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INCLUSION OF FERMENTED APPLE BAGASSE AND A PROBIOTIC (*Kluyveromyces lactis*) IN THE TILAPIA DIET (*Oreochromis niloticus*) DURING THEIR YOUTH STAGE

José Luis Guevara Valdez[1], Daniel Díaz Plascencia[1]*, Pablo Fidel Mancillas Flores, José Roberto Espinoza Prieto, Diana González López. Rosario Martínez Yáñez[2]

Universidad Autónoma de Chihuahua, Facultad de Zootecnia y Ecología. Periférico Francisco R. Almada Km 1. CP. 33820. Chihuahua, Chihuahua México.

[2]Universidad de Guanajuato, Departamento de Agronomía, División de Ciencias de la Vida. Ex Hacienda el Copal Km. 7. Carretera Irapuato-Silao. CP. 36500. Irapuato, Guanajuato México.

∗Correspondence Author: dplascencia@uach.mx

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SUMMARY

The aim of this study was to evaluate the effects of fermented apple bagasse (BMF) and yeast inoculum (IL) *Kluyveromyces lactis* in the tilapia diet. The variables evaluated were weight gain, length, Feed Conversion Ratio (CAT) and fillet yield. Four diets were prepared, including 10% BMF (MAN Diet), 3% IL (PRB Diet) and a mixture of both (MXP Diet), compared to a control (CTL). Measurements of the fish were taken weekly and then they were slaughtered to obtain the fillet. Fish fed with BMF had a significantly higher weight at slaughter (37.93 g) than control (34.92 g), while those fed with IL were significantly lower (32.42 g) (P < 0.05). Length gain had a treatment effect (P > 0.05), with the MAN diet (92.19 mm) having a greater effect than the control diet (88.38 mm), and the PRB diet (83.84 mm) (P < 0.05). Likewise, there was a difference with respect to the ACT (P > 0.05) because the MAN Diet had a ACT of 1.78, while the control obtained 1.91. Fillet yield was higher (P > 0.05) in CTL treatments and MAN diet (52.33 and 51.15%) than MXP and PRB (44.51 and 46.4%). It is concluded that BMF acts as a source of microbial protein of good quality and high digestibility, in addition, while yeast did not present positive effects on the fish, the antioxidant compounds present in BMF provided benefits that translate into an improvement in production parameters

INTRODUCTION

Aquaculture is the development of aquatic organisms under controlled or semi-controlled conditions, thus being one of the best economic options for obtaining protein of animal origin. Tilapia (*Oreochromis sp.*) is a species that has had a growing interest in the aquaculture industry in recent years, being the second farmed freshwater species in the world, after carp (Atwood *et al.*, 2003). It is important to mention that it is used in aquaculture production for its easy handling, for its rapid growth, its resistance to temperature changes, its adaptation to various environments and also for its ease of reproduction.

In tilapia production systems, a large part of the production costs correspond to feed, using animal protein, usually fishmeal, which considerably increases the cost of feed. Therefore, the search for good quality sources of protein of vegetable or microbial origin is a priority in fish production, as they are currently the most demanded source (Ribeiro *et al.*, 2014; Paz, 2019). In fish production by aquaculture, 50% or more of the expenditures are directed to feed, so it is necessary to develop a complete, balanced, highly digestible feed that presents added nutritional benefits and high protein quality, as well as additives that preserve the health of the fish such as nutraceutical and probiotic supplements in order to optimize the growth of the fish and achieve a greater productive benefit (Thiessen *et al.*, 2003; Idenyi *et al.*, 2022).

Consumers today are looking for a food that is nutritious and free of any chemicals, antibiotics, or heavy metals. This has led producers to look for alternatives such as natural additives to avoid rejection of their products in the markets. Several alternatives have been developed such as probiotics, enzymes and herbal extracts. Some of these alternatives, which have shown quite a few positive results, are nucleotides and probiotics. They are natural alternatives and are considered to contain important nutrients. They are involved in several biochemical processes that are necessary for the functioning of the body (Ringø *et al.* 2012). It creates positive effects in animals such as increased growth, intestinal morphology and immune responses (Barros *et al.* 2013; Paz, 2019).

The apple is a natural source of antioxidants, which in addition to providing a large amount of these, is an excellent substrate for the production of microorganisms through fermentation in solid state, which are capable of breaking down cellulosic structures (Dhillon *et al.*, 2012) and release the phenolic compounds contained in them to make them available to consumers of this ferment (Ajila *et al.*, 2011).

Apple bagasse (BM) consists of the residues from obtaining the juice, being composed of peels, seeds, hearts, stems and remains of pulp and soft tissues, which is why it is considered a product of high nutritional value (García *et al.*, 2009: Díaz-Plascencia *et al.*, 2011; Díaz and Rodríguez 2017). By using this material as an ingredient in animal diets, the producer faces several problems, such as its deficiency of digestible protein, its high moisture content and its low pH, so the development of microbial protein in this medium is presented as an excellent alternative to increase its protein level and modify its pH. so it can be used for animal feed as a high-quality food supplement, providing protein at a lower cost than animal meal, and with similar digestibility (Bhalla and Joshi, 1994; Rodríguez-Muela *et al.*, 2010).

Therefore, the objective of this study was to evaluate the effect of the inclusion of Fermented Apple Bagasse (BMF) as a source of protein and antioxidants and the use of yeast inoculum (IL) as a source of probiotics on tilapia production parameters.

MATERIALS AND METHODS

Localization

Faculty of Zootechnics and Ecology (FZyE) of the Autonomous University of Chihuahua (UACH), located near the city of Chihuahua, Mexico. The unit is located between parallels 28° 38' LN and 106° 04' LO, at an altitude of 1,440 meters above sea level. The average annual temperature is 18.6 °C with an average annual rainfall between 200 and 600 mm (INEGI 2017).

The study was carried out in the psyculture module of the (FZyE) was conditioned in temperature, light and a battery of fish tanks with a recirculating hydraulic system, which includes physicochemical filtering to maintain the biological conditions necessary to sustain the life of aquatic organisms and their optimal development throughout their productive stages.

The water was moved by means of a Pedrollo PKm 60 pump, with 0.5 hp power, at an average expenditure of 40 L/min for the entire system, which ensures adequate oxygenation. The filtration system consists of a 6 mm thick glass tank, which contains the filter media, compounds of activated carbon, pellet and zeolite, which carry out physical and chemical filtration, which allow the establishment of colonies of denitrifying bacteria, favoring an adequate level of nitrogenous waste compounds in the water. There was also an ultraviolet light system, which, through two 15 W tubes, irradiates the water in order to kill possible parasites and their vegetative forms, maintaining aquaculture health and water quality.

The water was constantly monitored for its quality in terms of Oxygen Concentration, Nitrate Concentration, CO2 Concentration, Temperature and Ammonium Concentration, so that they were always kept constant and within the optimal ranges for the species, and thus avoid influencing the results of the experiment.

Ethical aspects

The study was carried out in accordance with the internal code of bioethics and taking into account both the animal welfare regulations of the FZyE of the UACH and the regulations of the Official Mexican Standard on animal care published by the Ministry of Agriculture and Rural Development (SAGARPA 1999).

Yeast inoculum (IL). The yeasts used were from the *Kluyveromyces lactis*, recovered from the microorganism bank of the FZyE, of the UACH. To reactivate the yeasts, a nutritious broth of meat extract, soybean peptone, malt extract and necessary additives was used (Table 1), prepared expressly for this purpose. The yeasts were kept for 96 h in this broth at 25 ºC and a production of 5.7 x 10 was achieved[7] CFU/mL, by a simple count in a Neubauer chamber by the method described by Díaz-Plascencia (Díaz-Plascencia, 2011), where a sample of 1 mL of the inoculum is taken, and taken to serial dilutions, until the appropriate concentration is obtained.

The nutritious broth was then inoculated with these yeasts at a rate of 0.1% of the total weight. This already inoculated broth was left in aeration and controlled temperature for 72 h inside the incubator, in order to maintain a stable environment.

The formulation of the diet was made in the Nutrion 5 formulation software, based on the recommendations of the N.R.C. 2011, (National Research Council) for Tilapia (*Oreochromis niloticus*), making the necessary modification to include the levels of BMF without altering the nutritional aspects of the food. The diets were formulated as established in (Table 2), using the inclusion level of 10% BMF (MAN), 3% IL (PRO) and their interaction (MXP), contrasted against a control diet (CTL diet). These diets were prepared in the Animal Nutrition laboratory of the Faculty of Zootechnics and Ecology, where the ingredients were homogenized and moisturized at 24% w/w to be pelletized in a commercial meat mill. The pellets already formed were left to dry at room temperature on air-permeable trays for 48 hours until they reached a humidity of 8%, leaving a yellow food, of adequate consistency and pleasant smell and taste for the fish.

Once the diets were formulated, proximal analysis was carried out using conventional methods of the A.O.A.C. (Association of Official Analytical Chemistry) to evaluate their nutritional contents and determine if there are differences between them.

The fish used were Nile tilapia (*Oreochromis niloticus*) of one inch in average size, two weeks old, obtained at the fish farming center of La Boquilla, in the municipality of San Francisco de Conchos, in the State of Chihuahua, Mexico. The fish obtained were previously masculinized with 17α methyltestosterone in the food to avoid interference by the sex factor (García *et al.,* 2017).

Four treatments were established: the diet added with fermented apple bagasse (MAN), the diet added with yeast inoculum (PRO) and their interaction (MXP), as well as the control diet, this being the commercial one for tilapia (CTL). The following treatments remain:

1. MAN: Diet formulated with 10% BMF.
2. PRO: Diet added with 3% IL.
3. MXP: Diet formulated with 10% BMF and 3% IL.
4. CTL: Control diet.

A completely randomized design was used, where glass fish tanks were used with ten fish each as an experimental unit, with five replications for each treatment, giving a total of 20 fish tanks. Three fish were taken at random each week from each fish tank to obtain the measurements, having three replicates for each repetition.

Response Variables

Weight and length gain. Over 8 weeks, the fish were weighed and measured every 7 days, as described by Crivelenti (Crivelenti and Mundim, 2011), weighing them on an electronic scale in triplicate, taken randomly from each of the 20 fish tanks and measured in length, height and width with a millimeter vernier. To facilitate the handling of the animals, once removed from their fish tank, they were passed through cold water for a few seconds to reduce their activity and be able to be handled without risk of causing damage to the fish themselves.

The diet was given according to the mass of the experimental unit and the proportion marked by the NRC for optimal growth.

Fillet yield. After the trial was completed at 8 weeks, the fish were slaughtered by cephalic section after being weighed, measured, and sedated with ice. The fillet of each fish was obtained manually, weighed and frozen at -18 °C until chemical analysis (Fagbenro and Jauncey, 1998).

Survival rate. The fish were counted each day, noting in the log if there was mortality each day. In cases where there was mortality, the body of the fish was analyzed to detect possible disease marks, and it was noted in the logbook.

Experimental Design:

For the statistical analysis of the data, a one-way ANOVA test was used to compare the productive variables in each week and proximal analysis, in addition to a comparison of Tukey's means between treatments, considering the treatments statistically significant at $P < 0.05$. For the weight and length gain of the fish with respect to time, a study of means repeated over time was carried out with the Proc Mixed procedure, finding significant effects at $P < 0.05$. The statistical treatment was carried out with the SAS computational package, version 9.1.3 (SAS Institute, 2006).

RESULTS AND DISCUSSION

The proximal analysis of the diets showed that there was only a statistically significant difference in crude fiber levels, thanks to the inclusion of BMF, which has a significant amount of fiber, however, this inclusion does not modify energy levels or exceed the recommended amount of fiber for the species (FAO, 2010), so isocaloric and isoprotein diets were used for the experiment (Table 3).

In the present study, a significant effect ($P < 0.05$) was obtained from the inclusion of BMF in the diet of Tilapia hatchlings in the growth stage, presenting a greater weight gain ($P < 0.05$), greater gain in length ($P < 0.05$) and lower TCA ($P < 0.05$), (Table 4), while the inclusion of IL presented a negative effect on the same variables. On the other hand, the interaction of BMF and IL obtained a good response in the variables studied, although still lower than the BMF diet, but higher than the control diet (Tables 5 and 6). This effect can be explained by the presence of antioxidant agents in BMF, which was corroborated by what has been reported by other authors, where thanks to the inclusion of by-products from the fruit industry, antioxidants are provided to fish diets and improvements in their production parameters are obtained (Sahin *et al.*, 2014; Brenes *et al.*, 2016).

In the aspect of water quality control and physicochemical parameters of the environment in which the fish developed, it can be said that there was no statistically significant difference between the treatments ($P > 0.05$), however, there is a downward trend in temperature thanks to the normal change in environmental temperature throughout the year. since this experiment was carried out between the months of August and September. Despite this drop in temperature, the values remained within the appropriate range for the correct development of the species (22 to 32 °C), so no interference was observed with the experiment (Graph 1), nor with the oxygen concentration, which was maintained at adequate levels as provided by the FAO and SAGARPA technical data sheet for this species (Graph 2).

Regarding waste metabolites, constant monitoring of ammonium and CO2 shows that at no time were concentrations higher than those recommended for Tilapia (FAO, 2010), with ammonium always remaining below 1 mg/L and CO2 below 30 mg/L, (Graphs 3 and 4). Despite being below the maximum allowed, there was an increase in the concentration of ammonium in the fourth and fifth weeks, which can be attributed to the accumulation of waste in the filter, a situation that was corrected by cleaning it.

The pH of the water in the fish tanks showed variation throughout the experiment, showing that there was no difference between the treatments, so it can be said that the inclusion of BMF, IL and their interaction do not modify the pH of the water in the system where the fish develop outside the limits established for this species (6.5 to 8.5). and behaved in the same way as the control diet (Graph 5).

Mortality in the experiment remained low, with most deaths due to fish jumping out of the tank and being found hours later, ruling out the presence of parasitic or bacterial diseases upon review of the dead fish. Thus, survival remained between 92 and 100% per treatment (Table 7). This survival rate is adequate for the species, as it is managed between 90 and 100%, according to the FAO and SAGARPA technical sheets, in addition, it is similar to what has been found by other authors for the growth of tilapia (Reque *et al.*, 2010; Belo *et al.*, 2021).

Regarding the parameters of fillet production, it was determined that there are statistically significant differences between the treatments, that is, there was an effect produced by the inclusion of BFM and IL in the diets (Table 8). The diet that produced the best fillet yield was the control diet ($P > 0.05$), with 52.33% fillet by fresh weight, followed by the MAN diet, which had a fillet yield of 51.15%, very close to the control, while the MXP diet and the PRB were considerably different from the first two, as they had a fillet yield of 44.51% and 46.44% respectively. These results are consistent with those presented by (Rojas-runjaic *et al.*, 2011), where morphometric measurements agree for carcass weight, length and fillet yield (Salamanca-Carreño *et al.*, 2017).

This effect can be explained by the inclusion of BFM, which provides antioxidants to the fish, so that it decreases oxidative stress and allows it to produce more muscle mass, thanks to the fact that there is less free radicals, which generate less damage to muscle tissues and promote muscle development (Dhillon *et al.*, 2013).

CONCLUSIONS AND RECOMMENDATIONS

It is concluded that apple bagasse has antioxidant compounds, since the apple is rich in them, and these will serve to avoid oxidative stress in the fish due to oxygen free radicals, improving its productive performance with respect to weight gain, growth in length, feed conversion ratio and fillet yield.

It is concluded that the use of BFM provides antioxidant compounds of the polyphenol family in significant quantities, which improve the productive parameters of the fish, obtaining a better weight gain, growth in length, feed conversion rate and fillet yield, while the inclusion of IL does not produce a positive difference with respect to the control. Therefore, it can be said that yeasts *Kluyveromyces lactis* used in inoculum have a significant negative effect on production parameters.

The inclusion of BMF in the tilapia diet is a good way to attack the pollution produced by apple by-products generated in apple processing industries and give them productive use in aquaculture.

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Table 1. Chemical Composition of Yeast Reactivating Broth

Compound	Content per l of broth1
Meat extract	10.0 g
Soy Peptone	10.0 g
Sodium chloride	5.0 g
Dextrose	5.0 g
Malt Extract	3.0 g
Soluble starch	1.0 g
L-Cysteine	0.5 g

[1] 34.5 g are dissolved in an L of water and heated with stirring until dissolved. It is sterilized at 121 ºC for 15 minutes.

Table 2.- Composition of the diets used in the experiment

Ingredient	Diet1			
	Control (%)	MAN (%)	PRB (%)	MXP (%)
Soy paste	62.9	45.8	62.9	45.8
Vegetable oil	6.2	6.4	6.2	6.4
Milk powder	11.0	10.0	11.0	10.0
Ground corn	12.5	19.6	12.5	19.6
Meat meal	5.0	5.0	5.0	5.0
Premix V & M	0.3	0.3	0.3	0.3
Ca Carbonate	1.1	1.1	1.1	1.1

Salt	1.0	1.0	1.0	1.0
IL	-	-	3.0	3.0
BMF	-	10.0	-	10.0

[1] MAN indicates treatment with 10% BFM, PRB treatment with 3% IL and MXP treatment with both (10 and 3% respectively).

Table 3. Proximal analysis performed on the diets used in the experiment

Treatment1	**Ethereal extract, %**	**Protein, %**	**Ash, %**	**Crude Fiber, %**	**E.L.N., %**
MAN	8.27a	34.31a	8.07a	8.04a	41.30b
PRB	8.49a	34.98a	8.67a	5.03b	42.80ab
MXP	8.77a	33.85a	7.20a	8.16a	41.98b
Control	8.34a	33.51a	6.73a	5.01b	46.39a

[1] MAN indicates treatment with 10% BFM, PRB treatment with 3% IL and MXP treatment with both (10 and 3% respectively).

Different literals between rows indicate statistical difference (P > 0.05)

Table 4. Feed conversion ratio (CAT) of fish per treatment1 during the experiment [2]

Week	**CTL**	**MAN**	**MXP**	**PRB**
1	2.3469 AB	2.1356 b	2.0791 b	2.8724 to
2	1.7044 AB	1.2061 b	1.8873 AB	1.9057 to
3	1.8944 to	1.6103 AB	1.4577 b	1.7866 to
4	1.8958 to	1.0950 d	1.5841 c	1.7500 B
5	1.5593 c	2.2320 to	1.6496 b	2.0565 AB
6	2.0058 C	1.9989 c	2.4841 b	2.9477 to

7	1.8993 to	1.6871 b	1.8531 AB	1.9200 to
8	2.0392 c	2.3483 AB	2.3062 AB	2.4100 to
Average	1.9181 AB	1.7891 c	1.9127 AB	2.2061 to

[1] MAN indicates treatment with 10% BFM, PRB treatment with 3% IL and MXP treatment with both (10 and 3% respectively).
[2] Different literals between columns indicate statistically significant difference in the comparison of Tukey means (P > 0.05)
Table 5. Average weekly weight in grams of fish per treatment1 during the experiment

Week	CTL(g)	MAN (g)	MXP (g)	PRB(g)	E.E.
0	7.14a	7.5a	8.15a	6.87th	0.50
1	8.30ab	9.46a	9.08ab	7.21a	0.52
2	10.54ab	12.65th	11.03ab	10.06b	0.67
3	12.76b	15.78th	14.05ab	12.30b	0.80
4	17.69ab	21.34th	18.26b	17.16b	1.05
5	21.5ab	24.75th	22.22ab	20.14b	1.28
6	25.32ab	27.7a	25.41ab	22.58c	1.50
7	30.25ab	33.44a	30.21ab	27.77c	1.57
8	34.92ab	37.93a	34.34ab	32.42c	1.80

[1] MAN indicates treatment with 10% BFM, PRB treatment with 3% IL and MXP treatment with both (10 and 3% respectively).
[2] Different literals between columns indicate statistically significant difference in the comparison of Tukey means (P > 0.05)
Table 6. Average weekly length in millimeters of fish per treatment1 during the experiment

Week	CTL(mm)	MAN (mm)	MXP (mm)	PRB (mm)	E.E.
0	48.13th	48.90th	49.68th	48.72nd	2.17
1	52.62nd	56.08b	52.90th	52.00a	2.25

2	56.90ab	60.86a	58.29ab	55.44b	2.13
3	62.91ab	65.07a	61.81ab	58.65b	2.18
4	68.03ab	71.38a	67.82ab	65.26b	2.50
5	72.68ab	77.84a	73.06ab	70.48b	2.48
6	76.73ab	81.37a	76.14ab	75.08b	2.26
7	82.60ab	86.51a	82.86ab	80.28b	2.53
8	88.38ab	92.19a	89.51ab	83.84c	2.64

[1] MAN indicates treatment with 10% BFM, PRB treatment with 3% IL and MXP treatment with both (10 and 3% respectively).
[2] Different literals between columns indicate statistically significant difference in the comparison of Tukey means ($P > 0.05$)

Table 7. Survival of fish by treatment during the complete experiment

Treatment1	**Starter Fish**	**Fish at the end**	**Survival**
1.- Control	50	46	92 %
2.- MAN	50	49	98 %
3.- MXP	50	50	100 %
4.- PRB	50	47	94 %

[1] MAN indicates treatment with 10% BFM, PRB treatment with 3% IL and MXP treatment with both (10 and 3% respectively).

Table 8. Average fillet yield of fish per treatment at slaughter

Treatment1	**Fillet yield, %** [2]	**D.E.**
CTL	52.33rd	3.83
MAN	51.15ab	3.84

MXP	44.51c	6.06
PRB	46.44bc	2.71

[1] MAN indicates treatment with 10% BFM, PRB treatment with 3% IL and MXP treatment with both (10 and 3% respectively).
[2] Different literals between columns indicate statistically significant difference in the comparison of Tukey means (P > 0.05)

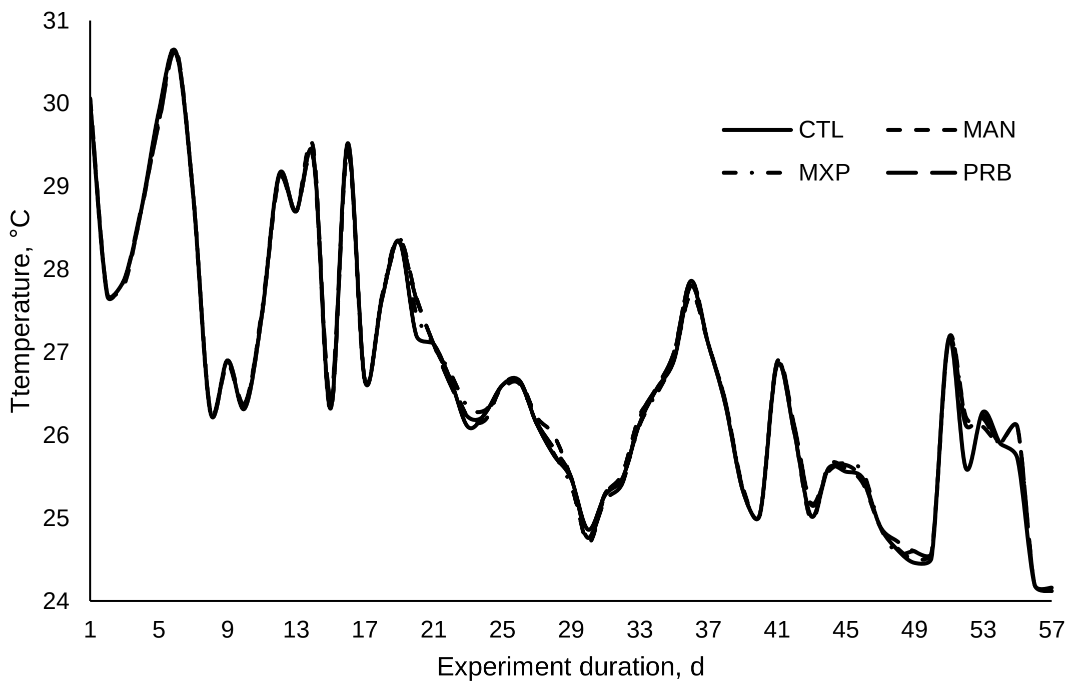

Figure 1. Water temperature of the fish tank system with respect to the time of the experiment.

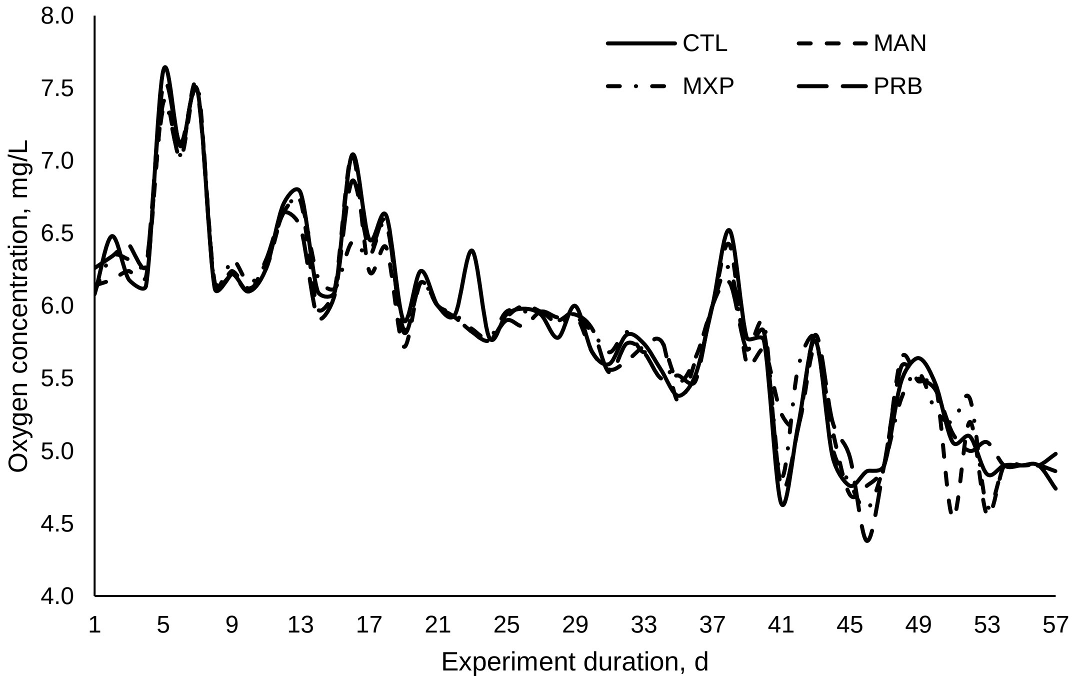

Figure 2. Concentration of oxygen in the water of the fish tank system with respect to the time of the experiment.

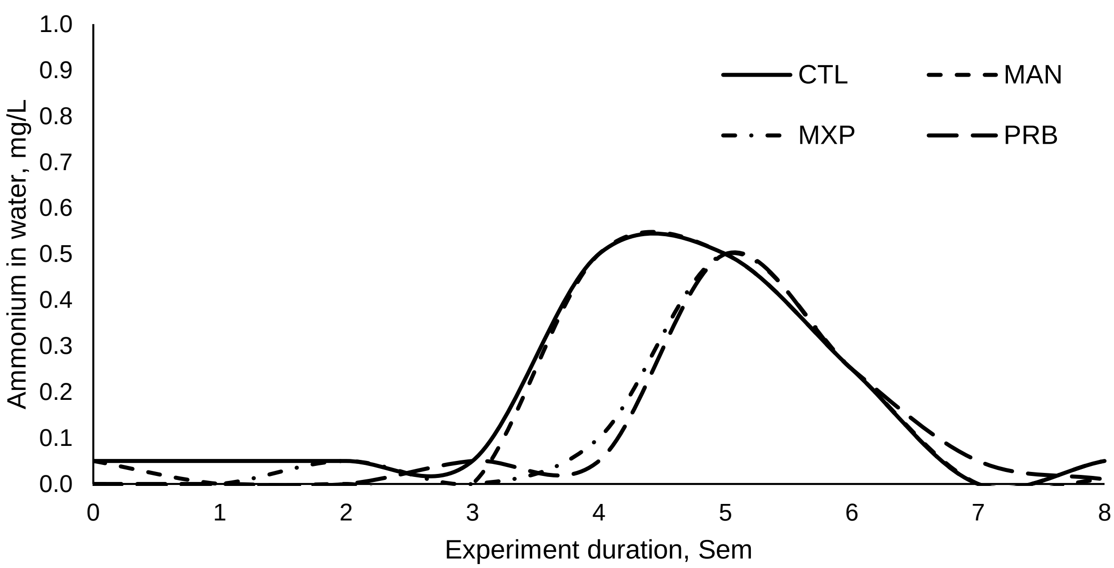

Figure 3. Concentration of ammonium in the water of the fish tank system with respect to the time of the experiment.

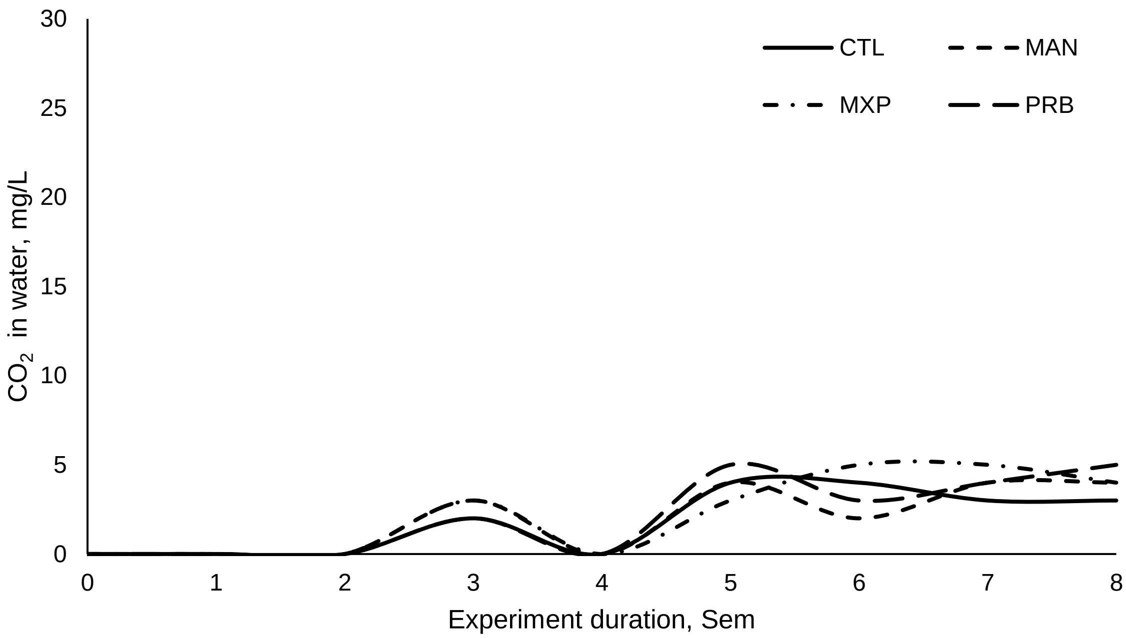

Figure 4. Concentration of CO2 in the water of the fish tank system with respect to the time of the experiment.

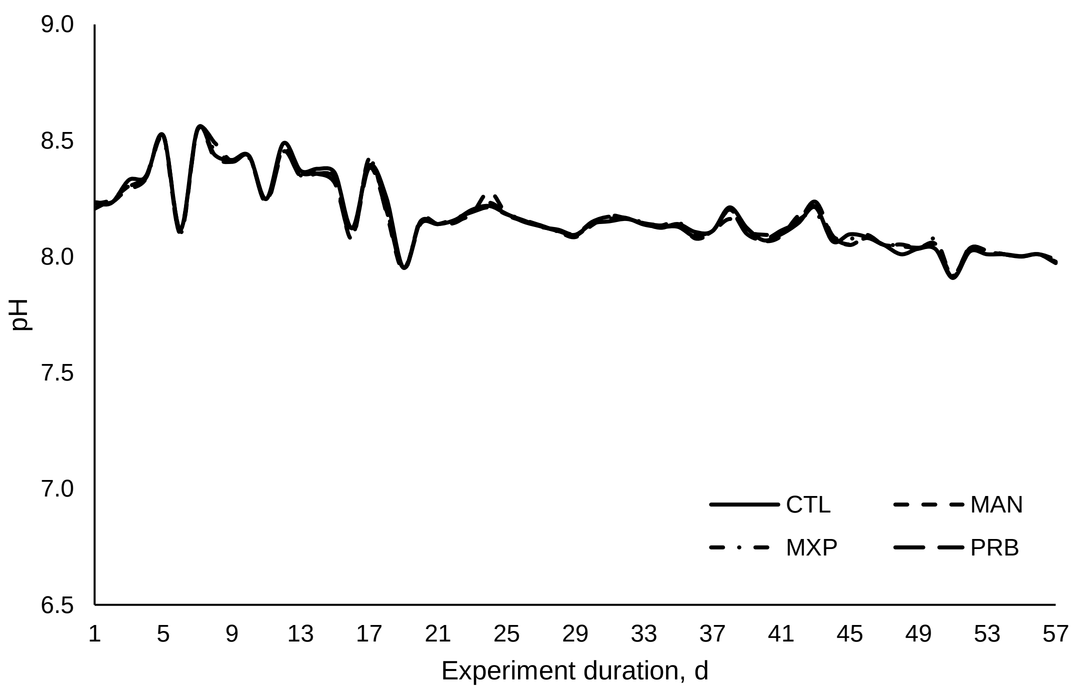

Figure 5. pH of the water in the fish tank system with respect to the time of the experiment.