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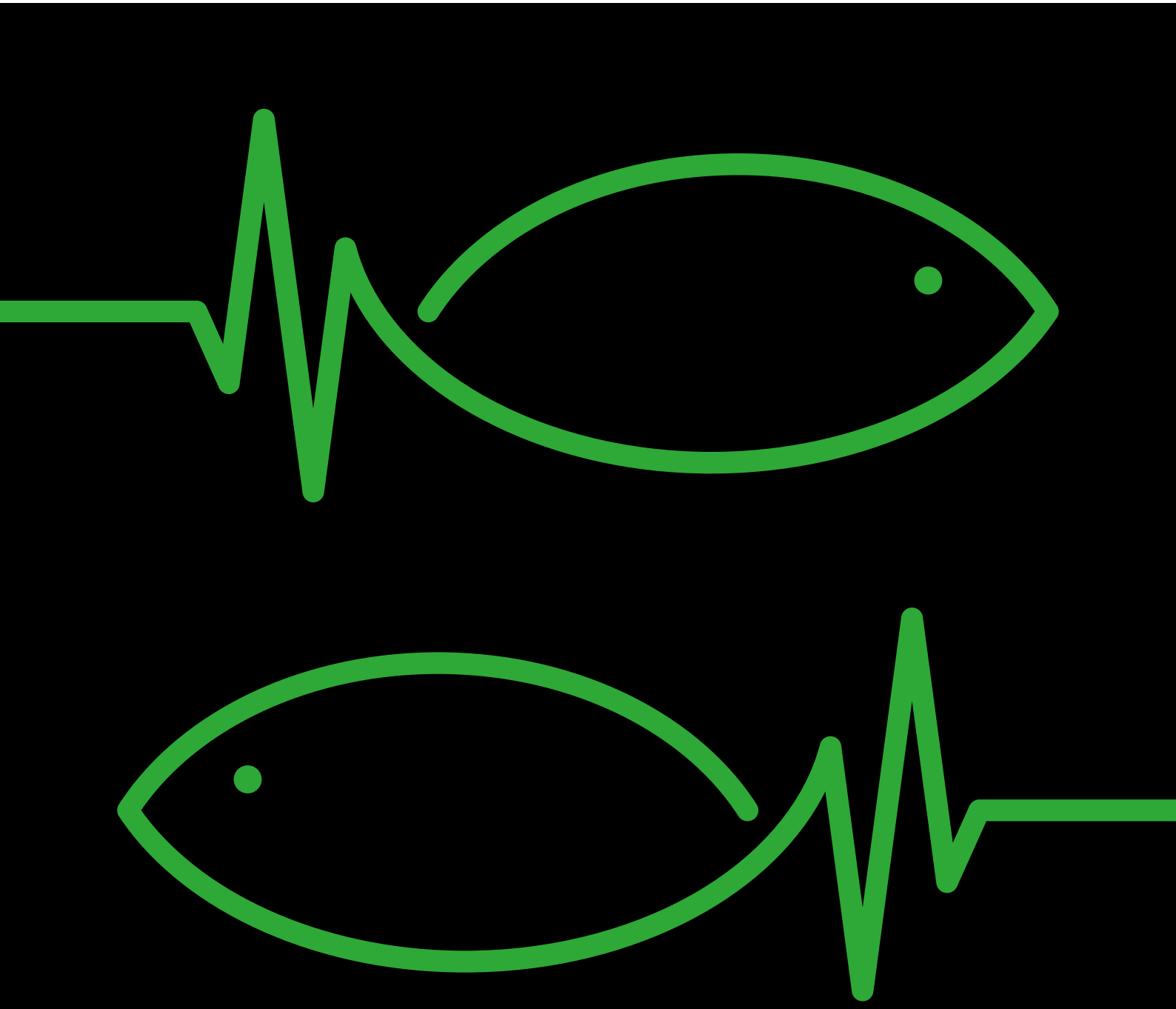
Keeping you naturally informed | Issue 54 | Aquaculture

Probiotics to boost immune fitness and gut health

Fusarium mycotoxins continue to threaten Southeast Asian aquaculture

Taking the perfect sample for mycotoxin analysis



How fit are your fish?

How fit are your fish?

Today's market place is highly competitive, making it extremely important for aquaculture producers to stay naturally ahead. One way of doing this is to ensure that your fish and shrimp are as homeostatically fit as possible.

Maintaining homeostatic fitness includes controlling the many variables of aquaculture production. Measuring and managing environmental factors or the biological, chemical and physical factors related to water quality has become standard practice.

New technologies promise to take this fitness to the next level. Thanks to advances in nutritional awareness and knowledge, and increased accessibility to raw materials from all around the world, diet formulations can now be more precise than ever before. At the same time, advances in technology have made a wide and exciting range of products available to producers. Such products can help overcome disease challenges, boost performance levels and ensure sustainability, all while being profitable.

In this issue of Science & Solutions magazine, we revisit probiotic use. The rigorous processes involved in aquafeed manufacture previously restricted probiotic use, but post-pelleting technology now makes more widespread application possible. Benedict Standen explains why probiotics constitute such a valuable addition to the diet.

Rui Gonçalves shares some results of the BIOMIN Mycotoxin Survey with you. He looks specifically at samples from Southeast Asia to dispel the myth that only aflatoxins are present in aquafeeds. Global trade of commodities has increased the range of mycotoxins found in this region, highlighting why it is so important to regularly check your feed for mycotoxin contamination.

Today's mycotoxin detection tools are quite reliable. However, some errors can and do occur—the majority of the total error in mycotoxin testing stems from improper sampling. If you are not sure how to take a good sample for mycotoxin analysis, read our guide on page 11.

Enjoy reading this issue of Science & Solutions, keeping you naturally informed.

Anwar Hasan
Regional Technical Manager - Aqua

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Probiotics to Boost Immune Fitness and Gut Health

Benedict Standen PhD
Product Manager

Despite growing trends in probiotic use, their application in aquafeeds has been constrained by the aggressive processes used during feed manufacture, which kill or maim heat-sensitive bacteria. Advances in post-pellet application and other technologies have overcome this constraint, enabling probiotics to deliver their benefits to the global aquaculture industry.

Probiotics offer feed millers the opportunity to produce value added functional feeds. Once the feed is consumed, a successful probiotic will colonize the intestinal tract and exert a number of benefits, often relating to enhanced immunity and disease resistance.

The intestine is one of the main portals of entry for invading pathogens. In order to successfully infect the host, a pathogen must navigate and survive multiple obstacles and attacks, executed by the host's immune system.

IN BRIEF

- Heat-sensitive probiotics can be successfully added to feeds thanks to advances in post-pelleting and other technologies.
- Probiotics deliver a number of immune and disease-resistance benefits.
- An increase in goblet cell and IEL numbers was recorded in probiotic-fed fish. Goblet cells and IELs are important components of the immune system.
- Probiotics support immunity in the whole animal, as well as locally in the gut.

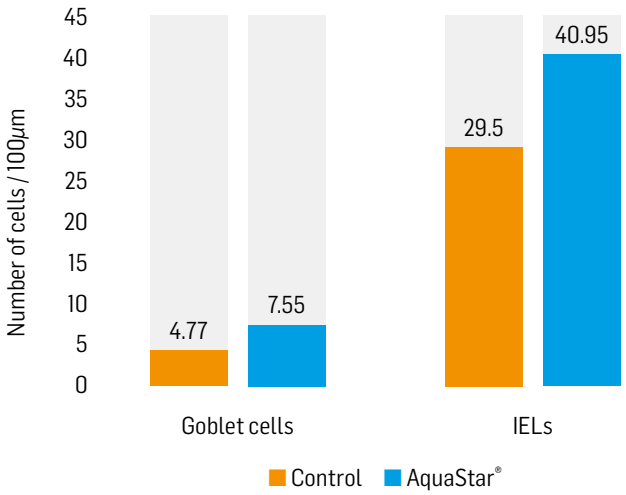
Innate immune response

As with mammals, the immune system of fish can be separated into innate (non-specific) and adaptive (specific) responses. Compared to mammals, fish are more dependent on the innate immune response for two main reasons. First, the innate immune system has developed to be non-specific and is therefore capable of mounting an immune response against a wide range of pathogens. Second, due to the ectothermic nature of fish, adaptive immunity can take considerable time. For example, antibody production in salmonids can take up to six weeks, compared to just hours or days for the innate immune system.

Reinforcing the first line of defense

The mucus layer produced by goblet cells provides the immediate line of defense. The mucus functions to trap and remove pathogens by providing both a physical and chemical barrier, since it contains a number of antimicrobial compounds. This mucus layer can be modified by the commensal microbiota as well as probiotic bacteria. For example, after feeding tilapia with a commercial probiotic (AquaStar®) for five weeks, there were approximately 60% more goblet cells in the intestine (*Figure 1*). These additional goblet cells could contribute to a greater production of mucus, thus providing a more impenetrable barrier, potentially retarding pathogens and preventing their attachment to the underlying epithelia.

Figure 1.
The abundance of goblet cells and intra-epithelial leucocytes (per 100μm) in the intestine of fish fed with and without dietary probiotics.



Source: BIOMIN

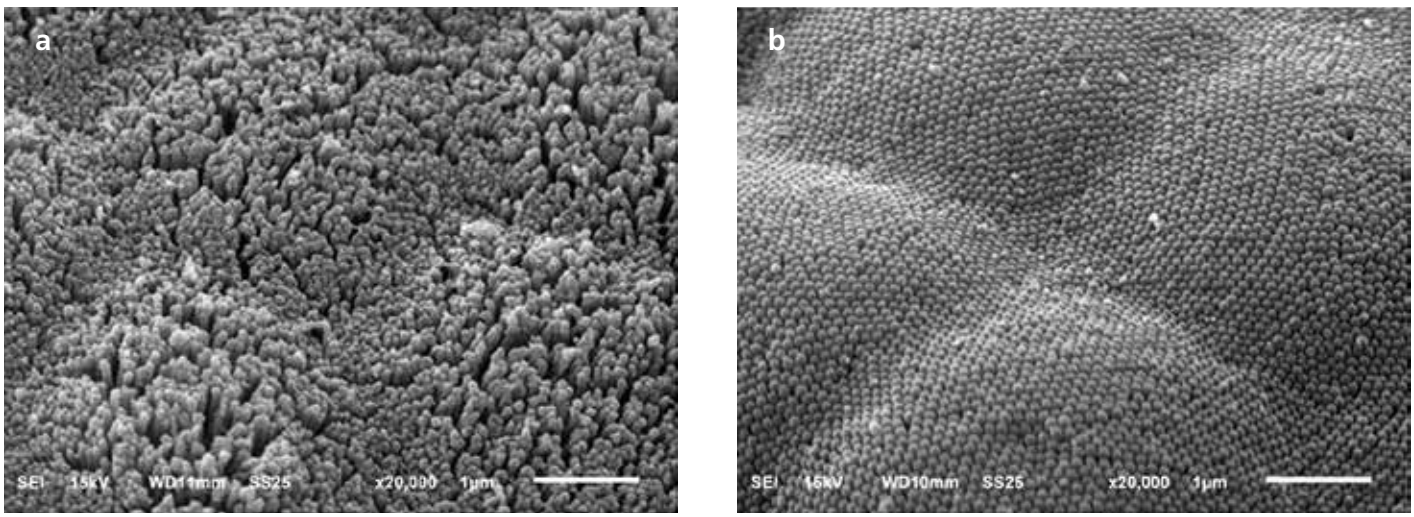
Strengthening the barrier: microvilli density

Beneath the mucus layer lies the epithelia, primarily consisting of enterocytes. These cells are lined with microvilli. Using electron microscopy, it was shown that feeding a probiotic significantly increased microvilli density in the gut (*Figure 2*). The benefit of this increase is two-fold. First, more numerous microvilli will increase the surface area so the host can acquire more nutrients from the feed. Second, any gaps between microvilli present an opportunity for pathogens to translocate the epithelia and infect the fish. Thus a higher microvilli density, resulting from probiotic inclusion in the diet, contributes to the gut barrier being more efficient and better able to block pathogens.

Larger leukocyte infantry

Assuming a pathogen was able to breach the epithelia, an army of white blood cells, collectively known as intra-

Figure 2.
Electron micrographs showing microvilli from fish fed (a) a control diet and (b) a probiotic supplemented diet. Gaps between microvilli as seen in micrograph a, provide an entry point for opportunistic pathogens. In micrograph b, the microvilli provide an impenetrable barrier, blocking pathogen entry.



Source: BIOMIN

epithelial leucocytes (IELs), would be waiting to attack. Research at Plymouth University consistently demonstrated that tilapia feeds supplemented with probiotics resulted in significantly larger populations of IELs. This increase was between 22 and 38% depending on the probiotic dosage, and the duration of feeding (*Figure 1*).

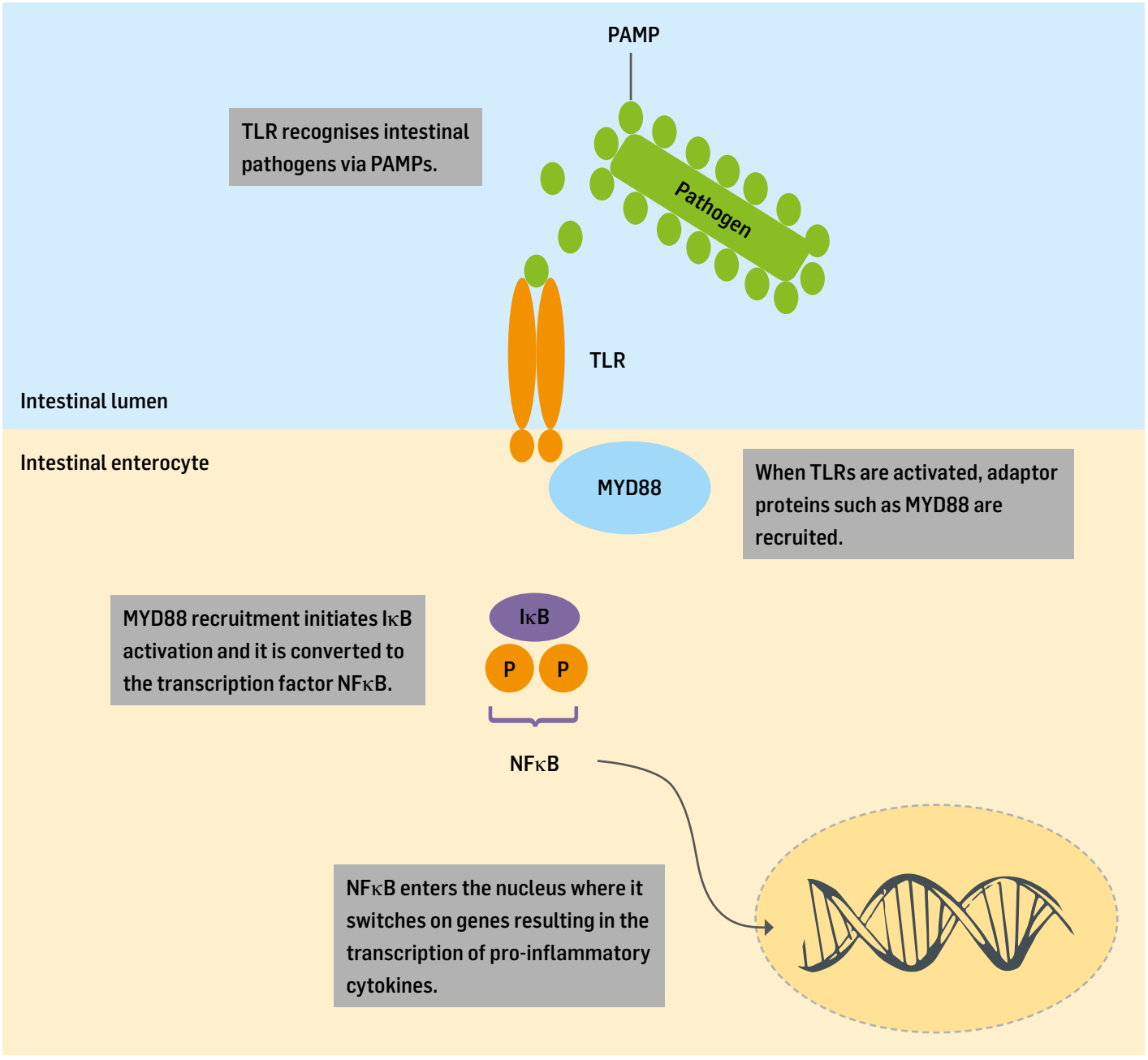
Better immune readiness

All pathogens express pathogen associated molecular patterns (PAMPs) on their cell surface. These are recognized by their respective receptor molecules such as toll-like receptors (TLRs) which notify the host on the pathogen type (i.e. bacterial, viral, fungal; *Figure 3*). Intestinal gene expression analyses show that probiotics can up-regulate the expression of TLR2 by approximately five-fold in tilapia. TLR2 is important for recognizing Gram-positive bacteria. This is particularly important because tilapia (along with many other warm water species) are susceptible to a number of Gram-positive infections, most notably *Streptococcus*.

Once activated, TLRs initiate a number of molecular

Figure 3.

Modulation of intestinal immunity through TLR signalling. Pathogens (and probiotics) bind to TLR. Upon activation, adaptor proteins such as myeloid differentiation primary response protein 88 (MYD88) are recruited. When this happens, IκB (inhibitor of nuclear factor kappa B (NFκB)) is phosphorylated (P) and degraded by the cell. This allows NFκB to pass from the cytoplasm into the nucleus of the cell where it initiates the cytokine transcription.



Source: Adapted from Cerf-Bensussan and Gaboriau-Routhiau, 2010

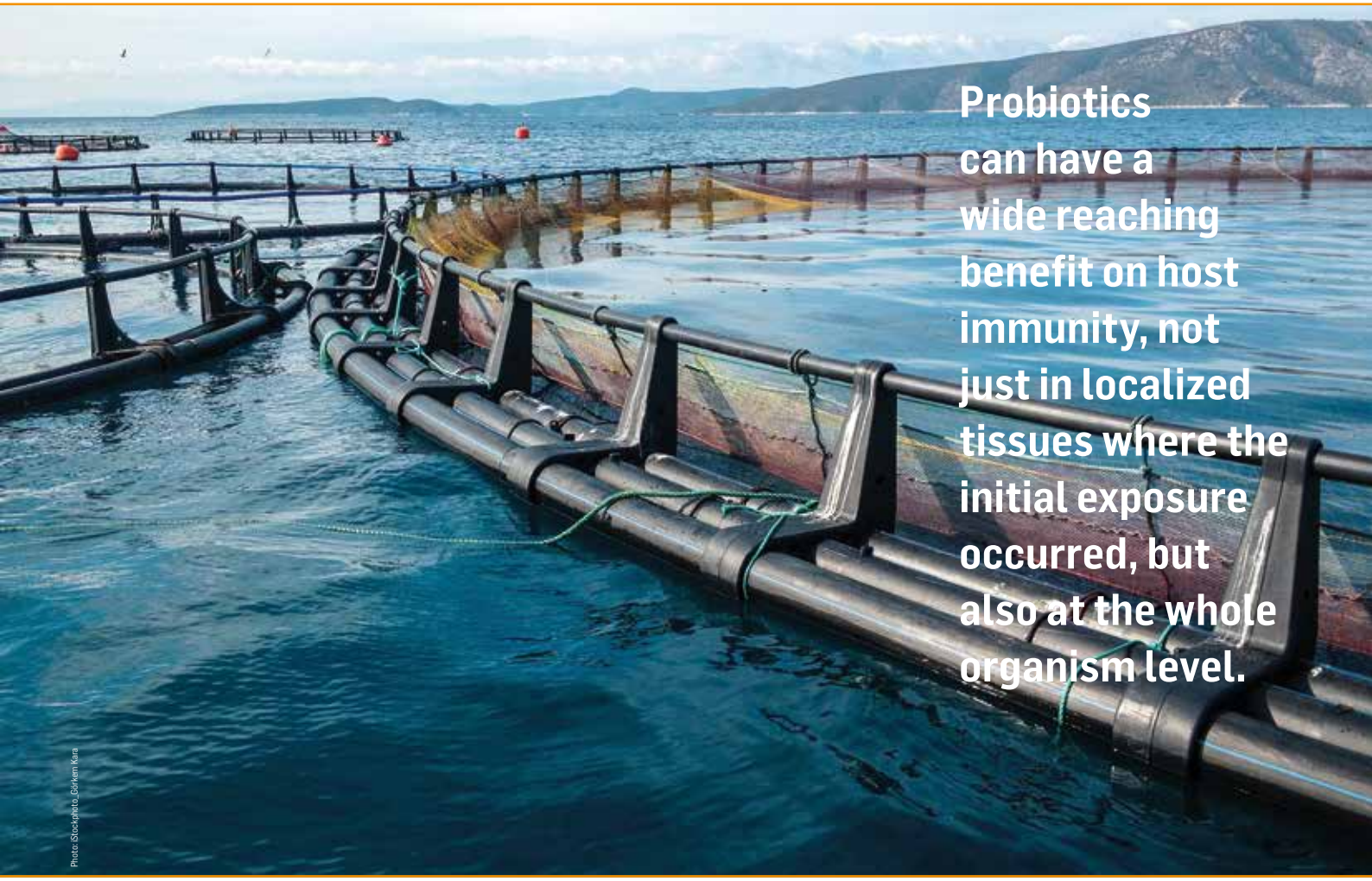


Photo: Stockphoto_Görkem Kara

pathways, which result in the production of pro-inflammatory cytokines. The same probiotic addition to tilapia diets caused an increase in pro-inflammatory gene expression, IL-1ß and TNFα. These data are suggestive of a fish that is more prepared to fight off potential future pathogens since the host can recognize and clear the threat much more rapidly, thus having greater immune readiness.

Getting the right balance

The gut is home to a large number of commensal microorganisms. It is important that these are protected by the host as they have important functions in intestinal development, nutrition and immunity. Anti-inflammatory cytokines are part of a tolerance mechanism that acts to de-sensitize the host, preventing an immune response to attack 'good' bacteria. Furthermore, they act to balance out the pro-inflammatory cytokines, thus maintaining an equilibrium within the mucosal immune system.

In vivo trials using tilapia demonstrated that the gene expression of two anti-inflammatory genes, IL-10 and TGFß can also be increased by the addition of AquaStar® Growout. The result tells us firstly that the host does not see the probiotics as a threat, and secondly that the probiotics can help promote and maintain mucosal tolerance.

Overall immune fitness: an extension of gut health

If a pathogen is successful in overcoming the localized immune system (i.e. within the gut), it is then at the mercy of the systemic immune system. In fish, this is controlled by the head kidney. Reverse transcription polymerase chain reaction (RT-PCR) analyses of head kidney and gut tissue demonstrated that the expression of immunity genes (TLR2, pro- and anti-inflammatory) were elevated in samples from both organs in probiotic fed fish. This reveals that probiotics can have a wide reaching benefit on host immunity, not only in localized tissues where the initial exposure occurred, but also at the whole organism level.

Conclusion

Probiotics can improve the intestinal barrier function, promote a state of superior immune readiness and enhance tolerance mechanisms, both within the intestine and other immuno-important tissues. This opens the door to healthier animals, fewer outbreaks of disease and less chemotherapeutical intervention in aquaculture production.

References are available from the author on request.

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Fusarium Mycotoxins Continue to Threaten Southeast Asian Aquaculture

Samples of plant-based meals and finished feeds from Southeast Asia were analyzed for the BIOMIN Mycotoxin Survey. The results help explain why mycotoxin deactivation strategies are so important for optimizing aquaculture performance levels. When was the last time you checked your feed for mycotoxin contamination?

Rui Gonçalves MSc
Scientist – Aquaculture

The contamination of aquafeeds and plant-based feedstuffs with mycotoxins is often neglected, but awareness of mycotoxin-related issues in the industry is growing. Manufacturers and producers now realize the importance of mycotoxins and their potential to impact production.

Myth or misinformation?

One of the main misconceptions in the aquaculture sector is that mycotoxin issues are the result of poor storage conditions leading to aflatoxin (AF) contamination.

IN BRIEF

- The majority of SE Asian raw material and finished feed samples contained *Fusarium* mycotoxins (DON, ZEN and FUM).
- 88% of samples analyzed for the BIOMIN Mycotoxin Survey were contaminated with more than one mycotoxin.
- Mycotoxins have different physical, chemical and toxicological properties, each requiring a different deactivation strategy.
- In order to remove all mycotoxins from feeds, a multi-strategy product is required.

This incorrect assumption results in the management of AF alone and, unfortunately, the management strategy for tackling AF is not suitable for managing *Fusarium* mycotoxins.

It is true that poor storage conditions can lead to contamination with AF and ochratoxin A (OTA). However, the BIOMIN Mycotoxin Survey data show that most of the mycotoxins came from the raw materials used to produce aquafeeds.

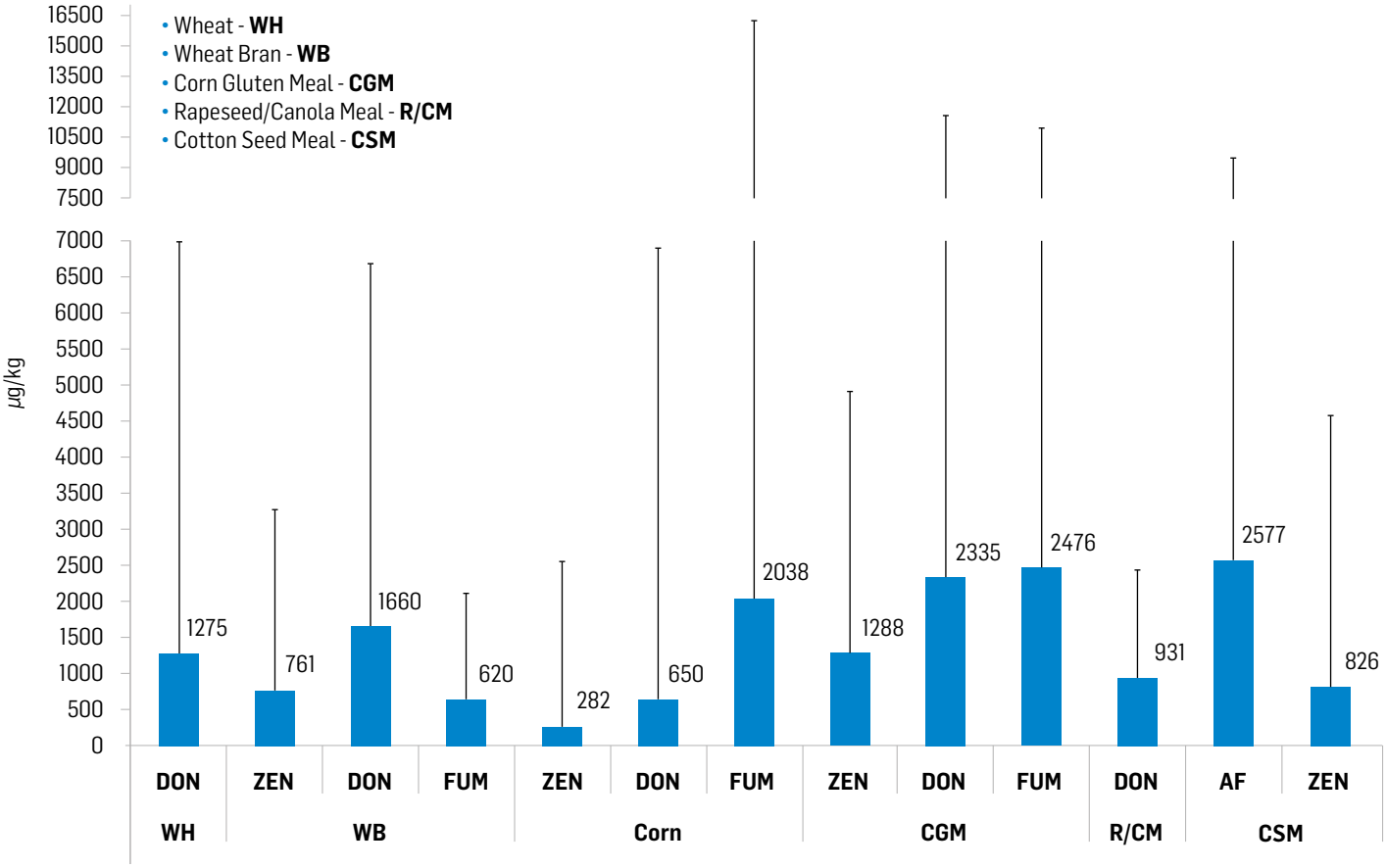
Widespread mycotoxin contamination

Assessments of mycotoxin contamination in the aquaculture sector (Goncalves *et al.*, 2016; 2017), in samples from SE Asia showed that wheat (WH), wheat bran (WB), maize (Corn), corn gluten meal (CGM), and rapeseed/canola meal (R.CM) were mostly contaminated with *Fusarium* mycotoxins such as zearalenone (ZEN), deoxynivalenol (DON), and fumonisin (FUM) (*Figure 1*). The only exception was cottonseed meal (CSM), which was contaminated with AF together with other *Fusarium* toxins (ZEN and DON) in considerable amounts. Finished feed samples were mainly contaminated with *Fusarium* mycotoxins, reflecting the inclusion of plant-based meals.

Mycotoxins not destroyed during processing

The high levels of *Fusarium* mycotoxins found in finished feed samples confirms that the contamination is

Figure 1.
Mycotoxin contamination in various plant ingredients



Source: BIOMIN

mostly related to the plant-based raw materials used in their formulation, because *Fusarium* fungi are generally a problem in the field rather than in storage. It is also important to remember that the mycotoxins commonly occurring in plant materials are not destroyed during most processing operations. On the contrary, processing concentrates the distribution of mycotoxins into the fractions that are commonly used in animal feeds (e.g. plant by-products).

Survey objectives

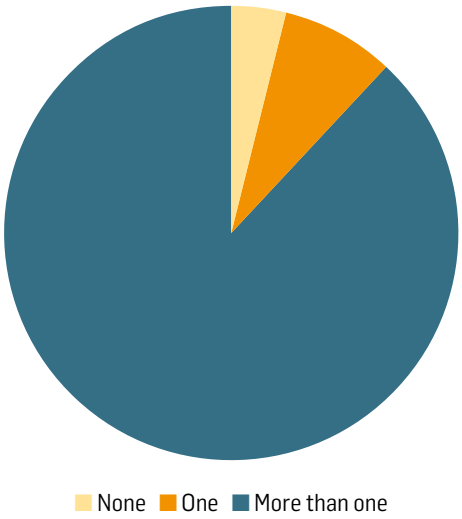
The objective of the BIOMIN Mycotoxin Survey was to analyze the level of mycotoxin contamination in the conventional plant ingredients used for aquafeeds, and in the by-products of these commodities, which are increasing in popularity due to price and availability.

The analysis looked for 18 mycotoxins per sample to account for masked mycotoxins and alternative metabolites as well as the more commonly occurring mycotoxins (*Table 1*). Finished feeds for fish and shrimp were included in the analysis. Due to increasing global trade and the incorporation of imported raw materials into aquafeeds, the mycotoxin contamination of locally produced commodities was compared to the same imported commodities.

Table 1.
Mycotoxins analyzed per sample

Type	Mycotoxin
Aflatoxins (AF)	AFB_1
	AFB_2
	AFG_1
	AFG_2
Zearalenone (ZEN)	ZEN
Type B trichothecenes	DON
	Nivalenol (NIV)
	3-Acetyldeoxynivalenol (3-AcDON)
	15-Acetyldeoxynivalenol (15-AcDON)
	Fusarenon X-glucoside (FUX)
Fumonisin (FB)	FB_1
	FB_2
	FB_3
Type A trichothecenes	HT-2
	T-2
	Diacetoxyscirpenol (DAS)
	Neosolaniol (NEO)
Ochratoxin A	OTA

Figure 2.
Number of mycotoxins present in each sample



Source: BIOMIN

The results

Over a twelve-month period, 175 samples of different plant proteins, aquaculture and fishery by-products, and finished aquaculture feeds from Southeast Asia were analyzed. All samples were tested for the mycotoxins listed in *Table 1*.

Within the analyzed samples, only 4% were free of detectable mycotoxins. Eight percent of the samples had only one mycotoxin and 88% of the samples were contaminated with more than one mycotoxin (*Figure 2*). This supports previous work that mycotoxin occurrence in plant-based meals, and consequently in finished feeds, is variable. It also confirms that AF is not the only mycotoxin to consider in aquafeeds.

How to fight *Fusarium*

Fusarium mycotoxins are a broad class of compounds all with different physical and toxicological properties, and chemical structures. Due to this diversity, a number of different strategies are required to detoxify them. Adsorption is the most common approach and many products using this strategy are already available commercially. However, as proven in several studies (Vekiru *et al.*, 2015; Hahn *et al.*, 2015; Fruhauf *et al.*, 2012), adsorption is not a feasible strategy for *Fusarium* mycotoxins; it is only effective towards aflatoxins and, to a lesser extent, ochratoxins. The reason relates to the flat chemical structure these mycotoxins have, which allows them to be captured between the layers of bentonite, a popular binder material. The non-planar chemical structure of other mycotoxins (e.g. DON or ZEN) results in less effective adsorption.

Some governmental authorities, particularly the EU Commission, have recognized this issue, which is why only aflatoxin binding claims are permitted in Europe. Complete mycotoxin deactivation requires the use of enzymes to deactivate or biotransform them. The enzymes provide a specific, effective and irreversible degradation of mycotoxins. BIOMIN is the only company to date that has feed additives legally recognized and registered in the EU for their ability to safely and effectively counteract mycotoxins.

The contamination of aquafeeds and plant-based feedstuffs with mycotoxins is often neglected.

Only the beginning

Drawing firm conclusions about the impact of mycotoxins in aquaculture is difficult and more research is still needed. Even so, it is clear that mycotoxins found in finished feeds negatively influence the aquaculture industry, affecting growth performance, feed efficacy and increasing disease susceptibility. Frequent monitoring of plant-based meals for the presence of mycotoxins, and a suitable mycotoxin management program should be employed.

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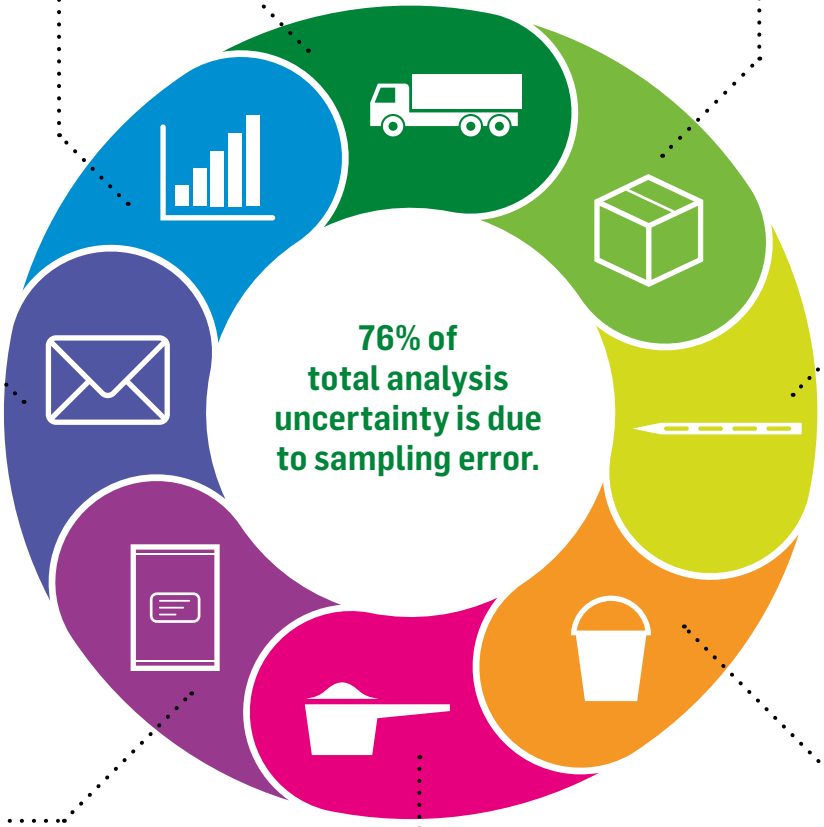
8 steps for taking the perfect sample for mycotoxin analysis

Mycotoxins are naturally inhomogenous in their distribution. There will be hot-spots of mycotoxins in an otherwise 'clean' batch. To get a true analysis result, sampling is really important. Follow these steps to get your sampling right.



1

Assess the size of your batch and work out how many sub samples you need to take.

For example, 1 ton of raw material = 1 kg of aggregate sample (10 x 100 g samples). The exact amount of sample depends on lot type and size according to EC Regulation No 401/2006.

2

Check the sampling method according to the material being sampled.

Your BIOMIN representative can provide you with the best practices for each material, making sure you collect and store your sample correctly.

3

Use the right equipment for the job.

There are lots of tools out there so do your research and make sure you have the right tool for the job.

4

Take incremental samples and mix them together thoroughly to form an aggregate sample.

Make sure incremental samples are thoroughly mixed together so that all components are homogeneously distributed.

5

Take the samples you will send off for analysis from your aggregate sample.

The laboratory carrying out the analysis will tell you how much sample they need. The general recommendation is at least 1 kg. Make sure you send them at least this amount, if not more. And consider storing some material for future reference too.

6

Make sure the bag or container is well sealed, and labelled with all the necessary information.

Your BIOMIN representative can provide sampling bags or advise on other sample containers that avoid altering the quality of the sampled material.

7

Post your sample off to the laboratory the same day.

Try not to send samples late in the week as they might be stuck in the post office over the weekend. Instead, store samples in a fridge or freezer and post them the following week.

8

Enjoy the benefits of receiving accurate and reliable mycotoxin analysis results.

But your sampling journey doesn't end there. Take regular samples to stay well informed about the mycotoxins in your raw materials or finished feeds.

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With 3 combined strategies

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- BIOTRANSFORMATION
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