detailed chemical fingerprints of cheeses can be obtained that can help prove product authenticity, for example, by showing accuracy of labelling for animal sources of milk or for cheese maturation age.

knowledge of the cheese grader, scientific developments in chemical analytic techniques and in omics technologies have progressed, leading to mapping of the many thousands of individual cheese components, including the microbial communities, proteolytic breakdown products, huge numbers of metabolites, as well as the fermentation primary end products. But how can all this information be applied in a meaningful way to help cheesemakers better assess and predict cheese ripening outcomes? This challenge can be tackled in part by the application of so-called omics technologies.[6,7]

Omics and cheese

The term omics refers to the scientific discipline of analysing the interactions and functions of large clusters of biological information molecules.[8] Omics technologies include metagenomics based on high-throughput next generation sequencing (NGS) methods, metatranscriptomics, metaproteomics, and metabolomics, targeting DNA, RNA, protein and metabolites, respectively.[9–11] In recent years, the application of omics technologies to study fermented food products, especially cheese, has greatly increased.[7]

Metagenomics encompasses amplicon sequencing and shotgun whole genome sequencing.[9] In amplicon sequencing, total DNA is extracted from an environmental sample, then a targeted region (e.g. within the *16S* rRNA gene for identification of bacteria) is PCR-amplified and sequenced. Due to inherent methodological errors that can occur using this approach, along with ongoing improvements in DNA sequencing and computer processing power, shotgun sequencing is gaining wider use. Here, total DNA is sequenced providing not only taxonomic identification results but also information on the total genes present in a sample and their potential corresponding protein (or enzyme) metabolic functions.[12] Such DNA-based approaches have helped to identify novel microbial species not identifiable using traditional microbiological culturing techniques in many environments, including cheese. An early pioneering study to apply metagenomics in cheese involved *16S* rRNA gene amplicon sequencing of 60 Irish soft cheeses.[13] In addition to common LAB species, many non-LAB bacterial genera were identified, such as *Prevotella* and *Arthrobacter*. The authors found that the bacterial community composition depended upon the cheese type, the origin of the milk, production technology and the ingredients used.

Metatranscriptomics and metaproteomics involve assessing the complete gene expression and protein complement (respectively) of multi-component biological systems. Both methods are difficult to apply in fermented foods, hence relatively few studies have been published to date.[7] Metatranscriptomics studies on cheeses have shown that regulation of microbial enzymes capable of impacting flavour development occurs during ripening, with one study on a Swiss-type cheese showing that regulation of central metabolism enzymes in cold ripening conditions varied depending on the species.[14] Metaproteomics studies have also revealed the functional roles of microbial proteins in fermented foods, however, there have been few studies on cheese, in part due to the complexity of analysing microbial and non-microbial milk proteins and their breakdown products together.[7,15] Despite these difficulties, these tools have the potential in future to provide exciting new insights into the functional aspects of the cheese microbiota.

Metabolomics consists of identification and quantification/semi quantification of all endogenous small molecules (metabolome) biosynthesised and modified in a cell, tissue or in a microbial consortium. Typically, there are two approaches: metabolite profiling (targeted analysis of specific groups of metabolites) and metabolite fingerprinting

(untargeted analysis of the global metabolome profile without the need for a prior specific hypothesis on a set of metabolites). Metabolomics relies on an efficient method for metabolites extraction, followed by application of analytical instrumentation – usually gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) together with nuclear magnetic resonance (NMR) and multivariate data analysis.[6,11] Other approaches may also be used; as one example, a group in the Czech Republic used matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) together with principal component analysis, or liquid chromatography coupled with electrospray ionisation and quadrupole time-of-flight mass spectrometry, to distinguish 27 cheeses made from milks of different animal species.[16] We also showed that spatially offset RAMAN spectroscopy (SORS), a fast, inexpensive and non-invasive method, can be used with chemometrics to distinguish cheeses made from different animal species.[17]

Multi-omics with data integration

Individual omics approaches have shown the enormous complexity of fermented food products at a biological level. However, there has been emerging interest in developing mathematical tools that analyse high-dimensional omics datasets obtained from multiple omics platforms applied to fermented foods. For example, new insights into the cheese microbiota were obtained from the combination of strain-level metagenomics with metabolomics, highlighting that different strains of the same species may produce different metabolites in cheese.[18] Samples of 55 artisanal cheeses from 27 Irish producers were analysed; the authors recovered 328 metagenome-assembled genomes, including 47 putative new species in cheese. In addition, numerous phage and bacteriocin genes were found. Most of the new species identified belonged to halophilic genera such as *Psychrobacter* and *Halomonas*, while other species belonged to genera known to be associated with cheese rinds (for example, *Brevibacterium*, *Corynebacterium*, and *Arthrobacter*). In another study integrated amplicon-targeted metagenomics and metabolomics provided the basis for the selection of cheese adjunct cultures for the accumulation of specific flavours in soft-type ripened cheeses.[19]

Multi-omics studies on Australian industrial and artisanal cheddar cheeses done by us have also revealed some interesting associations between cheese microbiota and metabolites. These studies further suggest the possibility of discovering new biomarkers for validating cheese age and brand authenticity and cheese quality. For example, some low abundant taxa such as *Pediococcus* spp. in artisanal cheeses correlated with the presence of 21 metabolites that may influence cheese flavour.[20] Another study showed how integration of metagenomics and metabolomics datasets could enable better differentiation of ten similar mass-produced cheddar cheeses of different brands and ages (Fig. 2).[21] In a further study we differentiated identical-style cheddars of the same age but of different quality manufactured by the same company.[22] By integrating multi-omics datasets much better resolution was obtained, giving more confidence in the results and thus proving (or disproving) authenticity. Other associations were revealed in these studies – for example, levels of phenylalanine correlated positively with the presence of *Thermus* spp. which have been implicated in the pink discoloration of cheese, while cheese cholesterol showed a negative association with *Streptococcus thermophilus*.[20] To our knowledge, this had not been previously reported. Potential cheese age- and quality-related biomarkers were also identified.

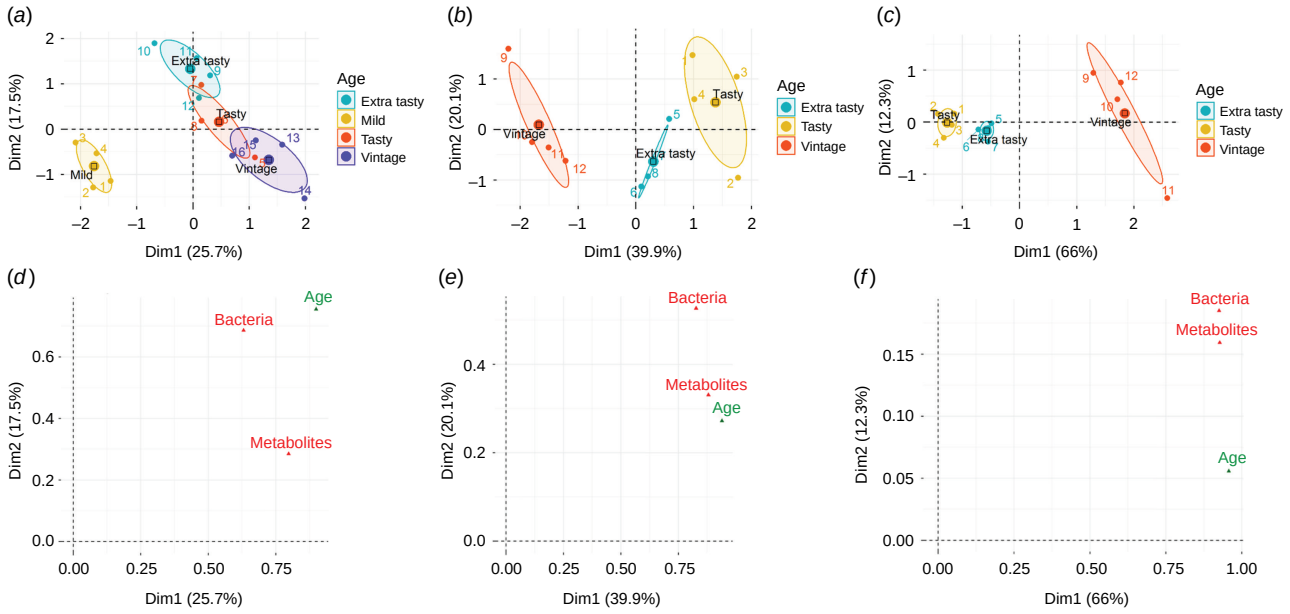

Fig. 2. Multi-factorial analysis of bacterial community composition and metabolite omics datasets obtained from ten similar style Australian cheddar cheeses. Analyses like these enable precise fingerprinting, and hence grouping and identification, of different cheeses, as well as identifying new microbe–metabolite associations. The cheeses represented in this figure are all mass-produced Australian cheddar cheeses of different brands and/or different ripening ages (maturity levels) made by three major local Victorian cheese manufacturers. Adapted from fig. 4 in Afshari *et al.* (2020).[21]

Challenges for multi-omics

Despite the potential of multi-omics to give new insights into cheese ripening, challenges remain. One major problem is cost. The technology is simply nowhere near the stage where it can be routinely applied. Other problems are heterogeneity across the same omics platforms, making data comparisons difficult, also challenges related to the large computational resources needed and a lack of any unified public repository where researchers can access multi-omics datasets.[23] As these limitations are resolved over time, multi-omics will become a major innovation for the food industry.

Prospects for real-time monitoring

Identification of novel biomarkers to predict cheese ripening outcomes, or detailed fingerprints to prove cheese authenticity, will only be useful for the industry if analyses can be done routinely, easily and affordably. Advances in sensor and real-time monitoring technology are bringing this goal closer. Such technologies have wider applicability in terms of achieving higher process efficiency, improved product quality, ensuring food authenticity and provenance e.g. through coupling with blockchain technology, reducing food waste and improving food safety through real-time pathogen monitoring. Some recent examples include biosensors for pathogen detection in food;[24] microbial potentiometric sensors (MPS) technology coupled with appropriate signal analysis tools and methodologies used to monitor kefir fermentation;[25] application of an electronic nose to accurately identify and quantify four yeast species (*Pichia anomala*, *P. kluyveri*, *Hanseniaspora uvarum* and *Debaryomyces hansenii*) in fresh soft cheese;[26] the development of biosensors for analysing fermentation-related parameters,[27] and monitoring the microbial quality of raw milk.[28] The latter is already being done in some parts of the dairy industry.

Together, these observations suggest that we can expect to have new highly sensitive tools for real-time monitoring of cheese quality in the future to complement traditional cheese grading practices. Coupled with the new insights provided by omics and multi-omics, this will lead to better prediction and management of cheese quality, as well as improvements in food safety and in ensuring product authenticity.

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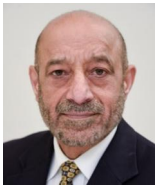
Dr Roya Afshari is an Honorary Research Fellow at RMIT University. She was awarded her PhD from RMIT University in 2020. Her research showed that multi-omics together with data integration analysis represents a powerful new approach for gaining deeper insights into the microbiota–metabolite interactions that underpin cheese flavour and quality. In 2021, she joined a commercial pharmaceutical and food ingredients company in Melbourne. Her research interests include molecular microbiology, metabolomics and use of multi-omics technologies to study food and natural products.



Harsharn Gill is a Professor of Food and Health Biosciences at RMIT University. He has over 25 years of experience in leading and managing food, nutrition and health R&D in the private and public sectors. Before joining RMIT, he held senior R&D leadership roles in Australia and New Zealand. He has received several major awards and has been appointed to international expert panels, including WHO/FAO, NIH and IDF. Professor Gill sits on the editorial boards for several international scientific journals and patent-protected products resulting from his research have been commercialised globally.

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The food microbiological analyst: pairing tradition with the future

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ABSTRACT

Choosing methods for the detection, isolation and identification of foodborne pathogens or spoilage microorganisms from foodstuffs is a complex task. Although there are standard food microbiology methods available around the world, many have changed little in decades, while some tests take many days to perform. The use of automated technologies, genomics and rapid methods are now replacing many traditional tests. Food microbiology analysts need to understand the underlying science and limitations of these methods for food analysis and the crucial importance of validation protocols for correct results reported to customers. This article discusses the current status of food microbiology analysis in commercial laboratories, what the future food microbiology analytic laboratory looks like, and the education changes that may be required to become a future food microbiology analyst.

Keywords: food safety, food spoilage, foodborne disease, genomics, method validation, PCR, rapid methods, WGS.

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Introduction

Food safety is important for all. The WHO has estimated that globally 600 million people get sick from foodborne illnesses each year including an estimated 420 000 deaths and loss of 33 million heathy years of life.[1] Apart from food safety, food spoilage and waste are also global issues. It has been estimated that one-third of all food produced globally is lost or wasted.[2] Not only is a large-scale spoilage issue detrimental to a brand but discarded spoiled food products cause losses in waste of energy input, land resources, water, shipping costs and more.[3] Therefore, the role of the food microbiology analyst is critical in ensuring foodborne outbreaks are solved, food is constantly monitored for microbial safety and spoilage of food is minimised. This has a major effect on people's health, safety, and the economy. However, identification of microbes in food presents many challenges that need to be understood by food microbiology analysts. Food often contains many microorganisms in a complex food matrix. Finding the elusive pathogen that caused a foodborne outbreak or caused spoilage in a batch of food can be like finding a 'needle in a haystack'. Critical decisions need to be made by the trained food microbiologist to ensure the laboratory has the ability to choose the appropriate method to produce accurate, sensitive and specific results that truly reflect the microorganisms present in the food sample submitted for analysis.

Choosing the appropriate method

The decision about which method to use will be based on the food microbiologist asking the right questions such as: what is an appropriate sample size; is the sample homogenous; what is an appropriate subsample for analysis; how consistent are the subsamples; which portion or area of the sample to target or include in the process; and is there matrix interference such as with garlic or spices, that requires inhibition mitigation or extra dilution, due to their antimicrobial effects on the target organisms. Decisions also need to be made if qualitative or quantitative analysis is required based on infective dose and pathogen virulence. An important consideration for qualitative analysis is the type of enrichment performed to ensure enough of the pathogen is present to be detectable in subsequent steps, especially for severe pathogens with a low infective dose. Uniquely in microbiology, the test target can exist in a wide spectrum of viable states, that is, target

cells may be damaged to varying degrees, and this is often related to the effects of food processing. This, in turn, means it is important to consider method choices to ensure maximum recovery of stressed microorganisms, such as using broths versus plates, or adding selective steps later rather than sooner in the method. In addition, the design and use of selective and differential agars can have limitations related to visually separating the irrelevant microbes likely to be present in a food sample from the possible culprit. Measurement Uncertainty (MU) is another issue to be addressed.[4] All routine test methods have MU, and as such, any numerical result should be seen as existing within a range of possible values within which the true value of the measurement lies. This must also be considered when obtaining a result, including the MU overlap of a specified or applied guideline. Numerous methods may be available for the same target organism. Does one method have lesser uncertainty (e.g. measurement of *E. coli* by acid production versus presence of a specific enzyme in chromogenic agars)? It is strongly advisable to consider MU when applying specific guidelines to produced results.

Food microbiology analysts need to be able to identify these issues when developing or implementing new technologies by being well trained in strict validation protocols. Although these questions have been addressed in designing international standards for the detection and identification of a handful of well-documented foodborne bacterial and viral pathogens, the CDC state that researchers have identified at least 250 foodborne diseases caused by a mix of bacteria, viruses and parasites,[5] many of which would not have current validated standard food microbiological methods. This requires future method development addressing many of the questions above and deciding which of the developed and emerging methods might be appropriate.

Automated rapid technologies, genomics and the future

Over the last few decades, analytic food microbiology laboratories have embraced automated technologies using culture-independent diagnostic platforms based on immunological principles or principles of nucleic acid amplification. The advantages of pathogen detection kits that rely on nucleic acid amplification techniques (such as but not limited to PCR) include significantly increased throughput, the potential to combine testing for multiple pathogens at one time, and the ability to detect viable but non-culturable (VBNC) microorganisms and others that are difficult to isolate by traditional culture techniques.[6] Procedures for use of the 'black box' equipment for rapid automated technologies can be quite straight forward with good instructions, but there is still a very important need for training of analysts to understand the underlying science and method limitations. As an example, the sensitivity of a PCR test in foods can be greatly reduced in a complex food matrix, such that a false negative could be reported if the food contains ingredients that are PCR inhibitors.[7] Another implication of automated technologies for the food microbiologist is to be able to change from observing a colony growing on a plate to reading a response, spike, or curve on a screen.

Many medical laboratories now use fully automated culture-independent diagnostic techniques, but most food microbiology analytic laboratories still do many tests using the traditional standard methods supplemented by modern automated methods. This is because microbial analysis of food can be significantly more challenging due to the mix of microorganisms typically present and possible low numbers of target pathogens compared to, say, identification of an infectious agent in normally sterile urine, CSF, or blood. In most cases a negative food pathogen result using the above technologies, such as a PCR test of sample taken directly from an enrichment broth, is enough to report the food sample result as negative and therefore the food as safe or compliant (notwithstanding the overriding importance of eliminating the likelihood of a false-negative); but if the test is positive, it is necessary to go back to the food sample (or at least the enrichment broth) and retest or confirm the result via an approved standard method that relies on isolating viable colonies.[8,9] This retesting or additional analysis is necessary as it proves the food sample contained a living pathogen and not just left-over strands of genetic material from a pathogen captured using genetic analysis. This would lead to a false positive result, when in fact the food processing steps may have been correctly designed to kill any microorganisms in the raw or pre-processed food. Therefore, again, understanding the importance of validation and intended use and consequences of using these methods cannot be understated.

In further advancement, genomic technologies are now rapidly replacing culture methods[6] as this technology advances and the cost of sequencing is continually decreased. Sequencing the genes that are diagnostic for a presumptive positive foodborne isolate growing on an agar plate is now routine in public health laboratories using commercially available sequencing equipment, but the interpretation of the sequencing reads requires people trained in bioinformatics. The advantage of genomics is that it can rapidly detect multiple genes or transcription products, which is invaluable in subtyping bacteria and for collecting epidemiological data. This is now critical for tracking foodborne outbreaks locally, nationally, and internationally in sufficient time to act. As the genomic analysis of SARS-CoV-2 has highlighted, gene sequencing is very important in assessing the evolutionary pathway of strains. In the same way, the sequencing of isolates to link clinical, food and environmental samples is invaluable in providing information about the origin of outbreaks, the path(s) of the pathogen from farm to fork,[6] and ultimately in implementing change to improve food safety in a timely way.

The GenomeTrakr Network is an international collaboration of government, public health and academic laboratories that collect and openly share genomic and geographic data from foodborne pathogens for the benefit of public health. It is the most extensive and best-known application of Whole Genome Sequencing (WGS) to food safety.[10] It includes the U.S. Food and Drug Administration (FDA), Centers for Disease Control and Prevention (CDC), U.S. Department of Agriculture, U.S. National Center for Biotechnology Information (NCBI), state health departments, and international partners.[11,12] It is vital to support public health and for the diagnosis and epidemiology of

emerging pathogens, microbial genome variation and evolution, and new gene discovery.[13]

One recent technology that sits between traditional methods and genomics is matrix-assisted laser desorption-ionisation time of flight mass spectrometry (MALDI-TOF MS). Again, food microbiology analysts who rely on the results of this technology should understand the underlying science and limitations. Its advantages are being useful for screening presumptive pathogenic or spoilage colony isolates as it generates rapid results, is cost effective and easy-to-use. However, identification of the isolated target relies heavily on the database of peptide mass fingerprints containing the spectra of known organisms[14] and while databases are improving, they are not perfect, due to limitations of lack of sufficient spectra in the database and an inability to discriminate between some related species.[15]

There is a need for more rapid and precise methods for microbial food analysis and the use of genomics will eventually become more mainstream. However, this requires ongoing education around sequencing platforms and bioinformatics analysis to be able to correctly interpret the results. These methods, as with any new method including novel molecular methods, will require robust validation to determine the sensitivity, selectivity and in particular, reproducibility, to be applicable in a global framework. The end goal is for regulators, manufacturers and public health authorities to make clear and confident decisions based on these results. However traditional culturing also remains important to determine pathogen viability, for enumeration, and as a proof of experimental concept for data obtained from genomic methods.[6]

Conclusion

Being a food microbiologist is a truly fascinating career path with many strings to its bow, but to ensure the upcoming student who wishes to become a specialist in food microbiological analysis has the widest possible career options, tertiary education must now encompass not only traditional food microbiology analysis but also cutting edge molecular, genomic and associated bioinformatic technologies.

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Vibrio parahaemolyticus: an Australian perspective

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ABSTRACT

A recent outbreak of gastroenteritis caused by *Vibrio parahaemolyticus* following consumption of South Australian oysters has brought to the forefront an increasing hazard for the Australian oyster industry and consumers. Vibrio pathogens have been sparsely implicated in illnesses in Australia; however, rising sea temperatures and varied weather conditions delivered by climate change could be driving the increased prevalence of these pathogens in our oyster growing regions. This is a brief overview of *Vibrio parahaemolyticus*, the risk it presents to our industry and consumers, and an insight into the limitations involved with detection and identification of the pathogen in foods.

Keywords: Australia, foodborne diseases, food safety, microbiology, oysters, qPCR, *Vibrio parahaemolyticus*.

Vibrios as human pathogens

Vibrios are ubiquitous in marine and estuarine environments and the genus encompasses over 140 species, with many involved in symbiotic relationships with marine life.[1] The comma-shaped, Gram-negative bacteria with polar flagella generally present no risk to humans; however, within the genus there are a small proportion, at least 12 species, that are human pathogens.[2] These pathogens are capable of causing infections ranging from ear or wound infections to foodborne illness and sepsis.[2] The most notable of these is *Vibrio cholerae,* a water and foodborne pathogen that is the causative agent of cholera. Presently, however, it is non-choleragenic vibrios causing concerns for the Australian seafood industry. The species of greatest concern in Australia currently is *Vibrio parahaemolyticus*. Some strains of *V. parahaemolyticus* are pathogenic and capable of causing gastroenteritis that is primarily associated with consumption of raw seafoods, particularly oysters.[3] In Australia, oysters are a significant commodity and in 2019–2020 it is estimated that over 11.2 million retail dozens were sold, to a total of over $100 million for the industry.[4]

In many major seafood producing and consuming countries such as the United States, China, Japan and Korea, *V. parahaemolyticus* is recognised as a leading cause of seafood-associated illness.[5–7] Concerningly, the number of infections in many areas appears to be increasing.[7]

Virulence qualities and the changing climate

Like other species within the genus, there is a high degree of genetic diversity among *V. parahaemolyticus* strains and importantly, not all strains are pathogenic. The molecular markers typically utilised to indicate pathogenicity are the thermostable direct haemolysin (*tdh*) gene and the TDH-related hemolysin *(trh)* gene or a combination of both.[8] Clinical isolates have been found to contain these markers approximately 90% of the time whereas environmental or food isolates carry a low likelihood of possessing these genes with only 1–10% of isolates carrying the markers.[9] In terms of distribution in seawater, Vibrio species have an increased presence in coastal tropical areas due to their preference for warmer water.[10] There are also indications that changing salinity levels and algal blooms can contribute to a greater risk of pathogenic vibrio species being present in certain areas.[1]

It is this predilection of vibrios for warm water which has prompted concerns about changing climates and warming sea temperatures.[11] In recent decades, incidences of vibrio-associated illnesses have been occurring in cooler climates where there had previously been no known cases.[12] Studies have revealed an upward trend in case numbers in already affected populations and many have positively correlated large outbreaks to warm weather events.[10] The persistence of this pathogen overseas has prompted surveys in many marketplaces and oyster farming regions to understand its prevalence.[13–15] Models have also been developed, such as the surface sea-water temperature monitoring system by the European Centre for Disease Prevention and Control (ECDC) to enhance risk prediction.[16]

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Vibrio parahaemolyticus in Australia

In Australia, the prevalence of vibrio infections historically has been intermittent, with many of the reported infections linked to overseas travel or the consumption of imported foods.[17,18] However, in the recent decade, outbreaks of *V. parahaemolyticus* infections associated with locally produced seafood appear to have been increasing.[19] The most recent of these outbreaks occurred over November–December 2021, where reports from this time period indicated that over 250 people had fallen ill across multiple states after consuming oysters harvested in Coffin Bay, South Australia.[20] In 2016, there were two separate outbreaks of *V. parahaemolyticus* infections in Australia, one in Tasmania associated with consumption of Tasmanian grown oysters involving 11 cases and one in Western Australia associated with consumption of South Australian oysters involving nine cases.[19] This increase in infections could be correlated to warming sea surface temperatures and weather events, with Australia not being spared effects of climate change.[21] There are other factors that might be contributing to an apparent increase in prevalence and it has been noted there is potential that infections are being under reported due to the bacteria previously not being included in clinical panels.[19] There are also varying State and Territory requirements as to whether *Vibrio* spp. infections are notifiable illnesses which can leave gaps in epidemiological data collection.[19]

V. parahaemolyticus is not a new pathogen; however, its increasing prevalence with many unknown contributing factors does complicate risk management practices. There are few guidelines available for safe levels of *V. parahaemolyticus* in food as the virulence is unclear. Food Standards Australia New Zealand (FSANZ), the Australian statutory authority which develops and maintains the Australian Food Standards Code, provides recommendation for levels of the bacteria in Ready to Eat (RTE) foods.[22] This serves as a guideline only and does not provide impetus for regular screening of seafood products. There are also some state guidelines in place to mitigate risks, such as control plans implemented by Tasmanian state authorities.[23] Despite these controls, there is still much to learn about the distribution of pathogenic strains of *V. parahaemolyticus* in Australian oyster growing areas. This prompts the need for further research to understand variations in the Vibrio populations during weather events and as sea water temperatures rise. Improvements in detection and identification methodologies would also assist in routine monitoring of the bacteria levels during varying weather conditions and assist in the development of appropriate risk management procedures.

Methods of detection and identification of *V. parahaemolyticus*

Questions have also been raised about the suitability of currently recommended methodologies for the detection and enumeration of both total and pathogenic *V. parahaemolyticus* in food. To adopt routine or surveillance testing and ensure testing results are consistent across multiple areas, access to robust, validated methods that are easily adopted by laboratories with a range of capabilities are required. Available methods for use by laboratories are described in a joint document by the Food and Agriculture Organisation (FAO) and the World Health Organization (WHO).[24] The traditional culture-based method of detection for vibrios is laid out in the Bacteriological Analysis Manual (BAM) by the United States Food and Drug Administration (FDA) as well as in the International Standards Organisation (ISO) standard ISO21872-1.[25,26] Briefly, 10–12 oyster specimens are homogenised and weighed into a selective broth for enrichment and then plated onto selective agar. Suspect colonies are then analysed using probes or transferred to non-selective agar for biochemical testing to identify bacterial species. Enumeration can be achieved by combining this method with a Most Probable Number (MPN) protocol to determine counts per gram. Many MPN methods for detection of *V. parahaemolyticus*, including the current Australian Standard AS 5013.18, utilise a three tube technique.[27] This provides a limit of detection (LOD) of 3 MPN/g, which may not be sensitive enough in some applications. There are alternative methods available that can provide far greater sensitivity, such as the US FDA BAM method that utilises a polymerase chain reaction (PCR) MPN method that can quantify down to 0.3 MPN/g. The increased sensitivity confers many benefits and can improve low level detections of pathogenic bacteria.

The MPN technique for quantification is the most accessible for laboratories but carries limitations, including lengthy result turnarounds and numerous confirmation tests for suspect colonies. Confirmation testing is vital for characterising the pathogenicity and virulence of *V. parahaemolyticus* isolates and can include detection of genes which indicate pathogenicity, sequence typing and serotyping. The time to detection and identification is a key aspect for the oyster industry and regulators who require rapid information in order to deliver safe products to the marketplace. Conversely, this method provides the best opportunity for genomic investigation of colonies and for epidemiological tracing. Also of concern with culture-based methods is the potential for bacterial cells to be missed. Some research has indicated difficulties in detecting and identifying *vibrio* spp. in samples, which can be partially attributed to their ability to enter a viable but not culturable (VBNC) state.[28]

The preferred alternative to culture-based methods in terms of rapidity and sensitivity is quantitative PCR (qPCR). For qPCR applications, there are multiple options available in terms of methodologies or commercial kits to use, many using different gene targets and having varying sensitivities. For detection and enumeration of total *V. parahaemolyticus* numbers within a sample, the ISO standard, ISO-21872, utilises *VpToxR* for species level detection and *trh* and *tdh* as molecular markers of pathogenicity.[26] Recent research has indicated the potential for false negative results using the *trh* gene as a marker, due to high sequence variability among strains and suggests an alternative target, a urease gene (*UreR)* which is located directly upstream to *trh* and highly conserved.[29] With such rich genetic diversity among *V. parahaemolyticus* strains and high homology to closely related species, it is important to investigate the most appropriate gene targets for PCR testing. Recent advances in technology have also provided accessibility to previously out of reach techniques, such as whole genome sequencing (WGS) and next generation sequencing (NGS). Utilising these tools to understand more about the genetic diversity of isolates and their pathogenicity will confer great benefits, particularly during outbreaks of illness. As *V. parahaemolyticus* infections increase, so too does the data available, and further work is warranted to determine the most appropriate analyses for detection and identification and to ensure there are standardised methods available to allow for optimal sensitivity and accuracy of testing.

Looking to the future

As the climate continues to change around the world and microbial communities shift with it, further research is required to protect our industries and consumers. Improvement in methods of detection and identification of *V. parahaemolyticus* as well as increased surveillance with epidemiological tracing can ensure Australia's safe enjoyment of oysters for years to come.

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Biography

Claire Hedges graduated with a Master of Biotechnology (Food Science) from RMIT University in 2017 and is currently a PhD candidate researching pathogenic *Vibrios* in seafood products through a collaboration between RMIT University and the National Measurement Institute (NMI). Alongside her studies, Claire works as a Research Development Officer at the NMI and her research interests include facemask testing, molecular microbiology and bioinformatics.

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Biofilms of foodborne pathogenic bacteria: how important are they?

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ABSTRACT

Biofilms are recognised as an important mode of life in bacteria. All species of foodborne bacterial pathogens are known to form biofilms *in vitro* under the right growth conditions. This fact is often extrapolated to claim that biofilms are critical to the transmission of foodborne pathogens, particularly during processing. While this may be the case little direct *in situ* evidence, with some exceptions, is available to confirm this. This is because there are a number of difficulties in studying pathogen biofilms in food processing facilities. The reasons for these issues are discussed by comparison to work in the medical biofilm area, and by using species such as *Listeria monocytogenes* and *Campylobacter jejuni* as examples. A range of potential solutions and avenues for future research are presented.

Keywords: biofilms, *Campylobacter*, foodborne pathogens, food processing, industry, *in situ*, *Listeria,* pathogenic *E. coli*.

Introduction

Biofilms are generally defined as a community of microorganisms attached to a surface or interface, and to each other, and encased in a matrix that they have produced. The matrix consists of extracellular polysaccharides, proteins and extracellular DNA and provides protection from the environment, a nutrient trap and facilitates interaction between cells.[1] Estimates suggest that upward of 40% of prokaryotic life exist in biofilms confirming the importance of this mode of life and the need to understand it better in a range of contexts.[2]

It is widely asserted that biofilms are critical to the ability of bacterial foodborne pathogens, such as *Listeria monocytogenes*, *Salmonella enterica*, pathogenic *Escherichia coli* and thermophilic *Campylobacter*, to move through the food system (particularly during processing) and cause human disease.[3] For this reason, studies investigating biofilm formation by single strains or collections of foodborne pathogenic bacteria in model systems, some mimicking those seen in food processing, are legion (without identifying specific papers a brief search of any database will confirm this). A high proportion of these studies use a micro-titre plate-based (polystyrene) crystal violet assay to quantify the biofilms, sometimes under a range of incubation conditions. In these cases the bacterial strains used (or most of them) demonstrate an ability to form biofilms in the model system. Often conclusions are drawn about their capability (or potential capability) to use biofilms to survive, persist and transmit in food-related environments. Rightly the caveat that further investigations need to be conducted, ideally *in situ* during food production, before any strong conclusions can be drawn is sometimes included. Unfortunately, aside from providing evidence of the wide distribution of the biofilm formation trait, these studies often contribute very little to our understanding of the role of foodborne pathogen biofilms in the food system. The reasons these studies fail in this regard, the difficulties in conducting more relevant studies and possible solutions to this are discussed below.

Why is it difficult to study foodborne pathogenic biofilms *in situ*?

In primary or further processing food facilities, particularly those processing high risk foods, the presence of pathogens is generally monitored for daily using swabs and/or product samples. For example, *Listeria monocytogenes* is monitored for in a range of small goods, dairy, fish, and poultry production facilities. In many of these which produce ready-to-eat largely untreated foods, such as fresh fish, the concerns around biofilms of

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this (and other) pathogens are clearly greater than in others, such as dairy. If positives are encountered corrective actions are taken which may entail stopping production, tracing back potential contamination in raw ingredients and implementing extensive additional cleaning protocols.[4] Product recalls may also ensue. Often these actions will resolve the problem and production will begin again. In the case of *L. monocytogenes*, only if the problem persists and the same strain reoccurs will consideration be given to the potential of a biofilm reinfecting the plant. In most cases tracing the source of the potential biofilms is difficult and doing so requires dismantling equipment, stopping production and specialist testing. In many cases the strain of concern cannot be traced, and *L. monocytogenes* biofilms cannot be confirmed.[5] In the case of other pathogens the situation may be more difficult with single persistent strains not generally the only cause of contamination. A further complication is apparent in that a wide range of potential bacteria can and do form biofilms in processing facilities, including on floors and walls, and in drains, which create a lot 'noise' in locating the pathogen of concern.

A comparison to what may be entailed in establishing the presence of biofilms on a medical catheter, implants or on teeth can give us insights into the issues with food-processing facility related biofilms. Generally, an infection in a patient alerts clinical staff to a potential issue. In the case of catheters or teeth, for example, they may be removed providing a ready source of material to investigate *in situ* biofilms using microscopy and disruptive sampling for molecular techniques. In the case of implants the relatively sterile interior of the human body often means only a single strain or species will form biofilms and cause issues and this strain can be isolated. These approaches can establish without a doubt that a biofilm is contributing to their persistence.[6] In food production only a limited number of foods, such as heat processed dairy products, may provide similar scenarios. However, while heat resistant spore forming spoilage bacteria, such as *Geobacillus*, can be categorically shown to form biofilms in dairy processing pipes,[7] pathogenic bacteria are generally not a major issue in these systems and are controlled by the heat.

The above scenarios highlight the key issues in establishing a role for biofilm formation by foodborne pathogenic bacteria *in situ* during processing. Namely, (1) the availability of samples of equipment and infrastructure to investigate the categorical presence of pathogen biofilms; (2) the complexity of the microbiological populations during processing and the ability to identify pathogens among other species; and (3) the difficulty of establishing if pathogens are part of mixed-species biofilms or simply adhering to them as they might to other surfaces.

What are the potential solutions to conducting more relevant studies?

To establish the importance of biofilms in bacterial foodborne pathogen transmission, and particularly during food processing, some systematic changes in the way they are investigated are required.

The first of these changes is conducting *in vitro* studies which provide information on the ability of pathogens to form biofilms under conditions more relevant to food-related environments. For example, numerous studies examine biofilm formation by *Campylobacter jejuni* at 37°C or 42°C in microaerobic environments in monoculture using micro-titre plates and draw conclusions about their importance in processing. This is not really useful as *C. jejuni* is very unlikely to encounter these conditions in the processing environment.[8] Studies in air, at ambient processing temperatures, and together with other bacteria that form biofilms suggest that *C. jejuni* is far more likely a 'passenger' on surfaces and other biofilms than an active biofilm former or participant in the community.[9]

The second is the wider introduction of in-processing biofilm sampling equipment. Some studies have been conducted by adhering, for example, stainless steel slides onto equipment or in drains which are then removed at particular times. A more satisfactory approach is the design of equipment and infrastructure with removable and replaceable areas or sections which can be routinely monitored as 'sentinels' for the presence of biofilm formation and in particular pathogens forming or associated with the biofilms. The potential for in-line real-time monitoring of biofilms through digital means is a reality in some plants and situations but generally does not indicate the presence or absence of pathogens.[10]

The third is the development of markers for biofilm formation. Differentiating cells that are simply present or transitory from cells that have been growing in a biofilm is critical to understanding the broader role of biofilm formation in foodborne pathogen transmission. This is an area which is receiving a lot of attention in the medical biofilm sphere and in which little work has been conducted in the foodborne pathogen space. The presence of extracellular molecular components produced only in the biofilm matrix, including polysaccharides or extracellular DNA, using mass spectroscopy or other methods, for example, may represent a way to assess if cells are part of, or have been recently associated with, biofilms. Other potential options may include the presence or absence of flagella that are switched on or off in a biofilm. This approach is in its infancy but is likely to grow in importance as techniques for detecting molecules evolve.[11]

The fourth approach is to develop a better understanding of the relationship between foodborne pathogenic bacteria and non-pathogenic microbes that are strong biofilm formers. As indicated above, biofilms in most food processing facilities are unlikely to be monocultures and the complex biofilms that form in drains, for example, may provide environments that allow pathogen biofilm formation. An example of one such group of organisms of wide interest in this context are the pseudomonads. Psychrotrophic *Pseudomonas* species can form extensive biofilms on surfaces and on food themselves. They may also provide environments conducive to the survival of foodborne pathogens such as *C. jejuni*.[9] However, what is not clear is how they interact with pathogens at a physical and molecular level and how this impacts their survival. The advent of 'omics' technologies and more sensitive molecular detection techniques will allow a better understanding of these interactions and provide possible mechanisms to manipulate them to the positive.[12]

Conclusion

In short, the answer posed to the question in the title is that in most cases, with some exception, we don't know. What we do know is that, unsurprisingly, most if not all foodborne bacterial pathogens can form biofilms and occur in processing facilities. We need to move on from re-establishing this to work on understanding if and where biofilms play a role in individual pathogen/food processing combinations. This requires not only a better understanding of biofilms *in situ* but also closer cooperation with industry, both of which have their own challenges.

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Biography

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Are point of management assays relevant for food safety in the poultry industries?

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ABSTRACT

The current pandemic has ensured considerable attention has been paid to the role of the approach termed 'Point of Care' diagnostics. Indeed, the term 'RAT' (Rapid Antigen Test) and RAT hunting now have totally different meaning to that widely understood before 2020. In the veterinary field, including food safety, the term used for these types of rapid *in situ* assays is 'Point of Management' (POM) assays. In this article, we describe our recent research on low cost, low technology, in-house style POM assays in the field of food safety as applied to the poultry industries. We then discuss what are the advantages and disadvantages of these low cost, low technology POM assays.

Keywords: *Campylobacter*, chicken, food safety, isothermal, LAMP, point of management, poultry, *Salmonella*.

Key food safety pathogens for the poultry industries

As with all food production systems, the potential for food safety pathogens to enter the system and cause human illness is a key issue for the Australian poultry industries. Two genera dominate the food safety issues linked to the poultry industries – *Campylobacter* and *Salmonella*.

Over 96 million cases of food-borne illness linked to *Campylobacter jejuni* and *C. coli* are estimated to occur globally each year.[1] The Australian infection rate of 146.9 cases per 100 000 population[2] is one of the highest among the industrialised countries.[3] Importantly, while not the only source of *Campylobacter*, it is recognised that in Australia, undercooked poultry are a major source.[4]

Salmonella is second only to *Campylobacter* as the most notified enteric pathogen in Australia.[2] As with *Campylobacter*, while there are multiple sources of human infections, raw and undercooked foods, eggs, and to a lesser degree poultry meat, are often associated with *Salmonella* infections.[5]

Food-borne pathogens impose costs onto both broad society as well as the production system. While figures are not available for Australia, a recent report estimated that campylobacteriosis costs the US (population >10 times that of Australia but with 10 times lower *Campylobacter* infection rate[3]) in the range of US$1.5–US$6.9 billion per year depending on the calculation method.[6] The reduced income and increased expenses associated with *Salmonella* in the Australian egg industry were estimated to cost $7 million annually in 2015–2016.[7] The scale of these economic impacts to both broad society as well as the producers emphasises the need for improved control of food-borne pathogens in the poultry industries.

POM assays

Our work in the development of POM assays arose from the finding that dipsticks made from untreated cellulose-based paper can bind nucleic acids in seconds, retain them during a rinse step that removes the contaminants and then release the nucleic acids when placed in a reaction buffer.[8,9] We have combined the low-cost DNA extraction technology with isothermal amplification performed in another existing innovation – the 'Diagnostic Droid'.[10] The workflow involves a centrifugation of a 2 mL aliquot of carcass rinse, a treatment of the pellet with proteinase K and heat denaturation to release the DNA. Two dipsticks are added to bind the DNA and purify it away from the contaminants. Each dipstick is then given a single wash and the DNA eluted into a loop mediated

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Fig. 1. Work flow for the *Campylobacter* Point-of-Management assay. A 2 mL aliquot of the carcass rinse is centrifuged (5 min) and the pellet resuspended, treated with proteinase K and heated to lyse cells and release DNA. The two cellulose dipsticks are added to the crude lysate to bind the released DNA. The dipsticks are then placed in a wash buffer and then in the LAMP reaction mix (one for *C. jejuni* and one for *C. coli*). The LAMP reactions proceed for 100 min at 65°C in the 'Diagnostic Droid'. The Droid monitors the reaction and interprets the result.

isothermal amplification (LAMP) reaction mix for either *C. jejuni* or *C. coli*. The 'Diagnostic Droid' performs the amplification step and monitors the increase in turbidity associated with a positive LAMP reaction by illuminating the reaction tubes with an LED and measuring the amount of scattered light via a light sensor perpendicular to the LED light source. The results are automatically interpreted with no need for human involvement and are available within 2.5 h[10] (see Fig. 1 for an illustrative workflow of our POM for *Campylobacter*). The Australian chicken meat industry has a self-set target of ensuring that all chicken carcasses have less than 6000 colony forming units (cfu) which is equivalent to 12 cfu/mL in the 500 mL volume used to wash the carcass. The performance of the POM assay was evaluated using 29 rinse samples that were examined by the relevant Australian Standard method[11] (see Fig. 2 for an illustration of the work required by this Standard) as well as the POM assay.[10] A total of 26 samples were in agreement – 16 were high in the POM assay and contained more than 12 cfu/mL and 10 were low in the POM assay and had <12 cfu/mL in the culture method. While three samples gave different results in the two assays (i.e. an 11% disagreement), only two samples (i.e. 7% of samples) were a major disagreement where the POM assay result was low and the culture result was above 12 cfu/mL. It is worth noting that these two disagreements occurred on the edge of the 12 cfu/mL cut-off – being 23 and 37 cfu/mL. All samples above 45 cfu/mL in the culture method were positive in the POM assay. Overall, this work has shown the potential for a very low-cost POM assay that uses little in the way of technology to be used to semi-quantify the level of *Campylobacter* in chicken carcass rinses.

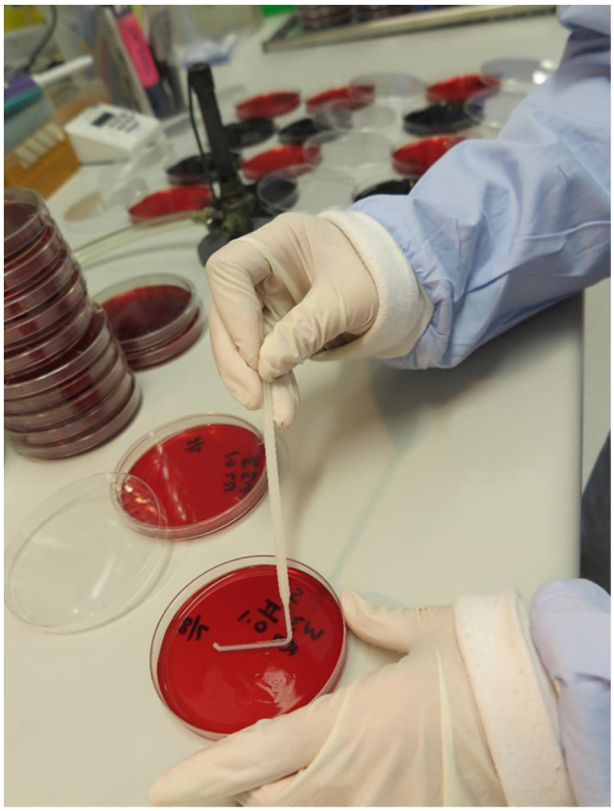

Fig. 2. Part of the work flow for conventional Australian standard methods for *Campylobacter*.

Who pays?

In evaluating the advantages/disadvantages of a POM assay there is a critical primary issue that first needs to be addressed – who pays? In a situation where a producer has a disease problem on farm, the solution of a vaccine that controls the disease results in an extra cost but returns an increased profit via less mortalities or an improved growth rate. In these circumstances, the producer sees a direct financial benefit from a new intervention or a new management tool. In contrast, producers or processing companies that introduce an intervention to reduce the level of *Campylobacter* have the cost of that intervention but no direct financial return. Society would benefit from the intervention with a lower level of campylobacteriosis in the population but the producer/processor bears the costs and gains no financial benefit. Hence, any interventions to increase food safety (such as POM assays to provide near to real time information on *Campylobacter* levels on carcasses) must be implemented with an understanding that society will benefit but that there will be no immediate financial benefit to the individual producer or processor. Clearly, an industry providing food to the population benefits from a public perception that they provide a high-quality product that is safe and healthy. The public expects safe and healthy food but often does not

understand the associated financial burdens. Hence, adoption of POM assays for food safety pathogens has to occur in a situation where no immediate benefit flows from the cost of adopting the technology.

Advantages of POM assays

In evaluating the advantages of the type of POM assay we have developed, our vision is that these assays are not seeking to replace the Australian Standard method[11] but rather add to the quality and relevance of the formal testing provided by the standard methods. The POM assay we have developed is as sensitive as the gold standard of culture but gives results in a little over 2 h vs 2 days required by the standard culture method. Hence, quality assurance/quality control staff can implement routine rapid, low-cost regular monitoring of the effectiveness of the processing chain in achieving the industry set target of <6000 cfu of *Campylobacter* per carcass. Given that there are around 50 processing plants for chickens in Australia and that over 663 million birds were processed in 2020, the need for cost-effective, low technology, rapid assays suitable for both the large multi-state processors as well as the much smaller state and regionally based processors is clear in our view. A POM assay would allow refinements and alterations in production parameters to be rapidly monitored for their impact on *Campylobacter* levels. POM assays should not be seen as replacements for the nationally certified standards but rather supplementary, additional tests that support the same goal – the production of a high quality, safe food product.

Disadvantages of POM assays

POM assays suffer the same generic problem that all food safety interventions suffer as outlined above (i.e. they are an additional cost). However, it should be noted that the cost of our POM approach is far cheaper than the currently available commercial instruments and assays.

There is a general acceptance that POM assays are not as sensitive as laboratory-based methods – as clearly shown in comparisons of RATs and RT-qPCR for COVID-19.[12] Our work is, at this stage, still too early to provide firm evidence about the relative sensitivity of culture and our POM assay. However, it should be noted that the three samples in disagreement all involved viable counts of less than 40 colonies on the two counting plates. As the reliable counting range for plate counts is 25–250 colonies,[13,14] it is clear that the disagreements occurred in a range where both technologies (plate counts and POM assay) were struggling to detect very low numbers of *Campylobacter*.

Conclusion

The importance of reducing the levels of *Campylobacter* on chicken carcasses for public health is well established. A European study has shown that a 2-log reduction in the number of *Campylobacter* on carcasses would result in a 30 times reduction in the incidence of campylobacterosis.[15] In our view, access to low cost, low technology, rapid POM assays is an essential requirement to ensure the level of monitoring required to achieve either the 2-log reduction suggested by the European study[15] or the industry-assigned target of 6000 cfu per carcass.

While this article has focussed on *Campylobacter* and chicken carcasses, there are other areas where POM assays for food safety pathogens could be effective tools e.g. in detecting the presence of *Salmonella* on layer farms. Few layer farms currently engage in *Salmonella* tests due to the costs of the assay, the delay in obtaining results and the distance from the farm to the laboratory. POM assays for on-farm detection of *Salmonella* would remove many of those barriers and could encourage a far more proactive quality assurance program.

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Data availability. Data sharing is not applicable as no new data were generated or analysed during this study.

Conflicts of interest. The authors declare no conflicts of interest.

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Biographies

Jillian Templeton is a microbiologist working for the Queensland Department of Agriculture and Fisheries. Jillian is a mid-career researcher who has led projects over the past 20 years to investigate the epidemiology of *Campylobacter* on Australian meat chicken farms; the development, validation and application of genotyping methods for *Campylobacter*; and the development of rapid diagnostic techniques for *Campylobacter*.

Jimmy Botella is professor of Biotechnology at the University of Queensland. Jimmy has eleven international patents in the field of Biotechnology, has founded two biotechnology companies and is a member of the Expert Scientific Panel for the Agricultural Biotechnology Council of Australia. Jimmy's research has been awarded with the Chinese Academy of Sciences Visiting Professorship for Senior International Scientists and the title of 'Pathologist of Distinction' by the International Society of Plant Pathology.

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Impact of photodynamic inactivation on microbial safety in foods

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ABSTRACT

Food-borne diseases caused by contaminated food products continue to pose a threat to public health, as well as causing major economic losses and a negative impact on companies' reputation among consumers. In the food industry, inactivation of pathogenic and spoilage microorganisms is conventionally performed through thermal- and chemical-based techniques, which can affect the nutritional and sensorial quality of food. Furthermore, the emergence of microbial resistance to conventional decontamination techniques has drawn increased attention to finding an alternative and sustainable approach for similar or higher decontamination efficiency. Over the past decade, photodynamic treatment has been introduced for inactivating food spoilage and pathogenic microorganisms as a promising cost-effective, chemical-free, environmentally friendly technique with no reports on toxic residues and microbial resistance. The application and efficiency of photodynamic treatment in various food matrices against a broad range of microorganisms demonstrates the potential of using this technology in the food industry.

Keywords: antimicrobial treatment, curcumin, food preservation, food safety, green technology, photodynamic, photosensitiser, reactive oxygen species.

Introduction

The key element in achieving good health and sustaining life is access to nutritious and safe food. According to the World Health Organization, over 200 different diseases with severity ranging from diarrhoea to cancers can be caused by unsafe food consumption, which contains harmful microorganisms or chemical substances.[1] Food safety is generally influenced by the growing world population, climate change, and globalisation of food trade. Therefore, it contributes greatly to global food and nutrition security, as well as to national economies.

Food industry traditionally uses conventional thermal-based processing such as dry-heating and steam-heating to reduce the microbial contamination of foods caused by vegetative cells, spores, and biofilms. However, this practice sometimes suffers from undesirable impacts on flavour, nutritional composition, and texture of treated foods.[2] Various non-thermal processing technologies such as ultraviolet light, irradiation, ultrasound, ozonation, cold plasma, pulsed electric field, and high hydrostatic pressure have been introduced to the food industry to reduce the microbial load while retaining the natural colour, flavour and nutrition. Nevertheless, the wide application of some of these non-thermal decontamination technologies is limited by strict processing conditions, expensive equipment, high energy consumption, and the emergence of microbial resistance.[3]

One of the recently introduced non-thermal decontamination technologies to the food industry is photodynamic treatment, which is also known as photosensitisation. For several decades, photodynamic treatment has been investigated and used for medical and dental purposes to treat tumour/cancerous cells and as antibacterial/antibiofilm treatment. Reactive oxygen species (ROS) are produced during the photodynamic treatment, which only requires the presence of oxygen, a photosensitiser, and light (at photosensitiser's λ_{max}). Photosensitisers become excited on illumination and generate cytotoxic ROS through subsequent de-excitation and collision with the surrounding oxygen molecules[4] (Fig. 1). The produced ROS exhibit a multi-target attack towards different intracellular components of microorganisms present in the food, such as their proteins, lipids, and nucleic acids, resulting in cellular death. Therefore, because of direct and non-selective oxidative damage to essential biomolecules required for cell integrity and function, there is also a low

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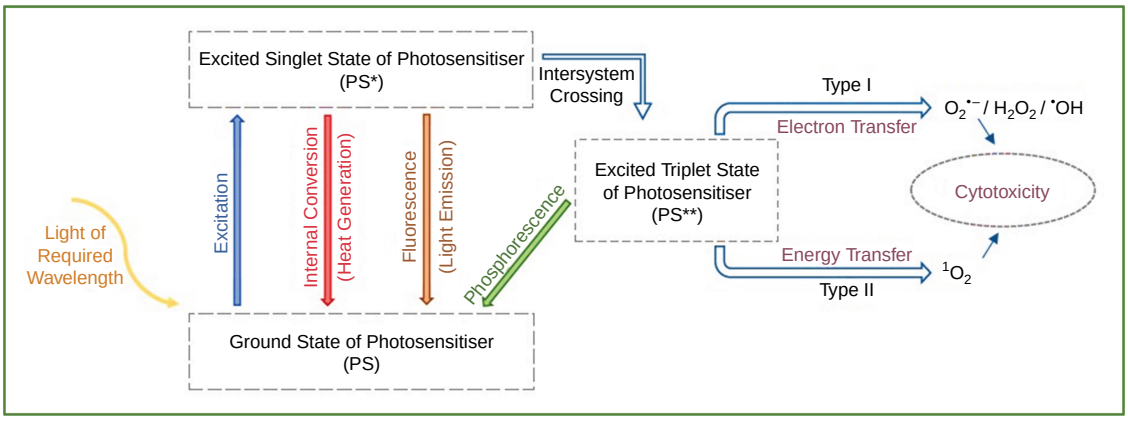

Fig. 1. A schematic overview of the photodynamic process.[6]

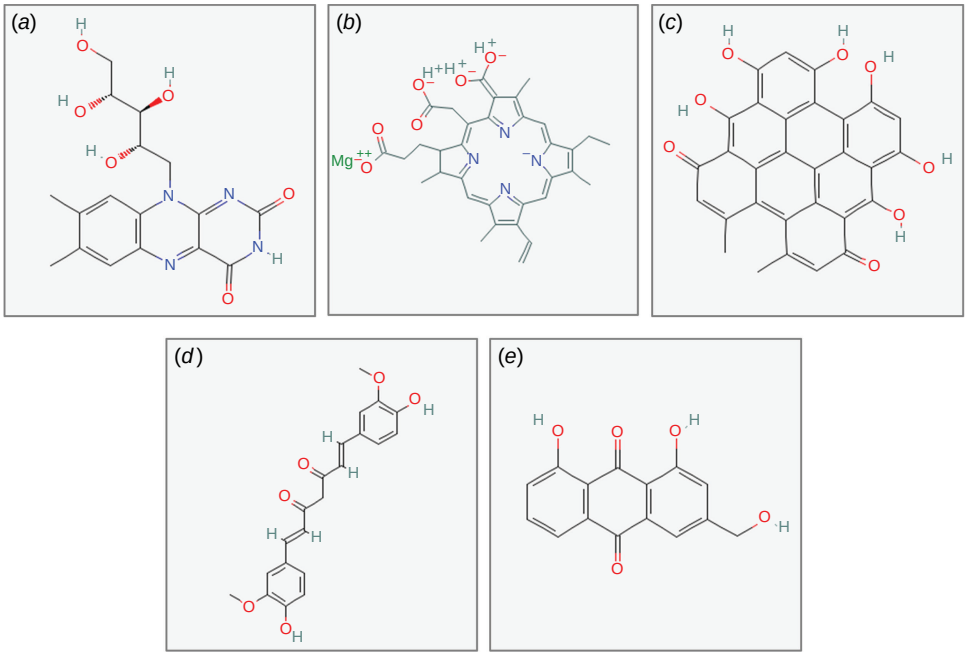

Fig. 2. Examples of natural photosensitisers: (*a*) riboflavin, (*b*) chlorophyllin A, (*c*) hypericin, (*d*) curcumin, (*e*) aloe-emodin.[7]

probability of the emergence of microbial resistance using photodynamic treatment.[5] Another advantage of this treatment is being chemical free that is in line with the growing demand for 'clean label' food products.

Generally, photosensitisers can be endogenous such as porphyrins, which already exist within some fungal and bacterial cells, or exogenous such as curcumin, chlorophyll, and riboflavin isolated from plant material (Fig. 2). However, the poor water solubility of some potent photosensitisers such as curcumin can limit the wide application of the treatment on different food products. This can be overcome with the aid of encapsulation technology using hydrocolloids isolated from natural resources. This results in better bonding of the photosensitiser with microbial cells and better accumulation in the vicinity of target cells and therefore an enhanced photodynamic antimicrobial effect. Furthermore, another feature of this photodynamic treatment is its low energy requirement and no toxic by-products or residue generation,[5] thus making it a safe and eco-compatible technology.

Photodynamic application for food decontamination

In 2004, Lukšien and colleagues explored the application of innovative and promising photodynamic inactivation for microbial food safety. The authors reported a complete *in vitro* photoinactivation of common food crop spoilage fungi, namely *Rhizopus oryzae*, *Aspergillus flavus*, *Trichothecium roseum*, and *Fusarium avenaceum*, using hematoporphyrin dimethyl ether as a photosensitiser.[8] Further studies have since shown the efficiency of photodynamic treatment in inactivating a wide variety of food spoilage and pathogenic microorganisms (i.e. vegetative cells, spores, biofilms) such as *Listeria monocytogenes*, *Salmonella enterica*,[9] *Vibrio parahaemolyticus*,[10] *Enterococcus faecalis*,[11] *Pseudomonas fluorescens*, *Shigella flexneri*,[12] *Staphylococcus aureus*,[13] *Bacillus cereus*,[14] *Candida albicans*, *Aspergillus niger*, *Penicillium griseofulvum*, *Fusarium oxysporum*, *Zygosaccharomyces bailii*,[15] *Aspergillus flavus*,[16] and *Botrytis cinerea*.[17]

Several studies have shown that photodynamic treatment has the potential to be applied on a variety of food products including fruits and vegetables, seeds and grains, meat and aquatic products, and juices. This also has the advantage of minimal influence on nutritional and sensorial properties compared to conventional thermal processing technologies. A few examples of decontamination efficiency of photodynamic treatment of various food matrices include reducing the population of *V. parahaemolyticus* on cooked oysters,[18] of *L. monocytogenes* on smoked salmon[19] and on fresh-cut pears,[20] of *E. coli* on fresh-cut pineapple,[21] of *Staph. saprophyticus* on fresh dough sheet,[22] of *P. fluorescens* on Minas Frescal cheese,[23] of *A. flavus* on maize kernel and flour[24] and on peanuts,[25] and of *Botrytis cinerea* on apples.[26] The use of photodynamic treatment in disinfecting the water in fish farming was also suggested by Wohllebe and colleagues. The authors successfully decontaminated the water containing larvae (of human pathogenic parasites) using low concentrations of chlorophyll acid[27] before introducing the fish. They further suggested chlorophyll-mediated photodynamic treatment as a practical and inexpensive treatment for controlling ectoparasites such as *Ichthyophthirius mulftifiliis* in fish.[28]

Furthermore, encouraged by promising antimicrobial efficacy of this treatment, photodynamic-mediated food packaging films have been recently investigated. The photosensitiser is embedded in the film forming matrix to add the antimicrobial activity to the food packaging material. Examples of photodynamically active food packaging are riboflavin-incorporated chitosan-based film as a salmon packaging material, with the ability to reduce *L. monocytogenes*, *V. parahaemolyticus* and *Shewanella baltica* populations,[29] carbon nitride-incorporated konjac glucomannan-based film as a cherry tomato packaging material with the ability to inhibit *E. coli* and *Staph. aureus* growth,[30] curcumin-incorporated cellulose laurate-based film as a pork packaging material with the ability to reduce *Staph. aureus* population,[31] β-cyclodextrin/curcumin complex-incorporated 2,3-dialdehyde cellulose-based film as a salmon packaging material with the ability to inactivate *L. monocytogenes*, *V. parahaemolyticus*, and *Shew. putrefaciens*,[32] and aloe emodin-incorporated poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)-based film as a packaging material for fresh-cut papaya, pork belly and pork bologna with the ability to inactivate *E. coli*.[33] It is also possible that the photosensitiser-incorporated food packaging material may improve the shelf life of the products by being exposed to artificial lighting in the retail stores. However, this needs further investigation.

Summary

In general, various studies have shown the efficiency of photodynamic treatment as a naturally based, cost effective, clean label and eco-friendly decontamination technique. The key benefit of this technology is that it is a non-thermal process and retains the nutritional and organoleptic qualities of food. It is easily scalable and can be implemented in the food industry as it can operate using an existing conveyor system. It does not require expensive equipment and the materials used such as curcumin and visible light are already approved and being used in the food industry. It is effective against a wide range of food related microorganisms including viruses, bacteria, yeasts, and moulds.

Besides the diverse applications of photodynamic treatment in post-harvest and processed foods from plant or animal sources it has applications in decontamination of fruit and vegetable washing water, making it attractive for adoption by industry. It can also be investigated as an antibacterial, antifungal, and possibly pesticide treatment for the photosensitiser to be applied directly on fresh produce and be photoactivated with the aid of artificial lighting for example in greenhouses, where the intensity of light and time of exposure can be controlled. However, the efficiency of the treatment is dependent on the processing conditions such as photosensitiser concentration, light dose, and wavelength, as well as the food matrix properties. Optimising and validating the treatment conditions, including photosensitiser formulation and illumination dosage, are the future challenges to effectively implementing the treatment in the food industry to replace the conventional decontamination techniques, while maintaining similar or higher decontamination efficiency.

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Rising stars in the bakery: novel yeasts for modern bread

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ABSTRACT

Bread is a widely consumed fermented food whose taste, aroma, and texture are partly determined by the choice of microbe(s) employed in dough fermentation. Consumer preferences and dietary considerations are currently changing; in addition to a desire for novel, complex flavour profiles, foods low in gluten and fermentable oligo-, di-, monosaccharides and polyols (FODMAPs) are becoming increasingly important. The potential of non-conventional yeasts to improve and diversify key aspects of breadmaking is highlighted in this mini-review. Researchers have investigated species from the genera *Kazachstania*, *Kluyveromyces*, *Lachancea*, *Pichia*, *Torulaspora*, and *Wickerhamomyces* to this end. Some species have demonstrated comparable leavening capacity to baker's yeast, as well as improved tolerance of baking-related stresses such as high salt and low pH conditions. Others have demonstrated valuable functional properties permitting the degradation of gluten and FODMAPs. Future research directions include the establishment of safe use status and the improvement of novel yeasts' baking traits through techniques such as evolutionary engineering.

Keywords: aroma, bread, fermentation, FODMAP, gluten, leaven, non-conventional yeast, sourdough.

The microbial fermentation of bread dough leads to the production of CO_2 and other metabolites that give bread its characteristic texture and aroma. Today, the chief microbe used by humans in this endeavour is *Saccharomyces cerevisiae*, also known as brewer's or baker's yeast. Its domestication to food environments occurred long ago,[1] and it has been the dominant breadmaking organism since the advent of purified *S. cerevisiae* monocultures in the 19th century.[2] Despite its predominance, *S. cerevisiae* has several major drawbacks as a fermenting yeast, namely its limited use of only a few carbon substrates and an inability to withstand certain stresses associated with baking, such as osmotic, oxidative, temperature, and ethanol stresses.[3] The range of nitrogen sources the yeast is able to assimilate is also relatively limited, due to a whole-genome duplication (WGD) event affecting several genera within the Saccharomycetaceae.[4] This may be due to WGD-driven gene partitioning: copies of nitrogen assimilation genes that each perform only a subset of an ancestral gene's function.[5] It is also likely that most *S. cerevisiae* strains used for breadmaking arose from interbreeding of an ale and wine strain, and while this has advantages for the consistency and speed of the fermentation process, it limits the taste and aroma complexity of the final products.[6] Therefore, there is a desire to seek out diverse breadmaking yeasts that can be used to make baked goods with improved technological and organoleptic properties, as well as those that can cater to the gluten-free and low-FODMAP (fermentable oligo-, di-, monosaccharides and polyols) demands of modern consumers.

There are many yeast species from the family Saccharomycetaceae (to which *S. cerevisiae* belongs) that are found in food environments. Non-*S. cerevisiae* yeasts are involved in the production of cocoa, kefir, fermented vegetables, wine, and beer.[7] Bread was historically leavened using traditional sourdough starters: flour and water mixtures that are left at room temperature for several days to ferment 'spontaneously', i.e. without a starter culture.[2] This practice continues today, and sourdough microbial composition – specifically unique functional properties of constituent microbes – continues to be an active field of study. A large proportion of non-*S. cerevisiae* yeasts are commonly found in sourdough starters, and recent research suggests that the fungal diversity of sourdoughs may be greater than previously thought.[8]

Mature sourdough starters tend to contain only one or two yeast species. The most common non-*S. cerevisiae* yeasts found in sourdoughs are *Kazachstania exigua*, *K. humilis*, *Candida glabrata*, *Torulaspora delbrueckii*, *Pichia kudriavzevii*, and *Wickerhamomyces*

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anomalus.[9,10] Novel non-conventional yeasts continue to be isolated from sourdoughs, such as *K. saulgeensis* which was first described in 2016.[11] It is important to note that while *S. cerevisiae* is also found in many sourdoughs, it remains unclear whether this is due to contamination from purified baker's yeast often used in the same bakeries[12] and industrial contexts or due to its autochthonous presence there. Sourdough yeasts occupy an environment described as 'specific and stressful' due to cereal dough forming an environment with low pH, low oxygen tension, and carbohydrates (mainly maltose) needing to be shared with fermenting lactic acid bacteria.[10] Sourdough starters are therefore a significant source of novel yeasts with interesting applications.

In addition to using sourdoughs themselves as reservoirs of novel breadmaking yeasts, the original sources of sourdough microbes (such as soils, plants, and insects) have yielded non-conventional yeasts of interest. Madden *et al.*[13] found that from a pool of yeast strains isolated from sugar-seeking insects, thirteen from the *Candida*, *Lachancea* and *Pichia* genera were able to produce bread loaves of comparable quality to those made with baker's yeast. Furthermore, it was found that some of the *Lachancea* strains isolated could grow in osmotically challenging conditions, meaning that isolates of this genus may be suitable for growth as purified monocultures on an industrial scale. Potentially, 'bioprospecting' in non-food environments could yield novel food fermentation organisms.

It is frequently observed that the taste, texture, and aroma profiles of sourdough bread are different to those of 'straight' dough breads produced with purified baker's yeast. While some of this difference must be attributed to the different process parameters involved in making sourdough bread and the presence of lactic acid bacteria in the sourdough ecosystem, attention has now turned to sourdough yeasts as a source of valuable aroma volatiles in bread. In fact, yeast metabolism has been reported to be the main source of aromatic diversity in fermented foods such as alcoholic beverages and bread.[14] When used as the sole fermenting yeasts in bread dough, *Wickerhamomyces supelliculosus* and *Kazachstania gamospora* were found to produce unique aromatic compounds.[15] These compounds may include volatile esters, associated with a fruity aroma, which have been reported in increased amounts when novel yeasts are used in bread fermentation.[6] A co-culture of *S. cerevisiae* and *T. delbrueckii* was found to improve production of succinic acid and acetic acid (Fig. 1) in steamed bread compared to dough fermented with mono-cultures.[16] Interestingly, when a strain of *S. cerevisiae* that had been isolated from an Australian sourdough was used to ferment bread dough, the resulting bread had a distinct, different chemical aroma profile compared to that made with commercial baker's yeast.[17] This suggests that even for *S. cerevisiae*, the derivation of yeasts from a sourdough environment may be related to important aroma- and flavour-generating properties.

Gluten and FODMAP contents in bread are, for those with coeliac disease, irritable bowel syndrome,[18] and other gastrointestinal disorders, significant obstacles to the consumption and enjoyment of bread. Recent research has shown that non-conventional yeasts may play a key supporting role in the predominantly LAB-mediated degradation of gluten in sourdough (Table 1). The presence of *T. delbrueckii* in co-culture with bacterium *Pediococcus acidilactici* was shown to enhance the latter's protein metabolism and accelerate its ability to degrade proteins.[19] Additionally, although not considered bakery yeasts, fungal proteases derived from *Aspergillus oryzae* and *A. niger* have been used in conjunction with sourdough lactobacilli to initiate primary hydrolysis of wheat proteins. Ultimately, these enzymes could detoxify wheat flour.[20] Interestingly, growth on a synthetic gluten-limited medium showed that strains of *W. anomalus* could be classified as 'gluten-degrading' in their own right, and that the extent of gluten degradation varied slightly between individual strains.[21] FODMAPs are a class of small, osmotically active carbohydrate molecules. These properties mean that they are not well absorbed in the small intestine and pass into the large intestine, where they undergo rapid bacterial

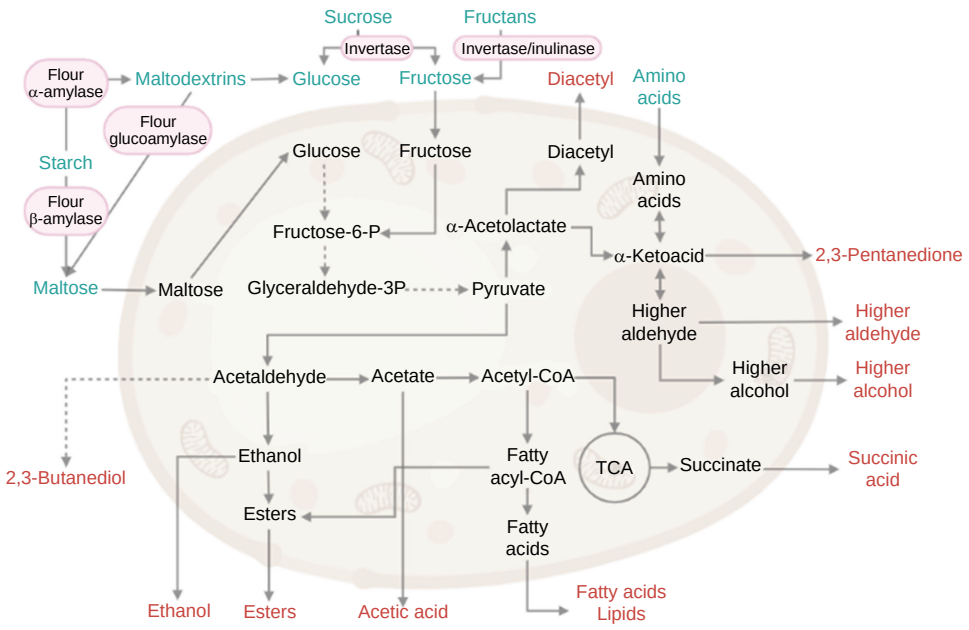

Fig. 1. Metabolic pathways of fermenting bread yeasts. These are well understood in *S. cerevisiae*, but may differ in non-conventional yeasts (e.g. *Kluyveromyces* spp.), in which they are poorly characterised. Green labels represent substrates, red labels represent secreted metabolites and pink bubbles represent extracellular enzymes. Dotted arrows indicate omitted metabolic steps. Based on De Vuyst *et al.* 2016.[10] TCA, tricarboxylic acid cycle. Created using BioRender.

Table 1. Non-conventional yeasts and their attributes and applications in breadmaking.

Species	Notable functions				Reference
	FODMAP degradation	Aroma volatile production	Stress tolerance	Gluten degradation	
Kazachstania bulderi		+ (as co-culture with *Lactobacillus plantarum*)			17
Kazachstania gamospora		+	+		15
Kluyveromyces marxianus	+	+			23
Lachancea fermentati			+		13
Saccharomyces bayanus (hybrid)		+			6
Torulaspora delbrueckii		+		+ (as co-culture with *Pediococcus acidilactici*)	6, 16, 19
Wickerhamomyces anomalus				+	21
Wickerhamomyces subpelliculosus		+	+		15

fermentation, which in turn causes abdominal swelling and luminal distention.[22] Novel yeasts derived from sourdoughs have been shown to have the enzymatic capabilities to break these carbohydrates down, notably fructans. As an example, *Kluyveromyces marxianus* was found to be able to degrade >90% of the fructan component of whole wheat bran, due to its ability to produce inulinase.[23] Struyf *et al.* have also demonstrated the successful use of co-cultures of *K. marxianus* and *S. cerevisiae* to ensure sufficient CO_2 production, producing bread with adequate loaf volume and ≤0.2% fructan content.[24] Non-conventional yeasts, both as mono-cultures and co-cultures, might therefore present an attractive opportunity to create high-quality bread that caters to the dietary needs of modern consumers.

An additional notable aspect of novel bakery yeasts is their capacity to form associations with bacteria. This is important in fermented food products such as kefir and kombucha that rely on a consortium of both yeasts and bacteria to produce their characteristic properties. These co-cultures have been shown to have interesting and valuable effects on final food products that their constituent monocultures are not capable of producing. When inoculated in bread dough as co-cultures alongside sourdough-derived lactic acid bacteria, novel sourdough-derived yeasts are capable of producing distinctive aroma profiles (with predominant *sour* aromas) and crumb structures preferred by sensory panels.[17] It is likely that interactions between yeasts and lactic acid bacteria affect the manner in which the bacteria use carbohydrates to produce metabolites.[25] It has been suggested that the oft-documented association between *Kazachstania humilis* and the sourdough heterofermentative LAB *Fructilactobacillus sanfranciscensis* may be driven by cross-feeding, as maltose metabolised by *F. sanfranciscensis* into glucose may provide a source of nutrition for the maltose-negative yeast.[26] Although the molecular mechanisms underpinning this interaction are yet to be fully elucidated, the frequent detection of established yeast-bacteria pairs in food environments suggests that there is a strong natural tendency for such partnerships to form.

Ongoing research is required to render novel non-conventional yeasts suitable for baking, especially in an industrial context. For instance, *Kluyveromyces marxianus* cannot ferment maltose,[23] so it requires added sucrose or an enzyme (i.e. amyloglucosidase) to release glucose from amylose to produce sufficient CO_2 to fulfil its leavening requirements. This emphasises the need to consider the metabolic demands of novel yeasts, and to consider whether they might function best as co-cultures, or in conjunction with certain substrates or enzymes. Co-cultures of yeasts or yeasts in combination with bacteria present an attractive area for future research into the applications of novel yeasts in breadmaking. Particularly for yeasts derived from non-food environments, safe usage status (generally-regarded-as-safe or qualified presumption of safety status) must also be established[15] before they can be approved for use on an industrial scale.

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Exploring lactic acid bacteria diversity for better fermentation of plant-based dairy alternatives

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ABSTRACT

Plant-based foods have risen in popularity in recent years including a number of dairy alternative products. Fermentation has the potential to support the development of innovative plant-based foods with enhanced flavour, texture and nutritional quality. Lactic acid bacteria (LAB) have been used for thousands of years to carry out fermentation of a wide variety of food substrates through production of organic acids and flavour compounds. However, LAB strains used in dairy fermentations are commonly found to be suboptimal in their metabolism of plant substrates, so efforts to identify alternative strains are needed. We provide an overview of the plant-based milk alternative category and explore screening approaches (including citizen-science efforts) to identify new LAB that hold potential in acidification and flavour formation of plant-based substrates.

Keywords: almond, alternative protein, citizen science, dairy-alternatives, fermentation, flavour, lactic acid bacteria, new foods, plant based.

The rise of plant-based foods and the non-dairy category

Consumer interest, preference, and market share for plant-based foods has grown significantly in recent years. This has been due to greater health conscientiousness in consumers, concerns around animal welfare and environmental impacts of greenhouse gas emissions, health-related issues such as dairy allergies and lactose intolerance, as well as the increased promotion of plant-rich diets and interest in veganism.

Plant-based food products include natural (fruits and vegetables) or processed (plant-based meat and milk alternatives). As milk is one of the most widespread and nutritious food sources, its plant-based alternatives have also seen high demand over the past decade.[1] This article will focus on plant-based milk alternatives (PBMA) and explore the potential of fermentation to enhance their flavour, texture, and nutrition.

In 2021, the worldwide PBMA revenue was US$19 billion.[2] The most common sources of PBMA include soy, almond, coconut, rice, and oat, with others including macadamia, walnut, pea, banana, and flax being less common. In Australia, the most popular PBMA currently is soy-based; however, demand for almond PBMA is growing rapidly.[3,4] The PBMA market share captured by almond-based PMBA increased from 18% to 44% between 2015 and 2020, while soy-based PBMA reduced from 69% to 48% in the same period. Australia is the world's second largest producer of almonds with ~$1 billion grown each year, and almonds are our most valuable horticultural export,[5] making this an attractive target for fermented PBMA product development.

In addition to being naturally lactose-free, PBMA contain bioactive compounds that are absent or have low bioavailability in cow milk, such as dietary fibres, antioxidants and phytoestrogens.[6,7] Furthermore, legume- and seed-based PBMA are promising plant-based alternative protein sources.[8] However, PBMA can have several disadvantages including undesirable flavour profiles (e.g. beany flavour in soymilk), a less comprehensive nutritional profile compared with cow milk, anti-nutritional compounds and potential allergenic activity. Fermentation is one potential option that can be investigated to improve PMBA nutritional properties and sensory attributes, and reduce allergenicity.[9]

Lactic acid bacteria (LAB) and their potential in PBMA fermentations

The most common group of bacteria used in food fermentations are LAB. For industrialised (controlled) fermentations, defined LAB strains are used as inoculants. LAB can be grouped into 'starter cultures', whose primary role is to produce lactic acid or 'adjunct cultures' that are used for flavour formation. Many fermented dairy foods including cheese, yoghurt, sour cream, cultured buttermilk, kefir and kumis all utilise LAB (such as *Lactococcus*, *Lactobacillus* and *Streptococcus*). Large culture supply companies provide a wide range of well characterised strains in live freeze-dried powders for dairy applications. However, these strains are adapted to dairy substrates and can be less suited for dairy-free plant bases due to differences in sugar, protein and sensitivity to growth inhibitors.[10,11] In preliminary work, we compared the ability of a cheese starter culture strain (strain A) to acidify cow milk and almond milk (a model PBMA). It acidified cow milk but was incapable of lowering the pH of almond PBMA (Fig. 1). Therefore, there is significant interest in identifying new strains better suited for acidification and flavour formation in plant-based substrates. LAB are commonly found on plants and wild strains exhibit greater metabolic capability than industrialised (domesticated) strains.[12] Therefore, searching for strains from plant niches suitable for plant-based fermentations appears logical.

In some preliminary work using the almond PBMA model, we have screened a collection of ~600 LAB, sourced from a wide variety of vegetables, fruits, and herbs for their ability to acidify almond PBMA. Previous work has shown that this LAB collection can be a novel source of diverse strains that have beneficial anti-bacterial activity, anti-fungal activity or food flavour production.[13–16] While >90% of the plant-derived strains of LAB acidified almond PBMA poorly, several strains possessed medium-to-strong almond PBMA acidification. As an example, strain B was identified as a strong acidifier, strain C a medium acidifier and strain D a non-acidifier (Fig. 2). Further work identifying the strains and also understanding why different LAB acidify almond PBMA differently using whole genome sequencing, metabolic and genetic methods is underway.

Citizen science as a tool for microbial discovery

Obtaining and screening LAB strain collections, as we have done previously as described above,[16] prior to determining their potential in plant-based food fermentations is however both costly and laborious. There is a need to first collect samples, grow, isolate, and identify microbes before even beginning to filter through the strains for applicable candidates. One way to raise efficiency and lower the costs is to make use of Citizen Science (CS), where the public is involved in some or all of the processes. CS is a strong tool for massive sample and data collecting on a low budget and in a short time span, exemplified by the Danish 2018 project Mass Experiment[17] (http://www.bacteriadanica.dk/) where school children collected over 30 000 environmental samples. The project ended up identifying ten new species of LAB. In another study[18] researchers were able to collect more than 500 unique sourdough starter culture samples from four different continents within 3 months, showing the potential of wide geographical coverage when using the public for sampling. CS has even been used to strengthen the possible origin of ancient yoghurt production, which includes the initiation of fermentation in milk, by adding twigs and leaves of specific plants.[19] Here, researchers collected hundreds of plant samples, from which the traditional yoghurt species *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* were isolated. CS in natural sciences is already well established, even though it might be called different names. A well-known phenomenon is the BioBlitz, where both experts and hobbyists contribute to the identification of species in a defined area within a short period. This type of CS is often used for identification of plants, insects, or larger animals, but could in principle just as well be used for microbial identification. Today's social media platforms, global connectivity, and technological advances in terms of e.g. genomic screening, microbial identification and characterisation can enable much faster discoveries, than if CS was used just 20 years ago. This further strengthens the arguments for implementing CS in more research-based work.

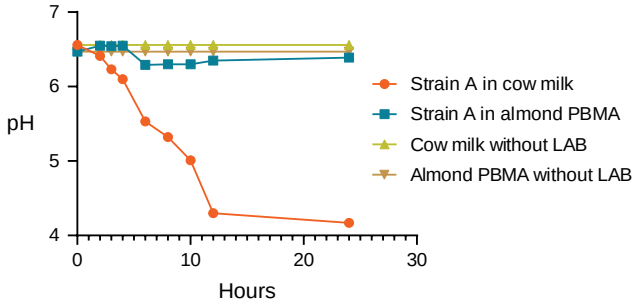

Fig. 1. Acidification bystrain A incubated in cow milk and almond PBMA held in non-shaking tubes at 30°C. Data are representative of at least two independent trials.

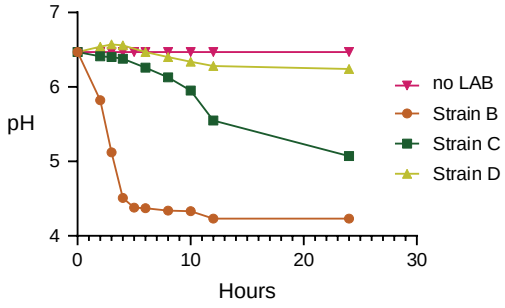

Fig. 2. Acidification bythree plant-derived LAB strains incubated in almond PBMA held in non-shaking tubes at 30°C. Data are representative of at least two independent trials.

Importance of microbial fermentation for flavour formation in plant-based alternatives

To optimise a plant-based fermentation for animal-based alternatives, flavour is key to success. As an example, various compounds produced during lactic acid fermentation are integral factors in obtaining a flavour profile like that of dairy yoghurt. This includes lactate and acetate (acidic), acetoin, diacetyl (buttery), and acetaldehyde (green apple), and even ethanol to name a few.[9,20] The production of these compounds relies on specific precursors in the plant base, as well as LAB capable of fermenting these precursors. Lactic acid fermentation can also decrease the amount of 'beany' off-flavours found in soy products, while also providing dairy related flavour compounds.[21] Madsen *et al.*[11] proved the importance of specific bacterial strain selection, and showed that dairy-adapted yoghurt starter cultures were unable to compete with plant-adapted ones in a mixed soy and malt base. The number of studies characterising flavour development of fermented PBMAs are much fewer than that for meat alternatives.[22] Kaczmarska *et al.*[23] investigated the chemical composition and sensory profile of various meat analogues, as well as fermented plant products such as tempeh and tofu, which are often regarded as meat-like substitutes. The results showed that only a few alternatives had a sensory profile vaguely resembling a meaty taste, and vastly different flavor profiles. Still, the study argued the possibility of using natto and tempeh as an ingredient in a meat substitute.

Future opportunities/challenges

With the recent public surge in interest of plant-based alternatives, the outlook of better quality products is promising. Still, there are several hurdles that need to be overcome, in order to create 'true alternatives'. Nutritional values are hard to reach without additives such as B12 and calcium,[9] texture of cheese alternatives is difficult due to the lack of casein proteins in plants. Likewise, it is complicated to correctly balance the flavor profiles of meat analogues due to the complicated landscape of various contributing compounds.[24] Several start-up companies and non-profit organisations, such as Perfect Day and Real Vegan Cheese, are already trying to tackle many of these obstacles through genetically modified yeast or bacteria producing the main milk protein casein, enabling a way of producing vegan alternatives. But this is still very much on a theoretical stage, and a complicated procedure. The use of plant-derived, non-modified microbes could still prove useful in this regard. Projects such as the Mass Experiment and large sourdough starter culture study[18] has proven that CS is a strong tool, and that nature still holds more potential in terms of new tools for food fermentation. Hopefully future plant-based fermentation research will pave the way for new applications.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. The authors declare no conflicts of interest.

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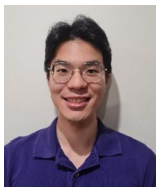
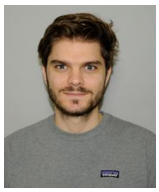
Biographies



Wenkang Huang is a PhD student in the School of Agriculture and Food Sciences at the University of Queensland. His research focus is on plant-based milk alternatives fermentation using lactic acid bacteria. He is currently working in the collaborative project between the University of Queensland and Technical University of Denmark.



Anders Peter Wätjen is a PhD student, with an alliance scholarship between Technical University of Denmark (DTU) and University of Queensland. He is investigating flavour production in plant-based dairy alternatives through lactic acid fermentation.

Dr Prakash is an academic at the University of Queensland with extensive experience in processing, physical characterisation and sensory profiling of food ingredients and products, including proteins (dairy and plant), hydrocolloids, dairy products (milk, yoghurt, custard, cream cheese, and dairy beverages), rice and meat. Her research interest also extends to 3D food printing and the digestibility of food ingredients in the human gastrointestinal tract.

Claus Heiner Bang-Berthelsen is a senior scientist at DTU National Food Institute. He is the lead investigator in a research team exploring plant-based dairy alternatives and novel food fermentation and valorisations of side-streams by fermentation. He teaches food microbiology and biochemistry courses at DTU.

Mark Turner is a Professor in food microbiology and Deputy Head of the School of Agriculture and Food Sciences at the University of Queensland. He currently leads a research team exploring food fermentation, quality and safety and teaches food microbiology courses.

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Congratulations to Professor Peter Timms on being honoured as a Queensland Great

The ASM congratulates Professor Peter Timms on being listed as one of eight Queensland Greats for the 2022 award round. Peter, who is based at the University of the Sunshine Coast, is a world expert in chlamydia infections in both humans and animals. He is leading a vaccine development program to safeguard koalas from extinction.

This year alone, as many as 1000 wild koalas in south-east Queensland will be given a single-shot vaccine developed by Peter and his team as part of the largest-ever trial to combat the sexually transmitted disease chlamydia, which can lead to painful urinary tract infections, loss of bladder control, infertility, blindness and death. The vaccine provides three levels of protection to koalas: reduced infection levels circulating in the population, reduced progression to clinical disease, and even reversal of existing cases of the disease.

The Queensland Greats Award recognises extraordinary Queenslanders who have made a long-term contribution to, or whose achievements have significantly impacted, the history and development of Queensland. Peter's achievements have been recognised with a commemorative plaque displayed proudly at Roma Street Parkland, Brisbane.

Peter has also been a long-time member of ASM, serving on ASM's National Council and then on the National Examinations and Qualifications Board for 10 years, initially as a member and then as Chair.

Professor Peter Revill receives Queen's Birthday Honour

Microbiologist and virologist Professor Peter Revill has received an AM (Member in the General Division of the Order of Australia) in the Queen's Birthday Honours for 2022. Professor Revill, Molecular Research and Development Group Head at the Victorian Infectious Diseases Reference Laboratory (VIDRL), Royal Melbourne Hospital, at the Doherty Institute, was honoured for his significant service to microbiology and immunology research.

Doherty Institute Director Professor Sharon Lewin congratulated Professor Revill, noting his important contribution to hepatitis research. 'Professor Revill's research into the molecular virology of hepatitis B virus has advanced our understanding of this virus and his global leadership and advocacy has highlighted the importance of finding a cure for hepatitis B', Professor Lewin said.

Professor Revill's work has focused on the molecular virology of hepatitis B virus (HBV) and played a key role in establishing the International Coalition to Eliminate Hepatitis B (ICE-HBV), in partnership with Professor Stephen Locarnini at VIDRL and Professor Fabien Zoulim at France's National Agency for AIDS Research (ANRS).

A Principal Research Fellow at the Department of Microbiology at the University of Melbourne, Professor Revill is also an executive member of the Australian Centre for HIV and Hepatitis Virology, a member of the Australian Centre for Hepatitis Virology, the Australian Virology Society and the Victorian Infection and Immunity Network. Peter, along with Adam Taylor, is theme leader of virology for The Australian Society for Microbiology.

This announcement is an edited version of the Doherty Institute's media release.

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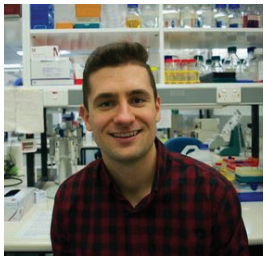

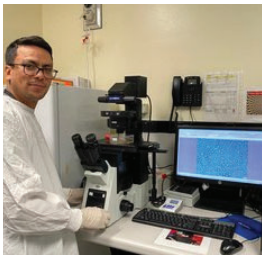

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