Innovating Alternatives

a podcast about AMR in food-animal production and the researchers around the globe who are working to reduce it.

Episode: The Curious Case of Aquaculture

Featuring: Prof Sophie St. Hilaire, Associate Prof. Dr Natrah Ikhsan, Prof Brian Dixon, Dr. Ha Thang Dong, Dr. Le Hong Phuoc

Justin Kemp (JK) – Did you know that some air bubbles in water are so small that they remain suspended in the water column, never floating up to the surface. That is until they implode and unleash highly reactive free radicals in every direction. Turns out these bubbles might be quite useful for disinfecting water.

> Prof Sophie St. Hilaire
> [*I think if we find technology that reduces bacteria in the water column, aquaculture systems, that can be used while the fish are in the water, that would be a success, especially if we can get more than 1000- or 10 000-fold reduction in bacterial counts*.]

Evelyn Baraké (EB) – Well, disinfecting the water in an aquaculture system is one way to deal with bacterial outbreaks.

> *[Sound clip from Spaceballs movie – "Radar about to be jammed!"]*

EB - Another might be the old war time trick of jamming the communications, of the pathogens that is, not the fish.

> Dr Natrah Iksan
> [*The idea is by quenching the signal molecules, all this behavior that somehow led to the pathogenicity of that bacterial pathogen can somehow be inhibited*.]

JK – Or maybe the solution to controlling pathogenic bacteria in aquaculture systems lies in a naturally occurring protein found in most animals.

> Prof. Brian Dixon
> [*It's their structure that gives them their activity. It binds directly to the membrane on the outside of the bacteria and punches holes in it. So that the bacteria leaks out its internal contents and dies*.]

EB - I'm Evelyn Baraké.

JK - And I'm Justin Kemp and this is Innovating Alternatives – a podcast about AMR and the researchers around the globe who are working to reduce it. In this episode we meet research teams adopting diverse approaches to tackle bacterial pathogens in aquaculture.

[Theme music]

JK – Ever heard of the United Nations?

EB – Oh, you mean the organization founded post second world war in 1945 to essentially prevent future wars. The one that developed the UN Charter laying out the organization's objectives including maintaining international peace and security, protecting human rights, delivering humanitarian aid, promoting sustainable development, and upholding international law? That United Nations?

JK – That's the one. So as part of the larger UN family, there are also several specialized agencies, including the FAO – the Food and Agricultural Organization.

EB – Right, they lead international efforts to achieve food security for all. They also have an active program on antimicrobial resistance if I'm not mistaken.

JK – You aren't mistaken, but that's not why I bring them up. Having 190 global members means that you are in a unique position to collect a lot of data. So, if you are interested in global scale questions like say, what animals do we humans prefer to eat, their databases are a great place to go digging.

EB – So what animals do we like to eat?

JK – Well, what seems like a fairly simple question becomes a lot more complex the more you dig into it. Let's start with the simple answer. We eat considerably more animals with lungs compared to those with gills.

EB – Huh, I've never really thought of it like that.

JK – It's a useful distinction - this above the water, below the water thing. The way we go about getting nutrition from terrestrial as opposed to aquatic animals has some unique differences. So total terrestrial animal production in 2019 was in the region of 1.3 billion tonnes of animal products. Were not just talking meat, but also eggs, milk and even honey. By contrast, we produced about 178 million tonnes from aquatic animals.

EB – Ok, so we could say that the animal component of our food system is heavily skewed towards terrestrial production.

JK – Exactly, and your use of the word "production" is key here. We rely almost entirely on animal husbandry – a technical term for farming domestic animals basically– to create the animal products we eat. I'm sure you know what these animals are. Any children's barnyard story book should feature most of the key characters.

EB – Sure, I mean cows, pigs, chickens, sheep.

JK – Exactly, those four animals account for 94 % of all the terrestrial animal products we eat. Add goats and buffalo and you're well over 98%. That's just six species that provide virtually all of the animal products grown on land today. And it's been that way for a long time. We've relied on these domesticated animals for thousands of years.

EB – Ok, so that's what's happening above the water, what about under the water. You mentioned some unique differences.

JK – Under the water, that's what we call aquaculture – the farming of aquatic organism. The one thing that really differentiates aquaculture, is the number of species farmed. So about 60 years ago, in the early 1960's, the FAO collected aquaculture production data for 86 groups in total. Back then it took 33 species to get to the 98% level of production reached by just six terrestrial species.

EB – Right so we farm a much bigger diversity of animals under water.

JK – The second story is that aquaculture has grown rapidly in the last 60 years, both in terms of the diversity of animals being farmed and the total production output. Although the origins of aquaculture go way back - the first texts on the subject appear in China around 500BC - the modern growth of the industry has been remarkable.

EB – What sort of growth are we talking about here?

JK – Well to put it into perspective, between 1960 and 2019, the production of terrestrial animal products increased from around 430 million tonnes to the 1.3 billion tonnes today, that's a 204% increase. Human population growth over the same period was 150%. Food animal production was keeping up with human population growth, and some change.

EB –I'm guessing that spare change means we eat more meat per capita than we did 1961.

JK – Yeah, but that's a whole other can of worms. Ok, so back to aquaculture - growth between 1960 and 2018? A whopping 5500% increase! And the number of species has also increased, we're up to almost 400 now.

EB – OK, so if I'm thinking of aquaculture in 1961 as a small basket of groceries at the supermarket, then aquaculture today is a car sized trolley carrying one of everything in the store.

JK – Pretty much – this growth of aquaculture is often referred to as the blue revolution. But this rapid growth, both in terms of total production and the expansion of species diversity has not come without costs - and unfortunately the emergence of antimicrobial resistance, or AMR, is one of them.

EB – Hang on, that's a bit of a jump - what's the exact causal link or mechanism between production growth and AMR?

JK – Any aquaculture production happens along a continuum from extensive farming on one end through to intensive farming on the other.

EB – On the extensive end of the scale, I'm guessing you might have a farmer who adds some fish to a natural pond and returns at some point to harvest whatever has survived and grown. On the other extreme, a highly intensive system might involve a farmer having to provide all the necessities of life to his fish.

JK – Exactly, a recirculating aquaculture systems or RAS as it is sometimes known would be an example of the latter. By using a RAS, the farmer provides for all the needs of the organism he is growing, including water treatment systems, supplemental oxygen, all their food, lighting and heat control, disinfection systems. It's basically like a life-support system for fish.

EB – And the link with AMR.

JK – A lot of the growth in aquaculture production has been due to the increased intensification of production, not just RAS systems but also those one rung down the intensity scale so to speak, like intensive cage and pond culture. Intensification increases animal density and can negatively impact water quality. Crowded conditions and suboptimal water quality equals physiological stress and impaired immune function which ultimately favours disease emergence. And what options do farmers have to turn to in the face of disease outbreak and economic loss.

EB – Now I'm with you – antibiotics - and from the first episode of this series, we know that antibiotics, if used inappropriately, provide the selective pressures that favour the emergence of antimicrobial resistant bacteria

JK – Exactly, but aquaculture has some additional unique quirks that facilitate AMR emergence and create distinct challenges for addressing this emergence.

EB – I've got an inkling that the species diversity might have something to do with this.

JK – Yeah, aquaculture is very much an evolving food production system. Hundreds of species across diverse culture systems over a broad geographical area. In many instances we are still figuring out the optimal way to farm these organisms. While we figure it out the potential for less-than-ideal culture conditions is high, and that invites disease outbreaks. Also, when they occur, we don't necessarily have the knowledge and tools to diagnose and treat emerging disease threats.

EB – So we're very much on a steep learning curve. OK, what else.

JK – There are a whole host of factors, but here's the executive summary of some of the main ones. (1) most aquaculture production occurs in sub-tropical and tropical regions – these are the same regions that are prone to rapid and severe disease outbreaks, (2) there are no antibiotics specifically for aquaculture, so we borrow from those used for humans and livestock, some of which are critically important for human medicine, (3) now that we have an antibiotic to use, we generally incorporate it into feed and administer is metaphylactically, a big word meaning that if some fish are showing signs of disease, everyone gets treated. Unfortunately, fish don't metabolise antibiotics very well and keeping track of what's happening with feed consumption underwater is difficult, so (4) a large proportion of the antibiotics and their metabolites land in the aquatic environment whose microbiome, due to its large variety of mobile genetic elements, is particularly prone to genetic exchange and recombination. The term "genetic reactors" has been used to describe aquaculture systems in this context. Add to this the fact that aquaculture systems are often directly linked to aquatic ecosystems, which are themselves the sink for antibiotic produced and used on land and you get an idea of the scope of the issue. Oh, and (5) the regulatory framework around antibiotic use, think monitoring and enforcement is limited in many of the countries that are major producers.

EB – Wow, that's quite the laundry list of challenges. How do you even go about finding a solution?

JK – Well, I don't think there is one single solution that will just instantly solve AMR – it the kind of problem that needs action on multiple fronts to start moving the situation into a more favourable direction.

EB – Disease is obviously a major driver, so interventions that reduce disease can directly impact antibiotic use. I'm thinking improved biosecurity on farms to limit disease spread, improved veterinary support in the form of diagnostics and treatment plans, maybe also improved farm management to limit the conditions that lead to outbreaks in the first place.

JK – Exactly, and on the other side of the coin is trying to directly manage the availability and use of antibiotics. There's a bit of the stick and carrot thing going on here. So, when we talk about the stick, we are talking about regulation. Top-down legislation governing who can buy what antibiotics and when and how they can use them. The rules are all good and well assuming they can be enforced of course, and that requires strong institutions and funding. On the other side is the carrot so to speak are audited certification schemes. If certification schemes have limits on antibiotic use, and having certification means farmers can access new and lucrative local and export markets, then they directly drive how animals are farmed. Somewhere in the middle is the carrot-shaped stick that is import legislation, things like monitoring antibiotic residues at the point of import. Having a valuable shipment of shrimp turned away from the receiving country can quickly influence behaviour in the country of origin.

EB – OK, but despite the best intentions to reduce disease outbreaks, they do obviously still happen. If the idea is to limit antibiotic use, what alternatives do farmers have to control disease.

JK – Well, alternatives. Antibiotic alternatives I mean. And as they used to say in Silicon Valley in the early 2000's, that's a nice Segway (segue). Because now we get to visit a number of research projects that are looking to do just that, develop alternatives to antibiotics in the world of aquaculture. And be forewarned, they are as diverse as aquaculture itself.

EB – So how should we go about this? rock, paper scissors?

JK – Ok, let's go: rock paper scissors – shoot. Rock beats scissors. You win. So where to first?

EB – OK, Nanobubbles.

JK – Nano what?

EB – Nanobubbles, as in really tiny bubbles. So, this project is a collaboration between the City University of Hong Kong, Suan Sunandha Rajabhat University in Thailand and the Research Institute for Aquaculture in Vietnam.

Prof Sophie St. Hilaire
[My name is Sophie St. Hilaire, and I am a veterinary epidemiologist. I work at City University of Hong Kong.]

[So antimicrobial resistance occurs in bacterial populations, including fish pathogens. We have seen some antimicrobial resistant pathogens of fish here in Hong Kong, and there's certainly reports in other aquaculture industries around the world. The reason that we have antimicrobial resistance is multifactorial, there's likely pressures from a number of different sources of antibiotics. One of them may be their use directly in the aquaculture industry.]

Dr Ha Thanh Dong
[My name is Ha Thanh Dong. I am a fish pathologist working at Suan Sunandha Rajabhat University in Thailand.]

[Antibiotics are normally used when disease outbreaks occur and there's a lot of misuse with antibiotics in Asian aquaculture. When a lot of farmers use antibiotics and they did not know that it's used for only treatment, is not for prevention, and some farmers use it for prevention. Also, in a lot of cases that we also experience, they use antibiotics without sufficient diagnostic information, so they didn't know whether the fish got infected with bacteria or with viruses. So, whatever, they use antibiotics. That is the one kind of serious misuse and it can produce more AMR in the future. Therefore, I think the research should focus on not only disease prevention or something to replace

antibiotics, but something to reduce the risk of disease in aquaculture system, for example, that would reduce the concentration of pathogens in aquatic system.]

Prof Sophie St. Hilaire

[One of the ways to reduce bacteria in water is to disinfect water. However, if the fish are in the water, which they are in aquaculture systems, it's sometimes hard to find the right disinfectant that won't hurt the fish. The nanobubble technology could be used while fish are in the water body to disinfect and reduce the amount of bacteria while maintaining fish health.]

EB –Maybe before we get into the nitty gritty of the project, a very short introduction into what constitutes a nanobubble and how it is able to disinfect water might be useful?

Prof Sophie St. Hilaire
[So a nanobubble is a very, very small bubble less than 100 nanometers in diameter. Nanobubbles are negatively charged in neutral pH so they do not coalesce together to make larger bubbles. They remain in the water in solution. And so, they have special physical characteristics that macrobubbles or larger sized bubbles don't have. When these bubbles collapse on themselves they then generate highly reactive hydroxyl free radicals which is why they are potentially useful for disinfection.]

[We also can incorporate different gases in the nanobubbles. We can actually put ozone in the nanobubbles. And ozone itself has disinfection properties. So, we don't need to use as much ozone if we put it in a nanobubble versus a larger-sized bubble like an aeration stone or macro bubbles.]

[We make our nanobubbles through a process of hydrodynamic cavitation. We do this by running compressed air and high-pressure water through a venturi tube, the change in pressure then results in the formation of bubbles.]

[Nanobubble technology has been researched for a couple of decades, mostly with wastewater treatment. More recently, nanobubble technology has been explored for uses in aquaculture systems, namely reducing bacteria, reducing algae, biofloc on the sides of systems and also increasing dissolved oxygen.]

EB – Sophie mentioned incorporating different gasses into the nanobubbles. This was the first step on the journey so to speak, trying to figure what gas would potentially provide the best disinfection rates.

Prof Sophie St. Hilaire
[We were expecting nanobubbles with just air to start and to see if the bubbles themselves had measurable disinfection properties. We found that at the scale that we needed to disinfect; we couldn't detect an effect on a regular, consistent basis. So, we moved to oxygen. And oxygen has an added benefit in that if you have high densities

of fish, and you add oxygen, oftentimes, that reduces stress, which improves the aquaculture system. So even if nanobubbles didn't have disinfection properties, if they increase oxygen in the water, that has benefits. Nanobubbles do that very effectively, you don't lose a lot of the oxygen, it stays in the water. So, it's a good environment for growing fish. That was a nice finding. And one that we certainly had anecdotal evidence of, by fish farmers that were using nanobubbles in their ponds and just reporting that the growth of the fish was improved. So, we confirmed that in the lab.]

[But the disinfection properties of the oxygen nanobubble was not as effective as what we require when you have disease outbreaks. When you have a disease outbreak, the pathogen load goes really high. If you can only get rid of a tenfold level of bacteria, it's not adequate to reduce or stop an outbreak from happening. That's when we thought, well, let's try the ozone. Because we know ozone is a good disinfectant. It's just that ozone can be quite toxic to fish. This technology, we believe, allows us to use a lower level of ozone, it remains in the water for longer at a lower concentration. So, it seems to be a viable option, at least in the short term. We haven't exposed fish in the long term to the technology. So that's another step that we have to take. But short term, it doesn't seem to have a negative impact on the fish at the level that we would require to have a 1000-to-10,000-fold reduction in bacteria, which is pretty good for reducing bacterial loads in the water during an outbreak.]

EB — So figuring out which gasses are best at reducing bacterial loads during an outbreak was the primary aim of this piece of baseline work, but the researchers also discovered something that they hadn't necessarily expected when they exposed fish to the nanobubbles.

Dr. Ha Thanh Dong

[You know, in our project we investigated different gasses and we found that ozone worked very well for disinfection and then we also found that it can disinfect both Gram-negative and positive bacteria and it's relatively safe for the fish in the aquaculture system. And then we also have some evidence that ozone nanobubble technologies can reduce mortalities caused by bacterial infections, and they also modulate the immune system to fight again bacteria. We discovered that when the fish is exposed to ozone nanobubbles, there's a vast array of innate immune genes that are upregulated. So basically, the genes turn on. We investigated several genes including stress response genes. After that, we challenge the fish with bacteria and we found that the fish fight bacteria better, so they survive more than the ones in the control group. After the fish were exposed to ozone nanobubbles, then we can say that it's like armies, so they are ready to fight with bacterial pathogens, ready to fight with enemies.]

JK — Wow, that's pretty cool. So, it seems like the nanobubbles have this kind of trifecta effect—removing bacteria, adding oxygen and stimulating the immune system. But I'm guessing a lot of this work is happening in the lab. Did Sophie mention how they plan to scale things up to find out how the tech would work in larger aquaculture systems?

EB — She did, the lab experiments are just the beginning of a series of trials trying to see how the nanobubbles will perform in both pond and recirculating aquaculture systems, each with own unique set of questions. And then, of course, scaling up for large-scale farming and regulatory issues.

Prof Sophie St. Hilaire
[What we're doing is we're testing the technology on bacterial pathogens of fish, we do that in the lab. We've seen a decline in the bacteria when we use nanobubbles, especially ozone nanobubbles, on the bacteria. Once we determine this, then we evaluate that level of nanobubble on fish themselves, we do fish studies under controlled conditions. Then we look at the nanobubble impact on ponds. So, the microbial ecology of ponds and how that changes with the use of nanobubbles. And then we look at on a larger scale, so pond water in tanks, and then on a larger scale in natural existing ponds.]

[For the pond culture where you might be growing species that benefit greatly from the microbiome and the microbial ecology of the pond, we also have this question of whether we could crash the system if we use this technology. We've been looking at the microbial ecology, pre-and post nanobubble exposure on a small scale, and what we find is we get a reduction in the bacteria, but we don't get a complete wipe out and it eventually comes back. We're looking at the genus of the bacteria pre-and post—a16S analysis of the microbial community—and we're looking to see is there something that's being removed more than others and what's the rebound bacterial population look like to see if we are skewing the bacterial population in the pond by adding these ozone nanobubbles. One option, if we see that that's the case is we only use the nanobubble technology when a problem is starting. So, it's basically a treatment. We use it when the fish start to show signs of a bacterial problem. We use nanobubble technology with ozone to reduce the level of bacteria in the pond and try to get rid of the pathogenic bacteria, which tends to monopolize during an outbreak. When there's no outbreak, we just switch to oxygen nanobubbles, which would benefit the community and the fish population.]

[The other thing that's interesting is that when you inject ozone in nanobubbles, the oxygen level in the water increases quite a bit. So, we're getting both the benefit of increasing oxygen, dissolved oxygen, and we also get the benefit of the disinfection. We think this technology will be useful in recirculation systems where your bacterial levels can increase, I mean, you have to watch for your biofilter, of course, but you can get rid of the ozone before it hits the biofilter. So, we think that this type of system will benefit from this technology. And the last step is to look at fish in a natural environment when

we apply the nanobubbles, so in a pond situation or a recirculation system, where there's high densities of fish, and there's high volumes of water.]

EB — And what does success look like in this project?

Prof Sophie St. Hilaire

[I think if we find technology that reduces bacteria in the water column of aquaculture systems, while the fish are in the water, that would be a success, especially if we can get more than 1000- or 10,000-fold reduction in bacterial counts. If we can increase dissolved oxygen, I think that's also a success. I should also add, a method that's cost effective for the farmer to do this on a large scale. All three of those have to be met in order for us to really call this a success. If we find that we can reach one or two of these then we have more work to do to improve the technology but it's relatively new technology. As we know, innovations always take a while to really transform the way that we culture fish. It'll take some time before the cost comes down and the system is perfected.

The question even if we find that this technology works on a small scale, can it be scaled up for aquaculture systems to reduce bacterial populations and can it reduce pathogenic bacteria to a level where we don't see the disease outbreaks and we don't need to treat with antibiotics?]

EB — And as always there are going to be barriers that need to be overcome before getting there. Fortunately, given that nanobubble machines are already employed in the wastewater treatment industry, in this case regulatory issues are unlikely to be one of them. But there are other challenges…

Dr. Ha Thanh Dong

[I think nanobubble technologies have been used for wastewater treatment. Therefore, I think there won't be major issues with approval regulations. It's already there, you know, so I think to be applied in aquaculture is not a big problem. But the big problem is, how to produce the big capacities of the machine that make it suitable for mass-scale farming. And you know, there are many companies that provide the device, and they advertise the technologies. But it's not easy to prove that the machine is really producing nanobubbles or if they produce microbubbles, which are totally different. Nanobubbles can stay longer in water compared to microbubbles. I think that currently the price for a nanobubble generator is still quite expensive for farmers. So, I hope in the future that manufacturers can lower the price and make it more affordable to farmers. The other issue is maybe in the future, if it's possible, they can adopt these technologies together with solar cells so it can reduce a lot of electricity and use clean energy.]

EB — So COVID.

JK — Um. Yes, COVID, kind of hard to ignore right now.

EB — Well, as a silver lining it might be spurring innovation in some less expected places…

Prof Sophie St. Hilaire
[There's a number of steps that have to happen when we use the nanobubble technology. And one of them is that the water has to be relatively clean going through the machine. And that has allowed us to look at different filtration systems to clean the water in terms of the large particles.]

[We've been looking at different materials to filter the water before it enters the nanobubble generator. With the issues with COVID, we have several engineers at our university that have looked at different materials to incorporate in face masks to clean air. And one of our colleagues at City U has looked at graphene materials to incorporate into masks. So, we've been exploring the use of this material, graphene, to disinfect the water and augment the disinfection properties of the nanobubble generator. By using this novel technology that's incorporated in masks, we've been able to create filters for water that kill some bacteria. So, we can stack that technology with the nanobubble technology and I think it increases the disinfection properties of the system.]

JK — I love that—even the nanobubble machines are masking up. I think as we begin the long debrief from this crazy time, we're going to discover all kinds of unintended outcomes of COVID, many will likely be negative, but hopefully some positive one too.

[crowded room sounds]

JK— OK, interesting fact, did you know that bacteria like to chat with each other? And that that conversation can regulate the expression of some of their genes?

EB — I can honestly say I did not know that!

JK — OK, so when pathogenic bacteria express certain genes during the infection process, like the so-called virulence determinants, the host organisms get sick. The thing is many of these products also trigger the immune system of the host. So, if bacteria can wait until their numbers are high enough before making a coordinated attack, they can potentially produce enough virulence products to overwhelm the host's defences.

EB — Wow, bacteria are sneakier and much more organized thank I thought!

JK — But, in order to do this, they need to figure out how many of themselves are around, and they do this is using a cell-to-cell chemical communication system known as quorum sensing. It works like this: bacteria release chemicals called autoinducers into the environment, the more bacteria there are, the higher the concentration of the autoinducers floating around. Once the bacteria sense that the concentration has reached a threshold level, the activation or repression of certain genes is triggered.

EB — OK, so if we wanted to control bacterial infections, could we, how do I put this—jam the signal? Prevent the bacteria from talking to each other so they can't coordinate their attack and cause disease?

JK — Exactly, and that's just what our next project is trying to do in the context of shrimp aquaculture. This is a project centred at the Department of Aquaculture at University Putra Malaysia.

> Dr Natrah Ikhsan
> *[Hi, I'm Associate Professor Dr. Natrah and I'm from the Faculty of Agriculture at UPM (Universiti Putra Malaysia). I'm the principal investigator of the project "Integrated Quorum quenching strategies to reduce AMR in shrimp aquaculture." With this project we have also collaborators from Universiti Kebangsaan Malaysia, International Islamic University Malaysia, UniversityMalaysia Terengganu and we also have international collaborators—Swansea University UK, University of Ghent, Belgium, and also Institute Pertanian Bogor, Indonesia.]*
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> *[Shrimp is one of the most important commercial species in Malaysia and it is also a very important sector for food production and food security. Of course, in terms of production in Malaysia, most of the challenges basically comes from disease outbreaks. And in terms of the usage of antibiotics, our group has conducted several questionnaires and interviews and most farmers basically tell us that they are not using antibiotics, but then, of course, it is very hard to get an honest feedback on this antibiotic usage in shrimp farms.]*

EB — I guess that's always going to be a limit of self-reporting surveys. Did they do any studies on AMR levels on shrimp farms?

JK — They did but pinpointing the cause of AMR in a particular location is extremely difficult.

> Dr Natrah Ikhsan
> *[Yes, our team did quite a number of surveys in terms of looking at the occurrence of antimicrobial resistance, particularly in shrimp farms around Malaysia. And we did notice some* Vibrio *species,* Vibrio *species are among the common pathogens for shrimp disease in aquaculture. And we found that a number of the species are resistant to several antibiotics, including tetracycline, ampicillin, penicillin and kanamycin and we noticed that most of them are basically resistant to ampicillin. However, it is not surprising because ampicillin was the first generation of antibiotics and basically what we used. It can be that this resistance to these antibiotics, it developed over time and it can be the misuse of antibiotics was from a long time ago but we can only see the effect of the antimicrobial resistance now. And then there is also the possibility that these antibiotic resistant bacteria actually didn't come from the antibiotics that are being supplied by the farmer, it can also originate from the water as most of the*

sampling sites are located near to other aquaculture and agriculture farms. So it is impossible to really pinpoint the real cause of these antibiotic resistance in shrimp farms.]

JK — So we talked about quorum sensing earlier and you mentioned that interrupting this bacteria chatter might be useful. Well, that process has a name—quorum quenching.

EB — Sounds very sci-fi!

Prof Natrah Ikhsan
[So, quorum quenching of quorum quenchers, also known as quorum sensing inhibitors, are basically any organisms or any compounds that can quench or basically inhibit the signal molecules that are being produced by bacteria. The idea is by quenching the signal molecules, all this behavior that somehow led to the pathogenicity of that bacterial pathogen can be inhibited.]

[Is because most of the disease outbreak in shrimp aquaculture was caused by the pathogenic Vibrio *species. And for example, we have the EMS or the Acute Hepatopancreatic Necrosis Disease (AHPND) outbreak, which led to huge economic losses to shrimp aquaculture industries, and AHPND are known to be caused by different* Vibrios *and we know also that* Vibrios, *the pathogenicity of* Vibrios *are known also to be regulated by quorum sensing mechanism. So that is why to counter this pathogenicity our invention basically contains high quorum quenching properties, that could halt the quorum sensing in pathogenic bacteria and reduce this transcription of the virulence genes.]*

[For the project, we are basically focusing on three main different quorum quenching strategies. The first one is on the use of probiotics in the form of feed formulation and also in the form of mature microbiota. Right, and the second one is on use of natural antibodies, while the third one is on the use of synthetic antibodies.]

JK — OK, so for this episode we are just going to focus on the probiotic component of the project, that's Natrah's speciality after all. So, in simple terms, probiotics are basically microorganisms that when ingested provide a health benefit.

Dr Natrah Ikhsan
[For the probiotic part, the focus is more on micro algae and its associated bacteria.]

[For probiotics there can be a lot of mechanisms. As a probiotic, it can be in terms of colonization, it can also produce antibiotic-like properties. But in our case, it is mostly on silencing the signal molecules.]

[So, in order to test for quorum quenching activity, our lab is having a lot of biosensors that are specifically designed to look at the activity. So, these are mutants. We can supply them with a synthetic signal molecule that we can buy from chemical supplier.

From that, we can see whether the organism that we are testing can inhibit the signal. So, this shows the ability of the quorum quenching.]

[So, we screen quite a number of microalgal species. Microalgae are basically used a lot especially in shrimp farms, because microalgae are known to have all these beneficial polyunsaturated fatty acids. And we noticed that a number of microalgae are also having these quorum quenching properties. And other than microalgae we basically selected some of them where we further investigate the associated probiotic bacteria that are also having quorum quenching properties because we think that microalgae cannot be alone. They are in symbiosis with other species. So that's why we would like to have this combination of microalgae and bacteria together for them to become a stronger team. And at the same time, of course, this micro algae and bacteria, they need nutrients for them to grow. So, our team has successfully formulated media to enable this probiotic to grow. And at the same time, we have the other strategies is to incorporate the quorum quencher with live feed. In our case, a type of live feed known as copepods. Copepods are basically a very nutritious live feed. Our collaborators have successfully mass cultured this copepod. These baby shrimp, they love this copepod, you can see, we have a video, the shrimp they really like the copepod. At the same time the copepod really loves the micro algae that we supply them.]

EB — Right, so the researchers screen different microalgae and their associated bacteria for their quorum quenching abilities. They then figure out how to mass culture the best ones. Then they get copepods to eat the microalgae so that when the copepods are released as live feed into shrimp culture systems, they essentially deliver the microalgae, and all its quorum quenching abilities, right into the shrimp's gut.

JK — Exactly, it's kind of like the copepod is the tortilla in a taco. I should also mention that the copepod encapsulation method is not the only approach the project is exploring to use their quorum quenching microalgae and associated bacteria. They are also exploring using them to seed green water culture systems—this is where you grow shrimp in water that has a high density of microalgae cells. This has a number of advantages including feed provision, water quality improvements and potentially more stable microbial communities.

EB — We're always keen to know how research teams plan on taking it to the next level. How are they looking to move this towards a viable product that farmers can use?

JK — Well, it turns out that University Putra Malaysia has an incubator program called the Putra Science Park that functions as a centre for innovation management and technology transfer, so it seems they have some great resources to turn to for help on this.

Dr Natrah Ikhsan
[In our University we have what we call Putra Science Park. They are a unit in UPM. They are capable enough to advise us on the next step. Just a few days ago, we had an interview organized under the Putra Science Park to secure a kickstart grant to start a

start-up company. And from there, the Putra Science Park would offer programs for us to learn more on market strategy and product support in order to bring our product to market. Our main issue is that as academicians, as scientists, we have really little knowledge in business and marketing.]

EB — Very cool that Putra University is providing the kind of support researchers need to turn their ideas into products that have the potential to have real impact in the world.

JK — Shall we move on to our next project?

EB — I still have so many questions about quorum quenching, but for the sake of keeping this episode under four hours, let's do it!

JK— Ok, so the next stop on our tour of Southeast Asia is Vietnam. This project is focusing on the freshwater catfish, *Pangasius*, a major contributor to global aquaculture production.

EB — I wasn't familiar with it before; I did a bit of digging and the numbers are pretty amazing. Production ballooned from 60,000 tonnes in 1998 to over 1.2 Million tonnes in 2008—that's a 20-fold increase. It has sort of levelled off in the last 10 years, but that's a huge amount of fish being grown. And the majority of that is being exported, the last count was to over 160 countries

JK—Yeah, talk about a fish with a global distribution, at least in fillet form. It's those nice simple white fillets that everyone is after. It's the kind of fish product that isn't too fishy, a subtle meat that works with any recipe that calls for white fish.

EB — But unfortunately, *Pangasius* culture hasn't escaped the bacterial disease issues associated with intensive culture and the use of antibiotics that follow. But the development of new fish vaccines and novel ways to administer them might just be the help everyone needs.

JK — Vaccines are really the MVPs of the decade. Helping us stave off everything from global human health threats to antimicrobial resistance in aquaculture.

EB — And in pork production too! But we'll save that for next episode. Fish vaccines for *Pangasius* are the focus of a collaboration between the University of Stirling in Scotland and the Southern Monitoring Centre for Aquaculture, Environment and Epidemics in Vietnam.

Dr. Le Hong Phuoc

[My name is Le Hong Phuoc. I am a Director of the Southern Monitoring Center for Aquaculture, Environment and Epidemics (of the Research Institute for Aquaculture No2 [RIA2]. RIA2 is under the management of the Ministry of Agriculture and Rural Development.) My background is microbiology and immunology.]

[Aquaculture in Vietnam has developed quickly now. We have two main culture species in Vietnam: Pangasius *catfish and (marine) shrimp. Every year we have high production of* Pangasius *catfish, about 1.4 million tons every year. We have the shrimp, also with high production around 700,000 tonnes (in 2020). Other species we have include Tilapia, Seabass, Seabream, Cobia and so on.]*

[There are two major pathogens of Pangasius *culture, two species of bacteria:* Aeromonas hydrophila *and* Edwardsiella ictaluri. *In the past, people used antibiotics to fight the problem of bacterial disease. In the past 5–6 years we also found antibiotic resistance in the fish culture, especially in the* Pangasius *catfish. But recently, many people found the harm of antibiotic use in aquaculture.*[1] *Many people try other alternative products, like herbal extractions and other antimicrobial products.]*

JK — Are we talking big commercial farms here?

EB — There certainly are big integrated farms, but there are other models as well, including small household farms and something that you see quite often in poultry farming—contract growing.

Dr. Le Hong Phuoc
[Normally the small farms are for the family. For example, in the family they have just one pond or two ponds in a farm with a small culture area. Nursing ponds can be around 1,000 to 3,000 square metres. In the grow-out pond, they'll have only one or two ponds of around 3,000 or 5,000 square metres each. But in the large scale, normally people have more than three ponds with a culture area totalling 3 to 4 hectares. We also have the commercial companies here. [2] *We also have a different form where the pond belongs to the farmer, but the farmer will sign the contract with the company. So, the company, will provide them with the feed, then after that when they have the fish (grown out), the family we collect the fish from their pond (which will be bought by the company).]*

EB — Ok, so this project is looking to develop new vaccines for bacterial disease in *Pangasius* culture, but as it turns out, this is round two of this process. Some vaccines do already exist but uptake has been limited. So the researchers have been surveying farmers to try to understand the barriers to adoption.

Dr. Le Hong Phuoc
[In the survey, we try to collect the information about how to deal with the disease in the farm, their knowledge about the vaccine. We also ask them if they are willing to use the vaccine later if the vaccine is available. We ask farmers about the barriers to

[1] The use of most antibiotics is no longer permissible in aquaculture in Vietnam.
[2] Culture areas of 3,000 to 5,000 square metres each.

applying the vaccine. We also ask the farmer if they have ever used chemicals or any antibiotic in their farm.]

[Recently, we have commercial vaccines for Pangasius *culture available in Vietnam, especially for the prevention of* Aeromonas hydrophila *and* Edwardsiella ictaluri *infections in* Pangasius *culture. But that kind of vaccine is only an injection vaccine. So that is the barrier in our culture system here, because with injection vaccines, we have to catch then we have to inject fish one by one. By that way the fish is very stressed. The farmer has spent a lot of time and the labour consumed in catching the fish one by one. The farmers, they don't like that way. So, in our project, we try to produce immersion vaccine that's much more convenient than injection vaccine. In our project, we also try to produce a bivalent vaccine. It means one vaccine that can prevent two diseases, two pathogens.]*

JK — Wow that's pretty epic, a bivalent immersion vaccine. Immersion vaccines make complete sense if you are a fish. I mean, imagine getting your head held under water while you wait for your annual flu vaccine—that's not going to do much for keeping your stress levels down.

EB — I like that you automatically took the perspective of the fish there! While I was listening to Phuoc speak, I was imagining how much of a struggle it would be for farmers to catch, handle and individually inject each fish. Much better to just deposit the fish in the vaccine, rinse and repeat. But first you have to develop and test the vaccine.

Dr. Le Hong Phuoc
[For the immersion vaccine, first we have to produce the vaccine in laboratory conditions. So how to develop the vaccine? First, we have to collect many different bacterial strains from many years. We have many bacterial isolates of Aeromonas hydrophila *and* Edwardsiella ictaluri. *After collecting the isolates, we will do the molecular biology work to select which strains we are going to use for producing a vaccine. Sterling University will develop the protocol for this, and after that, they will share that protocol. After selecting the isolate for producing the vaccine, we are going to culture and enrich the bacteria in the appropriate culture medium. Then after that, we are going to use the formalin to kill the bacteria to produce the inactivated vaccine. We have to kill them to make sure that all bacteria will die in the vaccine product that we call inactivated vaccine, our killed vaccine.]*

[First, we have to try with the small scale to see if we see any effect. We will do the preliminary experiments. We will try to immerse the fish in the short term, it depends, if we use a high dose of vaccine then we can shorten the time. We will try to standardize that. We hope that we will find some good results on the immersion vaccine, especially for the bivalent vaccine for the prevention of two diseases.]

[Later on, we will show the farmer how to use the immersion vaccine, then they will know how convenient it is compared to the injection vaccine. In our project we also plan to have the farmer training workshop. In the training workshop we are going to give the farmer information about the bacterial disease in Pangasius *culture, and how to prevent and treat the disease, then something related to vaccination. That information we are going to provide to the farmer in the training workshop.]*

EB — There's one aspect of the project that wasn't mentioned, and that's the idea of robotic vaccination!

JK — This is really our sci-fi episode, isn't it? First quorum quenching, now robotic vaccinations for fish!

EB — I kid you not. The team is working with a commercial partner that has already developed robotic arm technology for vaccinating salmon. The aim is to adapt the tech for *Pangasius*, but this requires giving the robotic arm the information it needs to recognize the right spot to vaccinate on the fish. These datasets are generated by image analysis of *Pangasius* fish as they grow and are used to develop an algorithm to guide the arm. Then onto pilot testing and ultimately, on to demonstrations to potential early adopters in the industry.

JK— Right, so time to move on to last project we get touch base with today. Here's a nice simple term for you: Pituitary adenylate cyclase-activating polypeptide.

EB — Seriously, please tell me there's an acronym for that!

JK — There always is, this one is PACAP. So, as you might have caught from the name, PACAP is a polypeptide. You know DNA, that long genetic code made of just four bases.

EB— Yeah, A, C, G and T, they provide the instructions for making proteins, right?

JK— Each group of three bases is called a codon, and that encodes for a specific amino acid, which are the building blocks of proteins. So, codon of three A's, AAA, gives you the amino acid lysine, for example. When multiple amino acids are linked together into more complex molecules, you're into the territory of peptides and when the number of amino acids gets above 10–15 in your chain, you're into polypeptide country.

EB — So what's so special about this specific polypeptide, PACAP?

JK — Well, it has several really useful features if you're looking for an alternative to antibiotics. An international team from the Universities of Waterloo and Prince Edward Island in Canada and the CIGB institute and Universidad de la Habana in Cuba are exploring how PACAP can be used to tackle bacterial disease in aquaculture.

Prof Brian Dixon
[Hello, I'm Brian Dixon. I'm a professor of biology at the University of Waterloo in

Ontario, Canada and I'm the Canada Research Chair in fish and environmental immunology.]

[Simply put, PACAP is a naturally occurring protein in animals, including aquaculture animals, and it has two specific effects. The first is that it binds directly to the membrane on the outside of the bacteria and punches holes in it. So, the bacteria leaks out its internal contents and dies. But PACAP can also bind to very specific proteins on the surface of our cells or our immune cells and activate them. So, it has a sort of a double effect in helping get rid of the bacteria by destroying them directly and helping to activate immune responses.]

[PACAP is part of the natural defence, there are several of these antimicrobial peptides that all bodies produce in response to pathogens, and this is one of them. So, it's a natural form of inducing antibacterial activity. So, PACAP stands for pituitary adenylate cyclase activating peptide. And that name reflects the fact that it has several other functions in the body, including intestines, intestinal activity, and some neural effects. So, it has quite wide-ranging effects beyond the immune system.]

EB — Now I've got an image stuck in my head of PACAP peptides punks walking up to bacteria and punching holes in them.

JK — Wow, what an alliteration! Well, now you're definitely going to be stuck with that image.

Prof Brian Dixon
[It's their structure that gives them their activity. They're usually either very positively charged along one side because of their shape, or they have these sulfur groups attached to cysteine in their amino acid sequence and sulfur is highly reactive. Either way the sulfur can bind to the membrane of the bacteria and start the process of punching a hole disrupting the membrane, or the positive charges are attracted to the bacterial membrane because it is overall negatively charged. So, it's like an ionic interaction. It's just a function of the structure of the antimicrobial peptide that allows it to bind. And then once several of them bind, they just gather together and punch a hole and disrupt the membrane.]

EB — So now it's a pack of PACAP peptides punks teaming up on bacterial cells. How's that for an alliteration? I guess we're OK with that kind of vandalism when the target is bacteria. If PACAP occurs widely in animals, does that mean that this antibiotic alternative can be widely applied to multiple species?

JK — It may just be, but you have to start somewhere, and in this case, they are focusing initially on salmon, tilapia, catfish and shrimp. But considering how popular those species are, there's already scope for significant impact.

Prof Brian Dixon

[Our project deals with controlling antimicrobial resistance in fish and shrimp aquaculture. Our partners are in Cuba and they grow a lot of a fish called tilapia, a type of catfish and shrimp for consumption.]

[The Cubans have lots of expertise with the PACAP peptide itself, and some connections to the shrimp, tilapia, and catfish industries. They've got expertise in the species that they grow there. In Canada, because we're largely focused on salmon, we're taking our techniques and investigating the immune responses in fish from salmon and adapting them to the tilapia and the catfish. In fact, the species we're testing in Cuba are tilapia, and they're grown around the world, particularly in Southeast Asia, but also in the Middle East and in Poland, and some tilapia aquaculture is global as well. I think that if we can get this to work in Cuba, and we get some parallels with salmon aquaculture in Canada, it will help a global industry that's worth about $6 billion dollars a year to the global economy.]

[Shrimp have a completely different immune system. They don't have antibodies the way we do, although they do share some of innate immune mechanisms like the amoeboid, macrophage cells. PACAP has been shown to have effects in shrimp, and we're really hoping that PACAP will enhance their version of immunity as well.]

JK — It turns out that not all forms of PACAP act the same way. You can potentially tweak it to improve performance. So, in addition to standard forms of PACAP, the team is also looking to test slightly modified versions to see if they offer improved bacterial-fighting or immune-activating characteristics.

Prof Brian Dixon

[We've been looking at modified versions, because it's known that if you change an amino acid, or if you modify the side group on an amino acid chain, you often get slightly different activities. And for PACAP, they have investigated five different variants that have had various different abilities to interact with the host and increase immunity. That being said, we've looked at the five variants in our systems and it turns out that the first one, and one with a slightly different amino acid sequence that's not too modified, are the best ones, certainly so far, in fish. We still have to do the work in shrimp and it may change there, but the simplest ones seem to be the best ones.]

[When we're testing these peptides, the first thing that we do is just test them directly against the bacteria. As I said, it can punch holes in the membranes of bacteria and destroy them ideally. So, the first thing we do is just mix it with the bacteria and look to see if it actually does that, if it destroys the bacteria and prevents its growth. The second step is to go in vitro with fish cells. We're getting quite good at growing fish cells in tissue culture dishes. We'll add the PACAP or the variants onto those cells, and then look at the immune activation and see if there is an actual enhancement of the

immune response. And quite often, we'll do that by mixing the bacteria in with the cells to see the cells' reaction to the bacteria. And then the final stage is to do it with live fish in tanks. So, we would take the fish and expose them to the PACAP, look to see if there is an increase in the basal level of immune response and then challenge the fish with bacteria and see how well they respond, if there's better survival or more intense immune responses in a live fish. And once we've determined all that, then we can figure out doses that we might use in a much larger field trial, which is the final step of our grant that will happen in Cuba.]

EB — Did Brian just say growing fish cells? Did he mean like growing just some of the cells, without the rest of the fish?

JK — Yup, he was talking about using tissue cultures of cell lines. It turns out, you can keep individual types of cells alive and reproducing indefinitely, without the original body they were collected from, and well beyond that body's eventual death. These are sometimes called immortalized cell lines, kind of like the HeLa cell line.

EB — Immortalized cell lines. Now we're moving from the sci-fi genre to straight up fantasy! But what's the HeLa cell line?

JK — Yeah HeLa, as in He for Henrietta and La for Lacks. Henrietta Lacks was an American woman, who unwittingly became the source the of one of the most important human cell lines in medical research. Her cells, taken during a biopsy for cancer treatment, were placed into a Petri dish with some medium by George Gey of Jon Hopkins University in 1952—it turns out they did quite well and became the first immortalized cell line capable of renewing itself in artificial culture indefinitely. It's estimated scientists have grown twenty tons of these cells since they were isolated. This story is also a study in issues of consent and privacy—Lacks never gave permission for her cells to be harvested or used for research or commercial purposes, family medical records and the genome of the cells were also released into the public domain.

EB — Wow that's really fascinating. You just opened up a really interesting can of medical ethics worms.

JK — Oh yeah that could be the subject for a whole other podcast though. Back to fish cell lines and PACAP.

Prof Brian Dixon
[A cell line is a particular set of cells, say skin cells or cells from the spleen, that have been taken out of an animal and we were able to grow them in a plastic dish using artificial media with nutrients. They don't always grow, they're not easy to make. But when you get one, they're very valuable because you've got an isolated cell type that you can experiment on in isolation, and we try in science not to use live animals where we don't have to. And they're also valuable because you can isolate one particular cell

type. So, we have cell lines from fish that are epithelial, so like skin cells, we have cell lines from fish that are immune cells, and we're able to isolate the effects of PACAP in inducing different types of immunity by looking at these different cells in isolation. The value of having cell lines is you can isolate specific effects and see what to look for when you start looking in vivo, because vertebrate animals are quite complex and there's mixes of different cell types there. It's hard to isolate the effect, specifically from a whole tissue like a whole spleen, or a whole kidney.]

[One unique finding is that these antimicrobial peptides are also valuable in antiviral resistance. They're well known for helping fight off bacterial infections. But it turns out that they can, in some cases, inhibit viral replication and induce antiviral responses, which I think makes them even more useful.]

EB — It all sounds pretty promising, fighting off bacteria and viruses. But how do you regulate a product like this? I mean, it's a naturally occurring peptide, so can you patent it? And how would regulators view it once it gets to the point where you try to get a product registered?

Prof Brian Dixon
[The regulatory approval will be the tricky part. Licensing this will require it being treated as a drug. It will have to be treated as a drug because it's an intervention. Even though it's naturally occurring, we're using it as a treatment. And so, we'll have to get approval as a drug intervention. So, the regulatory process will be quite complicated, and I think it will take several field trials and lots of evidence of its efficacy and its safety to get past that. I think that that will be the major hurdle before we get to using it as a treatment. We're just starting to go down that route. The Cuban group has patents on its use as a therapeutant, and that was the first step in the process. And so, we're just actually getting to the regulatory approval part.]

[I think that these are safe, we haven't seen negative effects. Because they're natural compounds, they don't have negative effects on the host, you don't have side effects. And because they're proteins, when they get in the environment, they're broken down quickly. The other side effect of antibiotics is that bacteria can develop resistance to them. But because this is just punching a hole in the membrane of the bacteria, it's very hard for them to develop resistance. So far, we've seen very few negative effects. But we're hopeful that we won't see any in the large-scale trials.]

[A successful outcome from my perspective would be showing that this antimicrobial peptide can be used to increase survival in an aquaculture setting and prevent diseases from causing economic losses. And I think also showing that it can replace antibiotics so that we don't have to dump as many antibiotics into the ocean, or into freshwater systems, to get the same level of survival after disease outbreaks in aquaculture.

EB — It's really good news that this alternative to antibiotics isn't really one that will contribute to developing more resistance.]

JK — Yeah, and maybe that's a great place to end our science fiction-y tour of the underwater world of alternatives to antibiotics.

EB — So that means that there is just one more topic for us to cover! One last episode in our four-part series. In the final episode of our series, we'll be looking at *Streptococcus suis*, a bacterial pathogen found in pigs that can be incredibly deadly, especially for piglets.

JK — And humans aren't safe from it either.

EB — And because it's so dangerous and its economic impacts on the pig farming industry are so high, it drives a whole lot of antimicrobial use. If scientists can find an alternative way to deal with *Strep. suis*, that will be a major win for AMR reduction.

[Theme Music]

EB— For everyone wanting to learn more about the podcast, read the transcript or get in touch, visit us on the podcast's home page linked in the show notes. We'd love to hear your thoughts. Also, don't forget to subscribe.

JK— Until next time, and thanks for listening.

[End]

*Note that some of the quotes throughout this transcript have been lightly edited for readability.

SHOW NOTES

Nanobubbles, bacterial small talk and a pack of PACAP punks punching holes in bacteria. In this episode we explore the diversity that is aquaculture, and the diverse approaches that researchers are adopting to develop alternatives to antibiotics for farming, the underwater type, that is.

Innovating Alternatives is a serialized podcast that will delve into the issue of antimicrobial resistance, a slow-moving pandemic that risks erasing the last 80 years of modern medicine's progress. We will take you right to the cutting edge of science, where researchers are developing new and surprising alternatives to antibiotics and innovative solutions to reduce the use of antimicrobials in livestock and aquaculture production.

Get in touch and let us know what you think of the podcast by email innovetamr@idrc.ca or on twitter @Livestock_IDRC

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