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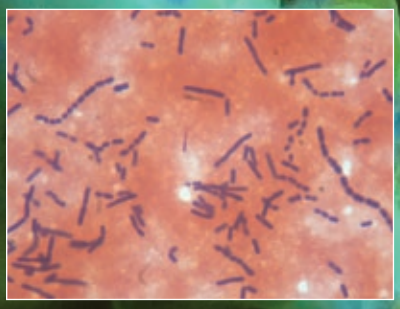
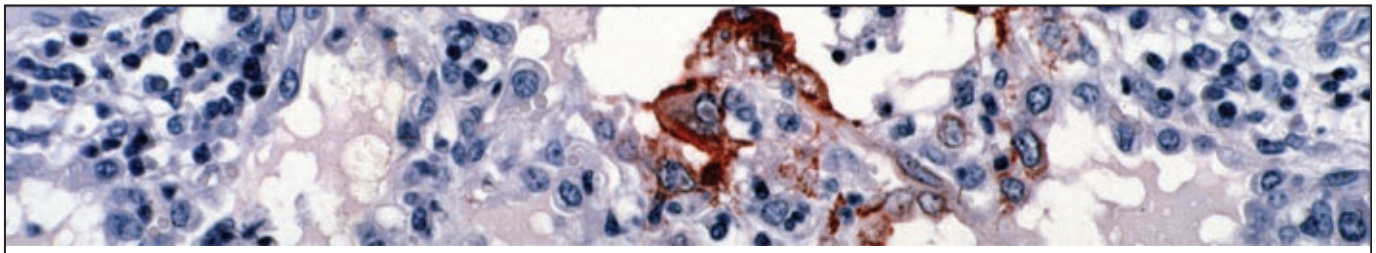


One Health Probiotics

plus Hot Topic: Australian COVID-19 vaccine



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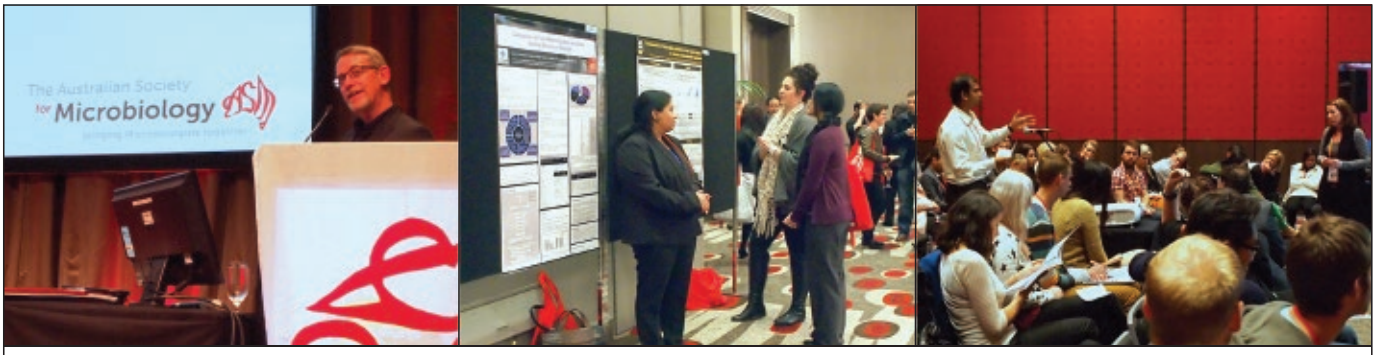

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Contents

Cover image: Coral polyps from the Great Barrier Reef (courtesy of Dr Justin Maire, The University of Melbourne); inset photos: Gram-stained yoghurt bacteria (Dr Joe Liu, Probiotics Australia); probiotics in capsules and foods; Yarra valley cattle; caesarean-born infant on probiotics (courtesy of Fransedo Risanto).

Dena Lyras
President of ASM

I will begin this Vertical Transmission by hoping that you and your families are all healthy and well, and coping during this very confused and frightening time. Microbiology and public health are certainly at the forefront of community and government attention at this time, and rapid solutions are being sought to deal with the SARS-CoV-2 pandemic. Unfortunately, funding for discovery research that will provide the required solutions for this and other microbial threats is lacking. For this reason, the ASM Executive (Dena Lyras, Roy Robins-Browne, Kate Seib, Anthony Baker, Rebecca LeBard), together with Mark Schembri, Jonathan Iredell, Priscilla Johanesen, Enzo Palombo, Cheryl Power, and Deborah Williamson, have prepared the following statement to share with you and the broader community on this matter.

Discovery research: a foundation for pandemic preparation that we must not neglect

The SARS-CoV-2 pandemic has infected 4.71 million people worldwide, resulting in over 315 000 deaths. The USA and UK have had 1.52 million and 244 000 infections, with the devastating outcome of over 89 932 and 34 636 deaths, respectively, as of 18 May. These numbers are increasing every day. The global mortality figures are so large that it is easy to lose sight of the fact that each of those deaths represents someone's friend, family member, mother, father or child; someone who is dearly loved and will be missed. By contrast, the effects of the pandemic in Australia have thankfully been relatively minor, with 'only' 7056 cases and 99 deaths.

We might consider that we are lucky in Australia, but it is not luck that has protected us. Our relatively low infection rate is the result of an evidence-based and co-ordinated Federal and State response. Central to this response has been the involvement of diverse members of our scientific community – epidemiologists, clinical microbiologists and microbiology scientists, nurses, GPs and hospital clinicians – who have worked to identify and treat people with infections, and to minimise community transmission. They are our heroes and deserve our gratitude every day. For the first time in living memory, science is constantly in the media, and the work of our scientists is highlighted daily in news and social media outlets. Indeed, the value of discovery research has never been more apparent. And rightly so – scientists will discover and deliver solutions to the SARS-CoV-2 pandemic, and the foundational backbone of these outcomes will come from basic discovery research across many fields, including microbiology, that was initiated well before we knew anything about the virus.

What about the next infectious diseases public health crisis? History shows us the value of discovery research related to infectious diseases. There is no better example than that of life-saving vaccines - smallpox has been eradicated worldwide, most regions have eliminated polio and other past scourges are historical footnotes. In fact, vaccines have been so effective that people have forgotten how devastating infectious diseases can be, with an increasing 'anti-vaxxer' movement facilitating the re-emergence of infectious diseases we had conquered. SARS-CoV-2 is a deadly reminder that infectious threats can emerge unexpectedly and can silently spread around the world before we can control them, wreaking the havoc we are seeing now. Distressingly, it was recently revealed that Australian researchers had been close to developing a potential universal coronavirus vaccine a decade ago but their efforts were halted by a termination in their funding. Sadly, this is a common story – promising research is halted because of a lack of funding for discovery research once the crisis is over. Furthermore, continuity in funding for discovery research is lacking. We invest huge resources to tackle new research problems, but this is generally short term.

Tackling infectious diseases, including pandemic preparedness, requires continual effort. Waiting until a threat has developed into a full-blown emergency is too late. Preparedness starts with discovery research. The past 20 years has seen the unexpected emergence of pathogenic viruses, including SARS, MERS, swine flu, Zika and SARS-CoV-2. But the next pandemic could be caused by bacteria (e.g. bubonic plague re-emerged in China last year), fungi (e.g. the worldwide expansion of *Candida auris*) or parasites (e.g. drug-resistant malaria). Antimicrobial resistance is also one of the biggest threats to global health and will impact every aspect of medical care, including cancer treatments and all surgeries. Infection threats are increasing and we must be prepared for any of these possibilities. Scientists correctly predicted the emergence of new coronaviruses and are not surprised by the current situation. Likewise, the other microbial threats cannot be ignored. We must be prepared for the inevitability that they will become bigger problems – history (and epidemiology) tells us they will.

The value of discovery research in the context of infectious diseases is not immediately obvious and its importance is therefore undervalued. We simply don't believe a pathogenic microbe will infect us, infections happen to other people. When infections arise, we and authorities can't believe there is no treatment and no cure; it's just a microbe after all - remember the underwhelming 'it's just a flu' attitude of world leaders at the start of the current pandemic. But as SARS-CoV-2 has shown us, without the development of the necessary toolbox in advance, microbes quickly get the upper hand and leave us stunned at the rapidity of their spread.

In recent years, the quantum of research funds has remained static (at CPI) and has not increased as needed to maintain a thriving research environment. The number of competitive applications has increased yet these applications are mostly not funded, with an 11.1% funding rate for our major medical discovery research funds, NHMRC Ideas grants. NHMRC Investigator Grants, designed to fund medical researchers at all career stages, have a 13.2% success rate. We spend millions of dollars educating and training our scientists and researchers and then do not support them in the research efforts that will bring the future solutions we will surely need, and there is no support mechanism in place to protect researchers through periods of vulnerability. It takes years of effort and funding to build expert research teams; without a mechanism for continuity these teams are dismantled rapidly, and their skills lost to the community which has supported them. They will simply not be there when we may desperately need them. In addition to this, the 'brain drain' is often discussed, because many talented researchers leave Australia when they realise they cannot build careers or meaningful research programs here. We are therefore losing our intellectual capital and talent. Those that remain here spend up to a third of their time applying for funds, at the expense of conducting critical research.

The lack of consistent funding is eroding discovery research. What will happen when the next crisis hits? We seem to have been 'lucky' this time. . .so far. But the SARS-CoV-2 pandemic has exposed our fragility in the face of infectious disease, and it is far from over. The current pandemic has shown that funding bodies can act rapidly when necessary, and funding can be made available and distributed promptly. However, it is unclear whether COVID-19 funding is diverting more funds away from other discovery research efforts, highlighting the need for distinct pandemic preparedness policies within the government's strategic research priorities. Moving away from peer-reviewed and investigator-initiated science to increased political governance risks the diversity and depth that we need to face future unknown risks, from global warming to infectious diseases. Cancelling peer-reviewed grant schemes to pay for short-term crisis management is dangerous policy indeed, and now is not the time to undermine the foundations of Australia's medical and scientific research.

The wastage of health and research dollars during the swine flu pandemic was well documented. The Australian Government Review of Australia's Health Sector Response to Pandemic (H1N1) 2009: Lessons Identified (https://www1.health.gov.au/internet/publications/publishing.nsf/Content/review-2011-l/$File/lessons%20identified-oct11.pdf), emphasised the need for 'robust science-based decision-making' and concluded 'Pandemics are unpredictable and therefore there is a need to remain flexible and adaptable to respond to all levels of threat to the health of Australia's population'. A brief bonanza in specific funding tends to promote a large amount of opportunistic research at the expense of diverse high-quality research that may serve the nation much better in the future. Australia's research capacity is part of our nation's critical infrastructure and deserves our support and respect. It is very easily damaged and will take a long time to rebuild. We degrade it at our peril.

New vaccines and drugs cannot be discovered and developed overnight, as evidenced by the current scenario with COVID-19, and to make these breakthroughs requires a nationwide, public shift in the prioritisation of research funding. Australian research needs strong and consistent funding to ensure that fundamental discovery research, which is the basis of all new medical advances, is properly supported to enable us to make the inventions today that will safeguard our health in the future. The current pandemic may be a catalyst for this change.

One health probiotics

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This issue of *Microbiology Australia* is on the topic of probiotics. The word 'probiotic', meaning 'for life', is derived from Latin 'pro' and Greek 'bios'. According to the World Health Organization and the Food and Agriculture Organization of the United Nations, probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'[1], while a very similar definition, albeit with the term 'body' (i.e. not plant), is used by the US National Institutes of Health: '. . . live microorganisms that are intended to have health benefits when consumed or applied to the body.'[2]. The field of human probiotics is steeped in a long history. Over a century ago, the Nobel laureate (1908) Ilya Ilyich (Élie) Metchnikoff suggested that human health could be enhanced and senility delayed by modifying the gut microbiota with lactic acid bacteria from fermented milk products – he is generally known as 'the father of probiotics'[3]. However, reference to human gut benefits from consumption of fermented milk predate Metchnikoff by millennia (e.g. Pliny the Elder) and sacred texts from Christianity and Hinduism mention fermented foods and their benefits[4]. Probiotic research took a major uptick with the advent of next generation DNA sequencing leading to multiple host associated microbiome initiatives including the human microbiome project, which revealed the importance of lactic acid bacteria as part of human microbiomes in different parts of the body[5].

In this issue of *Microbiology Australia* on probiotics, we present a broad one health coverage of the topic. Several aspects of probiotics for humans including production and regulation are discussed. However, we also go outside the classical human probiotics field to cover application to production animals and plants as well as application to marine native animals.

Fittingly, the first article is on future probiotic foods by Van Ho and Mark Turner (University of Queensland). Although milk is a common probiotic carrier, lactose intolerance makes this unsuitable for all and Ho and Turner explore other carriers like encapsulation and other foods including juices and vegetables. A multinational group led by Tristan Yusho Huang have written an article on natural skin microbiota members, including *Staphylococcus epidermidis*, which produce short-chain fatty acids that suppress the growth of the pathogen *Staphylococcus aureus*. A great overview of faecal microbiome transplantation (FMT) is given by Holly Sinclair from the Royal Brisbane and Women's Hospital (RBWH) and Paul Chapman (Queensland Institute of Medical Research Berghofer Medical Research Institute). Treatment of *Clostridioides difficile* infections and other human gut dysbioses are covered. A second FMT article on gastrointestinal illness is presented by Hayley Reed and Jakob Begun from the Mater Research Institute with emphasis on immune homeostasis. The relevance of probiotics in caesarean-born neonates is covered by Hanna Sidjabat (The University of Queensland when this issue was prepared and currently with Griffith University Menzies Health Institute Queensland), her previous students, Adam Irwin (Children's Health Queensland Hospital and Health Service) and Pieter Koorts (Neonatal Unit at RBWH). The probiotics industry for human consumption is anticipated to reach ~US$69.3 billion by 2023 as reported by Joe Liu, Brendan Cook and Shaun Roux from Probiotics Australia. They have opened Australia's only regulation certified probiotics production facility and their article covers the full gamut of the challenges around the commercialisation of probiotics. The article discusses topics from strain selection, mass manufacture, downstream processing, and finally shipping.

The topics in the Probiotics issue then switch focus from humans to the use of probiotics in animals. Dairy cattle are the focus in an article by Divya Krishnan and colleagues mostly from the University of Queensland and including Timothy Olchowy from University of Calgary, Canada. Mammary gland dysbiosis in milk producing cows and calf growth improvements via gastrointestinal tract probiotics are covered. In the pork industry, piglet mortality is a major issue. Although antibiotics were the go-to

strategy, antibiotic resistance and bans on antibiotic use have motivated alternative approaches including probiotics. Nowland and Kirkwood (University of Adelaide) describe FMT as a potential future probiotic strategy for piglets, using the human FMT successes in treating *Clostridioides difficile* infections as a model (and described by Sinclair and Chapman in this issue). The nascent probiotic application in these two production animal fields (cattle and pigs) requires quite some work before efficacy will be proven and broad adoption by practitioners is achieved. Probiotics in freshwater farmed fish are covered by an international collaboration of Luisa Marcela Villamil-Diaz (Universidad de La Sabana, Colombia) and colleagues from Australia and USA. Growth promotion, pathogen inhibition and stress tolerance are among benefits that could be conferred to fish by probiotics. The article also covers probiotic application to the animals. Although not covered in this *Microbiology Australia* issue, extensive use of probiotics in broiler chicken production has been of longer application and quite efficacious compared to other food-production animals[6], and a recent paper[7] concludes excellent prospects for the application of probiotics and other microbiome-directed therapies in taxa ranging from horses to salamanders to bees.

We have one article on probiotics in plants by Rob Walker and colleagues from the University of Melbourne. One of the most classical 'probiotics' in plants is the use of *Rhizobium* inoculants[8]. Rob and his co-authors discuss many plant-related probiotic topics and they are anticipating a good future for this industry. Last, Australia has the world's largest coral reef system stretching over 2300 km and seen as the world's biggest single structure made by living organisms. Although coral reefs provide critical ecosystem services and substantial personal income, they are globally suffering from one substantial issue called coral bleaching. One novel way to preserve the reefs of the world is to assist them by using introduced microbes which have beneficial properties for the corals. This topic is covered by Linda Blackall and her colleagues from the University of Melbourne.

Not all topics in the field of probiotics are covered in this *Microbiology Australia* issue. The term pharmacomicrobiomics was introduced in 2010 to investigate the interplay of microbiome, drug response and disposition (absorption, distribution, metabolism and excretion)[9]. This model will potentially contribute to the efficacy of biotherapy including therapy with probiotics. There are many recent initiatives in Australia on microbiome research as it relates to gut and environmental health – these are driven by research institutions and commercial companies alike. Enthusiastic researchers, clinicians and academics resonate with the high hopes of probiotics and microbiome research. The probiotics space will be a busy one into the future and this *Microbiology Australia* issue encapsulates some of the recognised areas while also covering less familiar ground.

Happy reading!

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Biographies

Dr Hanna E Sidjabat is a molecular microbiologist with a strong industry link in translating her probiotic research to manufacturing. In addition to her probiotic expertise, she has a solid background in antibiotic resistance mechanisms including genome and proteome due to 15 years of research experience. She has strong research focus in the bacterial genome, proteome of pathogens and probiotics. To date, Dr Sidjabat has published 87 peer-reviewed articles in international journals. Dr Sidjabat has supervised and mentored 35 PhD students, Postdoctoral Research Fellows, Master and Honours students, Microbiology Registrars, local and international Infectious Diseases Visiting Academics following the completion of her PhD in 2007.

Professor Linda L Blackall is an environmental microbial ecologist, who has studied many different complex microbial communities ranging from host associated through to free living in numerous environments. Her research has covered mammalian microbiomes of marsupials, humans, ruminants and horses; and the microbiota of non-mammals including corals and sponges. Environmental microbiomes explored in Linda's research span wastewater treatment (aerobic and anaerobic), solid waste digestion (landfill and composting), bioelectric systems and microbiologically influenced corrosion. The numerous methods she develops and employs in her research allow elucidation of microbial complexity and function in these diverse biomes.

Future probiotic foods

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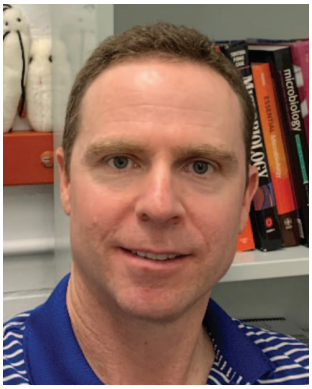

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Abstract. Foods containing edible probiotic bacteria, most commonly *Lactobacillus* and *Bifidobacterium* species, form a multi-billion-dollar industry worldwide. Currently marketed foods containing probiotics are mostly dairy based with yoghurts and fermented milks dominating the industry. Alternative foods as carriers of probiotics are being examined to reduce or eliminate lactose intolerance issues. Food categories including fruit juices, cheese, chocolate and even beer have been shown to be suitable for probiotic delivery. In addition, technologies such as encapsulation in food-grade alginate gels have allowed for improved probiotic survival in certain foodstuffs. We have explored the use of ready-to-eat vegetables such as baby spinach as carriers for commercial probiotics and found that high dose (>8 log CFU/g) can be achieved without having negative effects on appearance, taste or aroma. Leafy greens as well as other foods and beverages may be suitable probiotic containing new food products in the future.

The most commonly used definition for probiotics, initially proposed in 2001 by the Food and Agriculture Organisation of the United Nations (FAO) and supported by the World Health Organization (WHO) is 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'[1]. Most probiotics sold in edible products are *Lactobacillus* and *Bifidobacterium*, while products with *Bacillus, Escherichia coli* and *Saccharomyces* are less commonly available. Probiotic organisms are different to fermentation organisms and the health promoting effects may be only strain specific. More stringency around health claims of probiotics and functional foods in general in various countries has resulted in fewer unsubstantiated marketing claims, which have plagued the probiotic industry for the past three decades. In 2010, the European Food Safety Authority (EFSA) took the strict option of banning all health claims regarding probiotics and until now the only claim that is approved is regarding lactose intolerance prevention through yoghurt ingestion[2]. Nowadays, randomised, double-blind and placebo-controlled studies with high numbers of subjects are the bench mark to demonstrate probiotic efficacy. This is reasonable as probiotics are a major business activity with global sales expected to hit $50 billion by 2022[3]. Despite their controversial history, many scientific studies have demonstrated health promoting activities of specific strains in certain situations. In addition, with the explosion of microbiome insights, 'next generation probiotics', which are defined as 'live microorganisms identified on the basis of comparative microbiota analyses that, when administered in adequate amounts, confer a health benefit on the host', will likely be of significant commercial interest in the coming years[2].

Probiotic foods and beverages

Oral delivery of probiotics can involve a variety of different vehicles. Tablets and capsules containing high doses (e.g. 10 log CFU) of single or mixed strain probiotics are commonplace in pharmacies and supermarkets. The most common foodstuffs containing probiotics are dairy-based, including yoghurts and fermented liquid milks. Other dairy-based foods including Cheddar cheese and chocolate can also support viable probiotic bacteria[4,5] but are yet to make it to market. However, these products can contain high levels of sugar and with around 75% of the world's population being lactose intolerant, alternate non-dairy-based foods which can support probiotic bacteria viability have been investigated[6]. A leading probiotic food producer in the USA, Goodbelly, has developed snack bars containing 9 log viable *Bifidobacterium* BB-12 cells and fruit juice containing *Lactobacillus plantarum* 299v (https://

goodbelly.com/). A new juice product containing alginate micro-encapsulated *Lactobacillus casei* Lc431 cells, called PERKii, was launched in Australia during 2016 (https://perkii.com.au/). Encapsulation of probiotics improves viability during simulated gastrointestinal transit[7] and reduces fermentation of the fruit sugars in the beverage[8].

Other alternatives to dairy-based probiotic foods are cereal, meat and soy-based products. Cereal-based probiotic drinks containing >7.9 log CFU/mL of *L. plantarum* and *Lactobacillus acidophilus* were prepared from single and mixed flours of barley and malt[9]. Interesting recent research has identified that beer can support high survival of probiotic *Lactobacillus paracasei* L26 for 3 weeks before reducing to undetectable levels by 4 weeks[10]. Beer contains several antimicrobial compounds, including alcohol, acid and hops making it a challenging environment for bacteria to survive. A novel approach for the preparation of probiotic breads was developed by coating pan bread slices with sodium alginate film impregnated with *Lactobacillus rhamnosus* GG which could deliver up to 9 log CFU/30–40 g per bread slice[11]. Dry fermented meat products ingested without cooking are potential vehicles to transfer probiotics into the gastrointestinal tract as probiotic cells can be embedded and protected within the meat matrix consisting of protein and fat[12]. When added into fermented sausages, an initial population of 5 log CFU/g of *L. plantarum* 299v increased to 8 log CFU/g after fermentation[13]. Soy protein is also considered as a good protector for probiotics against harsh conditions in the intestine. *Lactobacillus acidophilus* LA-5 showed good growth and survival of >8.7 log CFU/g in a fermented soy beverage stored at 4°C for 21 days[14]. A mix of probiotic bacteria including *Lactobacillus acidophilus*, *L. rhamnosus*, *L. paracasei* and *Bifidobacterium lactis* incorporated into a non-fermented frozen soy dessert exhibited high viable populations exceeding 7 log CFU/g during 6 months storage while maintaining desirable sensory attributes[15].

New probiotic containing vegetable products

To further expand the range of probiotic containing foods, our group has examined fresh ready-to-eat leafy green vegetables as potential carriers. Several probiotic strains that were inoculated onto baby spinach by dipping the leaves for 5 mins in a bacterial suspension resulted in attachment of 7–8 log CFU/g spinach (Figure 1). Viability of probiotic strain A reduced slightly over 7 days, while probiotic strain B increased slightly to >8 log CFU/g. Based on a typical serving size of 60 g of baby spinach a dose of >9.8 log CFU could be achieved, making it equivalent to other high dose probiotic products on the market. We next determined if the probiotics affected the sensory properties of the spinach, such as appearance, aroma and taste. A panel of 40 volunteers, under controlled conditions in a food sensory laboratory, evaluated de-identified spinach samples stored at 4°C for 4 days. Using a triangle sensory test, it was found that there were no statistically significant differences ($P > 0.05$) in the appearance and flavour of spinach leaves inoculated with probiotic strain A or strain B to that of the control samples. Only 12 out of 40 people could differentiate the probiotic strain A containing spinach from the control spinach and 13 out of 40 could differentiate the probiotic strain B spinach from the control spinach. Spinach leaves with and without probiotics had a similar appearance over 7 days of storage at 4°C as shown in Figure 2. It may be concluded that the sensory quality of baby spinach was not adversely affected by the addition of two probiotic strains. In addition, we have found that washing the leaves in various types of salad dressings (e.g. French, Italian, Balsamic) does not detach cells or reduce their viability. Lastly survival of probiotics on spinach in simulated gastrointestinal digestion trials did not reveal any greater reduction in viability compared with

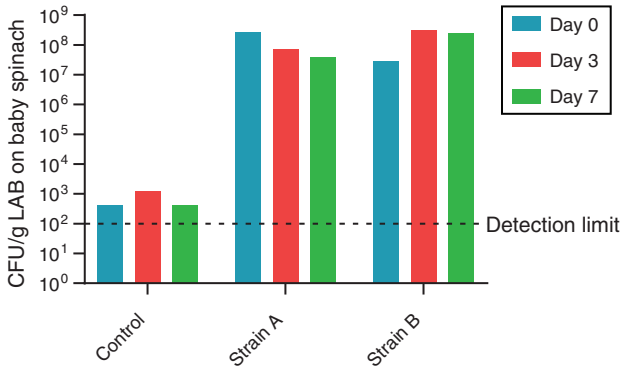

Figure 1. Survival of commercial probiotic strains A and B on baby spinach stored at 4°C. The control is uninoculated spinach, which has low levels of naturally occurring lactic acid bacteria (LAB).

Figure 2. Appearance of baby spinach with or without probiotic after 7 days of storage at 4°C.

probiotics suspended in milk. Leafy green vegetables with probiotics may provide an appealing alternative choice for health-conscious consumers in particular.

The future

Our work described here and that of other research groups suggest that there are many unexplored foods which could potentially support good survival of probiotic bacteria. Experimental and industrial trials using these foods are necessary so that factors such as water activity, pH and storage temperature can be optimised for adequate survival of the probiotic. In addition, negative effects on food quality in most cases due to growth and/or fermentation of the food are possible and should be evaluated chemically or using sensory trials. Physiological differences between probiotic species and even strains within species can exist which could mean that only certain probiotics can be incorporated into certain foods. With the explosion of new insights into human health coming from microbiome research, new probiotics and probiotic containing foods and beverages will likely be of significant interest for the food industry and consumers in the future.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

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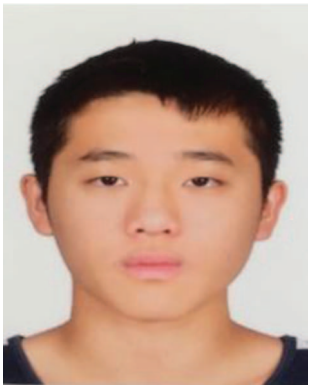
Amplification of probiotic bacteria in the skin microbiome to combat *Staphylococcus aureus* infection

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Abstract. ***Staphylococcus aureus*** **(*S. aureus*) is a Gram-positive bacterium. When pathogenic *S. aureus* colonises onto a skin wound or diabetic ulcer, it can cause a serious infection and lead to amputation or death. The current solutions (e.g. antibiotics and probiotics) are not sufficient enough to be a cure for this infection. To worsen the situation, the *S. aureus* bacteria continue to develop greater resistance towards antibiotics and are becoming more commonplace. An effective solution is to amplify the activity of probiotic bacteria in the skin microbiome by using selective fermentation initiators (SFIs) to induce fermentation. Our data demonstrated that the numbers of *Cutibacterium acnes* (*C. acnes*) and *Staphylococcus epidermidis* (*S. epidermidis*), two major bacteria in skin microbiome, on human skin did not vary significantly over the span of seven days. This stimulates probiotic bacteria such as *S. epidermidis* to produce sufficient short-chain fatty acids (SCFAs) to suppress the growth of *S. aureus*. The development of this new cure to *S. aureus* may reduce hospitalisation greatly as *S. aureus* accounts for the hospitalisation of more than five thousand people per year. Besides antibiotic, probiotics and bacteriophages, SFIs may become novel agents for treatment of infection.**

Skin microbiome and dysbiosis

The skin microbiome comprises the microbiota in skin that is home to millions of bacteria, fungi and viruses[1]. Skin dysbiosis refers to a condition in which microbial imbalances occur in the skin microbiome[2,3]. Mounting evidence indicates that the probiotic microbes in the human microbiome can employ bacterial interference[4] to rein in the overgrowth of opportunistic pathogens[5,6]. However, little is known about the interactions among probiotic bacteria within the human microbiome for maintaining homeostasis of the microbiome. Bacterial interference, used by probiotic *Staphylococcus epidermidis*, prevents growth of pathogens and has shown to be a promising modality for preventing and/or treating infections. Literature has demonstrated that *Cutibacterium acnes* and *S. epidermidis*, two major bacteria in the skin microbiome[7–9], can fermentatively metabolise

glycerol, a naturally occurring metabolite found in human skin[10], to repel the over-growth of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA). Our results showed the abundances of both *C. acnes* and *S. epidermidis* on the skin surface of the same person have no significant changes from Day 1 to Day 7 (Figure 1), indicating the stability of commensal bacteria in skin. The stability of abundances of commensal bacteria in skin will make it possible to apply a fixed dose of prebiotic to induce fermentation. SCFAs are one of metabolites of glycerol fermentation of *C. acnes* and *S. epidermidis*. Several SCFAs have been approved by the U.S. Environmental Protection Agency (EPA) or the Food and Drug Administration (FDA) as active compounds for use as antimicrobials[11–13]. It has been illustrated that a specific SCFA, butyric acid, can diminish inflammation via inhibition of histone deacetylase (HDAC) in host cells[14], suggesting the dual antimicrobial and anti-inflammatory abilities of SCFAs.

S. aureus infection in diabetic wounds

Infection of the skin by *S. aureus* is a major cause of hospitalisation and can cause death and organ failure. It is estimated to account for the outpatient visits of 12 million people per year, worldwide, and the problem continues to grow. Furthermore, doctors consistently rely on the use of antibiotics, resulting in the development of MRSA. MRSA is a major issue among people with diabetic ulcers[15]. Diabetic ulcers occur in 15% of people with diabetes, creating wounds that permit pathogens to enter the body, with one of the most common pathogens being MRSA. Already in a frail state, due to poor blood flow in the ulcer, a pathogenic infection impedes the healing of diabetic ulcers, and the spread of such infections to soft tissue or bony structures often results in the need for amputation. Considering these possible outcomes, the estimated 30% of diabetic ulcers that are colonised with MRSA means that MRSA is among the most common causes of amputation. *S. aureus* poses a potent threat not only to diabetic patients, but to healthy, normally functioning people as well. Not only can *S. aureus* enter diabetic ulcers, but also into traumatic skin wounds, which can lead to persistent tissue infection that occasionally progresses to systemic infection and death. Furthermore, MRSA is easily transferred. A mere touch of the infected skin or a touch of even an object that has come in contact with the infected skin can spread this infection. As antibiotics can only serve to be a temporary solution to this problem, scientists continue to propose new solutions to the ongoing issue.

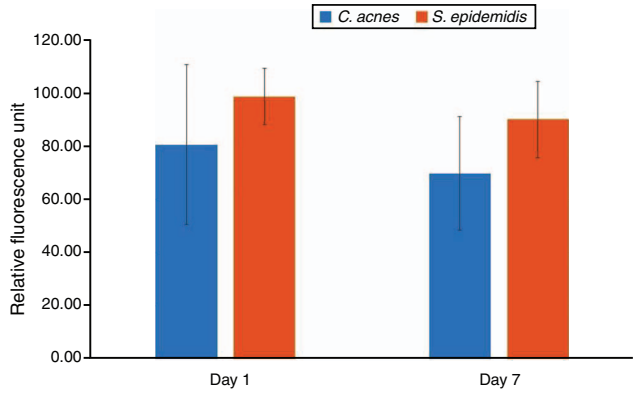

Figure 1. The abundance of *C. acnes* and *S. epidermidis* on the skin surface. Skin swabs from the arm skin surface (5 cm x 5 cm) were collected on Day 1 and Day 7 and submerged into 100 µl Saliva DNA lysis buffer (Norgen Biotek Corp., ON, Canada) immediately. The sample was diluted 10x with distilled water, loaded onto a GeneScan™ chip for bacterial identification using the 16s RNA sequencing. The fluorescence reading on the y-axis was generated by the GeneScan™ software based on fluorescence signal detected by the system (www.ameridx.com). The data was plotted manually by Excel software. Primers pairs for specific 16S rRNA gene amplification were GGGTTGTAAACCGCTTTCGCCT and GGCACACCCATCTCT GAGCAC for *C. acnes* and GCACGTAGTTAGCCGTGGCTTTCTG and CTTATAGATGGATCCGCGCCGCATT for *S. epidermidis*. The mean ± standard derivation for three separate samples was calculated. A two-tailed t-test was used for statistical analysis.

Possible problems of antibiotic, probiotic and bacteriophage for treatment of *S. aureus* skin infection

The use of antibiotics has provided an accessible and successful solution to almost all bacterial infections. However, antibiotics, if overused, can result in the development of antibiotic-resistant bacteria, which deems antibiotics to be undesirable for long-term management of bacterial infections. The emergence of MRSA provides a clear example of the shortcoming of this approach. The problems of antimicrobial resistance are discussed in the May 2019 issue of *Microbiology Australia*, while '*S. aureus*' drug resistance was part of the theme in September 2008. The use of probiotics represents a potential solution to this problem. Probiotics are essentially symbiotic microorganisms that outcompete pathogenic bacteria[16]. Adding probiotic bacteria to human skin will shift the course of infection leading to the balanced ratio of bacteria. As addressed earlier, *S. aureus* is an infection on the skin. However, the FDA prohibits the application of probiotics on the skin because probiotics are live bacteria and entrance of live bacteria into the bloodstream can cause other infections leading to death. Thus, probiotics can only be present in edible items such as yogurt and currently, does not represent a viable treatment for *S. aureus* infection. The last of the current solutions to combat dysbiosis would be the use of bacteriophage. Bacteriophage are viruses that selectively kill certain bacterial species[17]. Although this represents a creative approach to replace antibiotics, it has been reported that there are certain limitations inherent in bacteriophage therapy[18].

Prebiotic as a bacteria-specific carbon source for fermentation

The use of prebiotics represents a potential solution to the existing problems facing the management of MRSA infection. This approach essentially consists of assisting the beneficial or probiotic bacteria, while weakening pathological or undesirable bacteria. The fact that not all people who come in contact with *S. aureus* get an infection implies the existence of endogenous mechanisms preventing infection. In general, commensal bacteria use a carbon source derived from human cells (e.g. fibre or glucose) to make SCFAs such acetic acid and butyric acid via fermentation[19,20]. Among other things, these SCFAs can serve as 'microbial weapons' by which certain bacterial strains can inhibit the growth of competing species. If harmful bacteria overwhelm the probiotic bacteria, this may result in an infection or injury from pathogens. If the probiotic bacteria overwhelm the pathogens, the person would be safe from injury. The imbalance of bacteria in the microbiome is referred to as dysbiosis, resulting in pathologic infection. As current treatments proved ineffective against *S. aureus*, a new solution (Figure 2) to this problem would be to provide a defined prebiotic as a carbon source, also named a selective fermentation initiator (SFI), to selectively induce fermentation of probiotic bacteria. Pathogens and the probiotic bacteria in humans each have different enzymes to yield different SCFAs. This results from the fact that there are certain carbon sources that only the probiotic bacteria can ferment to combat pathogens. Due to differences in the enzymes of probiotics and pathogens, there are certain sources in which only the probiotics can utilise to ferment and produce SCFAs. Such carbon sources would be SFIs.

Different bacterial species make different enzymes that ferment specific carbon sources. All *S. aureus*, *S. epidermidis* and *C. acnes* can ferment glucose to SCFAs[21–23]. To gain maximum survival advantage, *S. aureus* and *S. epidermidis*/*C. acnes* that co-exist within a diabetic ulcer[24,25] exclude each other via production of SCFAs by fermentation of glucose. When *S. aureus* survives after competitive bacterial interference the infection *will* proceed to continue to damage the host. However, polyethylene glycol dimethacrylate (PEG-DMA) has been developed as a SFI that can specifically intensify fermentation activity of *S. epidermidis*, but not *S. aureus*[26,27]. The exclusive induction of the fermentation of *S. epidermidis* by PEG-DMA amplified the probiotic activity of *S. epidermidis* against *S. aureus*.

In a skin wound or diabetic ulcer, the microbiome is comprised of probiotic bacteria and *S. aureus* where probiotic bacteria act to inhibit the proliferation of *S. aureus*. The prebiotic strategy would result in the cultivation of fermentation specifically in probiotic bacteria such as *S. epidermidis*, amplifying their activity against *S. aureus* within diabetic ulcers. The probiotic bacteria metabolising these SFIs will create SCFAs via fermentation that prevent pathogens from entering skin wounds. SFIs do not eliminate all bacteria like antibiotics, therefore it would not leave the wound susceptible to opportunistic pathogens. Furthermore, since SFIs do not kill the pathogens directly, pathogens cannot develop resistance. SFIs also represent a more feasible solution compared to probiotics, since SFIs are not live entities, would not cause infection and therefore could be applied on the skin. Therefore, SFIs could be the most plausible solution to MRSA infections in diabetic ulcers. SFIs can potentially reduce hospitalisation, the need for amputations, and delays for healing diabetic ulcers.

Conclusion

The technology of bacterial fermentation has been widely employed in the development of various products including yogurt, wine, and vinegar. The concept of using SFI to activate the fermenting probiotic bacteria against *S. aureus* and restore the dysbiotic skin microbiome not only may inspire the next generation probiotic/prebiotic-based medicine but also defines novel roles of

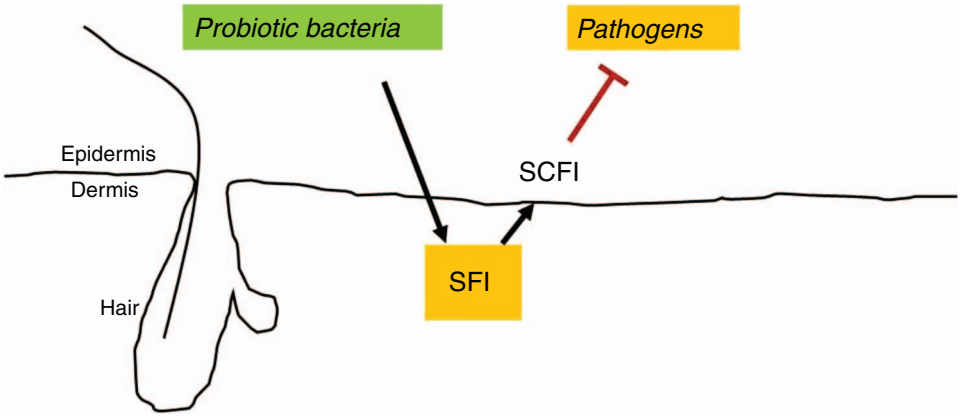

Figure 2. Probiotic bacteria mediate SFI fermentation to produce SCFA to decolonise pathogens in skin. Fermenting bacteria in skin can use SFI as a carbon source to undergo fermentation and produce SCFA which has antimicrobial activity to eliminate pathogens in the skin.

probiotic bacteria and their associated prebiotics in the innate immunity of the skin against *S. aureus* infections.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

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Yong Jiang is a CEO at America Diagnosis, Inc., San Diego. Dr Jiang has developed a DNA chip for high-throughput screening the relative abundance of microbes in the human microbiome.

Faecal microbiota transplantation: a review

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Abstract. Faecal microbiota transplantation (FMT) is the transfer of human faeces from a healthy donor to a recipient with a disease associated with gut dysbiosis. Here we review faecal microbiota transplantation as a treatment for *Clostridioides difficile* infection (CDI) and other conditions including decolonisation of multiresistant organisms. Donor selection and screening, adverse events, processing, administration and regulation of FMT are discussed.

Introduction

Faecal microbiota transplantation (FMT) is not a new concept, being first described in traditional Chinese medicine over 1000 years ago[1]. FMT delivered by faecal enema was successfully used in the treatment of pseudomembranous enterocolitis in 1958[2]. A timeline for FMT over the years is shown in Figure 1. FMT is now accepted to be the most effective treatment for recurrent or refractory *Clostridioides difficile* infection (CDI). Clinical trials have also been conducted using FMT in primary sclerosing cholangitis, non-alcoholic steatohepatitis, type II diabetes mellitus, irritable bowel syndrome, inflammatory bowel disease, hepatic encephalopathy, and eradication of multiresistant organisms[3].

Perturbations in the composition of intestinal microbiota occur after administration of antibiotics, other medications, dietary changes and travel. Antibiotic exposure decreases the alpha diversity with reduction in Firmicutes and Bacteroidetes phyla and proliferation of Proteobacteria including *Enterobacteriaceae*[4]. Following FMT there is reduction in Proteobacteria and expansion of Firmucutes, Ruminococcaceae, Lachnospiraceae, Clostridiaceae and Bacteroidetes[4]. Recipient microbiota engraftment has been demonstrated by day three after FMT[5]. This microbial community correlates with that of the donor's microbial community and has been observed to be stable for 4 months and up to one year[4–6]. Complete donor engraftment may not be necessary if functionally effective taxa are present and bacteria associate with secondary bile acid metabolism to provide resistance to recurrent infection[4].

C. difficile infection (CDI) and FMT

C. difficile is a Gram-positive anaerobic, spore forming and toxin-producing bacillus[1]. Spores are transmitted via the faecal–oral route and are an important cause of hospital-acquired infection. Between 15–70% of infants and 5% of adults are colonised, being more frequent in hospital and nursing home residents[1].

Antibiotic exposure, older age and hospitalisation are major risk factors for CDI[1]. Clinical spectrum spans diarrhoea, ileus and toxic megacolon, with severe CDI presenting with fever, haemodynamic instability and peritonitis. Recurrent CDI is classified as recurrence of CDI within 8 weeks of successful treatment and refractory CDI is defined as absent clinical improvement after 3–4 days of appropriate treatment[7].

FMT has been shown to be the most effective treatment for recurrent CDI and has repeatedly demonstrated superiority to comparators since the first randomised trial in 2013[8,9]. In a meta-analysis of seven randomised controlled trials and 30 case series, FMT was more effective than vancomycin (RR: 0.23) for recurrent and refractory CDI with clinical resolution rates of 92%[10].

The Australasian Society for Infectious Diseases published guidelines for management of CDI that includes FMT[7]. Australian therapeutic guidelines recommend FMT as preferred treatment for second and subsequent recurrences or ongoing refractory

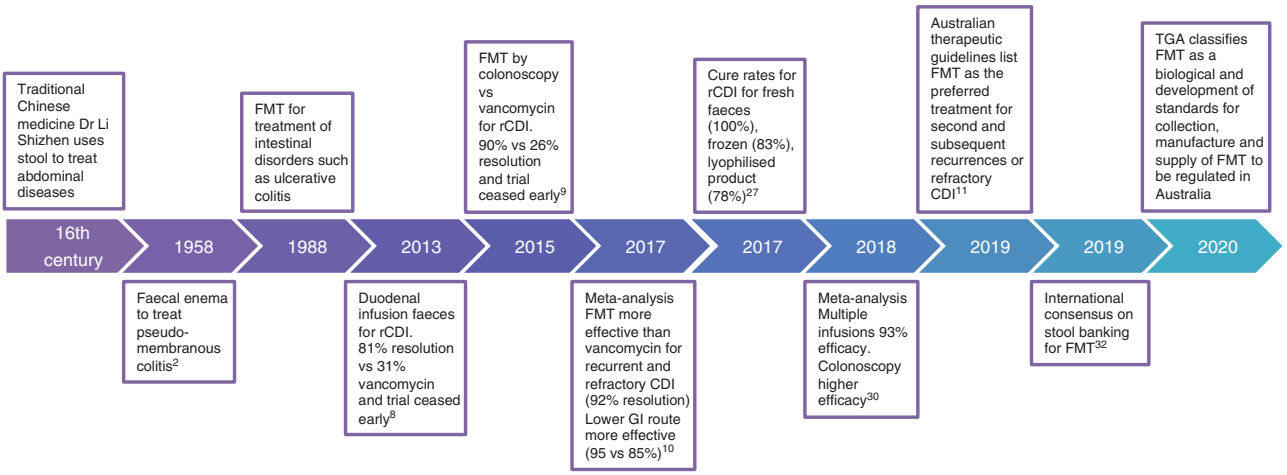

Figure 1. Timeline for faecal microbiota transplantation.

disease[11]. This is similar to American, European and British guidelines[12–14].

Adverse events

In general, FMT is considered a safe procedure with rare adverse events. Some of the common adverse effects include fever, abdominal pain, bloating and alteration to bowel habits[15,16]. Procedural complications include bowel perforation and mucosal tears[15,16]. Infectious complications including transmission of norovirus, Gram-negative bacteraemia and transmission of multiresistant organisms have been reported[15]. Deaths have been due to polymicrobial bacteremia in the setting of toxic megacolon, aspiration pneumonia as a complication of anaesthesia during colonoscopic FMT and regurgitation of faeculant material during endoscopic FMT[15–18]. Donor stool screening for multiresistant organisms is now mandatory following two cases of donor derived *Escherichia coli* Extended Spectrum Beta-Lactamase bacteraemia, resulting in the death of one patient[19]. The United States Food and Drug Administration (FDA) have recently issued a safety alert regarding FMT after cases of enteropathogenic *E. coli* (EPEC) and Shigatoxin-producing *E. coli* (STEC) infection in recipients possibly linked to a stool bank (www.fda.gov).

Food allergy with anaphylaxis is a contraindication to FMT[12]. FMT should be offered with caution in patients with decompensated chronic liver disease or immunosuppression and special consideration to donor screening (for CMV, EBV and *Strongyloides*) should be given for immunosuppressed recipients[12]. Elderly and debilitated patients have been treated with FMT for CDI with success, however they may have a lower primary cure rate and higher recurrence rate compared to a younger cohort[18,20]. Adverse events in the elderly population have included aspiration; therefore the colonoscopy route has been suggested as the preferred route of administration[18].

Limited data exists on long-term adverse effects. Jalanka *et al*.[21] found no difference in incidence of severe diseases or weight gain after 3.8 years of FMT and improved bowel habits and mental health were reported.

Donors

Traditionally, donors known to the patient were selected, however this could result in ethical and confidentiality issues if identifying a disease in the donor or a transmission event to recipient[22]. Alternatively, FMT is best sourced from a centralised stool bank from healthy unrelated donors[12]. Donors should be between 18 and 60 years of age and BMI between 18 and 30 kg/m^3 [12,23]. Donors are screened with a questionnaire followed by blood and stool testing with recommendations in Table 1. Woodworth *et al*.[3] recommend screening for carbapenem resistant *Enterobacteriaceae,* vancomycin resistant Enterococci and those with frequent contact with health care should be excluded. The risk of transmission of non-communicable diseases remains unknown; therefore, donors with cardiovascular disease, stroke, diabetes mellitus, obesity, metabolic syndrome and malnutrition are excluded[3].

Processing and preparation: impact on efficiency

Stool should be processed within 6 hours of defaecation. FMT material prepared in aerobic conditions has been effective for the treatment of recurrent *C. difficile* associated diarrhea[8]. However, ambient air exposure impacts on viable bacterial composition particularly for oxygen sensitive species[24]. Processing stool in an anaerobic chamber allows preservation of commensal species[24]. Freezing reduces the overall viability but the microbiota composition is not significantly different to fresh specimens[24], with viable

Table 1. Example of donor questionnaire and donor blood and stool testing.

Infectious diseases and risk factors	HIV, hepatitis B, hepatitis C, syphilis, HTLVI and II Current infection Risk factors for blood-borne viruses: illicit drugs, high-risk sexual behaviour, needle stick tattoo, piercing, acupuncture, blood transfusion <6 months Organ transplantation Recent hospitalisation or care facility High-risk travel <6 months Enteric pathogen <2 months Gastroenteritis <2 months Live attenuated virus vaccination <6 months Previous or latent tuberculosis
Medical history	Chronic gastrointestinal disease Systemic autoimmune disease Malignancy Recent gastrointestinal symptoms Neurological or psychiatric disorders or risk of prion disease Obesity, metabolic syndrome or diabetes Family history of colon cancer or other gastrointestinal conditions Atopy Chronic pain syndrome
Medication history	Antimicrobial drugs, immunosuppressants, chemotherapy <3 months Proton pump inhibitors >3 months Growth hormone, insulin from cows or clotting factor concentrates Experimental medicine or vaccine <6 months
Blood testing	Hepatitis A IgM HBsAg and HBcAb Hepatitis C antibody Hepatitis E IgM HIV-1 and HIV-2 antibodies HTLV-1 and HTLV-2 antibodies *Treponema pallidum* antibodies *Strongyloides stercoralis* IgG EBV serology (immunosuppressed) CMV serology (immunosuppressed) *Entamoeba histolytica* serology Full blood count and differential Creatinine and electrolytes Liver enzymes C-reactive protein
Stool testing	*Clostridioides difficile* PCR *Salmonella*, *Shigella*, *Campylobacter*, Shiga toxin-producing *E. coli*, *Yersinia*, *Vibrio cholerae* PCR +/- culture. Vancomycin-resistant *Enterococci* Methicillin-resistant *Staphylococcus aureus* ESBL *Enterobacteriaceae* Carbapenem-resistant and carbapenemase-producing *Enterobacteriaceae* Norovirus, rotavirus, adenovirus PCR Ova, cysts, parasite analysis *Giardia lamblia*, *Cryptosporidium*, *Isospora*, *Microsporidia* Protozoa and helminths *Helicobacter pylori* faecal antigen (upper route)

bacteria remaining after 6 months of frozen storage in 10% glycerol[22,25] and no difference in FMT efficacy observed when used for CDI[10].

There are a number of preparations for FMT including fresh, frozen and encapsulated faecal suspensions. Encapsulated freeze-dried preparations had 88% clinical success (49 patients) with no recurrence over two months[26]. In a randomised study of 72 patients with recurrent CDI, cure rates were highest for fresh faeces (100%), lowest for lyophilized product (78%; $P = 0.022$ vs fresh) and intermediate for frozen product (83%; $P = 0.233$ vs fresh)[27]. CDI recurrence was prevented in 84% receiving oral lyophilized microbiota capsules compared to 88% with FMT by enema ($P = 0.74$)[28]. In a non-inferiority randomised trial there was no difference after single treatment with capsule or colonoscopy delivery (both 96.2% without recurrent CDI at 12 weeks)[29].

Administration procedure: impact on efficiency

Bowel lavage is administered prior to FMT particularly for the lower gastrointestinal route. There should be minimum 24 hours free from antibiotics before FMT and at least 72 hours after FMT[12]. FMT can be delivered to upper (nasogastric, nasoduodenal or nasojejunal tube or upper endoscopy) or lower gastrointestinal tract (colonoscopic administration to caecum or terminal ileum or enema if not possible). Ianiro *et al*.[30] conducted a systematic review and meta-analysis of fifteen studies on different protocols of FMT for CDI. Multiple infusions increased efficacy compared to single infusion (93% vs 76%)[30]. Duodenal delivery had lower efficacy ($P = 0.039$) and colonoscopy had higher efficacy rates ($P = 0.006$). Lower faecal amount (≤50g) and enema had lower efficacy rates after single infusion[30]. Another meta-analysis also demonstrated administration by lower gastrointestinal route was more effective (95%) compared to upper gastrointestinal delivery (85%) with no difference between fresh or frozen FMT[10]. Consecutive courses after failure of first FMT showed incremental effect[10].

FMT services, stool banking and regulation

Historically, FMT has been performed with varying levels of sophistication across Australia, ranging from the *ad hoc* and infrequent preparation of fresh FMT material for recurrent CDI to specialised centres operating stool banks, such as the Biomebank (Adelaide, SA) and the Centre for Digestive Diseases (Sydney, NSW). In September 2019, the Australian Minister for Health determined that supply of faecal microbiota transplant products be regulated by the Therapeutic Goods Administration (TGA). The new regulatory model classifies most FMT products as class 1 or 2 biologicals depending on the extent of manipulation and whether manufactured in a hospital and used onsite. A Draft Standards for Faecal Microbiota Transplant Products is available with finalised FMT regulatory requirements expected in early 2020 (www.tga.gov.au). The American Gastroenterological Association (AGA) has proposed an FMT National Registry to collect outcomes to assess short- and long-term safety and effectiveness and current practices[31]. An international consensus on stool banking for FMT in clinical practice is available[32]. There are now Australian consensus statements for the regulation, production and use of FMT in clinical practice[23].

FMT for decolonisation of multiresistant organisms and treatment of other conditions

Small sample studies have shown that FMT was effective in reducing the number of antibiotic resistance genes in patients' resistome[33]. Huttner *et al*.[34] hypothesised that decolonisation could be achieved with oral antibiotics (colistin and neomycin) followed by recolonisation to restore intestinal microbiota. The results were only slightly in favour of the intervention group (OR 1.7). Nine uncontrolled studies with heterogeneity have evaluated the use of FMT for multidrug resistant Gram-negative bacteria decolonisation. However, the European guidelines suggest there is insufficient evidence for or against FMT in this context[35]. Similarly, UK guidelines do not recommend FMT as treatment for inflammatory bowel disease or other gastrointestinal or non-gastrointestinal disease[12]. Australian guidelines suggest FMT has been shown to be successful in induction therapy for mild to moderate ulcerative colitis however more studies are required before it can be implemented into standard care[23]. This is a developing research field and future treatment of conditions with FMT will be seen in the future.

Conflicts of interest

The authors declare no conflicts of interest.

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The future of faecal microbiota transplantation in gastrointestinal illness

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Abstract. The gut microbiome is made up of hundreds of trillions of microorganisms that reside in a state of homeostatic balance within the healthy individual. Next generation sequencing has provided insight into the diversity of these microorganisms that reside within our gastrointestinal tract; despite developments in metabolomics and culturing techniques, the functions of many of these bacteria remain largely elusive. As such, research into the capacity of the gut microbiome to regulate immune homeostasis has revealed the importance of bacteria in human health, with the potential for exploiting these bacteria only now coming into focus.

A number of diseases have been associated with 'dysbiosis', a term that denotes shifts in the relative abundance of the microbial communities in individuals with a disease relative to healthy individuals[1,2] (Fig. 1). This is generally characterised by a significant reduction in microbial diversity, and frequently a reduction in the abundance of beneficial commensals and an increase in pathogenic or pathobiont-like species. However, the characterisation of dysbiosis based on taxonomy is challenging, given the significant inter-individual variability at the microbial species level and the effect of environmental factors, such as diet and medications, on microbiome composition[3]. Additionally, the bioactive capacity of bacteria is not always phylogenetically conserved, with closely related microbes displaying variable immunomodulatory activity[4].

Faecal microbiota transplantation

Faecal microbiota transplantation (FMT) involves the infusion of healthy human donor faeces into the bowel of a patient most commonly via colonoscopy or enema, though oral routes have also been used (Fig. 2*a*). In administering FMT, the central hypothesis is that the contribution of the dysbiotic microbiome to disease can be overcome through restoration to one that resembles that of a healthy individual. The basis of this hypothesis is supported by increases in the Shannon diversity index that occur in responders versus non-responders following FMT in a number of diseases[6] (Fig. 2*b*).

Due to a plethora of successful research in the area, FMT is currently the recommended treatment method for recurrent *Clostridium difficile* infection (rCDI), with a cure rate of greater than 80–85%[7]. For the treatment of rCDI, FMT is effective regardless of the route of delivery, though lower GI delivery has demonstrated higher efficacy and less associated aspiration events; current consensus statements suggest that this should be individualized based on patient and disease characteristics, with careful consideration of the benefits and risks of each route of administration[8].

While antibiotics can be successful in eliminating the *C. difficile* bacterium, they also reduce the overall diversity of protective bacteria in the gut, creating an environment that encourages spore formation, vegetative growth, and toxin production. It is postulated that the reintroduction of a diverse array of bacteria through FMT restores the colonisation resistance potential of the microbiome, in which resident microbes able to out-compete *C. difficile,* thus preventing recurrent infection[9].

FMT has strong clinical evidence of efficacy for the treatment of rCDI, and emerging evidence for the treatment of a range of other pathologies.

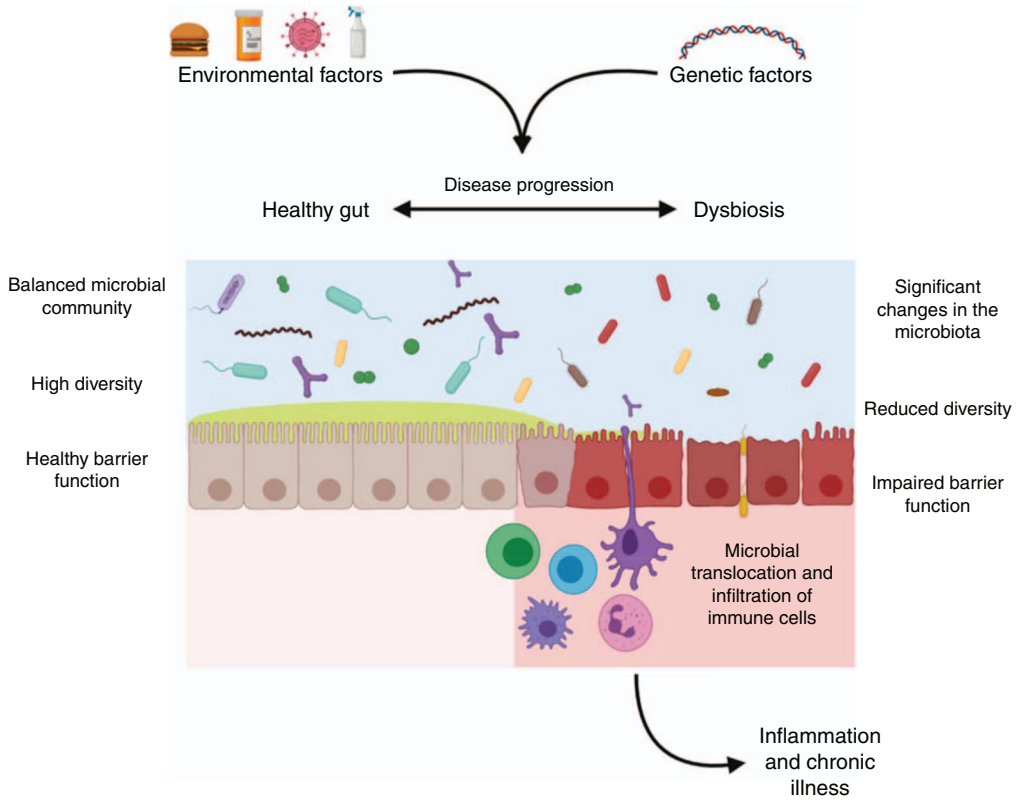

Figure 1. The healthy human gastrointestinal tract is made up of a diverse array of microorganisms, which contribute to the healthy functioning of the host. Healthy barrier function consisting of mucous layers and effective tight junction formation ensure the separation of these bacteria from the immune system. Shifts in the composition of the microbiota due to environmental and genetic factors lead to progression of gastrointestinal disease, which may be characterised by significant shifts in the microbiota associated with reductions in diversity. When coupled with impaired barrier function, this leads to microbial translocation and recruitment and infiltration of immune cells, resulting in the perpetuation of inflammation and chronic illnesses as a result.

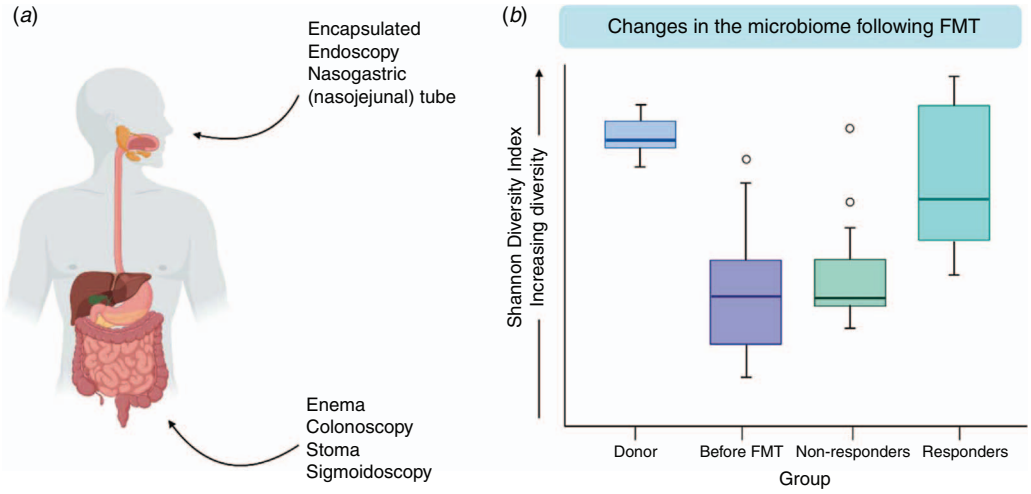

Figure 2. (*a*) Success rates of faecal microbiota transplantation (FMT) in clinical trials vary with disease, disease status and route of administration. In rCDI, Kao *et al*. (2017) found that FMT via oral capsules as not inferior to delivery by colonoscopy for preventing recurrent infection[5]. (*b*) The Shannon Diversity Index encompasses the species diversity and evenness of bacterial species within a community; an increased index being representative of communities with large numbers of equally represented taxonomically diverse microbes. Studies have found that in a number of diseases, FMT leads to an increase in bacterial diversity and abundance in responders but not in non-responders, with the composition of the microbiome shifting to one that resembles that of the healthy donor.

FMT in inflammatory bowel diseases

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract, of which the two main manifestations are ulcerative colitis (UC) and Crohn's disease (CD). In 2017, IBD was estimated to effect 6.8 million people globally[10].

IBD is underpinned by inappropriate immune responses to the commensal intestinal microbiome, in genetically susceptible hosts who are exposed to environmental factors that may trigger disease onset[11]. Current treatment paradigms for IBD rely on a variety of approaches including dietary therapy, the administration of

corticosteroids, immunomodulatory drugs, and biologic antibody-based therapies, as well as surgery for resection of the affected area of the gut. Despite the success of these approaches there still remains a therapeutic gap, with 10–30% of IBD patients being recalcitrant to medical treatment[12].

The microbiome of IBD patients is notably different to that of healthy individuals. IBD patients maintain significantly reduced taxonomic richness and a shift in abundance of key phyla, with general reductions in the abundance of members of bacterial families including Erysipelotrichales, Bacteriodales, and Clostridiales and increases in the abundance of Veillonellaceae, Enterobacteriaceae, Pasteurellaceae, and Fusobacteriaceae[13]. In addition, evidence supports the association of specific bacteria, including adherent-invasive *Escherichia coli*[14] and *Mycobacterium avium* subspecies *paratuberculosis*[15] with IBD, although it remains unclear if these organisms directly drive disease pathogenesis or are merely more abundant in the presence of underlying gut inflammation.

This taxonomic dysbiosis is coupled with functional dysbiosis, which has been increasingly explored in the literature. Non-targeted metabolomic analyses have revealed increases in metabolites including primary bile acids, amino acids and sphingolipids, and reductions in tetrapyrrole, triacylglycerol, cholesterol, and long chain fatty acids, in IBD patients when compared with non-IBD controls[16]. Changes in many of these metabolites are believed to be related to bacterial processes[17]. Therefore alternative treatment approaches including FMT, aimed at restoring an 'anti-inflammatory' gut microbiome, are gaining traction.

FMT in ulcerative colitis

UC presents as continuous, superficial inflammation and ulceration of the colon and rectum, in which symptoms occur intermittently as the disease flares and remits. Complications of UC can include toxic megacolon, colorectal cancer, and extraintestinal manifestations in the liver, eyes, skin, and joints[18]. Research into the use of FMT in UC has been promising despite disease heterogeneity. To date, four double-blinded placebo-controlled RCTs have been conducted in the area[19–22], accompanied by a large number of case reports, case series, and cohort studies. These studies generally involve multiple FMT treatments, up to five enemas per week over two months. These studies have demonstrated that FMT is efficacious in inducing remission in mild-moderately active UC, with primary remission rates following FMT reported in a meta-analysis to be approximately 30%[7], which is similar to that of many biologic agents studied in UC.

Generally these clinical trials have reported increased microbial diversity and altered composition in UC patients that achieve remission following FMT, when compared with pre-FMT samples or patients that do not respond[23]. Following a double-blind trial of 81 patients with active UC, Paramsothy *et al*. (2019) reported that patients in remission after FMT had increased abundance of *Eubacterium hallii* and *Roseburia inulivorans*, which contrasted with the higher abundance of *Fusobacterium gonidiaformans*, *Sutterella wadsworthensis*, and *Escherichia* spp. in patients that did not respond to FMT[23]. Significant changes in the functional capacity of the microbiome have also been reported to co-occur with the taxonomic shifts following FMT; Paramsothy *et al*. (2019) also reported that UC patients who achieved remission after FMT had higher levels of short chain fatty acid biosynthesis and secondary bile acids when compared with non-responders, who maintained increased heme and lipopolysaccharide biosynthesis profiles[23].

FMT in Crohn's disease

Research is ongoing into the use of FMT in CD, the subtype of IBD that exhibits transmural, discontinuous inflammation throughout the gastrointestinal tract. As of April 2020, five placebo- or sham-controlled RCTs were listed on the U.S. National Library of Medicine Clinical Trials website as being in the pre-recruitment or recruitment phases of study. The results of these studies will be informative; however, there is insufficient evidence at present to support FMT for treatment of CD[7].

The efficacy of FMT in IBD appears much lower than in rCDI, potentially reflecting the multifactorial aetiology of IBD, and the likelihood that bacterial species within this dysbiotic microbiome have well developed niches and therefore difficult to displace[24]. The higher variability of response seen in IBD studies when compared with rCDI is likely reflective of differences in study methodologies examined in IBD, and highlights the potential of donor and/or patient dependent effects.

FMT in irritable bowel syndrome

As with IBD, research into the use of FMT in other illnesses associated with gut dysbiosis is emerging. Despite affecting up to 1 in 5 individuals, the aetiology of irritable bowel syndrome (IBS) is poorly understood and treatment options are limited. Data collected through RCTs has been mixed, and when administration routes were analysed together, FMT did not consistently improve symptoms in patients despite positive results in some individual trials[25]. Many studies pool subtypes of IBS, which include constipation predominant, diarrhoea predominant, and mixed subtype, despite the potential for different underlying pathophysiologies, which may impact the analysis of efficacy in these trials. Available

RCT data appears to show more success in the diarrhoea predominant subtype; however, further studies are required to understand the characteristics of patients.

FMT for extra-intestinal illnesses

Changes in the gut microbiota have also been reported to co-occur with progression of chronic liver disorders such as non-alcoholic fatty liver disease (NAFLD)[26], non-alcoholic steatohepatitis (NASH)[2], cirrhosis[27], alcoholic liver disease[28], and hepatocellular carcinoma (HCC)[29]. In NAFLD for example, gut dysbiosis and increased gut permeability are associated with chronic and systemic liver inflammation that can increase the risk for developing HCC; the gut microbiota is therefore a potential target for managing this disease. As a result, clinical trials are currently underway to assess the efficacy of FMT in the context of liver disease (NCT02496390, NCT02469272).

As in other emerging clinical areas, there has been some preliminary success in the use of FMT in recurrent hepatic encephalopathy (HE), a complication of cirrhosis that manifests as an altered mental status. When compared with standard of care treatments, including lactulose and rifaximin treatment, those who received FMT had reduced HE recurrence and liver-related hospitalisation events, as well as improved cognition, demonstrating the promise of FMT in this setting[30].

Beyond faecal microbiota transplantation

Ongoing study in the emerging area of FMT therapy is clearly needed. Despite its preliminary successes, practical difficulties associated with FMT including donor recruitment and screening, manipulation of faeces, choice of delivery route, and lack of regulation, have encouraged research into the development of more defined therapies to overcome these barriers.

Research on products that contain either single microbial species, or a defined consortia of microbes, in an attempt to harness those bacteria with specific beneficial immunomodulatory capacities, is gaining traction. These products may contain live or dead bacteria, or their secreted bioactive products, and are designed to target specific pathways. In the case of IBD, these products may be developed to modulate aberrant immune responses or increase mucosal barrier integrity.

Examples of specific bacterial bioactive compounds include the microbial anti-inflammatory molecule (MAM) peptide produced by *Faecalibacterium prausnitzii*, which inhibits pro-inflammatory signalling in epithelial cells and reduces inflammation in murine models of chemically induced colitis[31], and polysaccharide A (PSA) from *Bacteroides fragilis*, which was found to suppress pro-inflammatory cytokines[32]. There is the potential for products such as these to be developed into single formulation 'probiotics' that can be taken orally to treat disease.

Probiotics and consortia products also offer the potential for regulated standardised treatments, though thus far these therapies have had limited success and require further trial in a clinical setting. Products such as SER-287 by Seres™ Therapeutics[33], and Rebiotix product RBX2660[34], are currently in the clinical trial phases for IBD and rCDI respectively.

Conclusion

Harnessing the power of the microbiome is an attractive therapeutic option for a number of diseases. However, as treatment approaches shift towards personalisation the use of FMT to manage disease may appear archaic. Nevertheless, its success in the treatment of rCDI and emerging successes in other clinical areas demonstrates its value within the treatment armamentarium. Regulatory pressures and a need for greater safety and reporting are resulting in a preference for FMT products originating from stool banks or commercial facilities. There is likely to be further evolution of microbial directed therapies; however, whether this will be single bacteria or consortia products remains to be seen, and whether these products will be superior to FMT depends on their success in clinical trials in the years to come.

Conflicts of interest

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Biographies

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The relevance of probiotics in Caesarean-born neonates

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Abstract. There is growing interest in the use of probiotics in neonates. In particular, *Lactobacillus rhamnosus*, *L. acidophilus*, *Bifidobacterium breve* and *B. longum* have been well studied. Caesarean-section (CS)-born infants often lack *Lactobacillus* spp. and *Bifidobacterium* spp., which showed increasing evidence in establishing the neonatal immune system. Furthermore, CS increases the difficulties for mothers in initiating and sustaining breastfeeding. Increasing evidence shows CS-born infants are more susceptible to allergy, infections and chronic inflammatory diseases later in life. The number of CS births has increased continuously, now accounting for 35% of all deliveries Australia wide. In this context, probiotics may have a role in establishing a healthy neonatal gut microbiome.

Introduction

'An ounce of prevention is worth a pound of cure' is an axiom by Benjamin Franklin, one that is relevant especially in the current COVID-19 pandemic. In Australia, rates of delivery by Caesarean-section (CS) have increased and reached 35% in 2017[1]. Antibiotics are used regularly for both prophylaxis and treatment of infections in mothers who deliver babies through CS[2]. This excess use is important for its potential role in driving antimicrobial resistance worldwide[3] and also has an impact on the establishment of the neonatal gut microbiome.

CS is associated with significant difficulties in initiating breastfeeding when compared with vaginal birth[4]. The microbiome of breast milk contains bacteria, including lactic-acid bacteria (LAB), and is

important in establishing the gut microbiome of neonates[5]. Breast-feeding helps to establish healthy gut microbiome. LAB were first described by Pasteur as part of fermentation to prevent spoilage approximately 70 years before the discovery of penicillin in 1928[6] (Figure 1).

CS-born infants generally lack LAB, i.e. *Lactobacillus* spp. and *Bifidobacterium* spp., which appear important in establishing the neonatal immune system[7]. Recent data support the theory that probiotic administration to CS-born infants may prevent allergy in children and young people[8]. Certain species of *Bifidobacterium* spp. may only be isolated from human breast milk within a few days after birth[9]. Early intervention through probiotic administration in neonates, especially in neonates born via CS may improve general health, given their susceptibility to various chronic diseases[7] as well as potential prevention of chronic inflammatory diseases, such as inflammatory bowel disease, rheumatoid arthritis, coeliac disease and diabetes mellitus later in life[10].

Probiotics, in particular *Lactobacillus* spp. and *Bifidobacterium* spp., are considered normal flora and part of human gut microbiota[7]. *Lactobacillus* spp. and *Bifidobacterium* spp. are considered generally regarded as safe, especially for oral administration[11]. In international guidelines such as the FAO/WHO guideline, probiotics are recognised as having a role in maintaining gut health and may modulate host immunity[11]. In this article, the genomes of *Lactobacillus* spp. and *Bifidobacterium* spp. are described along with the mechanisms of action of LAB in interfering against pathogenic bacteria.

Very recently the taxonomy within genus *Lactobacillus* spp. was re-classified into 25 genera[12]. As the changes were very recent, and these new genera have not been adopted to the WHO/FAO guideline for probiotics, genus *Lactobacillus* will be used for this article.

It is proposed that genus *Lactobacillus* of *L. casei*, *L. paracasei* and *L. rhamnosus* as genus *Lacticaseibacillus*[12]. *L. salivarius* and *L. fermentum* have been named as *Ligilactobacillus salivarius* and *Limosilactobacillus fermentum*, respectively[12]. Genus *Lactobacillus* of *L. acidophilus* and *L. gasseri* have not changed[12].

Probiotic use in neonates

There have been extensive studies of the use of probiotics in neonates including preterm infants[13–16]. In particular, these studies have focussed on the role of probiotics in reducing the incidence of necrotising enterocolitis (NEC) and sepsis. Most significantly, a randomised controlled trial of a symbiotic preparation including *L. plantarum* in 4500 term neonates in the community resulted in a 42% reduction in neonatal sepsis[17].

In addition to its impact on neonatal sepsis, probiotics may reduce gastrointestinal complications in neonates though the evidence is mixed[18]. The large Probiotics in Preterm Infants Study (PiPS) Trial randomised 1310 pre-term babies to treatment with *Bifidobacterium breve* BBG-001 or placebo and showed no reduction in rates of sepsis, NEC or death[16]. In contrast, the ProPrems trial, a randomised-controlled trial that included 1099 preterm infants from Australia and New Zealand demonstrated a reduction of NEC of approximately 50%[19]. The strains being used in the ProPrems trial were *B. infantis*, *S. thermophilus* and *B. lactis*. A metagenomic approach to characterise the gut microbiota was also used in a sub-study of ProPrems trial, which showed abundance of *Bifidobacterium* spp. in the infants administered with probiotic[15]. In neonates, while considered generally safe, cases of *Lactobacillus* bacteraemia have been reported including in a <1000 g weight pre-term infant following a laparotomy[20].

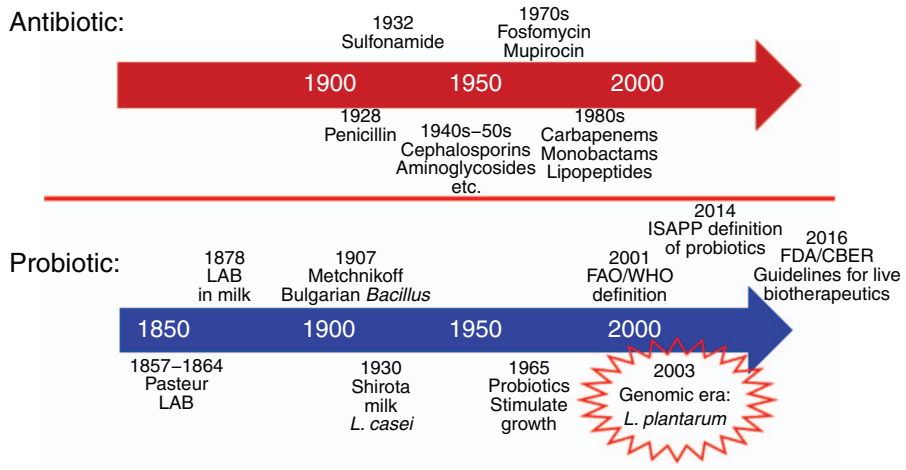

Figure 1. Timeline of probiotic development in comparison to the antibiotic development.

L. plantarum, *L. gasseri* and *L. salivarius* have been isolated from infants' oral and faecal samples[21–23]. Therefore, these three genera are considered normal infants' microbiota and warrant further research. Thus far, there is no published research study of using *L. gasseri* and *L. salivarius* in neonates. Further, these strains are not commercially available for infants or neonates yet. Research is required to include species not typically in the current formulation of probiotics for neonates. As *L. plantarum* may reduce atopic dermatitis[24], and *L. gasseri* and *L. salivarius* may have immunomodulatory effects[21,22], these should be considered for inclusion in probiotic formula for neonates. In the potential formulation of the probiotics, *Bifidobacterium* spp. have been reported as predominant genus in breastmilk microbiome[7]. Therefore, to add another strain to the formula would need to consider the species proportion in the breastmilk, i.e. with lower CFU than *Bifidobacterium* spp. Of note, *L. plantarum*, *L. gasseri* and *L. salivarius* are in the commercial formula available for adults.

Probiotic administration has also significantly reduced the length of stay in pre-term infants[23]. A cost-saving analysis in pre-term infants supplemented with probiotics showed a saving of €2000 per infant[25]. The clinical impact and cost effectiveness of probiotic administration require further well designed laboratory, clinical and cost analysis research.

Mechanisms of probiotics in interfering with pathogens and immune modulation

Probiotics interfere with pathogens through acid production, hydrogen peroxide production and bacteriocin activity[26]. Production of bacteriocins, small peptides with anti-bacterial activity of *Lactobacillus* spp. has been reported to inhibit pathogen growth[26]. Specific short-chain fatty acids (SCFA) have been studied to understand their beneficial properties, such as butyrate for the antagonistic activity against cancer cells and anti-inflammatory property[27]. SCFA production by *Bifidobacterium* spp. in the gastrointestinal tract results in a lower pH and inhibition of potentially pathogenic bacteria[28].

Bacterial exopolysaccharide has been known to possess immunostimulatory properties[29,30]. Extracellular vesicles (EV) in Gram-negative bacteria have been studied for their pathogenicity, host-pathogen interaction and potential targets in vaccine development[31]. Very recently, studies on EV were performed in probiotic strains and revealed potential delivery of bacteriocins and other beneficial properties through the EV[32,33]. Advancing research on EV of probiotics is highly recommended as it will provide further understanding on molecular mechanisms of probiotic bactericidal properties against pathogens and immune modulation. Evidence of *Bifidobacterium* spp. in boosting immune systems has been demonstrated mostly in the mouse model, marked by the stimulation of IL-6 and IL-10 in the ileal Peyer's patches and in weaned pig model, marked by the increase of IgA against the parasite and IgG[34,35].

Genomes of LAB

Genome data provides comprehensive data that might also help to determine the beneficial properties and the potential virulence determinants in the strains. Genome data enable the comparison of the strains with publicly available genome data. We limit the discussion of the genomes to the strains being used commercially in humans. The recent genus *Lactobacillus* name changes have not impacted the species and genomes, as we abbreviate the genera.

L. rhamnosus GG (LGG) has been the most commercially popular probiotic strain. More than 1100 studies on *L. rhamnosus* GG were found in NCBI (accessed 22 April 2020). *L. plantarum* 299v has shown beneficial properties such as effectiveness to treat irritable bowel syndrome[36]; regardless, only 112 studies on *L. plantarum* 299v versus 204 studies on *L. plantarum* WCFS1 were in NCBI. Very few studies were on *L. salivarius* with 39 studies of *L. salivarius* UCC118 found from NCBI. As previously described, the *L. plantarum* WCFS1 genome was first sequenced in the early 2000s and has been well described with its genome of 3 308 273 bp (GenBank accession number NC_004567.2) and a total of nearly 1200 identified proteins. The beneficial properties of *L. plantarum* WCFS1 include the ability of this strain to survive in a wide range of environments with temperature and pH changes[37]. The parental strain of *L. plantarum* WCFS1 is *L. plantarum* NCIMB 8826, which was isolated from human saliva[38]. *L. plantarum* NCIMB 8826 colonises the oral cavity well but not the human intestine, although it has been demonstrated to survive in the gastrointestinal tract, including faeces[39]. *L. gasseri* ATCC33323 (Accession Number of NC_008530.1) was the complete reference *L. gasseri* genome in the NCBI database with its genome of 1 894 360 bp. *L. gasseri* ATCC33323 is an autochthonous microbe in the gastrointestinal system[40]. Therefore, oral application of the *L. gasseri* ATCC33323 for intestinal colonisation may be well tolerated. For a comprehensive genome description of *L. salivarius* UCC118, the reference strain being used here is available through the study by Claesson and colleagues[41]. The size of the chromosomal genome of *L. salivarius* UCC118 was 1 827 111 bp (GenBank accession number: NC_007929.1). General probiotic properties of *L. salivarius* were the ability to eliminate pathogens and the adaptation to the

gastrointestinal niche[42]. *L. salivarius* UCC118 has broad spectrum activity versus Gram-positive bacteria[43]. Therefore, *L. salivarius* UCC118 has very strong probiotic properties and is autochthonous to the gastrointestinal tract.

Genomes of *Bifidobacterium* spp. have also been described, i.e. *B. longum* (n = 349), *B. breve* (n = 109), *B. bifidum* (n = 104) and *B. animalis* (n = 83) (from NCBI, accessed 2 March 2020). *B. longum* NCC2705, *B. breve* DSM 20213, *B. bifidum* PRL2010 and *B. animalis* subsp. *lactis* DSM 10140 are the reference genomes in NCBI with genome sizes of 2.257, 2.257, 2.215 and 1.938 Mb, respectively (GenBank Accession Numbers: NC_004307.2, NZ_JDUD000000000.1, NC_014638.1 and CP001606.1, respectively).

Current evidence

Probiotic supplementation in neonates has been frequently studied. In an era of interventional birth leading to high rates of CS, probiotics may have a role in establishing a healthy gut microbiome. The impact of probiotics in this setting may include a reduction in important acute complications such as neonatal sepsis, and NEC and longer-term impacts relating to the development of mucosal immunity and atopy. The heterogeneity of trial results may relate to the differing strains used. Genomic and metagenomics approaches to analysing the gut microbiome may improve understanding of gut dysbiosis and its role in these complications.

L. rhamnosus, *L. casei*, *L. acidophilus*, *L. plantarum*, *L. gasseri* and *L. salivarius* are listed in the three main regulatory bodies in

Table 1. Commercially available probiotics for infants including neonates.

Product (company)	Composition	Administration	Countries
Infloran (Laboratorio Farmaceutico)	*L. acidophilus* NCDO1748 and *Bifidobacterium bifidum* NCDO 2203	Neonates including premature infants up to 6 years	Product of Italy Available in Australia Widely used in neonatal units in Australia
Infant Probio (Health Aid)	*L. reuteri* NCIMB 30351 (200 million CFU per dose)	Drops (5 drops, 1/day), infants up to 3 years	Product of UK
Upspring Probiotic + colostrum (Upspring)	Six probiotic strains (*B. lactis*, *B. longum*, *B. breve*, *L. rhamnosus*, *L. acidophilus*, *L. reuteri*), 3 billion for Bifidobacterium and 2 billion for *Lactobacillus* spp. + colostrum	0–4 months (half pack per day) 4–12 months (one full pack daily)	Product of USA Available in Australia
Probiotic Baby (Jamieson)	*B. animalis* subs. lactis or BB-12 (1 billion CFU in 6 drops)	Drops 1–36 months	Product of Canada
Protectis baby drop (Biogaia)	*L. reuteri* DSM 17938 (100 million CFU in 5 drops)	Drops do not specify the age bracket, but for baby	Product of Sweden Available in Australia
Inner Health Baby Probiotic (Inner Health Plus)	*B. breve* (BR03 and B632) (2 million CFU in 5 drops)	6–36 months	Product of Australia
MetaKids Baby probiotics (Metagenics)	*L. rhamnosus* GG and *B. animalis* subs. *lactis* (BB12) (1 billion CFU in 6 drops)	0–12 months	Product of USA Available in Australia
Probiotics Baby Drops (Radiance)	*B. lactis* (BB12), 6 drops (1 billion CFU in 6 drops)	Pregnancy and baby including newborn	Product of New Zealand
Kids Smart Drops Probiotic (Nature's Way)	*B. animalis* subsp *lactis* BB12 (1 billion CFU per mL)	0–12 months – 0.5 mL daily (12–24 months – 1 mL)	Product of Australia
Baby probiotic colic drops (Renew Life)	*Pediococcus pentosaceus* and *B. longum* strains (1 billion CFU in 5 drops)	0–36 months	Product of USA Available in Australia
Flora Baby (Renew Life)	*B. breve* (600 million CFU), *L. rhamnosus* (500 million CFU), *B. bifidum* (400 million CFU), *B. longum* subp *infantis* (300 million CFU) and subp. *longum* (200 million CFU) in 500 mg	0–12 months (500 mg) >12 months (1 g)	Product of USA Available in Australia
Probiotic Powder for Infant (Life-Space)	Two types of probiotics that are naturally found in breastmilk	1–6 months	Product of Australia

European Food Safety Authority (EFSA), Canada and China as strains can be added in food[44], which may broaden the use of these strains as human food supplement in countries outside Europe, e.g. China and Canada. Europe has been the epicentre for probiotic development and generation so far. EFSA allowed 37 different *Lactobacillus* spp. for consumption through food[44]. Therefore, supplementation of *Lactobacillus* spp. and *Bifidobacterium* spp. to infants and neonates can be categorised as natural administration of beneficial microbes or probiotics to maintain gut microbiota and immune systems[45].

Infloran containing *Bifidobacterium bifidum* and *Lactobacillus acidophilus* is a commercial probiotic widely used in neonatal units in Australia. Other probiotics available in pharmacies are listed in Table 1. Many commercial preparations are not included in the table due to a lack of published data on the strain identity and CFU counts. Guidelines in choosing the right probiotics are available from International Scientific Association for Probiotics and Prebiotics website (https://isappscience.org/). Industry-related probiotic information can be found from the International Probiotic Association website (http://internationalprobiotics.org/). As probiotic administration is now becoming broader than oral administration, the use of the food-medicine interface guidance tool within Therapeutic Goods Australia (https://www.tga.gov.au/) is highly recommended in translating probiotic research to industry.

In summary, with the increasing evidence of CS births in Australia and worldwide, and antibiotic prophylaxis administration in CS births, as well as the potential delay of the breastfeeding initiation, it would be highly recommended to provide probiotics those commonly isolated from breastmilk, to CS born neonates. Probiotic administration mimicking the LAB of breastmilk will be likely a better option than inoculation of swabs originated from vagina, often called seeding. Future studies that include microbiome analysis, neurocognitive development as well as economic analysis of probiotic administrations are highly recommended.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

Dr Hanna E Sidjabat is a molecular microbiologist with a strong industry link in translating her probiotic research to manufacturing. In addition to her probiotic expertise, she has a solid background in antibiotic resistance mechanisms including genome and proteome due to 15 years of research experience. She has strong research focus in the bacterial genome, proteome of pathogens and probiotics. To date, Dr Sidjabat has published 87 peer-reviewed articles in international journals. Dr Sidjabat has supervised and mentored 35 PhD students, Postdoctoral Research Fellows, Master and Honours students, Microbiology Registrars, local and international Infectious Diseases Visiting Academics following the completion of her PhD in 2007.

Alaa Mohammed Ali Alsaggaf is a Master graduate in molecular microbiology from the University of Queensland, School of Chemistry and Molecular Biosciences. Alaa has worked extensively on the genome of *Lactobacillus* spp. within Sidjabat's team at the University of Queensland Centre for Clinical Research (UQCCR). She has clinical microbiology role in Saudi Arabia within Ministry of Health. She received the UQ SCMB Dean's award for her project in the second semester of 2018.

Akshatha Gopalakrishna has a Masters degree in molecular biology research and is equipped with extensive microbiology and biochemistry laboratory skills from the University of Queensland, School of Chemistry and Molecular Biosciences. She has worked extensively on *Lactobacillus* spp. for screening of probiotic strains within Sidjabat's team at the UQCCR. She has further extended her skills on histology, immunohistochemistry staining, various microscopic skills and sample preparation for MRI at the UQ Centre for Advanced Imaging.

Evelyn Nadar is a graduate of Master's in molecular biology research extensive with extensive laboratory skills from the University of Queensland, School of Chemistry and Molecular Biosciences. She has worked extensively on *Lactobacillus* spp. proteomic analysis of probiotic research within Sidjabat's team at the UQCCR. She has also expanded her animal handling skills and histology including microscopy skills.

Dr Adam Irwin is a conjoint Senior Lecturer and academic lead for Paediatric Infectious Disease at Children's Health Queensland and the University of Queensland. His research focuses on diagnostic evaluations to optimise the use of antimicrobials in children. Specifically, he is interested in healthcare-associated infections and infections resistant to antimicrobial therapy. He is also interested in the clinical and molecular epidemiology of invasive Gram-negative infections. Dr Irwin studied at the University of Birmingham Medical School and completed training in Paediatric Infectious Disease and Immunology in London. He was awarded his PhD by the University of Liverpool Institute of Infection and Global Health in 2016.

Dr Pieter Koorts is the Director of Neonatology Royal Brisbane and Women's Hospital since 2016, Acting Director of Neonatology RBWH (2015–2016), Deputy Director of Neonatology RBWH (2009–2015), Staff Specialist Neonatologist RBWH (2007–2009). Dr Koorts has affiliation as a Senior Lecturer with School of Medicine, University of Queensland since 2007. Dr Koorts started his career in paediatrics in 1998 and into neonatology as a Senior Lecturer and Senior Staff Neonatologist (2005–2006) at Pretoria University, South Africa. Dr Koorts was a neonatal fellow at Mercy Hospital for Women, Melbourne, in 2002–2005.

The challenges in commercialisation of Probiotic API manufacturing



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Abstract. The concept of probiotics is well known and has developed into a high value commodity in recent times. Despite the ever-expanding number of probiotic products on our pharmacy, health food and supermarket shelves, the probiotic culture active ingredient has always been imported until now. In 2019, Probiotics Australia Pty Ltd opened Australia's first and only Therapeutic Goods Administration/current Good Manufacturing Practice (TGA/cGMP) certified facility dedicated to the manufacture of probiotic active ingredients. This article outlines the significant export demand for Australian-made health products and the lengths to which Probiotics Australia have gone to create a facility to meet needs of the probiotics research, commercialisation and consumer market today and into the future.

'Product of Australia' or 'Made in Australia'?

Probiotics is one of the most focused topics in the functional food and complementary medicines markets. An international research company reported that the global probiotics market was valued at an estimated US$49.4 billion in 2018. This market is anticipated to expand at a compound annual growth rate of 7.0%, to reach US $69.3 billion by 2023. One of the primary driving factors for the market to grow rapidly is the increasing awareness of the probiotic health benefits among customers, especially in the Asia-Pacific region including countries like China and Japan[1]. Australian-made complementary medicine and functional food products are renowned for their quality and high desirability, especially in Asia. Much of this can be attributed to rigorous quality of Australian Standard for industry including probiotic manufacturing process. Parallel to this, the regulatory framework in Australia is compulsory in maintaining superior quality of Australian products. Unlike most countries in the world, Australia, through the TGA, categorises health supplements including probiotics as listed medicines instead of food. From the procurement of raw materials, quality control, manufacturing facilities and equipment, production processes, to the packaging and final quality control testing of finished products, all steps are subject to strict regulation and scientific guidelines.

By 2030, CSIRO predicts over $3.2 billion in export revenue to Australia derived from vitamins and supplements, including probiotics[2]. Nevertheless, customers seldom know that the active probiotics ingredient, the highly concentrated pure culture of dried probiotic powder, is imported from overseas, mainly from the US, Europe, China, Japan or India. The imported active probiotics ingredient is further formulated into the end products such as capsules or tablets, or sachets. Some other end products are in the form of functional foods including beverages and dairy products. Therefore, for commercial products containing probiotics, 'Made in Australia' labels do not mean that the probiotics were fermented, purified and tested fully in Australia.

Australia does not lack the technical and scientific knowledge or skills in the field of probiotics. However, most of the expertise is concentrated within the academic world, conducting research focused on the health efficacy and immune functions of different probiotic strains. Some novel probiotic strains have been developed in Australia and commercialised[3]; however, their marketing exposure is very limited on a global scale. This reflects the fact that commercialisation of novel probiotic strains or manufacturing of probiotic active pharmaceutical ingredients (API) has not been the focus in the Australia probiotic industry. On the world stage, major players in the probiotics industry have dominated the market with no significant Australian-made alternative. To compete in a global scale in commercialisation, Australia must focus on helping and bridging academic and industry to work synergistically in boosting the commercialisation of probiotics. Probiotics Australia, as a Queensland-based company is keen to be a key player in probiotic research, commercialisation and industry in Australia.

The challenges of up-scaling: is it just a larger fermenter?

Fermentation is an ancient concept. From wine, sour dough for bread and yoghurt drinks, humans have mastered this technique for thousands of years. In modern fermentation, temperature, pH, agitation and aeration control are just some of the fundamental and critical processing parameters that are closely controlled. In most of the research-focused laboratories, the bio-processing work is usually around optimising the fermentation media and conditions in lab-scale to pilot-scale bioreactors. Although the basic process of probiotic API manufacturing is well studied and familiar to scientists, researchers, processing engineers and technologists in Australia, the manufacturing scale of facilities and utilities are far more complex than the pilot processing of probiotic generation.

Overall, the probiotic manufacturing process can be divided into two main parts, upstream processing and downstream processing.

Upstream processing

Multiple fermentation lines are usually required with the fermenter size ranging from 500 to 100 000 litres in working volume. To achieve the required large commercial volume, fermentation is gradually scaled up from smaller to larger fermenters. In order to economically provide enough heating for sterilisation, industrial pure steam is usually provided from a boiler system that is capable of generating tons of pure steam per hour. As opposed to heating, the temperature maintaining and cooling of fermentation lines is just as critical. The cooling system throughout the process is essential to maintain accurate control over the viability of the cells. It is also crucial to incorporate well designed CIP (clean-in-place) and SIP (sterilisation-in-place) systems to comply with the cGMP cleaning validation requirement to a high standard.

Downstream processing

The downstream process starts from the centrifugation step (Figure 1). Depending on the strains and/or the bioactive components interested in harvest, the concentrating of the biomass is usually carried out by industrial scale filtration or centrifugation. For example, harvesting probiotic cells can be performed in a semi-continuous centrifugation system that is capable of processing hundreds of litres of fermentation solution per hour.

The by-product of the centrifugation is the supernatant. The volume of the supernatant can range from 80% to 99% of the fermentation volume, depending on the strain, fermentation media, equipment and conditions. Therefore, thousands of litres of supernatant can be generated from the process on a daily basis, which will need to be properly treated before disposal under the monitoring of the local city council in Australia. Although there are

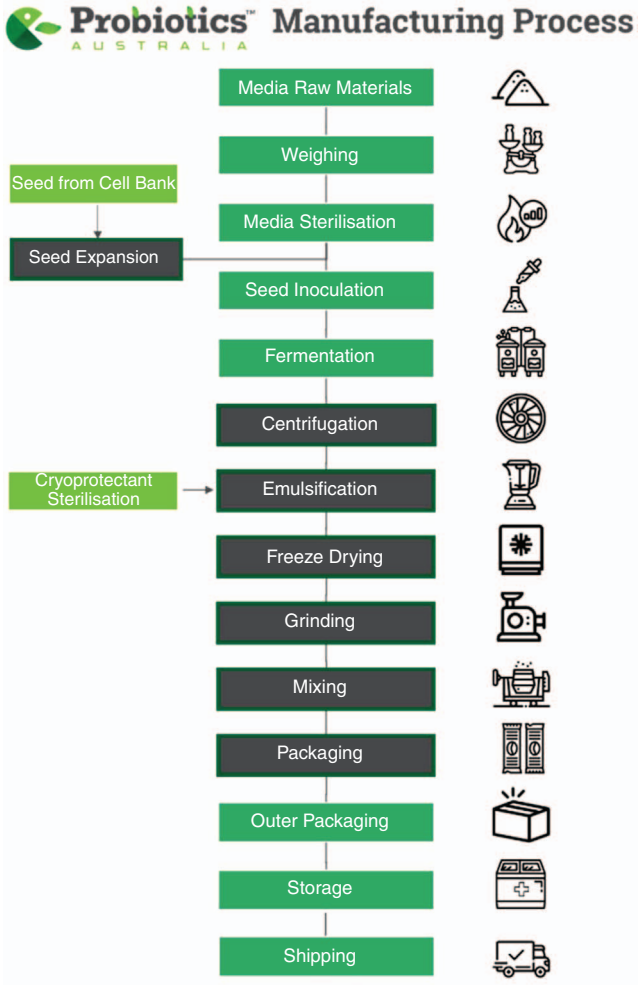

Figure 1. Probiotic manufacturing flow chart.

lots of literature showing benefits or good application of cell-free probiotic supernatants in different fields such as human health, animal health, bio-preservation, agriculture, etc., there does not seem to be many probiotic supernatant-base commercial products in the market.

Probiotics Australia is established and operating to enable all processes as illustrated in Figure 1, including for freeze drying. Freeze-drying cycle usually involves reducing the probiotic temperature down to as low as –190°C, depending on the type of freezing. The entire drying cycle could be more than 70 hours per batch. With multiple freeze dryers operating simultaneously, power consumption could translate into a huge economical problem for the company if the freeze dryer is not well designed, or the cryoprotectant formulation and processing conditions are not optimised.

After freeze-drying, there are multiple steps including harvesting the freeze-dried powder, milling, and mixing with excipients. The exposure of the products in the environment will require the processing facility to be cleanroom equipped with a HVAC system that can accurately control both the temperature, humidity and cleanliness of the air. The RH% (relative humidity) is ideally to be controlled under 30%. As handling probiotic powder can generate large amounts of particles (pure, concentrated and viable microorganism) travelling throughout the facility, high quality HEPA filters and differential pressure design between different processing areas must be an important part of the overall facility design to minimise cross-contamination, to ensure the strain purity of the products.

There are many day-to-day challenges in a modern probiotic API manufacturing plant. Other important utilities include the RO-water generation plant. Using high quality water in fermentation is critical for reducing the batch variation. QC analytical laboratory is also a fundamental component to assist the manufacturing plant for QC monitoring and troubleshooting.

The certification of TGA certified cGMP manufacturer

Probiotics are usually regulated as a food in the countries and regions that dominate this field. The US FDA lists probiotics that are suitable for use in their jurisdiction on a database known as GRAS (Generally Regarded As Safe). There are currently 29 records for 'Lactobacillus' and 17 records for 'Bifidobacterium' with GRAS Notices (as accessed 5 March 2020).

By comparison, regulations outside Australia are usually less strict than those that apply here. Across most of the world, HACCP-base systems from the food industry are usually employed for monitoring probiotics. However, in Australia probiotics could fall in the pharmaceutical category regulated by the TGA. The cGMP certification of a pharmaceutical API manufacturer by TGA is usually governed by the PIC/S (Pharmaceutical Inspection Convention – Pharmaceutical Inspection Co-operation Scheme) guideline Part 2, developed by the Internal Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use[4]. Using the PIC/S Part 2 as the guideline, usually BP (British Pharmacopoeia), USP (US pharmacopoeia), ISO standards are used to implement quality control plans, material testing methods, equipment calibration and validation plans etc. In the PIC/S guide Part 2, the fundamental QA components such as Documentation control systems, Processing parameters validation, monitoring and verification, Product recall systems, etc. are covered. Moreover, there are many additional requirements and system components very specific to the pharmaceutical and bio-processing or biotechnology manufacturers.

For example, cGMP certification for pharmaceutical and biotechnology manufacturers undertake Qualification and Validation of utilities, processing equipment, laboratory instrument, and manufacturing process as a critical part of their GMP certification. For all equipment used on site, from a laboratory thermometer to industrial bioreactors, documentation must be completed to qualify the equipment from its design stage – DQ (Design Qualification), to IQ (Installation Qualification), OQ (Operational Qualification), and PQ (Performance Qualification). The operational range of processing parameters is studied and identified in the OQ stage. This stage could be a very lengthy and expensive exercise for some of the equipment that are not stand-alone, but a set of systems consist of tanks, pumps, pipes with utilities of water, gas, steam connected to it. PQ is usually done through the actual manufacturing stage where real manufacturing data are collected to validate, evaluate and improve the process.

Another component that is quite specific to the biotechnology industry is the Cell Bank System Management. In the PIC/S guide Part 2 Section 18, the specific controls for APIs manufactured by cell culture is given. The starting active material for the fermentation is the 'seed' from the cell bank. The seed in the probiotic industry is the pure culture of intended probiotic strain. The discovery, isolation, purification, characterisation and banking of the probiotic strains involve traditional microbiology culturing methods and modern DNA sequencing identification methods. Other important components in the TGA/cGMP certification process include the quality systems required in the analytical laboratories, and the

quality systems required in the probiotic API manufactured for clinical trials.

The GMP certification process by the TGA is a highly technical, lengthy and extremely costly process. Many technical and regulatory hurdles are required to be overcome prior to the operation of the manufacturing plant.

Untapping the potential of probiotics

The concept for Probiotics Australia was born in 2009 when the opportunity for locally produced probiotic active pharmaceutical ingredients (APIs) was nascent. The vision was to construct a state-of-the-art facility to house research, development and production capabilities that would untap the potential of probiotics. One of the keys to unlocking that potential was to secure TGA certification for cGMP that would demonstrate that Probiotics Australia was delivering the highest quality product and give customers confidence in the probiotics they were consuming. In addition to TGA/cGMP certification granted in July 2019, Probiotics Australia also holds HACCP food license, ACO organic, USA NOP organic and FDA approval.

From their proprietary seed bank, Probiotics Australia can produce probiotic organisms for health, food, agriculture, aquaculture, veterinary and industrial applications. Fermentation capacities range from small scale bench-top experiments through to bulk cell-mass measured in tonnes and freeze dried in one of the largest lyophilisation sites in the southern hemisphere.

The research, development and manufacturing areas of Probiotics Australia are all located in the same building. This provides benefits in terms of rapid implementation of new techniques developed by the research team and offering a contract fermentation or manufacturing service. Through partnerships with universities and research organisations, Probiotics Australia has assisted to bring novel research from the laboratory and *en route* to clinical trials. The goal is for these trials to support the commercialisation of Australian research. Current studies pending publication encompass areas such as gastrointestinal health, Alzheimer's Disease, gut-brain axis, immune response and mother to baby microflora transmission.

An overnight success 10 years in the making, Probiotics Australia has evolved from a great idea to a thriving biotechnology research and manufacturing organisation with over 50 staff. The demand for Australian-made health products, determination of the founders and access to highly skilled scientists has led to the creation of a one-of-kind facility and business.

Conflicts of interest

All authors are employees at Probiotics Australia.

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Biographies

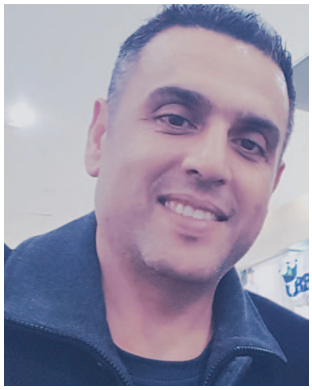
Dr Joe Liu, PhD, is the assistant general manager in Probiotics Australia Pty Ltd. He holds a Bachelor of Biotechnology, Master of Microbiology. Joe started his PhD in CSIRO Food Innovation Centre. His research focus was on novel processing technologies including Ultrasonication and Pulsed Electric Fields on the functional modification of dairy proteins. After graduation, Joe worked as senior microbiologist, technical services consultant and R&D manager in different analytical laboratories to provide technical supports and commercialisation consultancy to different industry sectors. In 2017, Joe joined Probiotics Australia and he was one of the key technical managers to design and construct the state-of-the-art TGA cGMP certified probiotic API manufacturing facilities. He is now leading the technical and R&D teams in Probiotics Australia with the focuses on novel strain discovery, probiotic functionality studies, and optimisation of probiotic API manufacturing technologies.

Brendan Cook, Sales and Marketing Manager, has over 15 years' experience in the Australian biotechnology, healthcare and pharmaceutical industries in manufacturing, technology transfer, sales and marketing roles. His dream to work alongside researchers to bring their innovations to market for the benefit of all is being realised at Probiotics Australia.

Shaun Roux, General Manager, Probiotics Australia, is a highly experienced senior manager with a proven track record leading multi-disciplinary, cross-functional teams. Specialising in technical business culture, complex projects and innovation. His role in international collaborations allowed a transfer of biotechnologies that formed Probiotics Australia, and that is now shaping the probiotic precinct of Australia. He leads the way in product development, innovation and has worked with most of the leading health care brands in Australia producing new and innovative products.

On the use of probiotics to improve dairy cattle health and productivity



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Abstract. Probiotics are genetically identifiable, live microorganisms that when administered in adequate amounts, confer appropriately sized health benefit (e.g. correcting dysbiosis, immunomodulatory effect) on a target host. In cattle, probiotics have shown promising results and long-term benefits in productivity when used on animals under stress. The health and production benefits of probiotics were attributed to improvement in fermentation in rumen and intestine, the stabilisation of rumen pH, and improvements in the intestinal barriers. In the bovine udder, a dysbiosis of the commensal intramammary microbiota and the presence of mastitis causing-bacteria has been linked to increased intramammary infections. Probiotic bacteria capable of biofilm formation inside the udder either serve as a barrier against pathogens or disrupt and replace biofilms formed by pathogens. Over the past two decades, several types of probiotics have been used as feed additives; however, the effect of probiotic use on disease prevention and cattle health and performance indicators, and characterisation of the immunomodulatory association between probiotic microbiota and host target system microbiota are yet to be quantified or documented.

The advent of the ban on the use of the antibiotics in agriculture in 1986 in Sweden, followed in 1999 by The Netherlands[1]. In 2003, the United Nations tripartite (World Health Organization, Food and Agriculture Organisation and World Organisation for Animal Health) released a joint report titled 'Non-human antimicrobial usage and antimicrobial resistance: scientific assessment report', which recommended strict surveillance and monitoring, and moderation of antimicrobial usage in the food-producing animal industry, specifically due to the public health implications entailed by zoonotic transmission of bacteria such as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., and *Enterococcus* spp. and antimicrobial usage or resistance risks[2].

The report raises many issues related to antimicrobial use patterns and their implications on animal and human health. The most important question arising from the animal health perspective was how health and productivity standards, on both animal and herd levels, can be maintained while reducing (or eliminating) the needs for antimicrobials? Noting that the definition of animal- and herd-level health indicator metrics such as mortality/morbidity rates, disease incidence, immune response and feed conversion efficiency vary between animal production systems[3]. Therefore, quantifying the effect of antimicrobial usage reduction on animal health and productivity across production systems remains a challenging task[4–6].

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host'[7,8], the microorganisms must be must be alive in an adequate number when administered, strains must be identified genetically and

appropriately tested on target conditions and hosts[8]. The probiotic interaction with host's system microbiota (e.g. udder, rumen, intestine) results in correcting system dysbiosis[9] and controlling several infectious inflammatory conditions through antagonism and immunomodulation[10]. Lactic acid bacteria (LAB) that are well known antibacterial producers and generally recognised as safe in the food industry offer a possible alternative to conventional antimicrobials[11].

The mammary gland contains unique microbiota[12,13]. The presence of bacteria not associated with mastitis in the healthy udder reinforces the concept of commensal mammary microbiota, and the ecological structure of the healthy udder microbiota may provide an understanding of the pathogenesis of intramammary infections (IMI) and offer opportunities for developing therapeutic or prophylactic products as an alternative to antimicrobials[14]. A dysbiosis of the commensal intramammary microbiota and the presence of mastitis causing-bacteria has been linked to IMI in dairy cattle[12]. The use of probiotics is proposed to correct the dysbiosis[9]. Studies have been conducted using viable cultures of LAB as intramammary infusions to successfully treat mastitis pathogens with the same efficiency as conventional antimicrobials[15]. Direct infusion with *Lactococcus lactis* in the udder has been shown to induce a rapid and considerable innate immune response with the greatest increase in immune gene expression coinciding with peaks in somatic cell counts (SCC)[10].

In 2015, a study was conducted to determine the effect of an intramammary infusion with a LAB-based probiotic mix in healthy lactating dairy cows[16]. The mix successfully elicited a massive inflammatory/immune response in the infused quarters[16]. The magnitude of the response is particularly noteworthy as the LAB-based mix did not colonise within the udder and bacterial counts recovered from milk decreased to zero 48 h post infusion. All animals experienced an increase in SCC and swollen udder quarters. The immune response was short-lived and SCC returned to pre-infusion levels within five days. It was hypothesised that the immune profile elicited by the LAB-mix was different from a pathogen assault and may prove to be a successful non-antibiotic treatment for mastitis because of the LAB-mix's ability to produce a bacteriocin with broad-spectrum antibacterial activity against gram-positive pathogens and elicit a rapid and substantial innate immune response. The 2015 study findings compare very favourably with other therapies recently investigated to treat mastitis[10].

Probiotic bacteria capable of biofilm formation have also shown promising results in the prevention of mastitis. The biofilm formation inside the udder either serves as a barrier against pathogens[17] or disrupts and replaces biofilms formed by pathogens[18]. The latter could have been driven by interspecies interactions: high growth rates and dominance of probiotic organisms over other biofilm formers[19] and substrate competition[20,21].

A controlled, crossover study was conducted in 2018 to evaluate the safety and efficacy of LAB-based probiotic applied as a teat spray in improving SCC of lactating dairy cattle[22]. On average, milk SCC in the control group was 66% higher (1.66, 95% confidence interval (CI) 1.08–2.56, $P = 0.02$) compared with the probiotics group (Figure 1)[22]. The study concluded that the probiotic bacteria may have produced a biofilm which could have hindered the colonisation of other bacterial isolates resulting in reduced bacterial counts on the teats. Our results compare favourably with the literature[17]. More work is needed to better understand the exact mode of action of the probiotic product tested. The successful identification of inflammatory modulators (pro/pre), antibacterial peptides and development of a new biological mastitis therapy could significantly reduce the substantial economic losses incurred by the dairy industry worldwide and improve animal health, productivity and welfare while increasing food safety[23].

In cattle, probiotics used as feed additives have shown health and productivity benefits when used when animals are assumed to be under stress[24]. In lactating dairy cattle, after controlling for the effect of days in milk, and cow parity, cows ingesting probiotics have been reported to produce an average of 1.21 L/day more milk (95% CI 0.34–2.08 L/cow per day; Figure 2*a*), more milk protein (0.03 kg/day; 95% CI 0.01–0.05 kg/day; Figure 2*b*), numerically lower average SCC and fewer clinical cases of

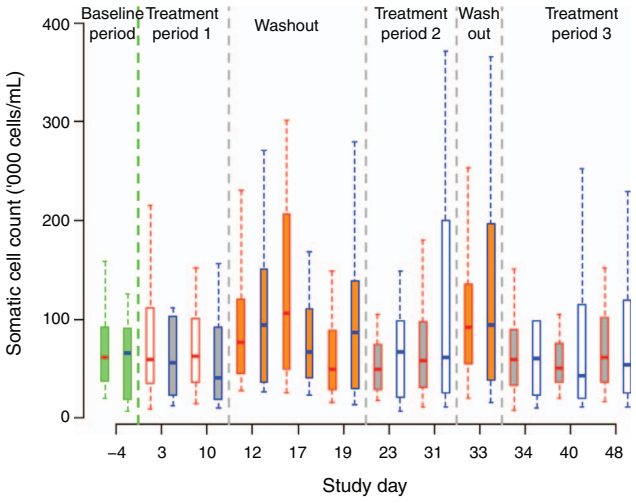

Figure 1. Box-of-whiskers plot of somatic cell count ('000 cells/mL) observed during baseline (green shaded boxes; experimental group A1 red border and red horizontal line; experimental group A2 blue border and blue horizontal line) and treatment periods (control article group in white shaded boxes; probiotics article group in grey shaded boxes; washout period in orange shaded boxes) of the study. The study design was a 3 × 2 randomised, controlled, crossover study conducted between June and December 2018[22].

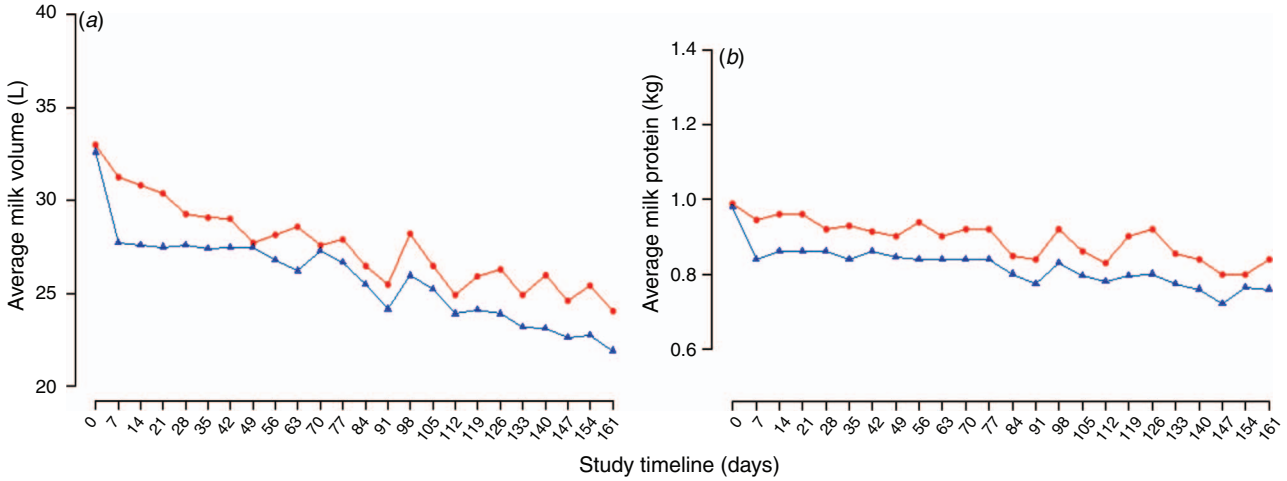

Figure 2. Line plot of average weekly milk production (L; *a*) and milk protein (kg; *b*) for the control (blue solid triangles and blue solid line) and probiotics group (red solid circles and red solid line). The results are from a randomised controlled study[25] conducted in 2018.

lameness and mastitis than the control cows[25]. Similar effects on calf health and productivity were also reported[26]. Calves on probiotics were heavier at weaning, and on average, rumen and intestinal organs' folding and crypts were more developed and more adapted compared with control calves[26]. These benefits were hypothesised to be linked to improvement in the ruminal and intestinal fermentation[27]. The current known mechanisms of action of probiotics in ruminants appears to be through a shift in the microbiota of rumen and rear-gut (small and large intestine), an improvement in fermentation or volatile fatty acids, the stabilisation of rumen pH, and improvements in the intestinal mucosal barriers through the probiotics competitively excluding pathogens and improving the local and systemic immune response[28,29].

Probiotic bacteria have also been isolated from soil[23], fermented green tea[30], the gastro-intestinal tract of various animals including poultry[31] and cattle[32]. The most common genera of bacteria used include *Lactobacillus* spp., *Bacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp. and *Enterococcus* spp. The intended application of probiotic bacteria varies. Studies that use bacteria like *Dietzia* spp., and *Megasphaera* spp., are more focused on their prophylactic uses. The choice of the interventions was based on the presumed mechanism of action of the probiotic strains, e.g. bacteriocin production, lactic acid production, oxygen scavenging, immune-modulation and more generally, their ability to establish a healthier microbial composition in the gastrointestinal tract. The general intent of probiotics is to replace the need for antimicrobials, but the combination of the two have been explored by few: Click (2011) used tetracycline with *Dietzia* spp. to prevent the development of Johne's disease symptoms in calf neonates[33], and Timmerman *et al*. (2005) used a prophylactic antibiotic and probiotic mixture to improve the health and growth of veal calves[34]. In more recent years, probiotic development has shifted from bacteria to using other organisms like yeast (*Saccharomyces* spp., *Candida* spp.) and mould (*Aspergillus* spp.). It has been identified that a mixture of organisms is more effective and are generally better for prophylactic therapy[34]. Some commercial probiotics are combined with other naturally isolated compounds such as allicin (e.g. Enteroguard®- Romvac Company) and medicinal plant mixes[35], which enhance the beneficial effects of the probiotic bacteria by acting synergistically. The most commonly observed or hypothesised mechanisms include the production of inhibitory substances like bacteriocins, organic acids and hydrogen peroxide, production of biofilms by changing bacterial population of gastrointestinal tract; 'stimulating faecal shedding of coliforms, decreasing concentration of stress hormones like cortisol, increasing in number of $CD3^+$, $CD4^+$, $CD45R^+$, $CD8^+$, T cells, $WC1^+$, $CD282^+$, detoxification of blood from heavy metals like zinc, cadmium and lead'[36,37]. There is a consensus in the literature further investigations into the exact mechanism of action of probiotics is required in order to maximise the outcome benefits that may be derived from probiotics bacteria.

Conclusions

Probiotics have been proposed as a viable alternative to antimicrobials to enhance animal health and productivity. Over the past two decades, several types of probiotics have been used as feed additives, however, the effect of probiotics use on disease prevention on cattle health and performance indicators (e.g. rumen health and development, growth rate, feed conversion), and characterisation of the immunomodulatory association between probiotic microbiota and host target system microbiota are yet to be quantified or documented.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

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Pangolins' purpose is pursuing ants not propagating peril

Pangolins, also known as scaly anteaters, appear to be a plausible link between horseshoe bats and humans in the coronavirus line of transmission. Pangolins are docile and reclusive creatures that live in tropical forests and are a native species of Southeast Asia. So how did they gain a role in disease creation and transmission?

Pangolins are the world's most trafficked mammals and are valued for for their meat and scales. Pangolin scales are made of keratin, like rhinoceros horns, and although they have no proven medicinal value they are used in traditional Chinese medicine to help conditions ranging from lactation difficulties to arthritis. Pangolins are very strong diggers and this ability to break through barriers and blockages is believed to reside in their scales.

In addition people pay up to $1000 for a live Pangolin to keep as a pet. They are very gentle and have no teeth. They carry their young on their back, like koalas, but could never be regarded as cute or cuddly. In Vietnam, pangolin flesh is an exotic food fetching up to $300 per kilo.

Many exotic animals are both poached and farmed, and subsequently eaten for novelty, therapy or for good fortune rather than for sustenance, as was the case originally. This practice, happily, is said to be slowly falling out of favour in mainland China.

Let us hope that the disastrous consequences of close contact with, and consumption of pangolins and other native species, are now fully recognised - for all our sakes - and that they are left to forage in protected tracts of tropical forest, rather than be captured and marketed in mixed markets where their viral passengers can find human hosts and subsequently cause widespread suffering and loss.

Maybe fortune cookie inserts could be printed to promote the message that good fortune follows preservation not pillage? Pangolins particularly.

Faecal microbiota transplantation: is it the future for pig production?

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Abstract. Piglet mortality is a major issue for the pork industry globally and until recently, the main method for improving growth performance and reducing disease in commercial practice is centred on anti-microbial use. Antibiotic resistance is a global concern and, as such, animal production industries are seeking alternatives to antibiotics. Different approaches under investigation include but are not limited to management of the intestinal microbial environment. The gastrointestinal microbiota is involved in a myriad of processes that impact host health and well-being. Recently, interest in maintaining a healthy microbiome in order to improve herd health is increasing. In this article, we focus on faecal microbiota transplantation as a method for manipulating and improving the gastrointestinal microbiota in pigs in order to improve health and performance.

Currently, 11–15% of all piglets born alive die prior to weaning within the pork industry globally[1–3]. This represents a major welfare concern and economic loss to industry. To date, much research has gone into reducing this loss but with varied success. The current management methods for reducing piglet mortality caused by sickness, such as diarrhoea, and improving growth performance in weaned pigs, is the administration of antibiotics, with their use often being both therapeutic and prophylactic. Organisations such as the World Health Organization, the US Centres for Disease Control and Prevention, and the European Centre for Disease Prevention and Control have identified antibiotic resistance as a global concern, as what were once common treatable infections are now becoming life threatening[4]. As such, alternatives to antibiotics need to be explored.

The intestinal tract houses a community of microorganisms that has a mutualistic relationship with the host, known as the enteric microbiome[5]. These microorganisms include bacteria, fungi, archaea, protozoa and viruses[6–8]. The enteric microbiome is involved in a myriad of processes, some of which include immune system maintenance and development, intestinal barrier function, nutrient metabolism and competitive exclusion of pathogens[8–10]. While antibiotics are effective in pathogen removal, they also impact the commensal microbiome[11]. If a healthy microbiome is maintained, the need for therapeutic interventions such as antibiotic administration will be reduced as the animal will be better equipped to cope with external stressors. This is where the interest surrounding methods for influencing the microbiome, through management such as pre- and pro-biotics and faecal microbiota transfers, has expanded.

In particular, one such method that has demonstrated efficacy in treating *Clostridium difficile* infections in humans is faecal microbiota transplantation (FMT). FMT involves the transfer of faeces from a healthy donor into the gastrointestinal tract of a recipient. This can be done either orally (Figure 1) or rectally via an enema[12]. The objective being that the beneficial bacteria within the healthy donors' faeces will competitively exclude the pathogenic bacteria within the unhealthy or sick recipient, therefore altering the microbiota and in the case of *C. difficile* infections, treating the disease[12] (Figure 2). This method can also be used for altering the microbiota of the recipient to resemble that of the donor for the

Figure 1. Oral administration of faecal microbiome transplantation via a gastric tube to a 20-day-old piglet.

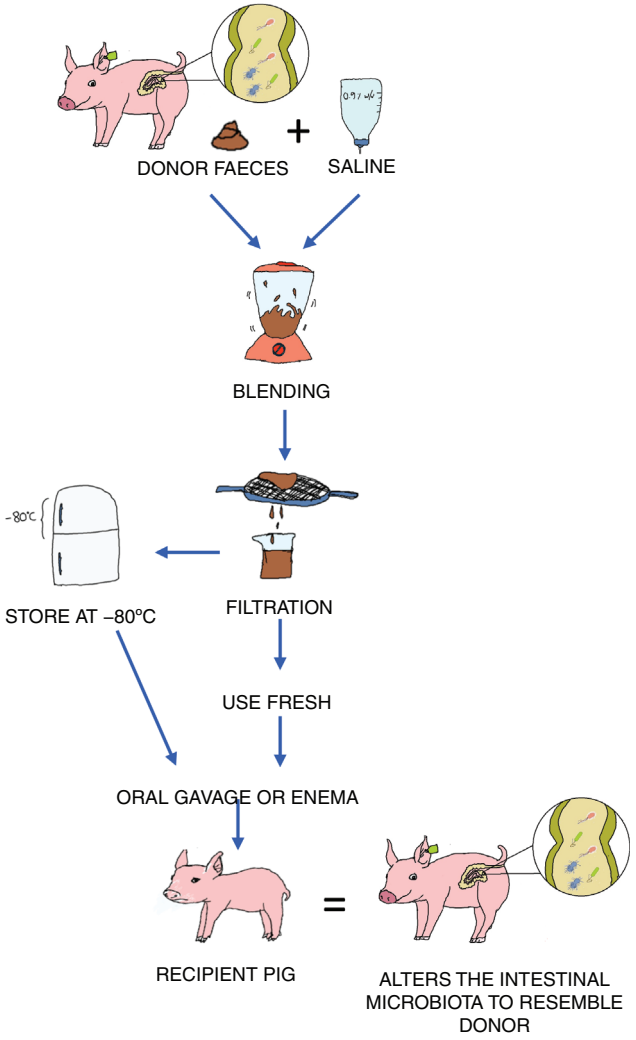

Figure 2. Schematic of faecal microbiota transplantation (FMT) in pigs.

objective of creating a phenotypic change[13]. FMT was first described by Ge Hong in 4th century China for the treatment of food poisoning and severe diarrhoea[12]. Today, FMT is commonly known for its efficacy for the treatment of *C. difficile* infections in humans. FMT has demonstrated a success rate of >90% in patients with reoccurring *C. difficile* where antibiotic use has been unsuccessful due to the formation of spores[14]. The use of FMT in other areas of human health and disease prevention are becoming increasingly popular; however, its efficacy in treating other diseases in humans to date is not as high. Although this is the case, investigation into its use within production animals such as pigs is increasing.

Recent studies investigating its use in pig production have shown promising but inconsistent results. Several research groups have demonstrated that the administration of multiple oral FMT to piglets from birth can increase average daily gain, reduce the incidence of diarrhoea and improve intestinal barrier and immune system function[15–18]. However, in contrast to this, others demonstrated a negative effect on intestinal integrity and growth when piglets received FMT directly or were reared on sows receiving FMT[19,20]. When examining the human literature, where additional phenotypic traits were transferred with FMT that mimicked the donor, it is evident that the donor used can significantly impact the results observed[21]. As such, particular care needs to be taken when selecting the appropriate donor as the risk of transferring undesirable traits is high. Further, Niederwerder *et al.*[22] found that FMT was an effective preventative effect against porcine circovirus associated disease in pigs co-infected with porcine circovirus type-2 and porcine reproductive and respiratory syndrome virus. The pigs that received one dose of FMT daily for seven days following weaning from healthy donor sows had a significant reduction in morbidity and mortality and increased antibody levels.

Studies where FMT in pigs was employed as a research model for humans have also found promising results that not only provide evidence for its effects on enteric microbiota modulation but also host metabolism. Wan *et al.*[23] demonstrated that oral FMT from 1 to 6 days of age reduced fatty acid oxidative catabolism and amino acid biosynthesis of piglets. Additionally, Brunse *et al.*[24] observed that rectal FMT from 10-day-old donor pigs to caesarean-derived preterm piglets changed their colonic carbohydrate metabolism from lactate to propionate production, increasing colonic pH. Rectal FMT also preserved goblet cell mucin stores and reduced the incidence of necrotizing enterocolitis. When comparing routes for FMT, it has been noted that when combining oral and rectal transplantation, piglet mortality increased. Conversely, those that

received only rectal administration did not suffer the same problems[24]. Further supporting the findings of the previous studies, Geng *et al.*[25] demonstrated that FMT reduced susceptibility to epithelial injury and modulated tryptophan metabolism in a piglet inflammatory bowel disease model. When taken collectively, it is evident that FMT in pigs not only alters microbial membership but also has effects on host metabolism, intestinal barrier function and the immune system.

Although FMT is a promising prospect it is not commercially applicable in its current form, with most studies administering multiple doses for 1–2 weeks in order to demonstrate an effect and fasting or stomach acid reduction protocols in place to improve post-gastric bacterial survival. Recently, our research group identified that the administration of a single FMT dose at weaning resulted in durable changes to 35 days of age (14 days post FMT) (T L Nowland *et al*., unpubl. data). To our knowledge, this is the first study to demonstrate changes to the microbiome of piglets after a single dose of FMT. However, whether this is possible in a younger pig and whether it lasts long term is yet to be determined. Additionally, some scepticism surrounds the use of FMT commercially due to the biosecurity risk that it entails as rigorous testing is needed in order to prevent the transfer of diseases[13]. If FMT is being considered in pigs for the treatment of a disease, then it is likely that the recipients are sick and probably relatively immunocompromised. Thus, the risk from possible transfer of pathogens will be increased. However, a possible refinement to FMT to minimise this risk is suggested by the work of Hu *et al.*[18]. These authors used a native Chinese pig breed with increased resistance to stress-induced diarrhoea to determine the identity of specific bacteria involved in this resistance. Such a targeted approach to disease control would have a major advantage over the 'shot gun' approach of conventional FMT. It is evident that research surrounding the use of FMT within pig production is still in its infancy. Although, an increasing number of studies are investigating the use of FMT as a tool for increasing growth, feed efficiency and treating enteric diseases in pigs, there is still a long way to go before it will be applicable to industry.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

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Current perspectives and applications in plant probiotics





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Abstract. **As agriculture and food security face unprecedented challenges, emerging agricultural innovations and existing practices require ongoing examination in the context of sustainability. In this review, we focus on the use of probiotic microorganisms for improved plant production. As plants are enormously diverse, emphasis is placed on the fundamental sites of plant-microbe interactions regarding benefits and challenges encountered when altering the microbiome of these locations. The soil, the external plant epidermis, and internal plant tissue are considered in discussion regarding the type of plant probiotic application. Plant probiotics range from broader soil beneficial microorganisms (such as *Trichoderma* spp.) through to specialised epiphytes and endophytes (such as root nodule bacteria). As each site of interaction affects plant growth differently, potential outcomes from the introduction of these exogenous microorganisms are discussed with regard to plant productivity. Finally, recommendations regarding regulation and future use of plant probiotics are points of consideration throughout this review.**

Introduction

Microbial communities (or microbiomes) are associated with all biotic systems, and the balance and function of a system can be altered by the metabolic activity and interaction of microorganisms within it. When the microbiome of a system is disturbed, it can result in changes in homeostasis in an organism or shifts in productivity in a system[1]. Depending on the change, this can lead to a deleterious or beneficial effect[2]. Probiotics is a term used when exogenous microorganisms are introduced, or endogenous microorganism populations are manipulated to elicit a beneficial change (for the purpose of this review we will conform to this nomenclature)[3]. The study of probiotics is an emerging field in mammals. For

example, an imbalance of the human gut microbiome has been shown to result in disrupted homeostasis (for reviews see [4] and [5]). However, other higher organisms, including plants, are more diverse in function and physiology therefore conclusions regarding the effects of probiotics are often species related[6].

Species belonging to the kingdom Plantae are enormously diverse and occupy most terrestrial surfaces on every continent on Earth. Given this diversity, it is difficult to generalise plant-physiology. For simplicity, sites where interactions between microorganisms and plant tissue occur are summarised in Figure 1. The site of infection and colonisation can occur internal to the epidermis (endophyte) and on the surface of the epidermis (epiphyte). All interactions between host (plant) and symbiont (microorganism) vary and the relationship is defined by the effect the symbiont has on the host, as illustrated in Figure 2.

Plant roots penetrate various layers of soil substrata in search of nutrients and water. During their exploration of soil, plant roots encounter millions of different microorganisms and have developed advanced genetic and metabolic mechanisms to both recruit and defend against microorganisms. Colonisation of roots involves a complex molecular communication between microorganism and roots. Attracted by root exudates, microorganisms migrate towards roots via chemotaxis and may colonise the root surface (rhizoplane), or in the soil aggregates that form around

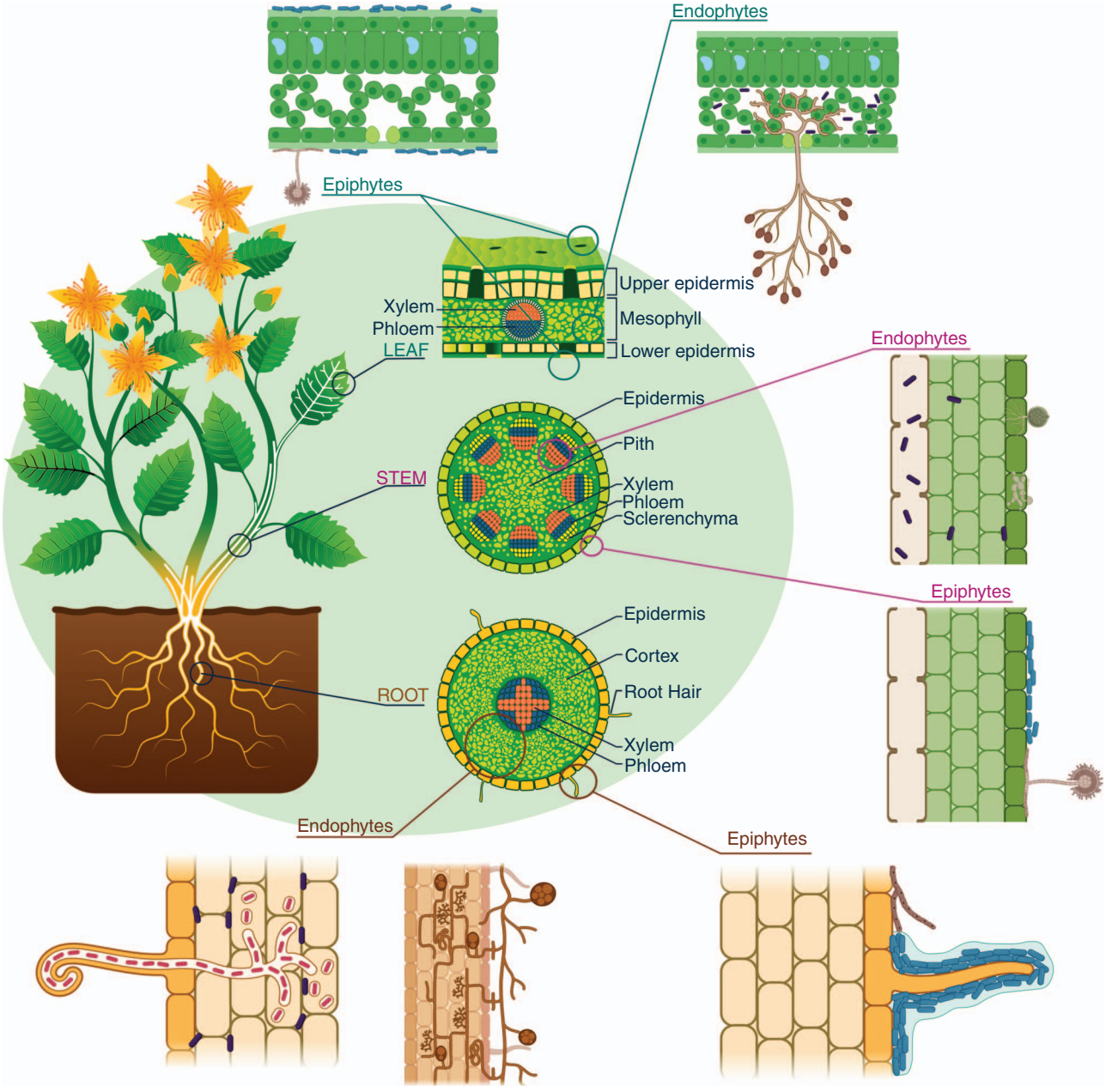

Figure 1. Diagram showing possible locations of interaction between epiphytes and endophytes on major types of plant tissue. Blue rods, bacterial epiphytes; dark purple rods, bacterial endophytes; red rods, root nodule bacteria; fungi shown in brown and grey. Not to scale.

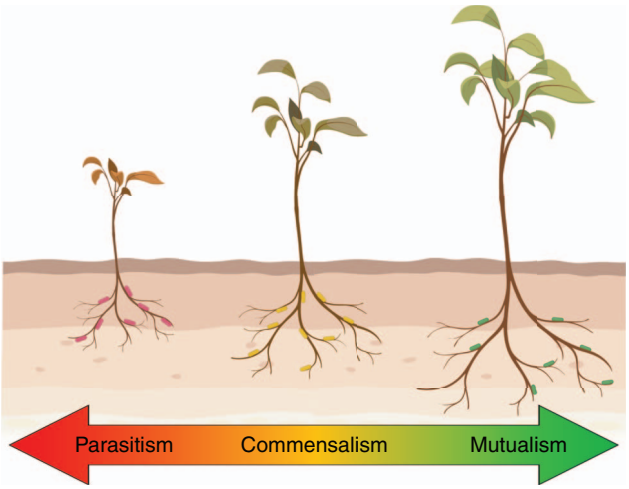

Figure 2. Overview of the types of interaction that occur between host and symbiont in plant-microbe interactions.

roots (rhizosphere), or both[7]. Most beneficial interactions between host and symbiont begin at the rhizosphere and should be considered the first point of manipulation for plant probiotics.

In this review, we will explore the microbiome of plants and the effect of changes in the plant status with the use of single or multiple species of microorganisms. Consideration will be given to discussions regarding host range and the use of promiscuous over narrow host range microorganisms as a point of critical consideration. The current applications in the use of plant probiotics will be described in the context of beneficial agricultural outputs under both biotic and abiotic stress conditions.

Soil probiotics: biofertilisers

The introduction of beneficial microorganisms to soil (biofertilisers) can result in improved plant growth. However, the mechanisms underlying improved plant health are different from the direct interaction between plant and host. Indirectly, microorganisms improve soil nutritional status and health through various mechanisms including: (1) increased phosphate availability through the solubilisation of occluded soil phosphates; (2) fixation of atmospheric nitrogen into bioavailable forms by free-living diazotrophs; (3) increasing the organic content in soil by cell turnover; (4) production of biofilms resulting in increased water retention; and (5) pathogen suppression (see reviews [8] and [9]). These microorganisms promote plant growth by indirect interaction with plants, and are, arguably, better characterised as soil probiotics. Research into increasing soil health through the introduction of microorganisms, or by a mixture of microorganisms and carrier, is apparent with over 713 patent filings regarding biofertilisers within the last ten years (source: Google Patents).

The beneficial effects of biofertilisers on crop yields has been documented extensively. A two-year study by Zhang *et al.*[10] is presented as a case study. The authors used a controlled fertilisation regime of composted cattle manure or composted cattle manure supplemented with a single fungal species, *Trichoderma rossicum,* and monitored soil chemistry, plant biomass and microbiota fluctuations. At the end of the trial, the authors reported a significant increase in plant biomass on land treated with composted cattle manure supplemented with *T. rossicum*. Interestingly, improved soil chemistry and fungal diversity were correlated with treatments, but bacterial diversity was not. However, DNA for metabarcoding were extracted from the bulk soil and changes in the rhizosphere were not monitored. Therefore, it is unknown if the rhizosphere microbiome had altered between treatments and elicited an effect on plant growth.

While biofertilisers offer an attractive method of soil amendment, Hart *et al.*[11] offer a cautious approach to the use of biofertilisers, in particular arbuscular mycorrhizal fungi (AMF). The authors contend that the use of aggressive generalists in biofertilisers may result in the loss of local AMF communities with unknown future ecological consequences. An additional point of consideration presented is the lack of regulation of biofertilisers compared to more traditional fertilisers. As the use of soil probiotics increases, consideration must be given to the greater biological implications – both positive and potentially harmful.

Plant probiotics: plant epiphytes – the generalists

Soil microorganism populations are more diverse than those found in the rhizosphere of plants, but soil microorganisms are much less abundant than the rhizosphere population[7]. For this reason, it is necessary to consider the ability of microorganisms to colonise plant roots for plant growth promoting properties. Within the rhizosphere, microorganisms play a crucial role in phosphate availability, they are also a source of nitrogen via diazotrophic nitrogen fixation and present a barrier (much like oral microflora in humans) to incoming pathogens (reviewed in [12]). The rhizosphere is an environment rich in organic acids, plant photosynthates and complex molecular signals. These plant compounds present selection pressure and may present a target for the development of plant probiotics intended for the rhizosphere. The current literature regarding plant growth promoting rhizosphere microorganisms is abundant. However, there are several key mechanisms that may elicit a positive plant growth phenotype.

The use of epiphytic microorganisms to alleviate abiotic stress is an emerging field of research especially considering arable land has

become increasingly impacted by climate change[13]. Other mechanisms of PGP in the rhizosphere include the solubilisation and mobilisation of occluded phosphates. Microorganisms can mine phosphate from soil and increase the amount of labile phosphorous available to plants. Research in this area of plant probiotics is extensive and will not be covered in this review, but for further reading see [8,12–15].

Salinisation of soil results in decreased agricultural outputs. Mukhtar *et al*.[16] explored the possibility of utilising halotolerant rhizosphere microorganisms on salt stressed maize. They isolated rhizosphere microorganisms from plant halophytes *Salsola stocksii* and *Atriplex amnicola* and screened them for potential PGP characteristics. The selected isolates were inoculated onto maize by seed coat and planted in saline soil. The results indicated a significant increase in root and shoot biomass of plants containing halophilic microorganisms. This study presents an example of plant probiotics by utilising microorganisms that are adapted to a stressed environment. For further reading regarding salt stress see [12].

A novel approach in the use of plant probiotics is presented in several papers discussing the bioremediation of heavy metals by rhizosphere microorganisms. By introducing organisms that can colonise root tissue and incorporate or metabolise heavy metals, reductions in heavy metal accumulation in plant tissue have been observed across multiple plant species[8,17–19]. Like the microorganisms isolated from saline environments, these plant probiotic heavy metal remediating species could potentially be sourced from contaminated land for use in agriculture.

Plant probiotics: plant endophytes – the specialists

Soil microorganisms and plant epiphytes confer PGP through a diverse array of mechanisms as previously discussed and generally these microorganisms can confer this benefit across multiple hosts. These are broad host range plant probiotic microorganisms. Endophytic microorganisms, in contrast, are much more selective and have a narrower host range. The most extensively studied plant endophytes are represented by the legume and root nodule bacteria interaction (RNB). For over a century, RNB have been used with their concomitant host to elicit a beneficial effect on plant growth by utilising the diazotrophic ability of the symbiont to increase plant nitrogen. However, this has presented a unique set of challenges due to genetic plasticity of RNB.

The inoculation of RNB onto a crop leads to an intimate symbiosis, but long-term exploitation of this symbiosis has led to unexpected consequences. Symbiotic genes are often located on plasmids or on symbiosis islands, and these genetic elements are susceptible to horizontal gene transfer. Transfer of symbiotic genes between similar species occurs at varying rates and, over time, can give rise to a population of native species that can outcompete inoculants and are ineffective nodule symbionts. This has been observed in several legume species including *Biserrula pelecinus*[20] and *Lotus japonicum*[21]. The rate at which horizontal gene transfer occurs between RNB may be greater than reported in the literature.

Conclusion

Plant probiotics is an area of research that is anticipated to gain much traction in the coming years. With agriculture productivity facing increased strain from urbanisation, climate change and land use, the augmented use of plant probiotics offers mechanisms to alleviate saline stress, heavy metal contamination, reduce plant stress responses, and increase agronomic outputs. However, all alterations to the microbiome of plants result in some changes occurring. Some changes are macroscopic, such as increased biomass, and others occur on microscopic levels that may accumulate unnoticed. The challenge facing agronomists, ecologists and biologists rests in harmonising the balance between existing plant and soil microbiomes with the introduced plant probiotics. By careful monitoring of not just agricultural outputs, but also the perturbations within the communities of microorganisms that share soil and plant tissue, plant probiotic treatments can offer a useful and powerful tool for plant growth promotion.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

Dr Robert Walker is plant and soil microbiologist at the University of Melbourne. His research interests include phosphorous and iron pathway regulation in plant growth promoting bacteria. He also works with fungal pathogens to understand plant defence priming using metabolomic and transcriptomic tools in Industrial Hemp with industry collaboration from Nutrifield.

Carl Otto-Pille is a Masters of Science student at the University of Melbourne. His research focuses on isolating bacteria from agricultural mixed-use land by trapping with the model plant, *Brachypodium distachyon*. These isolates are then screened for phosphate solubilising ability and their genome sequences will be available soon.

Sneha Gupta recently submitted her PhD thesis at the University of Melbourne. Sneha is a plant biologist who works with *Trichoderma harzianum* to elucidate biochemical interactions during salinity stress using advanced mass spectrometry imaging and analysis.

Martino Schillaci is a PhD candidate at the University of Melbourne. Martino studies the interaction between *Azospirillum brasilense* and the model plant *Brachypodium distachyon* under sub-optimal growth conditions. During his PhD, Martino studied plant phenotype using advanced imaging platforms at the Forschungszentrum Jülich (GER) and analysed the metabolome of plants using GC-MS and LC-MS at the University of Melbourne. Martino is due to submit his PhD thesis in 2020.

Professor Ute Roessner leads the Plant Biochemistry research group at the University of Melbourne. Professor Roessner developed novel GC-MS methods to analyse metabolites in plants. Together with the application of sophisticated data mining, the field of metabolomics was born and is today an important tool in biological sciences, systems biology and biomarker discovery. In 2003 she moved to Australia from Germany where she established a GC-MS and LC-MS based metabolomics platform as part of the Australian Centre for Plant Functional Genomics. Professor Roessner is currently in the position as Head of School, School of BioSciences, University of Melbourne.

Probiotics for corals




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Abstract. Coral reefs are found in warm, oligotrophic, euphotic marine waters and occupy <0.1% of the sea floor, yet support ~25% of earth's marine species. They provide critical ecosystem services to human populations including coastal protection, food (e.g. fish) and personal income by way of fishing and tourism. However, recent pan-tropical coral 'bleaching' (the paling of corals due to the separation of corals and their algal endosymbionts following exposure to environmental stress) has led to coral mortality, thus jeopardising the persistence of reef ecosystems. Consequently, it has been recognised that direct interventions may be needed for coral survival, and 'manipulation of the community composition of microbial organisms associated with the coral holobiont' has been proposed as one solution. Such probiotic strategies would allow corals to adapt rapidly (days to weeks) to changing environmental conditions, relative to mutation and selection taking many years. This review describes corals, and research that has demonstrated the potential of probiotic approaches to protect them from environmental stressors.

Coral reefs provide critical ecosystem services including coastal protection, a source of food (e.g. fish) and a source of personal income by way of fishing and tourism. They also suffer from many challenges including climate change, shading from sediment runoff, pollution (e.g. oils), overfishing, and attacks from crown-of-thorn starfish. As a result of climate change, sea surface temperatures are increasing and since 1901 by ~0.18°C per decade[1]. The summers of 2014-2017 saw heat-induced pan-tropical coral 'bleaching'[2], which is the paling of corals due to separation of corals and their photosynthetic Symbiodiniaceae often leading to coral mortality and eventually to the collapse of reef ecosystems[3].

Coral reefs are constructed by coral polyps as they secrete layers of calcium carbonate. These marine invertebrates (phylum Cnidaria, class Anthozoa) are typically found in warm, oligotrophic, euphotic marine waters, occupying <0.1% of the sea floor but supporting ~25% of Earth's marine species[4]. Each coral polyp is comprised of two cell layers (ectodermis and gastrodermis) separated by a largely cell-free mesoglea and include an external mucus layer[5], as shown in Figure 1. They have a tentacle-ringed mouth leading to the gastrovascular cavity. A coral polyp is connected to the next genetically identical polyp by the coenosarc. Corals engage in endosymbioses with single-celled algae from the family Symbiodiniaceae, which reside *in hospite* (in gastrodermal cells) surrounded by a membrane complex of host and algal origin, called the symbiosome[6]. The symbiosis is mediated by exchange of organic and inorganic compounds from which both partners benefit; critically, corals gain the majority of their fixed carbon from Symbiodiniaceae. Corals engage in sexual reproduction via either broadcast spawning (release of eggs and sperm to the water → larvae form in water) or brooding (larvae formed inside polyps → released to the water).

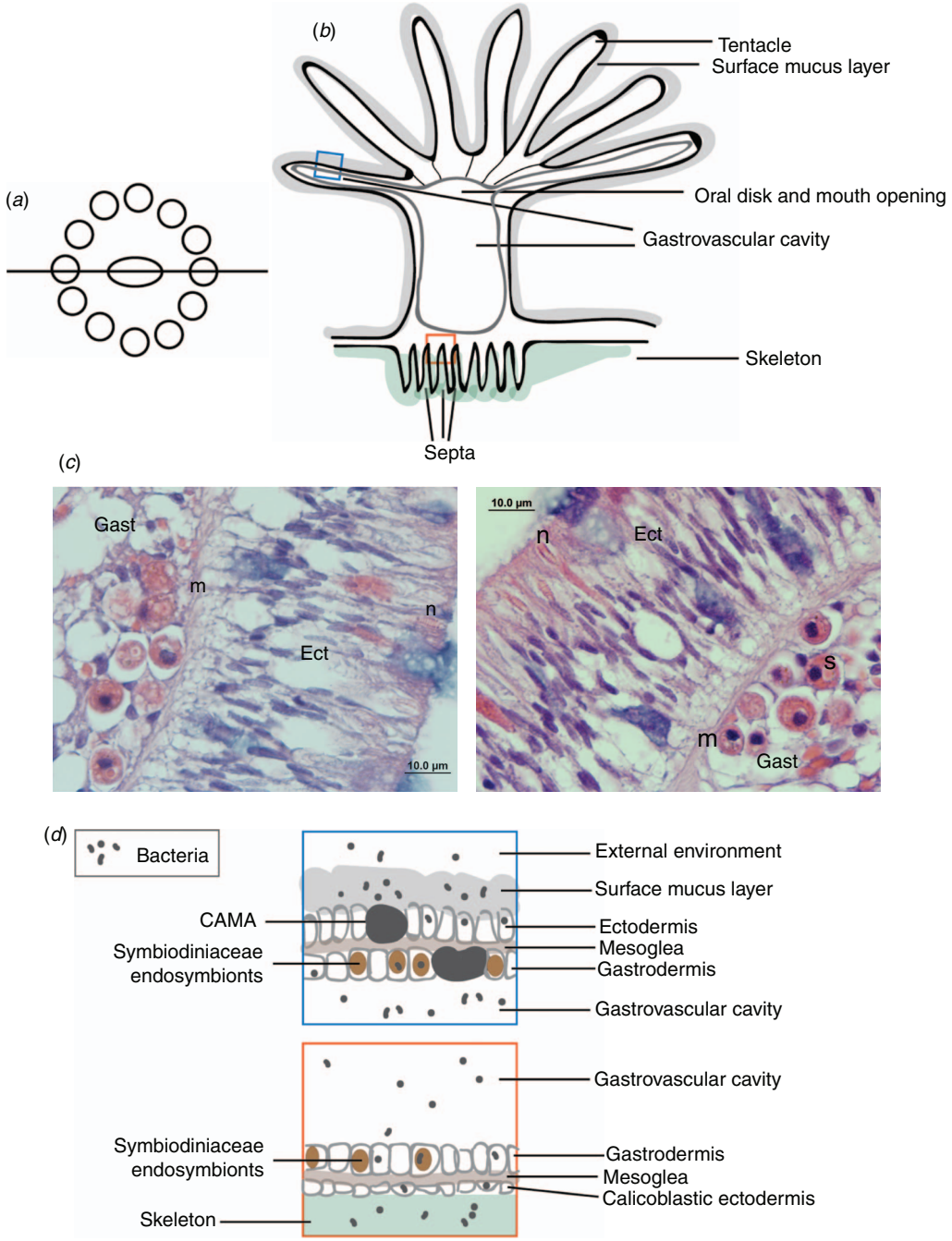

Figure 1. The body plan of a coral polyp, the location of bacteria within a polyp, and coral colony morphologies. (*a*) Plan view of a coral polyp with the horizontal line indicating the internal elevation view shown in (*b*); circles represent the tentacles and the oval represents the oral disk. (*b*) Internal elevation plan of a coral polyp showing the various microhabitats. Note that the gastrovascular cavity extends into the tentacles. (*c*) Brightfield microscopy image of a haematoxylin and eosin stained section through a coral larva (*Pocillopora acuta*) clearly showing the ectodermis (Ect), mesoglea (m) and gastrodermis (Gast) as well as Symbiodiniaceae (s), and cnidocyte showing coiled nematocyst (n). Photo credit: Katarina Damjanovic. (*d*) Diagrams of cross-sections through the tissue layers of a tentacle (top: blue boxed section from *b*) and the aboral part of a polyp (bottom: red boxed section from *b*) showing the various tissue layers and the location of bacteria. CAMA, coral-associated microbial aggregate.

Corals associate closely with prokaryotes (bacteria and archaea), viruses, microscopic eukaryotes, and, combined with Symbiodiniaceae, they are all collectively called the holobiont (for a review see [7]). However, there is scant knowledge on what controls the community structure and function of most of the microbes in the coral holobiont. Hypotheses for structuring include coral-produced chemicals in the mucus and natural coral-associated microbe-produced chemicals (see [8] for more information). Many functions of coral-associated bacteria are based on correlations between microbe identity and the phenotype of their closest relatives. However, proof for some phenotypic roles of bacteria have been provided, such as for nitrogen where nanoscale secondary ion mass spectrometry was used to show the incorporation and translocation of nitrogen from prelabelled bacteria into larvae of the coral *Pocillopora damicornis* and particularly into Symbiodiniaceae.

Probiotics for corals

'Manipulation of the community composition of microbial organisms associated with the coral holobiont' has been recognised as a direct measure needed to be a part of strategies to facilitate coral survival[9]. In line with this idea is the concept of probiotics for corals. Probiotics can be defined as, 'live microorganisms that are intended to have health benefits when consumed or applied to the body'[10]. In corals, probiotics are suggested as a rapid (day to weeks) natural strategy for corals to adapt to changing environmental conditions, relative to the alternative of mutation and selection taking many years[11]. They could also be applied to aquacultured corals. This initial report[11] specifically discussed the development of coral disease resistance by beneficial microbes in the naturally occurring holobiont. Teplitski and Ritchie[12] described the development and application of probiotics for several marine species including trout, shrimp and eels, as paradigms for coral probiotics. Other terms also encompass coral 'probiotics' including 'beneficial microorganisms for corals'[13] and 'microbiome engineering'[14].

Since probiotics are live microorganisms that should colonise the inoculated host, information about how corals normally acquire their microbiome is relevant. It has been shown that bacterial communities in corals are distinct from those in the contiguous seawater[15]. However, there are conflicting reports about whether specific corals associate with particular microbes[16], or whether the microbiome is shaped by the environment, location or weather. An experiment exploring whether adult corals are the source of bacteria for juveniles was carried out. Damjanovic *et al.*[17] exposed 'recruits' of the brooding coral *Poc. acuta* to adult *Poc. acuta* and adult *Platygyra daedalea*. The findings showed that *Poc. acuta* recruits harbour dynamic and diverse bacterial assemblages, which were not influenced by nearby adult corals. Another investigation showed that *Poc. acuta* maternally transmits members of the *Rhodobacteraceae* and *Endozoicomonas* spp.[18]. The feasibility of coral early life stage microbiome manipulation (probiotics) was investigated by repeatedly inoculating coral recruits (*Acropora tenuis* and *Platy. daedalea)* with a mixture of seven marine bacterial isolates, which had no specific targeted phenotypes[19]. The cumulative inoculations had a strong effect on the bacterial community composition and diversity in recruits of both coral species, compared to control recruits, despite being reared in the same environment. The conclusion from this set of experiments was that host factors, as well as the environmental bacterial pool influence the microbiome of early life stages of corals. Host factors may include microbe transmission mode (horizontal versus maternal) and host specificity. While the long-term stability of bacterial taxa as members of the host-associated microbiome remains to be evaluated[20], the findings provided support for the feasibility of coral microbiome manipulation, at least in a laboratory setting.

Use of coral bacterial inoculation

Two examples of practical applications of bacterial inoculation (probiotics) to corals are given below.

(1) *Disease mitigation*. Some strains of the necrotizing coral pathogen, *Serratia marcescens* form a biofilm and disrupt the normal mucus layer on corals leading to the disease condition known as 'white pox'. Pure cultures of bacteria from the coral *Acropora palmata* were found to produce anti-bacterial chemicals against a broad spectrum of pathogens, including *S. marcescens*[21]. This work was extended to clarify that the mucus layer of healthy corals contain chemicals that inhibit biofilm formation (a noted virulence phenotype) in white pox pathogenic strains of *S. marcescens*[8]. Several marine bacteria from corals or Symbiodiniaceae were capable of affecting biofilm formation and swarming (also a prominent virulence phenotype) in the white pox *S. marcescens*[8]. These so-called 'antagonistic' strains were inoculated along with the white pox *S. marcescens* to the sea anemone *Exaiptasia diaphana* (formerly *Aiptasia pallida*), a coral model. The progression of white pox disease was minimised by the antagonists potentially due to antimicrobial properties of the inoculated bacteria. Although it was tested on anemones, this method was deemed to hold promise for other cnidarians, like coral[8].

(2) *Bioremediation of oil*. A good example of how microbes can facilitate coral survival in the face of environmental impact is research by dos Santos *et al.*[22], where several bacterial species with the capacity to degrade water-soluble oil fractions were isolated from the coral *Mussismilia harttii*. The health of *M. harttii* subjected to petroleum hydrocarbons was negatively impacted according to photosynthetic efficiency; however, strictly this is a feature of Symbiodiniaceae, not corals *per se*. A single inoculum of an oil-degrading consortium composed of 10 bacteria (three *Bacillus* spp., *Acinetobacter calcoaceticus*, three *Paracoccus* spp., a *Psychrobacter* sp., *Vibrio alginolyticus* and *Pseudomonas stutzeri*):
   - improved the health *M. harttii* when it was exposed to petroleum hydrocarbons, and
   - the bacterial mixture accelerated the degradation of petroleum hydrocarbons.

Mitigating coral bleaching with probiotics

Oakley and Davy[23] provided a recent summary of the cell biology of coral bleaching. Although there are several hypotheses for coral bleaching[24–26], one common theme revolves around damage to the Symbiodiniaceae photosystem II leading to the formation of reactive oxygen-centered radicals[27,28] like singlet oxygen and superoxide[29]. This partially occurs because more oxygen is produced by the Symbiodiniaceae than is used in the milieu leading to toxic accumulation of reactive oxygen species (ROS). ROS have several cellular damaging mechanisms including to photosystem II reaction centres in the Symbiodiniaceae, which can result in

Symbiodiniaceae being lost from host tissue. Corals and Symbiodiniaceae have ROS managing mechanisms like catalase and superoxide dismutase, which degrade ROS to oxygen and water[29].

To test the ability of probiotic inoculation to mitigate ROS and disease-induced coral bleaching, Rosado *et al*.[30] isolated bacteria including five *Pseudoalteromonas* spp., one *Halomonas taeanensis* and a relative of *Colbetia marina* from the coral *Poc. damicornis* (grown in an aquarium) and its surrounding aquarium waters. Bacteria were screened for catalase activity, nitrogen metabolism (*nifH* and *nirK* genes via PCR), dimethylsulfoniopropionate demethylation (*dmdA* gene by PCR) and antagonistic activity against the coral pathogen *Vibrio coralliilyticus*; traits deemed relevant to protect corals against heat and disease stress. In controlled aquarium experiments, after a 10-day acclimatisation period, two stressors were evaluated. These were *Poc. damicornis* maintained in two temperature regimes, 26°C and 30°C (raised from 26°C over 9 days) and inoculation of *Poc. damicornis* with *V. coralliilyticus*. *Poc. damicornis* in both scenarios were inoculated with the seven-bacterial consortium on two occasions (days 10 and 15) and were maintained for 26 days. The method to determine bleaching was comparison of the coral tissue colour to a colour chart, and photosynthetic efficiency was also measured. It was concluded that the inoculated seven-bacterial consortium partially mitigated bleaching from temperature; although the reason was unclear as the inoculated bacterial consortium had diverse traits. In corals exposed to *V. coralliilyticus* and inoculated with the seven-bacterial consortium, no *V. coralliilyticus* were found after 26 days, demonstrating mitigation of this noted coral pathogen[31].

Future directions

The field of coral probiotics is at a very early stage and is currently limited by a lack of definitive information about the functional roles of coral microbiome members, apart from Symbiodiniaceae. Information that would aid development includes determination of bacterial phenotypes that are beneficial to the host. These might include ROS metabolism, although other phenotypes are likely valuable. Testing the maintenance of introduced bacteria in the host is also required. Given the perilous situation facing coral reefs, including the broad GBR bleaching over the recent 2019–2020 summer, addressing these knowledge gaps to advance probiotic strategies is critical.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

Professor Linda L Blackall is an environmental microbial ecologist, who has studied many different complex microbial communities ranging from host associated through to free living in numerous environments. Her research has covered mammalian microbiomes of marsupials, humans, ruminants and horses; and the microbiota of non-mammals including corals and sponges. Environmental microbiomes explored in Linda's research span wastewater treatment (aerobic and anaerobic), solid waste digestion (landfill and composting), bioelectric systems and microbiologically influenced corrosion. The numerous methods she develops and employs in her research allow elucidation of microbial complexity and function in these diverse biomes.

Ms Ashley M Dungan is originally from New York. Ashley completed her Bachelor of Science degree in Biology in 2011, conducting a senior research project in the field of environmental microbiology. She completed her Master's degree at Nova Southeastern University in Florida under Dr Nicole Fogarty, where she studied the impact of ocean acidification on the calcification of Caribbean adult and juvenile corals. After graduating in 2015, she worked for Mote Marine Laboratory in the Florida Keys as a Staff Chemist in the Ocean Acidification program; there she continued her work with corals and began working with *Diadema antillarum*, the long-spined sea urchin. Beginning early 2017, Ashley joined Dr Madeleine van Oppen and Linda Blackall at the University of Melbourne as a PhD student. Ashley's current research focuses on characterising the microbiome of the model organism for corals, *Exaiptasia pallida*, for future use in assisted evolution research. Her research interests are in the field of climate change, coral reef ecosystems, and assisted evolution.

Mr Leon M Hartman recently completed a PhD through Swinburne University as part of the Blackall and van Oppen research group at the University of Melbourne using the sea anemone *Exaiptasia diaphana* as a coral model organism. His research has employed molecular and bioinformatic methods to study bacterial microbiomes, their relationship with their hosts, and response to environmental perturbation.

Professor Madeleine JH van Oppen is an ecological geneticist with an interest in microbial symbioses and climate change adaptation of reef corals. Her work has been published in >200 peer-reviewed papers and book chapters. Her early career focused on evolutionary and population genetics of algae and fish, and subsequently corals. She obtained a PhD in the molecular ecology of macroalgae in 1995 (U Groningen, The Netherlands) and is currently an Australian Research Council Laureate Fellow with part positions at the University of Melbourne and the Australian Institute of Marine Science. Her team is using bioengineering approaches aimed at increasing coral climate resilience and the likelihood that coral reefs will survive this century. These interventions include coral host hybridisation and conditioning, directed evolution of microalgal symbionts and bacterial probiotics.

Probiotics for cultured freshwater fish

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Abstract. **Probiotic products are viewed as an alternative to the use of antibiotics in freshwater fishes farming. Probiotic organisms include bacteria, yeast, and filamentous fungi offering different benefits to fish including growth promotion, inhibition of pathogen colonisation, and improvement of nutrient digestion, water quality, and stress tolerance, as well as enhancement of reproduction. For these reasons, this review aims to identify the main trends in probiotic amendment in freshwater fishes. Strategies to incorporate the probiotic strains in the fish feed or pellets to allow optimal viability of the strains as they reach the fish gastrointestinal tract (GIT) are crucial in probiotic research and commercial applications for freshwater fish.**

Tilapia dominates the aquaculture industry in many tropical and subtropical countries and is one of the most important protein sources from freshwater fish[1]. Traditionally, antibiotics and chemicals have been used to treat infectious diseases in fish. As an alternative to the use of antibiotics, probiotics originated from the native gastrointestinal microbiota of fish have been increasingly common within the past two decades[2]. Probiotic organisms include bacteria, yeast, and filamentous fungi often originate from the GIT of fish, and can be applied individually or in mixtures or consortia.

In 2017, more than 150 million tons of fish were produced worldwide, with China being the largest producer country with 4 million tons of total product[3]. By 2030, it is expected that close to 62% of consumed fish will come from aquaculture and 38% from wild-caught fish[4]. However, one of the main difficulties in the commercial cultivation of aquatic organisms is the appearance of infectious diseases that hamper industry sustainability. Several researchers and producers point to disease as the leading cause of losses in production and economic resources[5].

Probiotic bacteria give multiple benefits to fish, such as growth promotion, inhibition of pathogen colonisation, and improvement of nutrient digestion, water quality, and stress tolerance, as well as enhancement of reproduction[6]. Yeast is the second group of microorganisms with probiotic potential. Yeast as probiotic supplements in the fish diet offer benefits that include modulation of the digestive microbiota, enhancement of immune responses, contributions to intestinal enzymatic physiology, and enhanced growth performance[7]. The third group of microorganisms with probiotic potential are the filamentous fungi. These fungi stimulate antioxidant response and the immune system, and additionally stimulate the production of various digestive enzymes including amylases, cellulases, β-glucanases, xylanases, proteases, and lipases[8]. Generally, these probiotic microbes are non-fish derived. In contrast, a multi-strain probiotic culture, maintained in continuous culture, was recently developed from Nile tilapia (*Oreochromis niloticus*) gastrointestinal microbiota. A *Cetobacterium* sp. was the dominant genus, and the multi-strain culture had *in vitro* antibacterial activity against fish pathogens such as *Streptococcus agalactiae* and *Aeromonas hydrophila*[9]. Results identified three bacteria within the continuous culture with distinct antibacterial activity against these pathogens.

It is essential to define the probiotic dosage for a specific fish and environment in order to avoid economic losses due to overdose or too low a dosage[10]. Most of the journal articles sourced suggested the use of probiotic concentrations of approximately 1×10^6 CFU/g of feed showed significant improvement in growth performance, resistance to infection, and immune modulation. Growth performance and health status improved in trout[11], and tilapia[12], with a probiotic microbial concentration around 1×10^6 CFU/g of feed. According to Merrifield *et al.*[13] that concentration of probiotics is necessary to ensure the colonisation of the probiotic in the intestinal tract of fish.

A very high concentration of probiotics, exceeding what is optimal, could result in wasted energy and nutrients, and, for that reason, it is necessary to test the functional dosage of probiotics in every particular situation[14]. Farias *et al.*[15] showed that a higher dosage than needed could partially suppress probiotic responses. The lower concentration tested (1×10^7 CFU/g) was enough to offer an improvement in growth performance and nutrient utilisation in *Oncorhynchus mykiss*[11] and *Salmo salar*[10]. Bhujel *et al.*[16] used regression analysis to define the optimal concentration of the two commercial probiotic formulations in *Labeo rohita,* showing that the effective dosages were higher concentrations than that suggested by the manufacturers.

Another critical factor for probiotic efficacy is the administration period. Researchers generally administrated multi-strain probiotics over periods of approximately 30 days (28–30 days), 45 days (42–49 days) and 60 (56–60 days). Addo *et al.*[17] found that growth performance was low at 21 days of treatment with a mixture of two *Bacillus* strains (SB3086 and SB3615). Similarly, *Bacillus subtilis, Saccharomyces cerevisiae* and *Aspergillus oryzae* did not show improvement in growth performance over 28 days of treatment[18]. However, the administration of *Micrococcus* sp. and *Bacillus* sp. enhanced growth performance of *Etropus suratensis* at day 28 in comparison to 14 days of administration. Other authors considered that more than 45 days are needed to confirm the probiotic potential offered by a mixture of probiotics. Giri *et al.*[19] showed that after 60 days, growth performance and immune modulation improved. Similarly, growth parameters improved with the administration of *Enterococcus faecium* and *Geotrichum candidum* to *L. rohita*[20] for 45 days and 90 days. The administration of a probiotic in *Piaractus mesopotamicus* showed an increase in survival and biomass production benefits offered by microorganisms administered over a more extended period[21]. Merrifield *et al.*[13] demonstrated that a continuous administration enhanced colonisation and probiotic activity in the rainbow trout (*O. mykiss*) gastrointestinal tract.

Generally, it is recommended that probiotics be applied in the earlier growth stages. The administration of two types of probiotic mixtures in *L. rohita* at different stages showed that growth performance and survival improved in hatchlings and fry, but administration only in the advanced fry stage did not affect survival and growth[16]. Likewise, Jha *et al.*[22] demonstrated that early administration improved survival and growth of *L. rohita* hatchlings and fry. In contrast, probiotic administration to advanced fry did not cause any effect. Similarly, Ridha *et al.*[23] showed that application of two types of probiotic mixtures improved growth parameters at the juvenile stage, more than when administered at the adult stage. However, the recommendation is to evaluate probiotics applications from earlier stages to market size with continuous administration and treatments being withdrawn at different periods[16].

The method of administration has been shown to affect probiotic performance[24]. The method most commonly used for administering probiotic mixtures is incorporation into the feed (92.8%), followed by direct incorporation into the water (4.8%) and in live food (1.6%). The process for incorporating the probiotic into feed has different stages: mixing the probiotic with feed, adding water, pelletising to the selected size, drying, packaging, labeling, and storing until feeding.

There are a few journal articles in which the authors measured the viability of the bacteria incorporated into the feed. Aly *et al*.[25] centrifuged and washed the probiotics with a buffer, and added a concentration of 1×10^9 CFU/g to feed. The feed was blended in an automatic mixer and pelletised. The pellets were dried in an oven at 45°C and the probiotic viability was measured weekly over five weeks of storage at 4°C and 25°C. Results showed that *B. pumilus* survived at both temperatures over the five weeks. Meanwhile, *Citrobacter freundii* and *B. firmus* survived at 4°C during the five weeks, but at 25°C, they were viable for one or two weeks, respectively. In another study, probiotics were centrifuged, and bacterial pellets were resuspended in nutrient broth[26]. The probiotic suspension was sprayed at $\sim 2 \times 10^9$ CFU/g on feed in plastic trays and air-dried in a microbial cabinet at room temperature (19°C). Viability was measured after three months of storage at 4°C, showing that *Exiguobacterium* JHEb1, *Vibrio* JH1, and *Enterococcus* JHLDc were viable at concentrations of more than 1×10^7 CFU/g of feed over the three months evaluated. Finally, *Bacillus amyloliquefaciens* 54A and *B. pumilus* 47B at three different concentrations (1×10^9, 3×10^9 and 5×10^9 CFU/g of feed) were mixed with feed and viability at 4°C was evaluated every week showing that the probiotic level decreased 10% after every three weeks of storage[27]. From the processing perspective to incorporate the fish probiotics, *Bacillus* spp. would be more suitable to be incorporated in the fish feed or pellets, since they are spore-forming bacteria, which allow heat resistance during the pellet compression process[28]. Floating fish feed is more desirable for fish. Therefore, probiotics that survive the preparation process for dry pellet would be more effective as fish feed.

Selection of probiotic strains should consider the potential unexpected transmission of the strains to human, or their genetic characteristics to other bacteria. Therefore, strains identified as probiotics for fish might not necessarily be suitable for commercial probiotics. Strains such as *E. faecium* and *C. freundii* are considered as opportunistic pathogenic bacteria in humans[29, 30]. Therefore, these strains are not recommended for future fish probiotics products.

In summary, probiotic microorganisms are a healthy, sustainable, and environmentally friendly approach in comparison with antibiotics to reduce the loss of aquaculture fish in disease outbreaks. Probiotics offer several benefits, including improved growth, immune modulation, and disease resistance. However, it is important to define the proper dosage, the administration period, fish stage at administration, administration method, and probiotic viability during production and storage in order to offer optimal probiotic efficacy in aquaculture fish. At the same time, it is important to evaluate its safety application on humans, other animals, and the environment.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

Javier Melo is a PhD student working on the evaluation of the probiotic potential of a competitive exclusion culture of the intestinal microbiota of tilapia (*Oreochromis niloticus*). Javier Melo has a Master's degree in Process Design and Management from the Universidad de La Sabana, where he studied the microencapsulation of probiotic spores and their inclusion in juices and dairy products. Javier has a degree in Medical Laboratory Science from the Universidad Colegio Mayor de Cundinamarca in Colombia.

Dr Ruth Ruiz is a Chemical Engineer from the Universidad Nacional de Colombia. Dr Ruth Ruiz has a Master's degree in Industrial Engineering from the Universidad de Los Andes. Dr Ruth has a PhD in Engineering where she studied the re-use of syrups from osmotic dehydration of fruits – cryoconcentration of high initial concentration sucrose solutions at the National University of Colombia. Dr Ruth Ruiz is an expert in industrial processes that include cryoconcentration, lyophilisation and production of microorganisms in bioreactors.

Michael Hume has a BS and MS from the Virginia Commonwealth University and a PhD from Oklahoma State University. His doctoral research investigated fluid transport across salivary gland membranes of the lone star tick (*Amblyomma americanum*) as affected by dopamine stimulation of the adenylate cyclase cytoplasmic membrane transport system. Dr Hume has over 30 years of service with the USDA, Agricultural Service investigating the development, discovery, and application of probiotics, prebiotics, and alternatives to antibiotics as dietary supplements to enhance the digestive microbiome to promote protection against invading human enteropathogens and to promote growth and production in food animals.

Dr Sidjabat is an expert in antimicrobial-resistant bacteria and probiotic development. Dr Sidjabat is currently an Adjunct Research Fellow within Menzies Health Institute Queensland, Griffith University, Gold Coast Campus. Prior to her Griffith University affiliation, Dr Sidjabat was a Researcher at the Infectious Diseases Theme at the University of Queensland Centre for Clinical Research (UQCCR) for 10 years working on antimicrobial resistance and probiotic development. Sidjabat's probiotic research work is within the agreement by Uniquest (https://uniquest.com.au/) with probiotic companies. Dr Sidjabat worked as Postdoctoral Research Fellow between December 2007 and 31 May 2009, at the Division of Infectious Diseases, University of Pittsburgh, Pennsylvania, USA. Dr Sidjabat's primary interest in probiotic screening and development is through phenotypic, genomic and proteomic approaches.

Dr Luisa Villamil is a Marine Biologist from Universidad Jorge Tadeo Lozano in Colombia. Dr Luisa Villamil has an MS and a PhD in Biological Sciences and Aquaculture from the Universidad de Vigo in Spain in the application of lactic acid bacteria in turbot (*Scophthalmus maximus*). Dr Luisa Villamil was a visiting Professor in the University of Rhode Island and the Woods Hole Oceanographic Institute. Dr Villamil's main activities are the development of new alternatives to antibiotics and promoting the growth and health of aquaculture organisms.

Disease X ver1.0: COVID-19

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Abstract. The SARS-Cov2 has presented the world with a novel pandemic challenge requiring a rapid response. This article provides a May 2020 snapshot from Professor Paul Young, who is part of a group working with urgency on Australia's leading COVID-19 candidate vaccine.

We first noted tweets about a new respiratory infection in Wuhan, China in late December 2019. At that time, we were about a year into a three-year grant funded by the Coalition for Epidemic Preparedness Innovations (CEPI). The goal of that grant was to establish a streamlined, Australia-based, rapid response vaccine pipeline to address the threat of emerging viral pathogens. The project was built around a patented platform technology that we had been developing here at the University of Queensland (UQ) for nearly 10 years[1]. We had already generated candidate subunit vaccines for 10 different viruses from a wide range of viral families and so were well placed to apply all that accumulated knowledge to this newly emerging virus. Initially, we viewed the task as an exercise to test the platform, not expecting the global spread that would follow. In those early days of January, we eagerly awaited the release of any viral sequence information. On 10 January 2020 the first full genome sequence of this new virus, a coronavirus like its predecessors SARS and MERS, was made public and overnight we had designed our first constructs.

We named our patented platform technology the Molecular Clamp. It was the brainchild of Keith Chappell, a post-doctoral scientist who had originally completed his PhD with me and then returned to my lab in 2011 after a post-doc stint in a leading respiratory syncytial virus (RSV) lab in Madrid. His task in Madrid, with the celebrated virologist José Melero, was to recombinantly engineer the RSV fusion protein F, to capture it in its pre-fusion form. The theory was that this form of the protein is what appears on the surface of the virus and so is the primary target of a protective antibody response. These proteins undergo a dramatic conformational change in driving the process of viral-host membrane fusion and in its post-fusion form, many of the epitopes recognised by antibodies on the native virion are hidden. Keith's work in successfully producing a constrained pre-fusion form of F was instrumental in Melero's team making the seminal observation that the majority of naturally acquired neutralising antibodies recognised the pre-fusion and not post-fusion form of F. This was a critical observation for vaccine design[2]. The problem was that his approach resulted in a protein that was not that stable.

When he returned to my lab it was to work in a relatively new area for us, virus-bacterial interactions, but he asked if he could also continue to work on the RSV F story. I had been involved with Biota for a number of years in the late 1990s, expressing RSV F as a target for antiviral drug design, and through that work we had discovered the second cleavage site for this protein. So, I was primed to be interested. Within that first year he came up with the idea of fusing the two heptad repeats of another fusion protein to the end of the target RSV fusion protein ectodomain. The highly stable six helical bundle that formed from their spontaneous folding and association provided a remarkably stable trimerisation domain. The irony is that it is the very stability of this post-fusion structural domain that we were able to re-purpose to stabilise the pre-fusion form of the protein. So began a long journey of unfunded research (consultancy revenue comes in handy), with Dan Watterson, another PhD graduate of my lab and returned post-doc, contributing substantially to what became the Molecular Clamp (MC). The three of us are co-inventors on the MC patent[1]. Despite numerous funding applications over subsequent years, including industry pitches, our first successful grant, specifically for this work was an NHMRC Project, submitted in 2017. Perseverance, or perhaps stubbornness is highly underrated, as so often is the basic science that underpins translational outcomes.

Also, in early 2017 I took a punt and booked a flight to Paris to attend the opening of a new organisation, CEPI, that I had only just heard about. It was a transformative experience for me. I have been passionate about contributing to neglected disease research all my working life, and had been involved in wonderfully collaborative and transformative research projects. But I had

never felt as much positive energy as I felt at that meeting, full of leading academic researchers, innovative NGOs and small biotechs alongside large pharma, all committed to finally answering the World Health Organization (WHO) call to deliver on a global preparedness strategy to deal with emerging pathogen threats. CEPI's mission was articulated at that meeting; to stimulate and accelerate the development of vaccines against emerging infectious diseases and enable equitable access to these vaccines for people during outbreaks. In addition to specific virus targets they also support platform technologies that could be applied to newly emerging pathogens, referred to by the WHO as Disease X.

On my return, Keith and I committed to an application to their first call for vaccine strategies targeting selected pathogens from the WHO Blueprint Priority disease list. This first application was not successful. However, CEPI liked what they saw in our proposal and asked us to submit to the next call, which was to support platform technologies that could be applied to multiple pathogen targets. The call had a number of key criteria that needed to be met, the most notable being a 16-week timeline from pathogen discovery to delivery of sufficient vaccine to enter a Phase 1 clinical trial. A challenging ask, but one we felt we could meet, given the seven years of development we had already put into our MC approach. Our application brought together colleagues from the ANU, the Doherty Institute, University of Hong Kong and CSIRO teams at both the protein manufacturing facility at Clayton in Melbourne and the AAHL facility in Geelong. To prove the technology, we needed to generate three separate vaccines, two for 'demonstrator' targets, i.e. ones for which existing vaccines or technology was already available to compare, and one emerging pathogen. We chose influenza and RSV for our first two targets and, fortuitously as it would turn out, the coronavirus MERS for the emerging pathogen. We also suggested in our grant proposal that in our last year of the three year grant we should be subjected to a stress test. We would be supplied with an unknown viral sequence, from which we needed to design, develop, test and manufacture enough vaccine to enter a Phase 1 clinical trial.

That was meant to happen in 2021, but we received that first, very real 'stress test' sequence on 10 January this year. The first constructs were designed within the first 24 hours. On 21 January we received a formal request from CEPI to begin full development and manufacture of a vaccine candidate. Within three weeks of receiving the initial SARS-CoV-2 sequence we had chosen a lead construct. We went on to design, express and test more than 200 different constructs by the end of the 4th week, but we ended up moving forward with that first excellent lead candidate. A model of the clamped, trimeric pre-fusion SARS-CoV-2 Spike protein that we have generated as our vaccine candidate is shown in Figure 1*a*. Figure 1*b* shows the UQ leadership team for the CEPI project.

The months that followed this early work have essentially been 24/7 for the whole team of about 20 UQ researchers, as well as all our colleagues in our partner institutions. It has been a revelation. Despite the immense workload, everyone has remained engaged and positive, and Zoom has become our constant companion. There have certainly been challenges along the way, but we have managed to keep to our original timeline of a start date for the Phase 1 clinical trial in early July 2020. Unlike some of the other candidate vaccines being developed globally, we have

Figure 1. (*a*) Structural model of the trimeric SARS-CoV-2 Spike protein ectodomain (prepared by D Watterson), stabilised by the Molecular Clamp (red). (*b*) The UQ CEPI leadership team (L to R): Dan Watterson, lead researcher; Christina Henderson, Project Manager; Paul Young, Project Co-Lead; Keith Chappell, Project Co-Lead; and Trent Munro, Project Director.

elected to complete all of our pre-clinical safety and efficacy studies prior to entering human clinical trials. At the time of writing, we have completed early mouse immunogenicity studies, which showed that the vaccine was able to induce highly potent neutralising antibody responses against live SARS-CoV-2, performed in collaboration with our colleagues at the Doherty. While mice are obviously not humans, the levels of neutralising antibody induced was substantially higher than that seen in recovered COVID-19 patients and so we are hopeful that we may be able to induce even higher levels of antibody with our vaccine than that induced by natural infection – it is early days, but the data are promising. We have been substantially assisted by large pharma (GSK, CSL and Dynavax) reaching out to us to offer their tried and tested adjuvants for this work. We have also now entered our vaccine into toxicology and animal protection studies, both of which should reach data points by June that will allow us to enter our Phase 1 study on schedule.

With the global race on, and more than 100 vaccines in development, we have also encountered challenges such as limited Australia-based capacity to support critical, high-level containment, animal challenge studies. CSIRO's AAHL facility had moved quickly to begin ferret protection studies on vaccine candidates from two international groups (Oxford University and Inovio) and so was not available for our work. However, we were able to reach out to Viroclinics Xplore in The Netherlands and at the same time, expand the number of species we could test, as well as the overall scope of the studies.

Like everyone else, we have had to adjust to COVID-19 reaching our shores. By mid-March, the university was starting to shut down as many began working from home and practicing physical distancing (I still prefer that terminology to social distancing). We obviously needed to remain at work and in the lab and so, on 20 March we met for the last time as a single group, with appropriate distancing, and split into two teams that would no longer physically interact. That way, if one person fell ill we would not lose the whole group to home isolation. It has been a strange time at the university, to be in the middle of semester with all teaching now online and virtually no one on campus.

The timeline for development of a vaccine for COVID-19 has been a topic of much debate. The typical timeline for vaccine development, from conception to licensure is anything from 10–20 years, with five years being the most impressive to date. Regardless, most commentators have been suggesting a 12–18 month timeline. This is a challenging ask, as there can be no corner cutting when it comes to safety and efficacy. Accelerated timelines and adaptive design for clinical trials, expedited regulatory approval, accelerated manufacturing and early emergency and compassionate use are all part of the strategies for the early delivery of viable vaccines. In early March we developed a strategy to uncouple manufacturing from the typical pipeline of vaccine development, with the intention to run full-scale manufacturing alongside the clinical trials and not wait for confirmation of efficacy. It is a financially risky strategy, but one that could deliver significant vaccine doses, initially for emergency use and then immediately once regulatory approval was received. This would require a significant early funding boost and so we submitted a proposal that outlined this parallel development plan (Figure 2). The proposal was jointly funded by the Queensland government, the Federal government, and generous philanthropic support from Foundations and the community. The overall level of support and positive feedback and encouragement we are receiving has been extraordinary. Everything from the major Foundation donations, a number of whom have not funded in the medical space previously, to the letters of support we have received from school children and the smaller, but no less important donations, such as the $6.50 that was sent to us by one child in Victoria from his 'share' jar, have all been truly inspirational.

The development of our vaccine is now the primary focus of the team and is continuing at pace, with all members of our consortium managing a range of variables that we continually need to adjust. What would normally take years to develop and

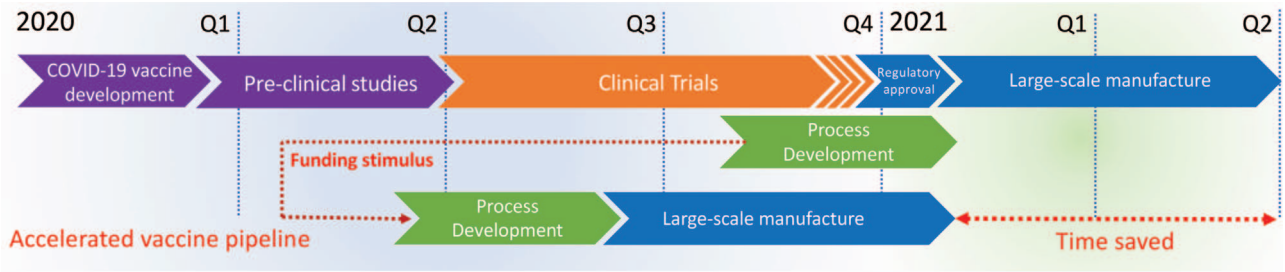

Figure 2. Schematic of the UQ COVID-19 subunit vaccine development pipeline. Funding stimulus has allowed us to advance and accelerate vaccine manufacture, cutting some 6 months off the expected vaccine delivery timeline.

finesse, we have only weeks and months to progress. But groups all over the world, developing the more than 100 candidate vaccines that are currently in play, will be having similar issues. The major triage point is coming soon: the shift to large-scale manufacture. There is limited global capacity available and it is likely that only 3–4 vaccines will make their way through this transition point. We are hopeful that ours will be one of those vaccines that makes it through the months ahead, with its use ultimately contributing to the control of this once-in-a-lifetime pandemic.

Conflicts of interest

The author declares no conflicts of interest.

Acknowledgements

There are too many people involved in this endeavour to acknowledge everyone, but special recognition of all the magnificent UQ research and development team, particularly A/Professor Keith Chappell, Dr Dan Watterson and Professor Trent Munro and our extraordinary professional staff support, our CEPI management team who have brought extensive wisdom and advice to the work, our partners in the CEPI project Consortium from the Doherty Institute, the ANU, CSIRO Protein Production Facility at Clayton, University of Hong Kong and our many commercial partners. And none of this would be possible without the generous support of our funders; CEPI, Queensland and Federal governments, NHMRC, Foundations and our amazing wider community.

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A therapeutic tipple?

When frightened and faced with overwhelming infectious disease, humans have invariably looked for miracle cures. Today we are frantically searching for anti-coronavirus activity in an antimalarial drug, a treatment for head lice and drugs used to treat HIV. The use of alcohol is officially limited to hand wash, although reports from some retail liquor chains suggest that alcohol consumption or 'self medication' has been adopted by many in the community. In 1918 the Spanish flu also drove US citizens to drink. In this case it was whisky, an unproven and, in many places, unobtainable remedy. It was the days of Prohibition and vast stores of bootleg liquor had been confiscated and either disposed of or impounded.

At the time the medical community was divided as to the medicinal value of whiskey. Along with brandy and wine it had been dropped from the US Pharmacopeia in 1916, and in 1917 the AMA joined ranks with the Prohibitionists and resolved that 'the use of alcohol as a therapeutic use should be discouraged'.

However, not all AMA members were convinced and continued to recommend, and even prescribe, whiskey for patients for a variety of ailments, especially influenza, believing it stimulated their heart and lungs and eased suffering.

But whiskey was not easy to obtain. Doctors could prescribe medicinal whiskey and pharmacists could dispense it, but there were strict limits on the amounts allowed. To circumvent this impediment to free trade, ever resourceful US entrepreneurs concocted a plethora of over the counter patent medicines, or changed the curative claims of existing potions to include influenza. What all had in common, aside from being entirely unproven, was a substantial alcohol content.

So what has changed? Unjustifiable claims for certain therapeutic agents are still made by people with agendas for profit or power. Countering this negative note though, a good whiskey, used in moderation of course, continues to dissipate many of life's stresses and strains. Cheers!

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MEMBERSHIP MATTERS

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Queensland

Without the contribution of all of you, our members, we would not be able to hold these great events, and we hope that while 2020 will have fewer events where we can meet in person, it will still be a successful year for everyone, and we will do our best to bring some great and relevant microbiology to all of you. The committee is always happy to receive suggestions for events especially from members are more remote locations.

Ulrike Kappler

South Australia/Northern Territory

At the SA/NT branch level, the Committee will continue to meet using teleconference options to plan for the near future. We will continue to provide monthly newsletter and updates, links to other matters of interest, and share updates on future meetings being planned. Our fundraising activities continue; and we'll provide our Branch members first option and access to these (details will be provided through the ASM SA/NT Branch newsletter). We'll continue to liaise with WA Branch around the next Tri-State meeting. We'll continue to represent the voice of the Branch members to ASM National Council, and National Executive, and work with them to deliver for the members we represent.

Peter Traynor

New South Wales/Australian Capital Territory

The NSW branch committee will continue to meet monthly via Zoom. We will continue to provide our newsletter Syntrophy and updates and links to other matters of interest. We are planning to have zoom seminars with guest speakers to replace face-to-face while these are not possible. We will provide our Branch members first option and access to these (details will be provided through Syntrophy).

Jim Manos

Western Australia

As we have entered the period of the "Great lockdown", all of us are reorganising our lives and careers to manage this unprecedented and historical event. WA branch remains enthusiastic about their mission to continue reaching out to microbiologists in Western Australia. We continue to hold committee meetings by Zoom and are planning new online events and novel ways for members to present their abstracts. We are excited by the prospect of inviting speakers to online meetings with ASM members and are using this opportunity to out-reach to our remote membership.

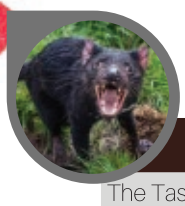
Please follow us on our website events calendar or our Twitter and Facebook as we announce our activities and keep in touch with your fellow microbiologists in WA!

Charlene Kahler

Victoria

As always, the ASM Vic branch committee is working hard for our members. We are committed to delivering value to members, and providing an opportunity for our members to connect and share ideas. We will be consulting with our membership to ensure that deliver the right events, on the right platform, to support them during the COVID-19 pandemic and beyond.

Catherine Satzke



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Tasmania

The Tasmanian branch committee will continue to represent Tasmanian microbiologists on the national stage. At this point, it's very unlikely that our 2020 BiState meeting with Victoria will proceed. As such we are investigating online options to hold an alternative function later in the year.

Please remember that the Tasmanian branch committee is here to represent you, our members. Should you have any requests or suggestions, please don't hesitate to contact either myself
or one of your local state branch committee members.

Belinda McEwan

Office Manager, Shona Kennedy, who is still at the end of the phone and is happy to help you to renew your membership or assist in how to defer payment but retain your membership if you are experiencing financial hardship.

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