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PROBIOTICS IN AQUACULTURE: FOCUS ON SHRIMP FARMING

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ABSTRACT

Probiotics are assemblages of living bacteria that colonize an animal's digestive tract with purported beneficial effects, which range from decreased susceptibility to certain types of bacterial diseases, to enhanced absorption of nutrients. Despite the widespread commercial availability of 'probiotics' for use in aquaculture, there is little evidence to support that these products are consistent with the traditional definition of the term. It is probably more reasonable to characterize those products that actually contain viable bacteria at levels that could affect some change as tools for manipulating the microbial ecology in ponds. The use of these microbial management tools in pond culture - with an emphasis on shrimp farming - will be discussed with a focus on peer reviewed literature and some general conclusions made about their use, their potential and what the direction of future research will be.

INTRODUCTION

Aquaculture is the production of aquatic organisms for the purpose of consumption as food. Quite simply, it is water based agriculture. Global production of protein by aquaculture has continued to grow unabated for decades and all indications are that it will continue to do so for the foreseeable future (FAO 2007). It is certain that protein from aquaculture will serve a critical role in the nourishment of a burgeoning global population estimated at 10 billion or more by the year 2100. Along with this industry growth there have come many of the same production issues that affect land based production of protein. Foremost among these are profit limiting diseases and environmental impacts, which are inherent and inevitable consequences of monoculture rearing practices in confined areas, commonly seen in the land based culture of animals as well. Some mitigation tools have been adapted from land based animal husbandry and applied to aquaculture husbandry practices, including vaccines for immunoprophylaxis and antibiotic use to reduce the impact of disease. Strategies to minimize environmental degradation, such as the use of closed systems and reliance on Best Aquaculture Practices (BAP), have reduced the overall footprint of many aquaculture operations.

In the last few years there has been a surge in the commercial availability of a set of tools that has the potential to positively impact both animal health and environmental degradation. These are an outgrowth of similar tools that are being used in animal husbandry and in humans as nutraceuticals, and the term 'probiotic' has been used to describe these tools. Unfortunately these tools, at least as far as aquaculture goes, in many cases contain bacteria that are not viable, are included at levels that are so low as to be virtually useless at the recommended levels of use, or have no scientific basis for use at all. This article is focused on what these tools are, what the reality appears to be, based on published studies, and where we can expect to go from here, primarily from the crustacean perspective.

WHAT IS A PROBIOTIC?

The modern concept of probiotics has it roots in the observations of Elie Metchnikoff, an early 20th century Nobel Prize winning microbiologist, who believed that bacterial species in soured milk could positively impact longevity by altering the flora in the gut (Metchnikoff 1907). This was not a novel concept at the time, as many cultures have, in fact for several millennia, ascribed positive health benefits to the consumption of materials that we now understand to contain viable bacterial cultures. The term probiotic was defined by Parker (1974) as "organisms and substances which contribute to intestinal microbial balance." Fuller (1989) expanded on the definition to include living organisms, "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance." This solidified the concept to focus on the intestinal microbial flora. Havenaar and Huis (1992) further refined the definition to "A viable mono- or mixed culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous flora." This further expanded the scope of the definition to include man and reaffirmed the focus on impacting the intestinal flora. Schrezenmeir and de Vrese (2001), upon reviewing the concepts and historical roots, modified the definition to: "A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the

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microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host." Further refinement of the definition is likely to come as probiotic research continues. Clearly, the primary concepts are that there must be viable organisms in the material and that they have an impact on animal health by affecting the indigenous flora in the digestive tract.

Based on what we understand are the properties of probiotics for humans, from the standpoint of effectiveness a true probiotic should have the following properties:

1. Be composed of living bacteria (or yeast) or spores that can germinate into living bacteria.
2. Administered orally in the food or in the water.
3. Site of action would be cell surfaces. This means binding or colonizing cell surfaces. It should be accepted by the host, e.g. through ingestion and potential colonization and replication within the host, and it should readily reach the location where the effect is required to take place.
4. Mechanism of action would be the direct or indirect inhibition of pathogenic bacteria.
5. Additional benefits would be a "healthier digestive tract," a stronger immune system and for agriculture, better growing animals.
6. It should not be harmful to the host it is desired for.
7. It must work *in vivo*, not just *in vitro*. In other words, even if experimental data show a positive impact in the laboratory, it must be shown to have an impact in the animal that it is being used on.
8. It should not contain genetic material such as virulence resistance genes or AB resistance genes.

If these properties are incorporated into an all encompassing definition, it precludes considering most of the products currently in use for aquaculture as true probiotics.

PROBIOTICS AND HUMAN HEALTH

The use of various bacterial mixtures (largely *Lactobacillus* based) has found a solid market in human health in the last decades. These mixtures are sold as dietary supplements and foods in many different formulations and presentations (NCCAM 2008). Selling under this designation does not require proof of efficacy in the United States. This is advantageous for those that market these products, because there is very little valid scientific data (reproducible, valid experimental design with appropriate controls) to support most of the claims that have been made (Tannock 2003, NCCAM 2008). Most products are being sold without specific, scientifically verifiable claims. Furthermore, the exact mechanisms by which those that have apparent efficacy function are not clearly understood (Walter 2008). Clearly, if this is the case for a market as large as the human market, then similar concerns exist for animals as well. In all likelihood, the same statements also can be made about aquaculture.

PROBIOTICS IN ANIMAL PRODUCTION

Recently there has been resurgence in the interest in and potential use of probiotics. This is a result of the increasing problems associated with the use of antibiotics in agriculture (Schwarz et al. 2001), including aquaculture. These include the relative ease of development and transfer of antimicrobial resistance, regulatory and health concerns about antibiotic residues in food, as well as a movement towards the use of more holistic approaches to the management of health and prevention of disease. The EU banned the use of antibiotics as feed additives in agriculture in 2006, opening a large potential niche for probiotics if they could be used to reduce disease susceptibility and improve growth. This has resulted in a proliferation of products and a growing body of scientific literature aimed at supporting the concept, and the use of specific bacteria in humans (NCCAM 2008) and in animals (Casey et al. 2007).

There is a great deal of literature examining the potential of probiotics to impact animal health (Schwarz et al. 2001, Hong et al. 2005). Probiotics have been successfully tested in poultry and are widely used (Lee et al. 2006, Lin et al. 2006), although only one product - a mixture of 29 bacterial strains - has been approved for use in agriculture by the USFDA (Tannock 2003). It is likely that this interest will continue and research is ongoing in many different animal species including those produced by aquaculture.

PROBIOTICS IN AQUACULTURE: FOCUS ON SHRIMP

The global shrimp farming industry has been expanding rapidly in recent years, with current global production of farmed shrimp surpassing 3 million MT per year (Jory 2008). There is a great deal of interest in the use of bacterial strains as beneficial health impacting tools in aquaculture in general (Gatesoupe 1999, Balcazar et al. 2006, Kesarcodi-Watson et al. 2008). These have been the subject of several reviews, including the excellent recent one by Kesarcodi-Watson et al. (2008). These authors are optimistic for the fruits of future research, and concluded that inherent limitations to current testing methodologies have not resulted in products that are active *in vivo* on a wide scale commercially. This implies that few, if any, currently available commercial products work. There are very few published field studies in shrimp and several are reviewed in this paper.

As in agriculture, the use of antibiotics in aquaculture has been restricted and residues are becoming an ever increasing source of regulatory concern. For fish, immunization is a valuable tool (Newman 1993), although this avenue is not open to shrimp as their immune systems are not phylogenetically evolved enough to ensure the same type of protective immunity inherent in fish (Newman and Bullis 2001). Being able to immunize fish has resulted in significant reductions in antibiotic use in marine fish aquaculture (Maroni 2000), although there are still many pathogens that have not been the subject of vaccine development and the use of antibiotics is still wide spread in certain sectors. There is an urgent need to replace or lessen use with other tools, of which probiotics could be one potential route.

Moriarty (1998) proposed that the definition of probiotics for use in shrimp farming encompass environmental manipulation, i.e. a water additive instead of a feed additive. This likely stems from the concept of using microbes for bioremediation, a common approach to dealing with certain types of deteriorated environments. The term probiotic is really a misnomer, because most of the products in the market today have no scientifically verifiable evidence to support that they act by changing the gut flora in a meaningful way. The vast majority of products in the market place today should be more appropriately referred to as "Microbial Ecology Management" tools (MEMs).

According to Kesarcodi-Watson et al. (2008), the literature on this use of bacterial amendments in aquaculture has shown few indications of success. The first reports of the use of live bacterial cultures that appears in the literature for aquaculture appeared in the mid 1980s (Tucker and Lloyd 1985, Boyd et al.1984, Boyd and Gross 1998). Several studies documenting the addition of bacterial inoculants to catfish ponds failed to show a consistent benefit, although Queiroz and Boyd (1998), by lowering the level of statistical significance needed to show correlation, were able to show a correlation with increased survival in one set of trials.

Indeed, great care must be taken when viewing studies that show correlative relationships in the absence of mechanisms that can plausibly underlie cause and effect. Correlative statistics can be misleading and they do not demonstrate cause and effect. The conclusions by Queiroz and Boyd (1998), based on increasing the p value to 10% from 5% (which is the norm accepted by most statisticians), are therefore likely not valid. Although the argument is made that the variability of the farming environment necessitates this, few statisticians would agree. Furthermore, the lack of a plausible mechanism further confuses the issue. Many variables affect survival and productivity in general, and with no data to support that the bacteria added to the pond even grow, these types of conclusions are, at best, tenuous. Approximately 112.5 L of a culture that ranged from 10^9 to 2 x 10^9 CFU per ml were added per ha per week. This was approximately 16000 CFU per ml. Since there was no difference in bacterial counts between control and treated ponds, it cannot be concluded that the bacteria that were added even grew in the ponds. Unfortunately, this is all too commonly what is seen in the field and is part of the dilemma that companies that market these types of product face.

There is a great deal of variation between the types of bacteria, the viability of these bacteria, and the real (not claimed) levels of them in products (Boyd et al. 1984), making it very difficult to make claims that any effect is attributable to the growth of the bacteria in the aquatic environment. In fact, many commercial products contain bacteria that are not shelf stable, such as *Lactobacillus* species and related bacteria, nitrifiers that are costly and difficult to culture, and anaerobic bacteria that are poorly characterized as to the actual content of viable organisms. Using bacteria that have been reported to be components of human probiotics and marketing them to aquaculturists as shelf stable products that can be milled into the feed or added to the water is a marketing ploy that has no basis in science.

HATCHERY USE

Some apparent successes have been reported, notably in the hatchery (Garriques and Arevalo 1995) and using on site cultured *Vibrio alginolyticus* species. This practice is widespread , despite being a form of roulette as there are no safeguards in place to prevent conjugation (exchange of genetic material) between bacterial species. Also, there are significant risks associated with culturing *Vibrio* species for mass inoculation in larval rearing tanks by untrained personnel. There are many pathogenic strains of *Vibrio alginolyticus* and it is not unrealistic for non-pathogenic strains of vibrios to become pathogenic, simply by acquiring a plasmid (Newman 1979). More than one hatchery manager has found this out the hard way. Enough replicate studies have been reported, however, to confirm that using bacterial amendments in the hatchery is a viable management tool, although there is little evidence to allow a scientific explanation as to the mechanism. It has been reported that the widespread use of this approach in Ecuador significantly reduced the use of antibiotics and improved overall hatchery survival, reducing the time needed between production cycles. It is theorized that the tanks are colonized with the seeded bacterial strains and that this effectively inhibits the growth of the "pathogenic" bacteria, but this has yet to be proven. Gómez-Gil et al. (2000) reviewed the then current state of the use of probiotic bacteria in larval aquatic organism culture and concluded that the quality of the data generated was not consistent with being able to make any solid, science based conclusions that this approach is actually a viable one.

It is clear that, while there are products that are being marketed as probiotics for use in shrimp hatcheries, the lack of a proven functional mechanism still makes their use more anecdotal than real. Hatcheries require clean water and it makes more sense to focus on ensuring that the shrimp are reared in a high quality environment than resorting to the use of questionable microbial amendments that may or may not work. Nonetheless, the concept of competitive inhibition of pathogenic bacteria is promising and deserves significant further exploration.

FARM USE - ORAL VS. ENVIRONMENTAL

Shrimp production ponds are highly variable environments. Many shrimp farms include large production ponds ranging in surface area from a few thousand m^2 to several dozen or more ha, and with depths typically from 80 cm to 2-3 m. Water sources are commonly oceanic or estuarine and water quality ranges from pristine to eutrophic. Ponds may have earthen bottoms or be lined with a variety of materials. Soil types are highly variable as well, with clay based soils offering the greatest degree of water retention. Shrimp are reared at densities ranging from a few to hundreds of individuals per m^2. Protein content in aquafeeds range from low (under 20%) to high (over 40%) levels. Feed composition varies as well, with no single formulation being consistently used. Fertilization strategies are as varied as the water quality. All this variability presents a challenge to purveyors of tools that are intended to impact the environment, either directly or indirectly.

In reality - despite numerous claims to the contrary - there are few, if any, products in the market place that can actually make valid claims that they function as traditional probiotics in commercially produced shrimp (or for fish for that matter). That is, that they affect the animal because of benefits that occur in the intestinal tract. Most products likely act as tools for bio-remediation, and while they may be ingested, it is very difficult to connect this with scientifically verifiable data that show that this is the underlying cause of any noted impact.

There are two types of approaches towards getting bacteria into these environments. One is in the feed and the other is by direct addition to the environment. As discussed, probiotics, by definition, are intended to impact animal health by altering the intestinal flora. This can be accomplished in any number of ways, including inhibition of pathogens by the production of antimicrobial substances by competition for nutrients; by occupying attachment sites and thus preventing the pathogen from establishing itself; by stimulating protective immunity (usually non-specific); and by improving the overall nutritional status of the host by improving feed digestibility and thus nutrient availability. The end result should be an impact on animal health. Traditional approaches entail using feed as the vehicle for this. The challenge with this approach in shrimp farming is the difficulty of delivering viable bacteria to animals to consume in the feed that will give the desired result. *Lactobacillus* species, by far the most commonly used oral probiotic bacteria in humans, require refrigeration for any realistic shelf life viability (Harry Lyle personal communication). They die quickly at ambient temperatures and will not tolerate the temperatures experienced in normal feed milling. They can, however, be applied as top dressing, although this method of delivery does not typically allow for the delivery of consistent dosages as it is likely that many of the bacteria diffuse off of the feed before it is ingested. It is well known that nutrients diffuse from feeds very rapidly as well. *Bacillus* spores show varying degrees of heat tolerance and may survive being milled into feed, although this is likely not going to be a consistent process across different feed milling technologies in use. At this time, there are no bacteria that can consistently survive the extreme stresses of being milled into feed.

Many different studies have been published (Kesarcodi-Watson 2008) that show the potential benefits from the oral application of varying species of bacteria. Unfortunately, all have similar shortcomings in methodology. Laboratory based trials with feeds that are top dressed or prepared in pilot size batches do not realistically approximate real world conditions. Although efficacy has been shown in a number of studies, there is little if any scientifically reproducible data to substantiate that these approaches will work under normal commercial conditions. The inclusion of heat sensitive bacterial species in premixes is common and, based on available data to date, not going to result in a benefit that can be attributable to the presence of living bacteria.

These limitations have encouraged the development of products to treat the environment with the hopes that this will result in changes in the microbial population that favorably impact the production process. Evaluating these types of approaches has little meaning in the lab environment, except perhaps to validate the ability of the bacteria to grow and perhaps to have a localized impact on the ecosystem that the tests are conducted in. Larger, real world tests are problematic for a number of reasons, not the least of which is that there are so many variables present that can confound the results. These include, but are not limited to, water quality variability (open vs. closed systems, water source), animal rearing densities and other lesser understood physiological parameters, individual management philosophies (perception of cost benefit), and sometimes the ability to even determine that there has been a benefit. Many shrimp farms do not keep records in a manner that is consistent with using hard numbers to evaluate cost benefits. Looking for consistent changes in survival, growth rates, water quality, etc. are not going to be universally effective in evaluating the usefulness of these tools. For these reasons, there have been very few published observations of real condition testing of these types of materials.

Moriarty (1998, 1999) first reported on the use of bacterial additives to commercial shrimp ponds. His studies with *Bacillus* species, which are in many respects ideal candidates for these types of applications (Hong et al. 2005), showed that constant application of *Bacillus* species with purported antibiotic activities against *Vibrio harveyi* could positively impact survival. It has also been observed by Sharmila et al. (1996) that *Bacillus* species comprise a significant component of the endogenous microbial flora of certain shrimp species, further making them suitable candidates for this approach. Unfortunately, certain experimental design errors impacted the scientific validity of the results. The authors compared different farms and did not have the same controls on the different farms. Furthermore, the constant application (every 1-3 days) of an on-farm cultured preparation of the *Bacillus* species requires a significant investment in technical know-how and infrastructure to ensure that it is done properly.

Large scale production of pure cultures of bacteria is best left to trained microbiologists, because in the hands of farm staff, even with cookbook systems, it can be a risky endeavor. Personal experience by this author, a trained microbiologist with significant fermentation experience, has shown that consistent and reproducible culture is challenging. Despite the lack of proper controls and the probable high costs associated with the application of this type of product, the data suggest that the use of this approach warranted further research. Comparing the performance between different farms cannot be used as indication of product effectiveness. This is a common problem in evaluating these types of products in the field. It is very difficult to get appropriate controls and very few farms can actually state with a high degree of certainty what the causes of mortality are. Furthermore, ponds with 100% survival are suspect. It is this author's opinion that this is a result of miscounting what has been stocked and cannot be taken as indicative of a real impact on survival. Moriarty (1999) speculates that the *Bacillus* have competed against the vibrios by interfering with attachment and by the production of antimicrobial substances. This would have occurred in the environment and not in the intestinal tract of the animal, making this a MEM. This sets the stage for what these products probably really do.

Rengipipat et al. (1998), using a *Bacillus* strain isolated from the intestines of *Penaeus monodon*, showed a protective effect in the laboratory against *Vibrio harveyi* challenges. These studies were based on laboratory observations, not field trials. These authors later published observations that suggested that the mode of action might be an impact of the immune system on shrimp (Rengipipat et al. 2000). It is known that a wide variety of materials impact the immune systems of shrimp (Newman and Bullis 2001), and this is probably a plausible mechanism of action for the observed effect reported by many of these types of products. In fact, it is likely that immune stimulation may be the primary impact explaining differences in disease susceptibility. Their approach differed from that of Moriarty (1998) in that the *Bacillus* used in their studies were fed to the animals. A suspension of viable bacteria was top dressed onto the feeds and air dried before being fed to shrimp held in laboratory tanks. This method could have allowed bacteria to diffuse off of the pellets and enter the animals through other portals, resulting in the observed immune stimulus. While they report that one potential impact may have been related to colonization of the bacteria in the gut, based on differential counts between controls and fed animals stable colonization would not have been required for this observed difference.

McIntosh et al. (2000) reported that the addition of a commercial *Bacillus* preparation, fermented on site and added five times a week, failed to produce any discernable impact on production or water quality in their system. This was tested in replicate tanks and conducted under well controlled conditions. A number of authors have published a variety of differing protocols with a variety of potential probiotic candidates (Shishehchian et al. 2001, Vaseeharan and Ramasamy 2003, Gullian et al. 2004, Venkat et al. 2004, Wang et al. 2005, Li et al. 2006) in recent years. Many are based on lab studies and only a few on field studies. The conclusions are consistent in only one respect-that they are inconsistent.

Based on these observations, it appears that while there are positive impacts in some rearing environments, we are still not at a point in time where off-the-shelf products will provide a consistent benefit. Given the variability of the shrimp farming environment, this is not surprising and the focus should probably be on cost benefits that take into account that not all treated ponds will see benefits.

WHAT LIES AHEAD?

As with any endeavor where there are any number of companies involved in marketing products, there is a great deal of "gray" literature on this subject. Companies have no real incentive to publish observations in peer reviewed literature if it entails revealing trade secrets or information that can be used to the advantage of their competitors. Much of the gray literature is actually marketing literature and should be considered as such.

In the years to come we are going to see more targeted and selected delivery of a combination of microbes that impact environmental quality in a more or less consistent manner. Some of these will find their way into the feed, likely through the development of heat resistant technologies like microencapsulation. We should not expect to see dramatic impacts on many disease processes, as it is not likely that there will be impacts on viral diseases, and given that many bacterial pathogens can find their way into shrimp via portals that are not intestinal, even if products that stably colonize the guts of shrimp are developed, they will not reliably always result in a cost effective reduction in disease impacts. The need to use antimicrobial agents may still be required.

The industry should also expect to continue to see a proliferation of products that are marketed based on poorly designed lab and field studies, and that contain bacteria that may or may not provide the types of results in their marketing claims. There are, however, indications of some very promising products in the pipeline that may be useful using non-traditional methods of applications such as top dressing.

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