IN VIVO STUDY OF CONJUGATED DIMINAZENE ACETURATE FOR ICHTHYOPHTHIRIOSIS OF FARMED CARP

Andrei-Cristian LUPU[1], Alin BARBACARIU[2], Constantin ROMAN[1], Raluca MÎNDRU[1], Gabriela-Victoria MARTINESCU[1], Andrei-Alexandru CÎMPAN[1],Liviu Dan MIRON[1]
[1]University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad", Faculty of Veterinary Medicine, Al. M. Sadoveanu, 8, 700489, Iași, România, lupuandrei@protonmail.com
[2]Stațiunea de Cercetare-Dezvoltare pentru Acvacultură și Ecologie Acvatică, Șos. Iași-Ciurea, Iași

Abstract
The aim of this study was to explore the efficacy of a veterinary drug, Diminazene aceturate (4,4'-(1-Triazene-1,3-dyil)-bis-(benzenecarboximidamide), in an inclusion complex with β-cyclodextrin as a suitable treatment for parasitic diseases caused by Ichthyophthirius multifiliis in farmed carp. The efficacy was determined by the reduction in the infection intensity. The complexes were prepared by the coevaporation method and were characterized by DSC and FTIR. The selected stoichoimetry for the chosen drug was 1:1. Administration of Diminazene aceturate and complex was carried out by including appropriate doses in animal feed. Our studies suggest that the Diminazene–β-cyclodextrin complex results in a reduction in the infection degree and decrease in the trophont size in the treated fish. The oral treatment of Diminazene aceturate in inclusion complexes may be an alternative to bath treatments in carp farming.
Keywords: *inclusion, β-cyclodextrin, diminazene aceturate*

Introduction

In recent years, the inclusion of cyclodextrins in drugs has been used as a mean to improve drug properties, such as low solubility and slow dissolution rate, in order to improve bioavailability and to reduce adverse effects. A large number of studies have been published on the inclusion complexes of cyclodextrin, many of them in order to establish appropriate preparation procedures. Thus, previous studies in our laboratory and those of another group in this field have been aimed at assessing the usefulness of the complexes formed with regard to aspects such as stability(Szejtli, 1988), bioavailability(Vila-Jato și colab., 1986) or reduction of unwanted properties, e.g., Gastrointestinal ulcer(Otero Espinar et al., 1991) or undesirable organoleptic properties (Szejtli, 1988; Anguiano-Igea și colab., 1996).

Most studies aim to improve medicines for people. In view of the public health problems that have recently occurred in livestock farms and in aquaculture, increasing importance is attached to the control and formulation of medicinal products used in the treatment of animals for human consumption. The high propagation density characteristic of aquaculture promotes the emergence of infectious diseases of all types (viral, bacterial, fungal and parasitic). Parasitic diseases are particularly important, especially those caused by life-cycle species, including fast-spread ectoparasites. Such infections can have serious economic consequences, mainly due to outbreaks that have caused high mortality rates. For many such diseases, effective vaccines and treatments are not available.

One of the most representative species in freshwater aquaculture is carp - *Cyprinus carpio*. Parasites that affect this cyprinid include *Gyrodactylus sp.*, *Ichthyobodo necator* and *Hexamita salmonis* and histophagus ciliate *Ichthyophthirius multifiliis*. In the next study we evaluated the efficacy of a complex drug used in the treatment of parasitic infections in other animals, predominantly by oral administration (Tojo et al., 1994). Administration of oral drugs is generally preferred, because administration by immersion often leads to environmental contamination.

In the oral treatment study, we evaluated the efficacy of Diminazene aceturate and diminazene acetaturate complex, which were mainly administered by incorporating them into animal feed. Diminazene, like other antiparasitic drugs, is poorly soluble in water, so it is poorly absorbed in the intestine, greatly reducing its efficacy. Medicines such as diminazen have poor organoleptic properties and some animals reject the food they contain. In addition, besides decreasing treatment efficiency, poor absorption and low solubility, water contamination problems and the waste container also occur. In the present study, we evaluated the use of cyclodextrin inclusion complexes for the delivery of active substance to *I. multifiliis* infected farmed carp (*Cyprinus carpio*). We also examined the taste of feed containing β-cyclodextrin inclusion complexes.

The purpose of this article is to explore the efficacy of a veterinary medicinal product diminazene aceturate in inclusion complex with β-cyclodextrin as appropriate treatment in some parasitic diseases caused by *Ichthyophthirius multifiliis* in farmed carp. Efficacy is determined by the reduction in infection intensity. The complexes were prepared by the co-evaporation method (lyophilization) and were characterized by FT-IR, DSC. The selected stoichiometry was 1:1 for the drug and β-cyclodextrin. Administration of the complex was performed by including the appropriate dose in animal feed. Our estimates suggest that these Medicated-Cyclodextrin complexes will result in a reduction in infestation, but also a decrease in trophont intensity in the treated animals (fish). Oral and complex drug treatment may be an alternative to carp curing treatments.

Material and methods

Solubility studies

Solubility studies of substances alone or in the presence of β-cyclodextrin will be performed by the Higuchi and Connors method (1965). The excess of drugs will be added to tubes containing 10 ml of water or an aqueous solution of β-cyclodextrin (0-16 mM). The tubes are shaken at 80 cycles / min for 5 days in a water bath at 20 ° C ± 0.5 ° C. When equilibrium is reached, the tubes are centrifuged for 5 minutes at 5000 rpm, then the supernatant is filtered through cellulose nitrate membranes (0.45 Amp Pore size) to remove the suspension of the materials. The amount of drug in solution is determined by UV spectrophotometry at 306 nm. The stability constant is calculated from the initial linear region of the phase solubility diagram as described by Higuchi and Connors (1965).

Host used for complexation

The host is represented by the molecule of β-Cyclodextrin (Fig. 1).

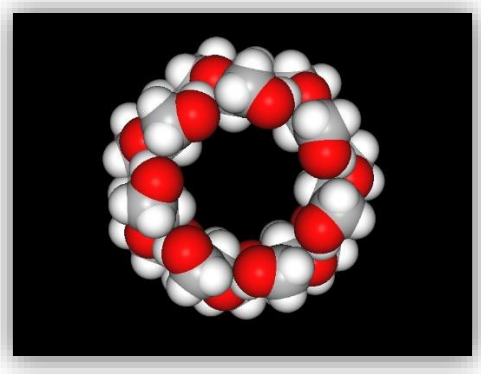


Figure 1 β-Cyclodextrin. Picture made with MarvinSketch

Cyclodextrins are substances composed of sugar molecules (-d-glucopyranose) linked together in a ring form, namely cyclic oligosaccharides. They are also called cyclo-amylose, cyclomaltose or Schardinger's dextrins and are non-reductive in nature. The CD's nomenclature is based on the number of units of glucose in its structure, so that the CD has 6 units of glucose called α-CD, the 7-linkage CD is called β-CD and the one with 8 units is called γ-CD. The CD-e glucopyranose units are linked via the 1-4 bonds. The formation of glucopyranose unit linkages gives the CDs a conical shape (Arun et al., 2008). There are hydrogen bonds between the 2-OH and 3-OH groups around the outer edge. These links are the weakest in α-CD and the strongest in γ-CD. Around the bottom edge, the 6-OH groups can also form hydrogen bonds, but the bonds are destabilized by dipolar effects and are not normally present in the CD crystals. In α-CD, the hydrogen bond is pure 3-OH (donor), 2-OH (acceptor). But in β and γ-CD, the bond changes between it and 3-OH (acceptor), 2-OH (donor). The CDs are amphipathic structures in which the 3-OH and 2-OH groups are exposed on the broader edge and on the outer edge of the 6-OH group. The outer cavity is lined with these hydrophilic groups and the inner surface is etched by the ether as anomeric oxygen atoms. Thus CDs have a hydrophobic inner cavity and an outer hydrophilic surface (Zhou and Ritter, 2010).
For our study, we considered working with β-cyclodextrin.

Prepararation of solid drug-β-CD complexes

After confirming and characterizing the formation of the complex in the solution, we proceeded to prepare the complex in a solid state. For this purpose, we used the co-evaporation method (lyophilization), which usually offers good inclusion rates (Blanco et al., 1991), which is attractive for industrial scale applications, given its simplicity. In addition, the characteristics of the lyophilization mixture provide the possibility of being easily incorporated into the animal feed at the time of manufacture (typically based on granulation and extrusion procedures).

As stated, inclusion complexes with β-Cyclodextrin were obtained by the lyophilization method (Szejtli, 1988). To determine the complexity efficacy, drug mixtures and β-cyclodextrin at different proportions were lyophilized using a 50:50 v/v mixture of water and glacial acetic acid as a wetting agent. Preliminary studies have indicated the need to reduce the pH of the wetting agent to facilitate partial dissolution of the drug and thus to improve complex formation; Indeed,

formation of complexes does not occur if the wetting agent is only water. Acetic acid was chosen in view of its high volatility so that it was rapidly removed from the complex, minimizing toxicity problems.

For the preparation of inclusion complexes, the drug and β-cyclodextrin are mixed in suitable proportions and then milled in a mortar. Subsequently, the wetting agent (Blanco et al., 1991) was added. The thus obtained paste was oven dried at 40°C for 24 hours and the 200-500 μm fraction was obtained and used for subsequent tests. Using this method, drug and cyclodextrin mixtures were prepared at a molar ratio of 1:1.

Complex characterization in solid state

Complexes obtained by the lyophilization method were characterized by, FT-IR, differential scanning calorimetry (DSC).


A


B

Figure 2 Diminazene (B) and complexed diminazene (A) in solid state

Differential scanning calorimetry

Aluminum tubes containing 1-2 mg of product were placed in a Shimadzu DSC50 DSC (gas air, temperature range 50-250°C, heating rate 10°C / min). DSC is a useful technique that allows us to determine temperature transitions, such as melting, boiling, dehydration or crystallisation, which may occur in the sample material, resulting in an endothermic or exothermic reaction.

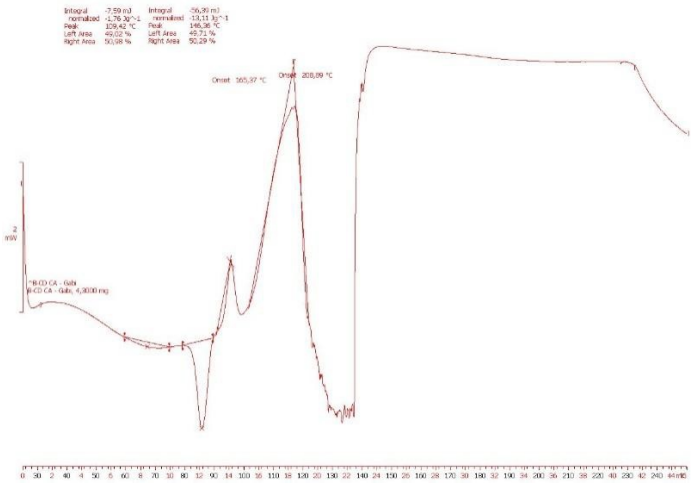
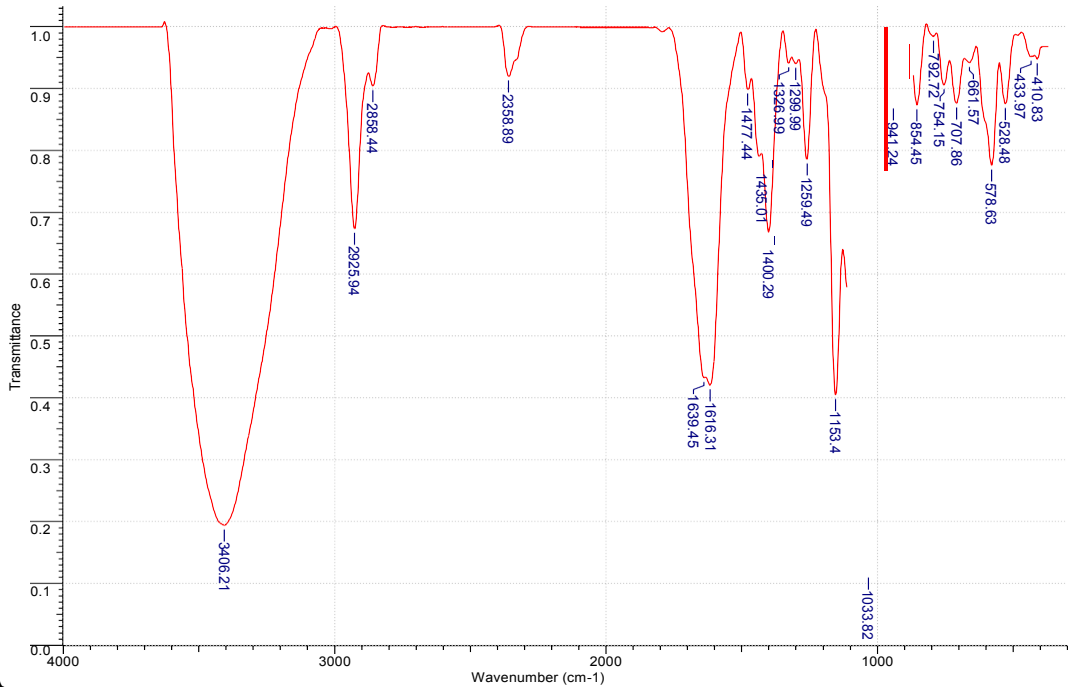


Figure 3. Thermogravimetric analysis of complex β-CD-Diminazene aceturate

Thermal curves obtained by DSC can provide information about complexing the drug with cyclodextrins and about their crystalline state.

FT-IR analysis (Fourier Transform Infrared Spectroscopy)



A

Figure 4. FT-IR analysis forthe complex Diminazen-β-CD

Variation of the shape, passage, and intensity of the IR absorption peaks of guests or hosts may provide sufficient information for the occurrence of inclusion. The graph showed the IR spectra of Diminazene, β-CD and their inclusion complex. The IR spectrum of Diminazene showed its characteristic bands. There was a very strong absorption band at 1616 cm^{-1} for C=O stretching vibrations.

The absorption band at 1400 cm^{-1} was indicated for the stretching vibration of C-C in the hexatomic ring. 941 cm^{-1} was for the C-H absorption band in the C-conjugated system. The IR spectra of the inclusion complex are similar to β-CD because of the reduced amount of diminazene in the system. However, some variations in the spectra were found. The absorption band at 1639 cm^{-1} disappeared or was shifted to the small wave numbers in the diminazene/β-CD inclusion complex, indicating that the C=O stretch vibration was restricted after the formation of the inclusion complex. 1400 (1477) cm^{-1} was strongly weakened, indicating that a majority of the diminazenhexametal ring was included by β-CD, but perhaps only part of diminazene was included, only one hexatomic ring of the two. In the present article, diminazen inclusion complexes with β-CD have been prepared and complex structures have been investigated by FT-IR. The experimental results showed that the module of the complex was the part of the diminazene molecules were included in the β-CD cavities.

Preparation of the feed containing the drug

Firstly prepare a homogeneous mixture with mortar and pestle, the drug complex and the commercial feed.


A

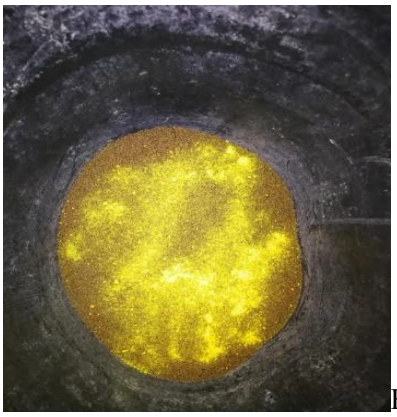

B

Figure 5. The feed used for the inclusion of the drug

The feed is previously ground in a blade mill. The mixture is moistened with water to obtain a suitable consistency for subsequent extrusion, then extruded (into an extruder) using the cylinder with a 2 mm orifice. The obtained pellets are dried in a furnace for 24 hours. They have an average weight of 0.2g and one kilo of feed contains about 5000 pellets.

Characterization of animal feed

The drug content in food can be determined by spectrophotometry at 306 nm. 15 minute release tests (15 minutes being the estimated maximum time required for fish ingestion) were performed to determine if significant amounts of drug can be lost after release into water before ingestion.

Determination of drug content

The drug-containing feed (15 mg) was weighed, milled and maintained for 24 hours in 500 ml of 1:50 v / v acetic acid / acetic acid (to ensure complete dissolution of the drug). The samples are then centrifuged at 2000 rpm for 10 min at room temperature and filtered through cellulose nitrate membranes (pore size 0.45 Am). The drug content was determined in the filtrate by UV spectrophotometry at 306 nm.

Release studies

The feed (15 mg) containing the drug was placed in a beaker containing 500 ml of water and subjected to slow magnetic stirring. After 15 minutes, a sub-sample was taken and centrifuged as in the previous paragraph. After filtration to remove the supernatant, the drug was quantified by UV spectrophotometry.

In vivo studies

Fish stock

The fish (*Cyprinus carpio*) is obtained from a local fish farm (Research and Development Center for Aquaculture and Ecology Iaşi from Al. I. Cuza University) and has been acclimated for at least 36 hours in a 250 l reservoir, with aeration and constant temperature (19 ± 1 ° C, pH 6,5).

The natural light-dark cycle is simulated (14-16 light hours, 8-10 hours dark). The fish will be fed on a daily basis with commercial food.

Infection

The fish used for the analysis are naturally infected with *I. multifiliis*, but also with other parasites. They show clinical signs, which are then confirmed by examining dermal microscope (Motic x20).

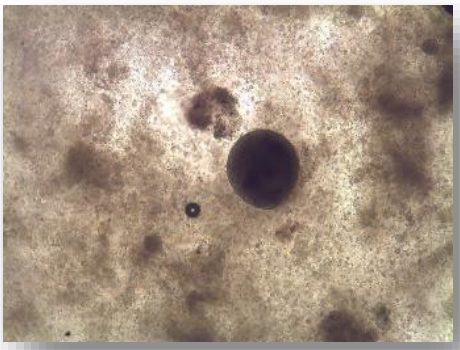


Fig. 6. Trophont of *I. multifiliis* collected from the skin of an infested carp (20x)

Drugs and analisys

The treatment trials were performed on groups of 7 infected and maintained in 180L tanks. Simultaneous control tests (also 7 fish: identical but not medicated feeds) were also performed. The tank conditions (water source, flow rate, aeration, pH, temperature, light/dark cycle) are the same as those of the acclimatization period. The treated fish received medicated feed (5g / kg feed) daily throughout the experiment. In all cases, the feeds provided will be 1.7-2% of body weight per day. Throughout the test period, fish have been regularly monitored to ensure that they eat food and to check for signs of toxicity.

The harvested fish are saplings of the *Cyprinus carpio* species with body mass between 78 - 101 g.

Table 1.

Body weight of the fish selected for the study

Nr. Crt.	FOR CONTROL	FOR DIMINAZENE ACETURATE	FORβ-CD-DIMINAZENE COMPLEX
1.	82,32 g	86,35 g	87,19 g
2.	78,83 g	91,02 g	101, 95 g
3.	93,24 g	88,33 g	87,95 g
4.	82,22 g	81,48 g	88, 04 g
5.	88, 37 g	95,66 g	95,15 g
6.	97, 82 g	99,07 g	94,22 g
7.	79,35 g	82,77 g	97,11 g

After the 24 hours completion of the test, the fish will be anesthetized to determine the intensity of the infection.

In the first series of analisys, we compared the efficacy of uncomplexed feed and feed containing the β-cyclodextrin-drug complex (in both cases with 5 g of drug/kg of feed) to treat *I. multifilius* infection. For these tests, we used naturally infected fish, allowing us to evaluate the effectiveness of the treatment.

For these tests, I started the treatment immediately and continued it for 10 days. Duration of *I. multifiliis* infection is variable and temperature dependent: duration is approximately 9-10 days (Tojo et al., 1994b, Tojo and Santamarina, 2001). These tests thus, test efficacy against the various stages of the parasite life cycle.

Determining intensity of the infection

The fish were anesthetized by immersion in water with clove oil (0.3 ml/l) in a 150 l separable basin until the breathing became weak. A mucus sample will be taken by gently scraping a part of the body surface of the fish (skin and fins). Mucus samples will then be examined under a microscope (100x). In the case of naturally infected fish, the intensity of the infection will be recorded on a five-point scale after examination of the entire field.

Results and discussions

Solution interactions between drug and β-Cyclodextrin were investigated by examining the diagram in the solubility phase. As can be seen, solubility increases with increasing β-cyclodextrin concentration, indicating that these inclusion complexes have limited aqueous solubility. However, the complexity solubility of the complex will be greater than that of the drug alone.

Compared to the values obtained for cyclodextrin inclusion complexes of other drugs, the stability constant in our case is high. This indicates that these complexes have a high stability in solution due to strong interactions between cyclodextrin and drug. Although high, this stability constant is within the optimal range to improve the bioavailability of poorly soluble drugs: Values below this range involve excessive dissociation in the solution leading to precipitation of the drug, while very high levels imply inappropriate dissociation, so that the free medication remain insufficient for effective absorption.

Analysis of the flat region of the diagram in the solubility phase indicated that the drug-β-cyclodextrin ratio in the complex in solution was 1:1.

To determine the characteristics of lyophilized products, I will use the differential scanning calorimetry. It shows DSC traces for Diminazene, β-cyclodextrin and various lyophilized mixtures. DSC traces for β-cyclodextrin show the thermal events characteristic of this excipient, resulting in the loss of water molecules inside the cavity (wide endotherm which occurs between ambient temperature and 140°C) and the reversible transition to 220°C. Cyclodextrin melts and decomposes at temperatures above 250°C. The DSC route for Diminazene presented an endotherm at different temperature degrees, corresponding to their melting.

All the mixtures tested (1:1) showed the drug fusion endotherm, indicating the presence of the free crystalline drug. In fact, the product is probably a 1:1 complex mixture and excess free cyclodextrin. Furthermore, the examination of the results indicates that the melting point decreases with the increase in the proportion of cyclodextrin, suggesting that a solid dispersion is formed between the two components. The presence of excess cyclodextrin can thus increase the dissolution rate, even if the drug is not complexed.

Based on these results, we selected the 1: 1 ratio for *in vitro* studies because this proportion assured that the drug was in complex form. In addition, the use of excess cyclodextrin is common in the preparation of inclusion complexes.

The selected dosage form (food containing the drug) requires that the feed pellets remain in the water until ingested. Under these circumstances, it is very important to have minimal medication loss from the dosage form in the first few minutes in contact with water: this would lead not only to drug loss but also to water contamination. The main feed components (flour, proteins, oils, etc.) and the procedures used to make it (typical extrusion) favor negligible or very slow release. Once ingested, the gastrointestinal environment favors disintegration and digestion, which results in the release of the soluble components contained in the pellet. This will confirm the amount of medicine released in the first 15 minutes in contact with water at a temperature close to that of the growth tanks (20°C) with a slight stirring. Our results indicate that neither drug-only pellets nor pellets with the complex have released significant amounts of drug during this period. It is important that the fish consume all the pellets within 15 minutes.

After confirming that the drug is not lost from pellets in water, we have conducted tests in which the infected carp was given food containing a drug or complex. The food that contained the complex was quickly eaten and I did not see any evidence of low palatability; by contrast, feed containing only the medicine (Diminazene) was sometimes rejected. This is, of course, an important aspect of a treatment given in animal feed. The ability of cyclodextrin to hide the unwanted taste of drugs such as bitterness, such as diminazen, is known.

Table.2.
Treatment results of carp with feed containing Diminazene aceturate (DIMA) or DIMA-β-Cyclodextrin (in both cases 5g drug/kg feed, after 10 days)

Carp no.	*DIMA-β-Cyclodextrin*	*DIMA*	*Control*
1	+	+++	+++
2	+	+++	+++
3	±	+++	+++
4	±	+++	+++
5	-	++	++
6	-	+++	**M**
7	-	++	**M**

Fish stock used for this test (7fish per group) were naturally infested with*I. multifiliis*. Legend: zero (-) no trophond detected; very low (±), a single trophont; low (+) 2-10 trophonts; moderate (++) 11-50 trophonts; high (+++) >50 trophonts; (M) dead fish.

Results of carp treatment with feed containing the drug or complex. As can be seen, untreated fish have maintained high infections, and 2 out of 7 fish died (Table 2) during the 10-day test period. In fish treated with uncomplexed Diminases, no mortality occurred, but the intensity of infections remained high in all fish throughout the test period. In fish treated with Diminazenecomplex, by contrast, the intensity of infections dramatically decreased to zero in 3 out of 7 fish and at very low levels in the other four fish.

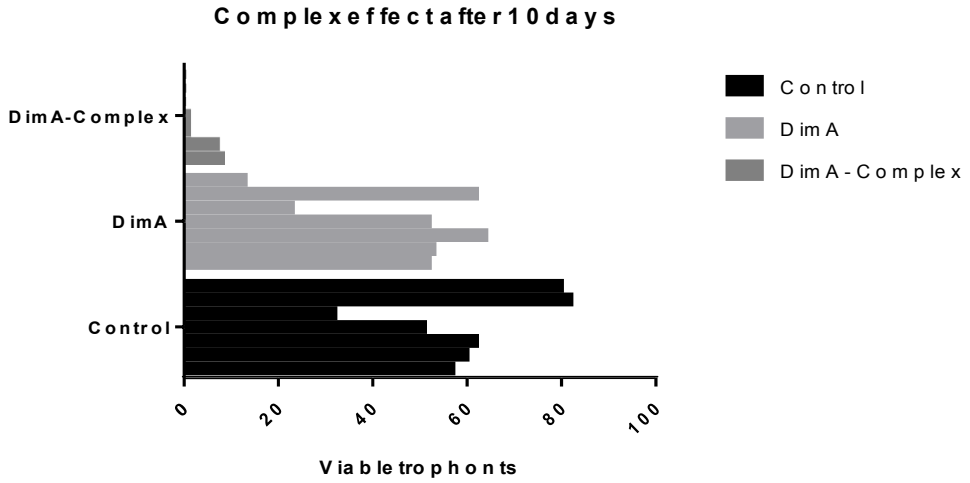


Figure 7. Viability analysis of the trophonts after 10 days of treatment with medicated feed

Figure 7 shows the very high intensity of the parasite load in the first two groups (of the 7 fish/group) than the group in which we used the Diminazene-β-Cyclodextrin complex in the feed.

Currently, our laboratory is conducting studies to investigate this phenomenon and to determine the doses and time needed for the treatment to completely eliminate the parasite.

In conclusion, preliminary results will suggest that these inclusion complexes of cyclodextrin with an antiparasitic are a promising option for the treatment of ichthyophtiriosis in farmed carp. Because *I. multifiliis* is a localized parasite in the skin, it is not easily accessed by drugs. Cyclodextrin inclusion complexes seem to improve accessibility and avoid the need to use immersion treatments, which often can not be authorized from the perspective of public health and environmental issues.

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