Use of a functional feed additive to reduce mortality from franciselosis and streptococcosis

Leonardo Mantovani Favero, Waldo G. Nuez-Ortín, Maria-Mercè Isern-Subich, Ulisses de Padua Pereira, Adisseo and Londrina State University

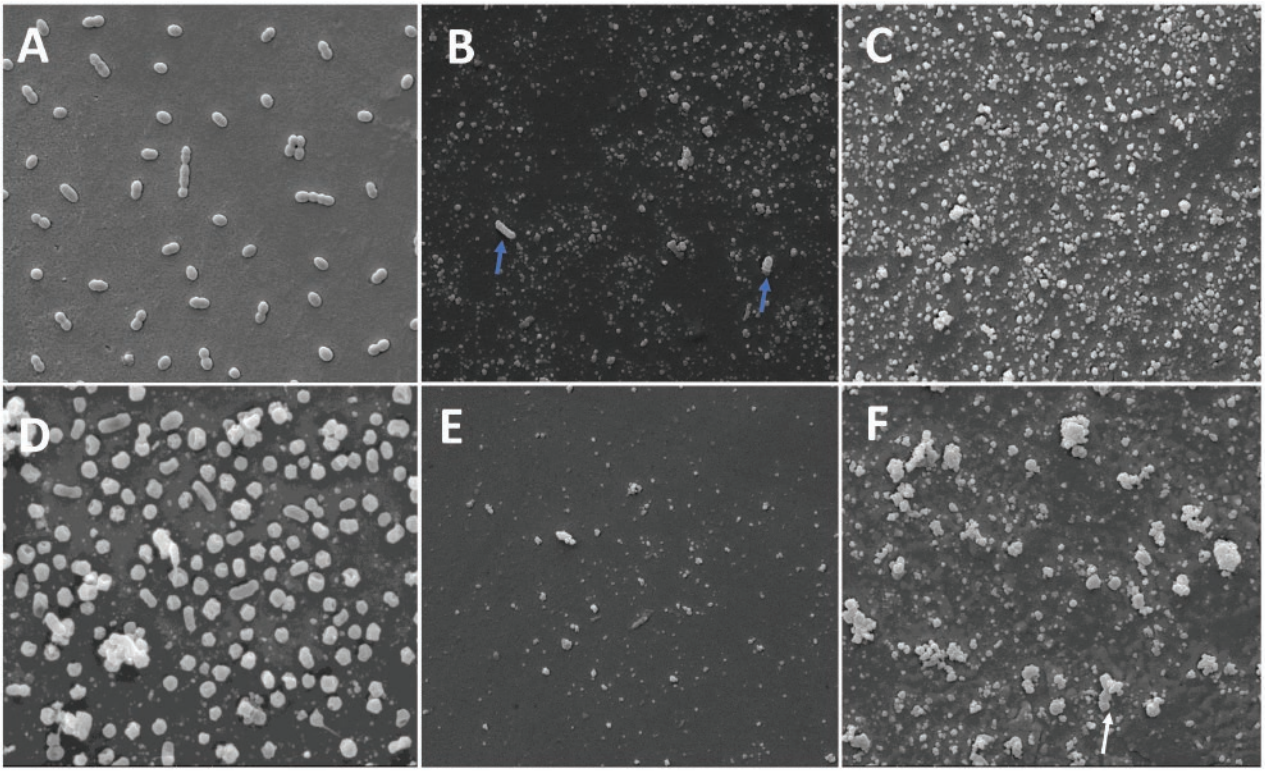


Figure 1. Scanning electron photomicroscopy of *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *orientalis* cells treated with different concentrations of Sanacore® GM. 10,000x magnification. A: control cells of untreated *S. agalactiae*. B: *S. agalactiae* cells approx. treated at 0.2% for 30 minutes. Reduction of the number of cells and alteration in coccus morphology (arrows). C: absence of *S. agalactiae* cells after treatment at 1% for 30 minutes. D: control cells of *F. noatunensis* subsp. *orientalis* without treatment. E: *F. noatunensis* subsp. *orientalis* cells treated at 0.1% for 30 minutes. Significant reduction in the number of cells and changes in bacterial morphology. F: *F. noatunensis* subsp. *orientalis* cells treated at 1%. Reduction in the number of cells and accumulation of the product on the bacterial surface (arrow).

Tilapia production is globally spread. In 2018, production was estimated at nearly 6.3 million metric tons (MT). Although tilapia tolerate adverse conditions and stressors better than most commercial aquaculture species, current rearing conditions (i.e. high densities, water temperature and quality, etc.) induce a stress level that is detrimental to the animal´s immune system and that, in combination with the presence of pathogens, results in disease outbreak.

Disease treatment may be ineffective since drugs are supplied through the feed and during periods of low intake as a consequence of disease. The indiscriminate use of antimicrobials is one of the main causes of bacterial resistance, and thus disease prevention must be prioritized. Prevention involves qualifying the farm operators for correct husbandry practices, acquiring fry and juveniles of good genetic quality and origin that certify the absence of pathogens

Group name	Challenge	Treatment	Replicates (nº)
CN	None	None	2
CNP	None	Sanacore GM	2
GF	F1 (10^9 CFU/mL of water)	None	4
GFP	F1 (10^9 CFU/mL of water)	Sanacore GM	4
GS	S13 (10^5 CFU/fish)	None	4
GSP	S13 (10^5 CFU/fish)	Sanacore GM	4

Table 1. Description of the experimental groups for the *in vivo* evaluation of the Sanacore® GM.

in these lineages, and stimulating animal health. Prevention will ameliorate pathogen pressure to establish itself and trigger mortality.

Fish farmers and feed manufacturers are aware of the importance of functional feed additives to promote health and strengthen the disease prevention strategy. The use of health promoting additives is recommended during the whole production cycle and in higher doses during stressful conditions such as manipulation of the fish or temperature fluctuations. Thus, it is important to adjust dosage based on the challenges of the production system.

Sanacore® GM is a health promoting additive specifically designed to enhance the resistance against possible diseases and performance. This functional additive is a synergistic mixture of botanical extracts with broad-spectrum bacteriostatic and bactericidal activity as well as ability to interrupt the communication system among pathogenic bacteria (i.e. quorum sensing). Sanacore® GM has been shown to induce a more stable and robust gut microbial flora, resulting in a better animal response to health challenges.

The Fish Bacteriology Laboratory at the Londrina State University (LABBEP, Brazil) tested the *in vitro* ability of Sanacore® GM to destroy bacterial cells and its *in vivo* ability to minimize the negative effects of infection in tilapia by *Streptococcus agalactiae* and *Francisella noatunensis* subsp *orientalis*. These bacteria are the cause of streptococcosis and franciselosis, respectively, two diseases that cause high mortality in tilapia culture worldwide. Outbreaks of *S. agalactiae* usually occur in fish farms among adult fish during the summer season when the water temperature is higher than 27°C, whereas outbreaks of *F. noatunensis* commonly affect fry and fingerlings during the winter season when the water temperature is lower than 24°C. However, under stressful or poor environmental conditions, outbreaks can occur in atypical temperatures and affect fish in other life stages.

Material and methods

Minimum bacterial concentration (MBC) of the product and scanning electron microscopy techniques were used to evaluate the *in vitro* destructive capacity of the bacteria. For the determination of MBC, suspensions of both bacteria were incubated by triplicate for 24 hours with different additive concentrations and then each suspension cultured in solid media to determine MBC. For electron microscopy, the suspensions of the two strains were exposed for 30 minutes to two different concentrations of the functional additive to evaluate their effects on cellular morphology.

In vivo testing was performed to assess the response of tilapia treated with the functional additive against *Streptococcus agalactiae* and *Francisella noatunensis subsp orientalis* infection. Tilapia juveniles (30g) were distributed in groups according to Table 1 and acclimatized for eight days. After acclimation, CNP, GFP and GSP groups received feed containing Sanacore® GM at 0.3% for 20 days. At the end of the

%	4	2	1	0.5	0.25	0.125	0.06	0.03	0.015	0.0075	0.0038	0.0019
µg/ml; g/kg; kg/mT	40	20	10	5	2.5	1.25	0.6	0.3	0.15	0.075	0.0375	0.0188
S. agalactiae	BC	BC	BC	BC	R	R	R	R	R	R	R	R
F. noatunensis subsp. orientalis	BC	BC	BC	BC	BC	BC	R	R	R	R	R	R

Table 2. Minimum bactericidal concentration of Sanacore® GM at different concentrations against *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *orientalis*. BC: bactericidal concentration; R: non-bactericidal concentration.

	Final weight (g)	Daily weight gain (g)	FCR	SGR
None	52.3	0.59a	1.35b	1.37b
Sanacore	55.5	0.77b	1.04a	1.67a

Table 3. Growth performance during pre-infection (20 days) supplemented (Sanacore® GM) and non-supplemented (none) fish. Data submitted to variance analysis (ANOVA), followed by Tukey's test, performed to evaluate the difference in treatment averages ($p<0.001$).

pre-infection period, and in order to assess the immunestimulant effects of Sanacore® GM, blood samples from nine fish per treatment were collected to determine serum lysozyme concentration (i.e. activity to break down the peptidoglycan layer in the wall of the pathogenic bacteria) and alternative component activity (ACH50) (i.e. amount of fish serum required to induce 50% hemolysis in red blood cells of rabbit). Additionally, and in order to evaluate changes in the gut microbiota, stool samples from nine fish per treatment were collected for 16s rDNA analysis. Finally, the GF and GFP groups were challenged with the F1 strain of *F. noatunensis* supbsp. *orientalis* via water immersion, and the GS and GSP groups with *S. agalactiae* via intraperitoneal route. The same feed fed during the pre-infection period was fed during the 20-day post-infection period. The water temperature was mantained between 27ºCand 29ºC in the tanks infected with *S. agalactiae*. In the case of infection by *F. noatunensis*, temperature was mantained below 20ºC for the first three days to induce infection and below 22ºC during the rest of the infection period.

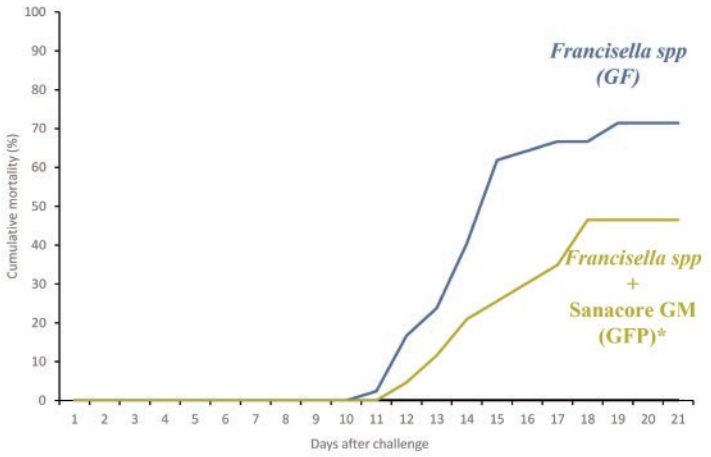


Figure 2. Cumulative mortality observed in groups after the experimental challenge with *Francisella noatunensis* subsp. *orientalis* (GF and GFP groups). n=2 and n=4 for non-infected and infected groups, respectively. Difference of 32% between GF and GFP with statistical significance. * $p<0.01$ significance in Fisher's test.

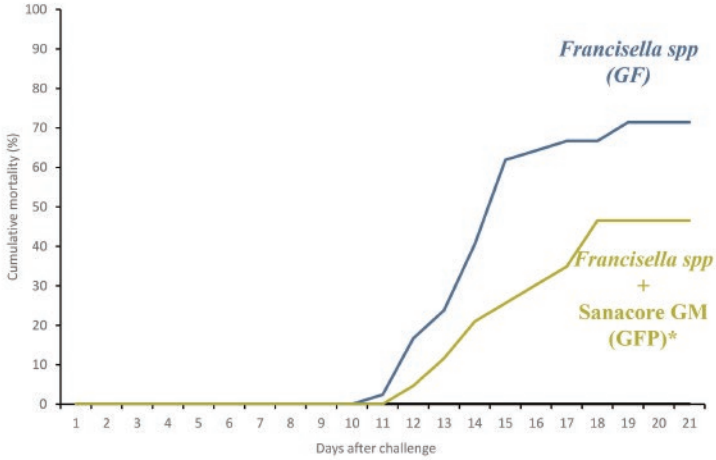


Figure 3. Cumulative mortality observed in groups after the experimental challenge with *S. agalactiae* (GS and GSP groups). n=2 and n=4 for non-infected and infected groups, respectively. Difference of 17% between GS and GSP groups without statistical significance.

Results and discussion

MBC for *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *orientalis* were between 0.25 and 0.5% and between 0.06 and 0.125%, respectively (Table 2). As shown in Figure 1, the additive reduced the number of bacterial cells and altered their morphology. The antimicrobial effects achieved by these concentrations are representative of cost-efficient dietary inclusions ranging from one to five kg per ton.

At the end of the pre-infection period (20 days), a growth-promoting effect was detected in fish supplemented with Sanacore®GM, with statistically significant improvements of approximately 30% in daily weight gain, feed conversion and specific growth (Table 3). The gut microbiome analysis revealed changes in the dominant populations, specifically for the genus *Cetobacterium* and

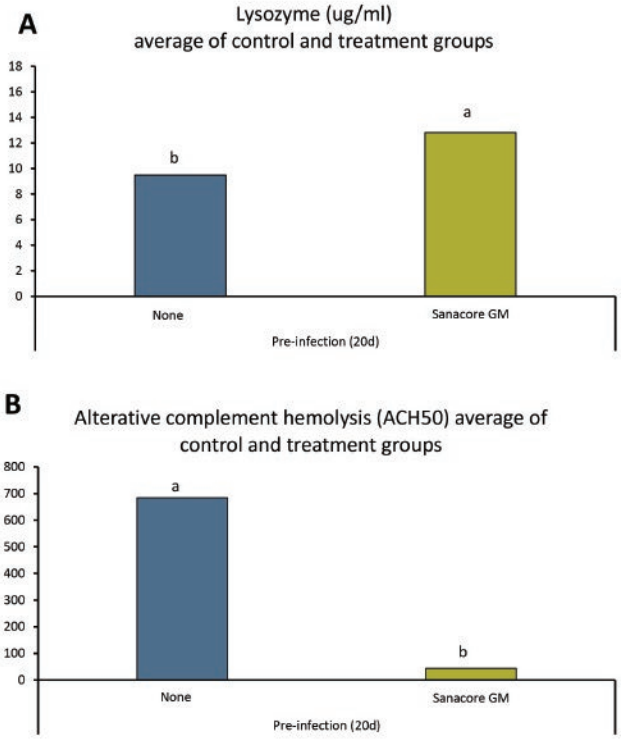


Figure 4. Innate immune assessment of control group (None) and treated groups (Sanacore® GM) after 20 days of supplementation with Sanacore® GM (end of pre-infection period). n= 9. A: Lysozyme breaks down the peptidoglycan layer in the wall of the pathogenic bacteria. B: ACH50 is the amount of serum required to induce 50% hemolysis in rabbit red blood cells.

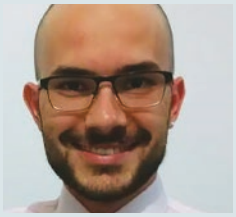
Romboutsia. *Cetobacterium* is a producer of vitamin B12 and short chain fatty acids (SCFA) as major end products of carbohydrate metabolism. Therefore, the growth promoting effects can be partly attributed to a positive modulation of the gut microbiota that increases the contribution of SCFA as energy supply to the enterocytes.

Following the infection challenge, the groups treated with the functional additive presented a significantly lower mortality compared to the untreated groups, of 32% and 17% for franciselosis (Fig. 2) and streptococcosis (Fig. 3), respectively. The lower reduction in mortality under infection by *S. agalactiae* can be attributed to infection route. The intraperitoneal infection is more aggressive and certainly not reflecting natural conditions, however, it constitutes the most viable route to experimentally induce disease in the case of this pathogen. The groups supplemented with Sanacore® GM showed a milder evolution of the disease and did not cease appetite completely. Improved disease resistance to both infection challenges can be partly explained by significantly higher lysozyme activity and significantly lower complement activity (i.e. less amount of fish serum required to induce 50% hemolysis in red blood cells of rabbit) induced by Sanacore® GM supplementation (Fig. 4). This immunestimulant effect may be also associated to a more stable and robust gut microbiota. This has been reported for Sanacore® GM in other species and reflected by the increased diversity of the gut microbiota within each fish, and also by the increased homogeneity across fish receiving the additive (Robles *et al*., 2017).

The dietary inclusion of Sanacore® GM showed advantages in tilapia production. *In vitro*, data here clearly shows its deleterious effects on *S. agalactiae* and *F. noatunensis* subsp. *orientalis*. *In vivo*, it promoted growth and disease resistance. The positive effects of Sanacore® GM are attributed to antimicrobial activity, positive modulation of the gut microbiota and immune modulation.

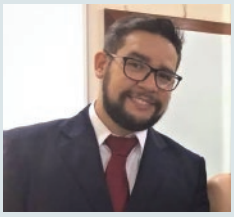
More information:

Leonardo Mantovani Favero
Postgraduate student
Fish Bacteriology Laboratory of
Londrina State University, Brazil



Maria-Mercè Isern-Subich
Product Manager
Aquaculture Health
Adisseo, Belgium

Ulisses de Padua Pereira
Adjunt Professor
Fish Bacteriology Laboratory
of Londrina State University,
Brazil



Waldo G. Nuez-Ortín
Lead Scientist Aquaculture
Adisseo, Belgium
E: waldo.nuezortin@adisseo.com