Doctoral (PhD) Thesis

APPLICATION OF HERBAL EXTRACTS TO IMPROVE HEALTH STATUS AND INNATE IMMUNE RESPONSE OF FISH

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1. Background and objectives of the study

According to a recent and generally accepted theory, the simultaneous presence of the following factors is required for the development of fish diseases: pathogen, environmental stress and the weakened organism of fish. Therefore there are three main directions in the prevention of fish diseases, which are based on the removing or elimination of one of these three factors.

The most commonly used method of removing pathogens is the application of various antibiotics and chemotherapeutic agents. The most important drawback of their application is that most of them accumulate in the fish meat or in the environment, therefore only a few chemicals or medicines are allowed to use in treatment of fish diseases. Another drawback of antibiotic treatments is that they promote the development of resistant pathogens and weaken the immune system of fish.

The second direction is to reduce the environmental stress. A method of this is the improvement of water quality; however, this is more and more hindered by the declining quality of natural, surface waters. Preventing the stress caused by crowding and transportation is often an impossible task in the circumstances of commercial fish rearing, especially intensive aquaculture.

The most promising method in prevention of fish diseases is the enhancement of resistance of the fish, which can be achieved by the application of vaccines and the so-called immunostimulants, which can enhance the innate (non-specific) immune system of fish. Vaccination is regarded as the most effective method; however, a vaccine is effective only against one kind of pathogens, and so far there are no effective vaccines against intracellular pathogens (e.g. *Renibacterium salmoninarum*).

In contrast to vaccines, immunostimulants enhance the innate immune response, therefore their effect is of short-term and they do not contribute to the development of immunological memory. In spite of this, they can be effectively applied to improve the disease resistance of fish. Immunostimulants are compounds of various structures. There are polysaccharides, peptides and alkaloids among them. In practice they can be applied by injection, bathing and orally, of which the latter method is the most commonly used and the most practical. In the case of some immunostimulants the exact molecular mechanism of their effect is known, and agents enhancing the innate immune response can be purchased as commercial products with various product names.

Immune-enhancing activity of certain medicinal herbs has been known for a long time in traditional medicine. However, isolation an identification of their active components started only at the end of the 19th century. Herbs have an especially important role in traditional Chinese and Indian medicine, in which they have been used for more than 4000 years. Traditional medicine in both countries has a greater emphasis on prevention than curing of diseases. It explains that many kinds of immunostimulant herbs are used in these countries. Recently there is a conjuncture in identification of the herbs' active components and research of their mechanisms of action. In recent years more and more experiments have been done with herbal extracts on various, economically important fish species, because herbal extracts as well as more traditional immunostimulants could be applied in fish culture to replace antibiotics and chemotherapeutic agents.

Group of immunology at the Research Institute for Fisheries, Aquaculture and Irrigation (HAKI) has been studying immunostimulants for nearly 20 years. In this field of study the research of immunomodulating herbs has been started in 2004. Results of this research are presented in this dissertation.

Main objectives of the study:

- Examination of three Chinese herbal extracts (*Astragalus membranaceus*, *Ganoderma lucidum* and *Lonicera japonica*) applied alone or in combination with each other on common carps and Nile tilapias

- Examination of the effect of herbal extracts on the effectiveness of vaccination

- Examination of the effect of herbal extracts and boron, a trace element on the innate immune response of fish

- Selection of the most effective herbal extract or herbal combination in the experiments

- Testing the practical applicability of selected herbal extract or herbal combination in half-industrial tests on common carps, Nile tilapias and African catfish.

2. Materials and methods

2.1. Experimental designs

Experimental fish were kept in the recirculation fish rearing system of HAKI, in 400 litre plastic tanks. Commercially available, powdered herbal extracts were used to make the experimental feeds. Extracts of *Astragalus*, *Ganoderma* and *Lonicera* were attached to the grains of the fish feeds using cornmeal oil. Feed additive containing boron was attached to the grains of the feed using a similar method. Feeds without herbal extract or boron supplementation were used as controls. Daily feeding rate was 2% of the fish biomass in each group. At the end of the experiments, fish were challenged with the bacterium *Aeromonas hydrophila* in an isolated recirculation system.

2.1.1. Effects of *Astragalus*, *Lonicera* and *Ganoderma* extracts on the innate immune response of tilapias

Three-month old fish with an average individual weight of 70.9±10.5 g were allocated into four experimental groups, with 50 fish per group. Fish were fed with feeds containing the following herbal extracts: *Astragalus* 0.5%; *Lonicera* 1.0%; *Astragalus* 0.5% + Ganoderma 0.5% and the control feed. Fish were being fed for three weeks. At the end of the experiment, 20 fish from each group were challenged with *A. hydrophila* ($3x10^7$ cells/ml, which equals LD_{50}, 0.1 ml/fish, intraperitoneal injection), and mortalities were recorded during one week.

2.1.2. Effects of *Astragalus* and *Ganoderma* extracts on the innate immune response of common carps and the effectiveness of vaccination against *A. hydrophila*

Eight groups were set up in this experiment, with 60 fish per group. Average individual weight of the four-month old fish at the start of the experiment was 56.9±7.9 g. Fish were fed with feeds containing the following herbal extracts: *Astragalus* 0.5%; *Ganoderma* 0.5%; *Astragalus* 0.5% + *Ganoderma* 0.5% and the control feed. In four experimental groups fish were injected with a vaccine against *A. hydrophila/A. salmonicida* two times, once at the start of the experiment and two weeks later. Fish of the other four groups were not vaccinated, only fed with the above described feeds. Fish were being fed for five weeks. At the end of the experiment, 30 fish from each group were challenged with *A.*

hydrophila ($1.2x10^8$ cells/ml, which equals LD_{80-100}; 0.1 ml/fish, intraperitoneal injection), and mortalities were recorded during one week.

2.1.3. Effects of two herbal extracts (*Astragalus* and *Lonicera*) and boron on the innate immune response of common carps

Four-month old fish with an average individual weight of 59.0±9.6 g were allocated into four experimental groups, with 60 fish per group. Fish were fed with feeds containing the following herbal extracts and trace elements: *Astragalus* 0.1% + boron (B); *Lonicera* 0.1% + B; *Astragalus* 0.1% + *Lonicera* 0.1% + B and the control feed. Concentration of boron was 4 mg/kg. Fish were being fed for four weeks. At the end of the experiment, 20 fish from each group were challenged with *A. hydrophila* ($5x10^7$ cells/ml, which equals LD_{50}, 0.1 ml/fish, intraperitoneal injection), and mortalities were recorded during one week.

2.1.4. Effects of *Astragalus* and *Lonicera* extracts applied alone or supplemented with boron on the innate immune response of tilapias

Three-month old fish with an average individual weight of 79±11.6 g were allocated into six experimental groups, with 50 fish per group. Fish were fed with feeds containing the following herbal extracts and trace elements: *Astragalus* 0.1%; *Lonicera* 0.1%; *Astragalus* 0.1% + B; *Lonicera* 0.1% + B; *Astragalus* 0.1% + *Lonicera* 0.1% + B and the control feed. Concentration of boron was again 4 mg/kg. Fish were being fed for four weeks. At the end of the experiment, 20 fish from each group were challenged with *A. hydrophila* ($3x10^7$ cells/ml, which equals LD_{50}, 0.1 ml/fish, intraperitoneal injection), and mortalities were recorded during one week.

2.2. Design of the half-industrial experiments

Half-industrial experiments were also done in the recirculation fish rearing system of HAKI. Experimental groups of common carps and tilapias with 200 fish in each were kept in 500 litre tanks, whereas groups of African catfish with 49 fish in each were kept in 20 litre tanks. In all three experiments, fish were fed with feeds containing the following herbal extracts and trace elements: *Astragalus* 0.1% + *Lonicera* 0.1%; *Astragalus* 0.1% + *Lonicera* 0.1% + B and the control feed. Concentration of boron was 2 mg/kg in these experiments.

Daily feeding rate was 4% of the biomass for the tilapias, 8% of the biomass for the common carps, and initially 8% of the biomass for the African catfish. The latter feeding rate was gradually reduced to the 3% of biomass during the experiment. Experimental feeds were produced by the fish feed mill of the institute.

At the start of the experiments, average individual weight of tilapias was 23±0.7 g. In the case of common carp, the average individual weight was 2.6±0.1 g, and in the case of African catfish, it was 6.6±0.4 g. Due to the small size of fish, blood samples were taken only once in these experiments, after six weeks from tilapias, and after four weeks from common carps and African catfish. In these experiments fish were not challenged with bacteria.

2.3. Laboratory methods for determination of immune response and disease resistance

2.3.1. Blood sampling of experimental fish

Fish of the laboratory experiments were sampled once a week, blood samples were taken from five fish in each group. In the half-industrial experiments there was only one blood sampling, from ten fish in each group. Volume of blood samples was 1 ml in all experiments. Fish were anesthetized before taking blood samples, using anaesthetics containing 40 g norcaicum and 10 ml adrenaline (Tonogen) per litre. Each fish were sampled only once to avoid the stress caused by multiple blood sampling. Laboratory tools used for sampling (injection needles, syringes, transfer pipettes, Eppendorf-tubes) had been treated with heparin before samplings to avoid blood clotting.

2.3.2. Isolation of leukocytes and blood plasma from the samples

Leukocytes and blood plasma was isolated from blood samples by density-gradient centrifugation. One ml of Histopaque 1.119 (Sigma) was added into siliconized glass tubes, and one ml of Histopaque 1.077 (Sigma) was layered on it. Both layers contained 120 µl Bacto Hemagglutination Buffer (Difco, USA). Blood samples were carefully layered on the top of the gradient, and centrifuged at 4°C with a force of 700 G. Centrifugation time was 30 min in the case of samples from tilapia and African catfish, and 35 min in the case of samples from common carp. Cells gathered between the two Histopaque layers after the centrifugation, and the blood plasma remained isolated on the top the gradient. Blood plasma was removed and stored at -20°C for further examinations. Leukocytes were also removed,

and they were washed in plastic centrifuge tubes by centrifugation, using Hank's Balanced Salt Solution (HBSS, Sigma). Following centrifugation, volume of cell suspensions was set to 1 ml using HBSS. Cells were counted in a haemocytometer, and based on this their concentration was set to $1x10^7$ per ml in each sample.

2.3.3. Measuring phagocytic activity of leukocytes

During this measurement cells phagocytise Congo-red stained yeast cells. Active phagocytic cells can be separated from inactive cells and the remaining yeast cells by their weight. One ml of leukocyte suspension from each sample was mixed to one ml of Congo-red stained yeast cell suspension in plastic centrifuge tubes. Cells were incubated at room temperature for 60 min, and then 3 ml of Percoll 1.055 (Sigma) was layered below the suspension. Samples were centrifuged at 20°C with a force of 850 G for 3 min to separate active phagocytes from inactive cells and remaining yeast cells. Leukocytes were then removed and washed in HBSS by centrifugation. Cells were suspended in 1.2 ml trypsin-EDTA solution (5.0 g/l trypsin and 2.0 g/l EDTA, Sigma) and incubated at 37°C overnight. Extinctions of samples were measured on 510 nm wavelength, using the trypsin-EDTA solution as a reference.

2.3.4. Measuring respiratory burst activity of leukocytes (extracellular)

Extracellular respiratory burst activity of isolated leukocytes was determined based on the reduction of ferricytochrome-c. Following the setting of cell concentration, cells were measured into the wells of a 96-well microtiter plate. A well contained 100 µl of cell suspension. Samples were measured in triplicates. Equal amount of 2 µg/ml ferricytochrome-c solution was added to the samples. This solution contained 1 µg/ml phorbol-12-myristate-13-acetate (PMA, Sigma). To check the specificity of the reaction, ferricytochrome-c solution containing 300 U/ml superoxide dismutase (SOD, Sigma), in addition to the PMA was added to another series of samples. Samples were incubated in darkness, at room temperature for 30 min, and their extinctions were measured on 550 nm wavelength. Extinction values of samples treated with PMA/SOD were subtracted from values of samples treated with PMA only. These values were multiplied by 15.87, and the results were the quantities of superoxide (O_2^-) radicals in nanomols.

2.3.5. Measuring respiratory burst activity of leukocytes (intracellular)

Intracellular respiratory burst activity of leukocytes was determined by a method based on the oxidation of nitroblue tetrazolium (NBT, Sigma). Cell concentrations were set to $1x10^7$ per ml using L-15 cell culture medium. Following the setting of cell concentration, cells were measured into the wells of a 96-wells microtiter plate. A well contained 100 µl of cell suspension. Samples were measured in quadruplicates. Cells were incubated for two hours at 19°C, and then the medium was replaced with 100 µl NBT solution (2 mg/ml NBT and 10 µl/ml PMA). After one-hour incubation at room temperature, the NBT solution was removed and the cells were fixed in 100% methanol for five minutes, then they were washed three times in 70% ethanol and dried on air under a fume hood. By the effect of oxidative free radicals, NBT taken up by the cells formed a water-insoluble, blue coloured formasan, which could be dissolved in 120 µl 2M potassium-hydroxide and 140 µl dimethyl-sulfoxide per wells. Extinction of the turquoise-blue coloured solution was measured by a spectrophotometer on 620 nm wavelength. Intensity of extinction is linearly proportional to the quantity of oxidative free radicals produced.

2.3.6. Measuring lysozyme activity of blood plasma

Micrococcus lysodeikticus bacteria (Sigma) were suspended in phosphate buffer (0.05M; pH 6.2) in 0.02% (w/v) concentration. Solutions of lyophilised hen egg white lysozyme in 0,5; 1,0; 2,5; 5; 10 and 20 µg/ml concentrations were used as standards. A new standard curve had been drawn before each measurement. Samples were measured in triplicates. 50 µl blood plasma or standard solution was added to each well of a 96-well microtiter plate and 250 µl *Micrococcus* suspensions were added to them. Extinctions of suspensions were measured on 531 nm wavelength two times, once immediately after adding the *Micrococcus* suspensions, and once 20 minutes later. Lysozyme kills the bacteria by lysing their cell walls; therefore the extinction values of the second measurement are lower than the values of the first measurement. The former values were subtracted from the latter ones, and lysozyme concentrations of plasma samples were calculated using the standard curve.

2.3.7. Measuring total protein level of blood plasma

Total protein levels of blood plasma samples were measured by a colorimetric method based on the Biuret reaction, using a diagnostic reagent kit (Reanal). Samples were measured in triplicates. 10 µl blood plasma or standard solution was added to each well of a 96-well microtiter plate, and then 300 µl diluted Biuret reagent was added to them. Samples were then incubated for 20 min at room temperature, and their extinction was measured with a spectrophotometer on 550 nm wavelength. Total protein concentration was calculated using the following formula: $y = Am / Ast * x$, where y is the total protein concentration of the sample, *Am* is the absorbance of the sample, *Ast* is the absorbance of the sample and x is the known protein concentration of the standard.

2.3.8. Measuring immunoglobulin-level of blood plasma

Total immunoglobulin levels of blood plasma samples were measured by a similar method. 50 µl blood plasma and equal amount of polyethylene glycol (PEG, Sigma) was added to each well of a 96-well microtiter plate. Samples were incubated for two hours at room temperature, and then the plates were centrifuged with a force of 1000 G for 15 min. Then total protein levels of the supernatants were determined using the method described above. Samples were measured again in triplicates. These values were subtracted from the total protein concentrations, and the results were the immunoglobulin-concentrations of the samples.

2.3.9. Challenge experiments with the bacterium *Aeromonas hydrophila*

At the end of the experiments (except for the half-industrial experiments) equal numbers of fish from each group were challenged with a virulent strain (B2/12) of the bacterium *A. hydrophila*. Before the tests, bacteria had been kept at 4°C, on solid medium. One day before the experiments, they were inoculated into 10 ml TSB (Tryptic Soy Broth, Fluka) liquid medium and incubated for 24 hours at 28°C. Liquid cultures were centrifuged; pelleted bacteria were suspended in 10 ml Phosphate Buffered Saline (PBS) solution, and then washed by centrifugation. Concentration of bacteria was set with PBS to the LD_{50} concentration by the extinction of suspension on 610 nm. This dose causes 50% mortality of the experimental animals. Its value had to be determined with preliminary experiments. 0.1 ml

bacterial suspension was injected to the peritoneal cavity of fish. Mortalities were registered during one week, and survival rates were calculated. Survivors of the challenge were killed at the end of the experiment by over-anesthetising. Dead fish (both killed by the bacteria and over-anesthetising) were disinfected with chloride of lime. During the experiments, water of the eight cubic meter recirculation system was being disinfected by the ultraviolet (UV) lamp built into the system. Before releasing, the water was treated for 24 hours with 14 litres of bleach.

2.3.10. Determination of specific antibodies against *Aeromonas hydrophila*

In one of the experiments done with common carps, effects of herbal extracts were examined not only on the natural immune response of fish, but on the effectiveness of vaccination against *A. hydrophila* as well. Levels of specific antibodies against the bacteria in blood plasma samples taken from vaccinated fish and non-vaccinated control fish were determined using an indirect ELISA (Enzyme Linked Immunosorbent Assay) method. Suspensions of *A. hydrophila* were added to the wells of 96-well microtiter plates. Optical densities of suspensions (on 610 nm) were set to 1.0 using PBS. Plates were incubated overnight at 4°C, during this time bacteria attached to the surface of the wells. Suspensions were then removed and plates were washed three times in the appropriate buffers. Serial dilutions were made of the blood plasma samples from 1/32 to 1/4096, using PBS. 100 µl of diluted blood plasma samples were added to each well of the plates, which then were incubated overnight at 4°C. Plates were then washed five times in the appropriate buffer, and then 100 µl antibody suspension was added to the wells. Monoclonal antibodies used in this experiments were developed against common carp antibodies and conjugated with horse radish peroxidise by the producer (Aquatic Diagnostics Ltd., Stirling, UK). Plates were incubated for one hour at room temperature and washed. Following this, 100 µl of chromogene solution (Tetramethyl benzidine hydrochloride (TMB) dissolved in phosphate buffer) was added to each well, and plates were incubated at room temperature for 10 min. The reaction was stopped by adding 50 µl 2M sulphuric acid to the wells. Extinction of the yellow coloured solution was measured with a spectrophotometer on 450 nm. A reaction was regarded as positive if its extinction value was at least three times higher than the value of the negative control. Endpoint (the highest dilution of the samples that gave a positive reaction) was used for the evaluation.

2.3.11. Statistical evaluation

Samples were measured in triplicates or quadruplicates, except for the phagocytic activity, which was measured only once with each sample. During the evaluation of data, the arithmetic means of parallels were used. Five fish were sampled at each sampling, and arithmetic means of their data were calculated. Differences between the results of experimental groups were evaluated using one-way analysis of variance (ANOVA) and Student-Newman-Keuls test, at a significance level of $p<0.05$, except for the values of the appropriate vaccinated and non-vaccinated groups, because these were compared with t-test. Results were presented on bar diagrams, where the arithmetic means of data and the standard errors of mean were described.

SigmaStat and SigmaPlot software, developed by SPSS, Inc. was used for the statistical evaluation of data and for diagram drawing.

3. New scientific results of the dissertation

1. In our first experiment with tilapias, the mixture of *Astragalus* and *Ganoderma* extract proved to be the most effective immunostimulant, because it significantly enhanced the phagocytic activity of leukocytes and lysozyme activity of blood plasma. Cumulative mortality after *A. hydrophila* infection was the lowest in this group also. *Astragalus* and *Ganoderma* extracts applied alone had a weaker, but still positive effect on the parameters of immune response and resistance against bacterial infection.

2. In one of our experiments with common carps, *Astragalus*, *Ganoderma* and the combination of the two herbs had a clearly positive effect on the innate immune response of non-vaccinated fish, which was demonstrated mainly by the enhancement of the respiratory burst and phagocytic activity of leukocytes and lysozyme activity of blood plasma. In the vaccinated groups, the positive effect on non-specific immune response was not that obvious. However, following *A. hydrophila* infection mortalities were lower in all treated groups than in the control. Level of specific antibodies against *A. hydrophila* was not significantly changed by the application of herbal extracts.

3. In our next experiment with common carps, fish feeds were supplemented with boron, a trace element, in addition to the two herbal extracts (*Astragalus* and *Lonicera*). Experimental feeds characteristically had a positive effect on the phagocytic and respiratory burst activities of leukocytes, and they reduced the mortality following *A. hydrophila* infection. Again, the combination of the two herbal extracts proved to be the most effective immunostimulant, because this treatment had the strongest effect on the enhancement of non-specific immune parameters (especially the phagocytic activity), and on the reduction of mortality following bacterial infection.

4. In our last experiment with tilapias, the effects of herbal extracts (Astragalus, Lonicera and the combination of them) applied with or without boron supplementation were compared. Astragalus extract applied with or without boron supplementation had the strongest positive effect on the innate immune parameters (especially phagocytic activity). Mortalities following *A. hydrophila* infection were lower in all treated groups than in the control. Similarly to the previous experiment, combination of the two herbal extracts (now with boron

supplementation) had the best effect on the enhancement of resistance against bacterial infection.

5. Based on the results of experiments described above, it was obvious, that herbal extract in combination with each other are more effective immunostimulant than applied alone. Combination of *Astragalus* and *Lonicera* extracts applied in 0,1-0,1 % concentration proved to be the most effective, therefore this combination was selected for the half-industrial experiments. Three half-industrial experiments were carried out, one with tilapias, one with common carps and one with African catfish. One control and two treated groups were set up in each experiment. In one of the treated groups, herbal extracts were supplemented with boron.

6. In the half-industrial tests, different fish species reacted differently to the treatments. In the case of African catfish, parameters of innate immune response characteristically changed positively. However, in the case of common carps, similar changes could not be observed in the given time point. In the case of tilapias, cellular parameters increased by the effect of treatments, whereas humoral parameters remained at a lower level. In the half-industrial experiments, feeds supplemented with boron increased non-specific immune parameters fewer times than feeds supplemented with herbal extracts only. However, in the case of common carps and tilapias, both feeds with or without boron had a positive effect on the growth parameters.

4. Practical applicability of results

In addition to the well known, commercially available immunostimulants (Ergosan, Levamisole, QAC, Vitastim, etc.), herbal extracts can also be applied to enhance health status and innate immune response of cultured fish. Similarly to the other immunostimulants, herbal extracts are of natural origin, cheap and they do not cause environmental pollution and do not develop resistance even if they are applied in large quantities. They can be applied using the simplest way (feeding) in practical aquaculture. Fish feeds supplemented with herbal extracts are especially recommended if the fish are attacked by a disease against which there is no vaccine available and against which the enhancement of innate immune response is sufficient.

In addition, application of fish feeds supplemented with immunostimulants, including herbal extracts is recommended in such cases when the fish are exposed to some kind of stress that can be foreseen. Stress has a serious negative effect on the disease resistance of fish. This effect can be significantly reduced by the application of immunostimulants. Some pathogens, like *Aeromonas hydrophila*, which has been chosen as a model by us, can cause disease only in those fish that has a weakened immune system. In pond aquaculture the most common of these stresses affects the fish in spring, when fish are moved into the rearing pond after the wintering period. This transfer causes a serious stress to the already weakened fish and makes them susceptible to infectious diseases. By the application of fish feeds supplemented with immunostimulants the risk of disease outbreaks can be lowered, or if it could not be avoided, the losses can be reduced. It is recommended to start feeding the fish feed supplemented with immunostimulants 2-4 weeks before the planned operation.

There are many operations in intensive aquaculture that causes a weaker or stronger stress to the fish. These include weighing, selection and transfer of fish. Their negative effects can be significantly reduced by a treatment with immunostimulants for an appropriately chosen period. However, it means a difficulty that various fish species react differently to treatments with immunostimulants, therefore the effective dose and duration of treatment has to be determined for each fish species, which has been proven by our experimental results.

5. The author's publications in the field of dissertation:

Publications in peer-reviewed journals:

Ardó, L., Yin, G., Jeney, Zs., Xu, P. és Jeney, G. (2007): Kétféle kínai gyógynövényt (*Ganoderma lucidum* és Lonicera *japonica*) tartalmazó haltáp hatása a nílusi tilápia (*Oreochromis niloticus*) természetes immunrendszerére (előzetes eredmények). *Agrártudományi közlemények 26. különszám, 9-14* (in Hungarian).

Ardó, L., Yin, G., Xu, P., Váradi, L., Szigeti, G., Jeney, Zs, Jeney, G. (2008): Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron enhance the non-specific immune response of Nile tilapia (*Oreochromis niloticus*) and resistance against *Aeromonas hydrophila*. *Aquaculture 275, 26-33.* IF: 1,678

Yin, G., Ardó, L., Thompson, K. D., Adams, A., Jeney, Z., Jeney, G. (2009): Chinese herbs (*Astragalus radix* and *Ganoderma lucidum*) enhance immune response of carp, *Cyprinus carpio* and protection against *Aeromonas hydrophila*. *Fish and Shellfish Immunology 26(1), 140-145.* IF: 3,161

Jeney, G., Yin, G., Ardó, L., Jeney, Z. (2009): The use of immunostimulating herbs in fish. An overview of research. *Fish Physiology and Biochemistry 35(4), 672-682.* IF: 1,232

Publication in proceedings of an international conference:

Yin G., Ardó, L., Jeney Z., Xu P. and Jeney G. (2007): Chinese herbs (*Lonicera iaponica* and *Ganoderma lucidum*) enhance non-specific immune response of tilapia, *Oreochromis niloticus*, and protection against *Aeromonas hydrophila*. *Proceedings of Conference of Diseases in Asian Aquaculture, 269-281*.

Publication in a non peer-reviewed journal:

Jeney, G., Ardó, L., Váradi, L., Jeney, Zs. (2010): Gyógynövénykivonatok alkalmazása a halbetegségek megelőzésére. *Halászat 103, 65-69* (in Hungarian).

Presentations and posters on Hungarian and international conferences:

Ardó, L., Yin, G., Pao, X., Váradi, L., Szigeti, G., Jeney, Z., Jeney, G. (2006): Gyógynövénykivonatokkal és bórral kiegészített haltáp hatása a nílusi tilápia (*Oreochromis niloticus*) természetes immunrendszerére. *XXX. Halászati Tudományos Tanácskozás, Szarvas, Hungary, p. 59-60* (in Hungarian).

Ardó, L., Yin, G., Xu, P., Szigeti, G., Jeney, Z. and Jeney, G. (2007): Effect of two Chinese herbal extracts (*Astragalus membranaceus* and *Lonicera japonica*) and two trace elements (boron and selenium) on non-specific immune response of common carp, *Cyprinus carpio*. *7th Nordic Symposium on Fish Immunology, Stirling, United Kingdom, p. 83.*

Ardó, L., Yin, G., Pao, X., Váradi, L., Szigeti, G., Jeney, Z., Jeney, G. (2007): Kétféle gyógynövénykivonat (*Astragalus membranaceus* és *Lonicera japonica*) és két nyomelem (bór és szelén) hatása a ponty (*Cyprinus carpio* L.) természetes immunrendszerére. *XXXI. Halászati Tudományos Tanácskozás, Szarvas, Hungary, p. 39-40* (in Hungarian).

Ardó, L., Yin, G., Jeney, Zs., Jeney, G. (2008): Pontyok természetes immunválaszának javítása gyógynövénykivonatok és nyomelemek alkalmazásával. *50. Georgikon Napok, Keszthely, Hungary, p. 61* (in Hungarian).