UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

SUPLEMENTAÇÃO DIETÉTICA DE MICROENCAPSULADO DE ÓLEO ESSENCIAL E ÁCIDO ORGÂNICO EM TILÁPIA-DO-NILO SOB CONDIÇÃO DE ESTRESSE

Autora: Jaísa Casetta
Orientadora: Dra. Eliane Gasparino
Coorientadora: Dra. Stefânia Caroline Claudino da Silva

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Tese apresentada como parte das exigências para obtenção do título de DOUTORA EM ZOOTECNIA no Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá – Área de Concentração Produção Animal.

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Autora: Jaisa Casetta
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TITULAÇÃO: Doutora em Zootecnia - Área de Concentração Produção Animal

APROVADA em 18 de fevereiro de 2022.

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Profª Drª Lorena Batista de Moura

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes.”

(Marthin Luther King)

Aos meus pais, Valdenir Casetta e Arminda Maria Casetta,

Pela vida e por me ensinarem a lutar pelos meus sonhos.

A minha irmã Jamille Casetta,

pelo carinho e amizade.

Aos meus sobrinhos Raíssa Casetta Guidelli e Raul Casetta Guidelli,

por me mostrar a simplicidade da vida.

Ao meu esposo Felipe Sabadini Facina,

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DEDICO

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A todos que, direta ou indiretamente, contribuíram para a realização deste trabalho.

BIOGRAFIA

JAÍSA CASETTA, filha de Valdenir Casetta e Arminda Maria Casetta, nasceu em 04 de julho de 1994, em Maringá no estado do Paraná.

Cursou graduação em Zootecnia na Universidade Estadual de Maringá, no período de 2012 a 2016, com defesa do trabalho intitulado "Avaliação reprodutiva de *Danio rerio* em condições controladas de cultivo", sob a orientação do Prof. Dr. Carlos Antonio Lopes de Oliveira.

Em fevereiro de 2019, obteve o título de Mestre em Zootecnia, pelo Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá, área de concentração Produção animal, com a dissertação intitulada "Efeito da metionina e o risco de predação na fisiologia, genética e sobrevivência do zebrafish (*Danio rerio*)" sob orientação do Professor Dr. Ricardo Pereira Ribeiro e coorientação da Professora Drª. Eliane Gasparino.

Em 14 de janeiro de 2022, submeteu-se a exame de qualificação como pré-requisito para obtenção do título de Doutora em Zootecnia. Em 18 de fevereiro de 2022, submeteu-se a defesa de tese para obtenção do título de Doutora em Zootecnia.

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ABSTRACT

In tilapiculture, the cultivation of single-sex animals is extremely widespread, as it allows for standardization of lots, in addition to improving the general performance of the animals. It is possible, however, that hormonal inversion interacts with other factors, such as environmental and nutritional management, and distinctly modulates the immune response and antioxidant system of animals. To test this hypothesis, an experimental trial was carried out with Nile tilapia larvae six days after hatching, divided into four experimental groups: NI (non-inverted animals), I (inverted animals with 17α-methyltestosterone), NI+M (non-inverted supplemented with microencapsulated (organic acid and essential oil) and I+M (inverted with 17α-methyltestosterone and supplemented with microencapsulate); half of which were subjected to transport stress after 28 days of the experiment. At the end, the survival rate; performance parameters (Gain in weight, standard and total length) were evaluated , feed intake and feed efficiency); gene expression (growth hormone - *GH*, growth hormone receptor - *GHR*, Myostatin - *Mstn* and myogenic differentiation - *MyoD*, heat shock protein - *HSP70*, interleukin 1 beta - *IL-1β* and cyclooxygenase-2 - *COX 2*) by RT-PCR; the activity of catalase (CAT) and superoxide dismutase (SOD) enzymes; carbonylated protein; total antioxidant capacity by DPPH (2,2-diphenyl-1-picryl-hydrazyl) and histology (muscle, liver and intestine). Animals I and I+M had higher growth parameters in the total evaluation period, higher feed efficiency, higher survival rate and muscle length ($p<0.001$). Differences in growth were observed only from the fourth week onwards, with a negative highlight for the NI treatment with the lowest growth in the period ($p<0.0001$). There was no difference between treatments for the number of hepatocytes. The histomorphometric evaluation of the intestine revealed that the greatest height and width was observed in the larvae of the I+M treatment. There was a statistical difference for the *GH* ($p=0.0092$), *GHR*

($p<0.0001$), *MyoD* ($p<0.0001$), *Mstn* ($p=0.0102$), *HSP70* ($p<0.001$), *IL-1β* ($p<0.0001$) genes and *COX-2* ($p<0.0001$). The highest concentration of carbonyl protein was found in the NI treatment ($p=0.0006$). Transport decreased the total antioxidant capacity of DPPH in all treatments ($p<0.001$). The highest SOD activities were observed in groups I and I+M (without transport, $p=0.0004$), and I (with transport, $p<0.0001$). The highest CAT activity was observed in the NI+M treatment (no transport, $p<0.001$). Inversion may represent a potential oxidative stress factor and cause damage to animals in the short, medium, and long term. Dietary supplementation of natural antioxidant compounds represents an interesting alternative to combat such damage. To test this hypothesis, an experimental trial was carried out with inverted and non-inverted Nile tilapia fingerlings, supplemented or not with a blend of organic acids and essential oils protected by microencapsulation. The animals were divided into four experimental groups: NI (non-inverted animals), I (inverted animals), NI+M (non-inverted animals supplemented with microcapsules) and I+M (inverted animals supplemented with microcapsules). Blood parameters were evaluated (WBC - White Blood Cells; LY - Lymphocytes; RBC - Red Blood Cells; HGB - Hemoglobin; HCT - Hematocrit; MHC - Mean Corpuscular Hemoglobin; MCV - Mean Corpuscular Volume and MCHC - Mean Corpuscular Hemoglobin Concentration), oxidative stress markers (catalase enzymatic activity - CAT and superoxide dismutase - SOD; and total antioxidant capacity - DPPH) and gene expression (HSP70 - Heat Shock Protein). The parameters HGB ($p<0.001$) and HCT ($p=0.005$) were reduced beyond the recommended limits for animals in group I. The MCV varied statistically between groups ($p<0.001$), however, all values were within the range recommended for the species, jointly indicating normocytic anemia in group I fingerlings at the time of collection. CAT activity, SOD and DPPH capacity differed statistically between the experimental groups ($p<0.0001$), with lower SOD and CAT activity and higher DPPH in animals supplemented with microencapsulated. Expression of HSP70 was lower in I+MI animals ($p<0.001$). Taken together, these data support the conclusion that sex inversion improves productive performance, immune response, antioxidant profile, reduces protein oxidation in the larval stage, and that microencapsulated dietary supplementation is effective in improving the performance of inverted larvae and decrease protein oxidation in non-inverted larvae. On the other hand, in the fingerlings of inverted animals during larval stage, they have a lower total antioxidant capacity, which reflects a worsening in the hematological and enzymatic

parameters related to immunity; and that microencapsulated dietary supplementation is sufficient to improve the immune response in inverted and non-inverted fingerlings.

Key words: Hormonal inversin; immune response; monosex male herds; *Oreochromis niloticus*; prevention of oxidative damage; supplementation

I – LITERATURE REVIEW

1. Nile Tilapia (*Oreochromis niloticus*)

It is estimated that by 2030, fish farming will account for 60% of all fish destined for human consumption (FAO, 2018), and this promising scenario is due in part to the development and intensification of all stages of development, including precocity, as well as the ease of obtaining larvae during the initial stage of production, especially those of tilapia (Brabo et al., 2016).

Tilapia (*Oreochromis niloticus*) is intensively produced worldwide and is currently the most important species in Brazilian fish farming, accounting for 60.6% of the total fish farming in this country in 2020 (802.930 tons), with a growth of 12.5% (486.155 tons) compared to the previous year (432.149 tons). Being e real highlight of the national scene, tilapia's performance was the best among all farmed fish, putting Brazil as the 4th largest producer in the world in 2020 (PeixeBR, 2021).

Apart from the above, tilapia has ideal zootechnical indices for commercial production, such as short production cycle, ease of reproduction with high prolificacy and fast growth, good feed conversion and weight gain rates (Watanabe et al., 2002). In addition, this species is resistant to different handling techniques, tolerates high densities and low levels of dissolved oxygen (Meurer et al., 2002) and adapts to the supply of artificial feed from the larval stage (Pezzato et al., 2004).

2. Obtaining of Nile Tilapia larvae

Tilapia presents early sexual maturation with an approximate size of 152 mm and a weight of 40 grams in nature without genetic improvement (Peña-Mendonza et al., 2005), with genetic improvement programs for the species, sexual maturation ended up

being delayed (Eknath et al., 2007). While its reproductive management can basically occur in two distinct ways (Rakocy, 1990; SENAR, 2017):

1. Breeding in hapas: The fish are housed in net-tanks called hapas, which allows for better control and easier access to fingerlings under similar conditions, as the eggs can be collected directly from the mouths of the females, ensuring a more efficient hatchery in a controlled environment of incubators.
2. Breeding in nurseries: Animals are allowed to roam free but breeding in this case is not well controlled as animals can breed freely while inspection is more difficult. Progeny collection occurs in loose post-larvae (cloud collection).

Regardless of the chosen system, as long as there are adequate conditions, such as genetics, photoperiod, temperature and available food, tilapia can reproduce naturally throughout the year (Puttaraksar, 2004), following some common steps (Figure 1).

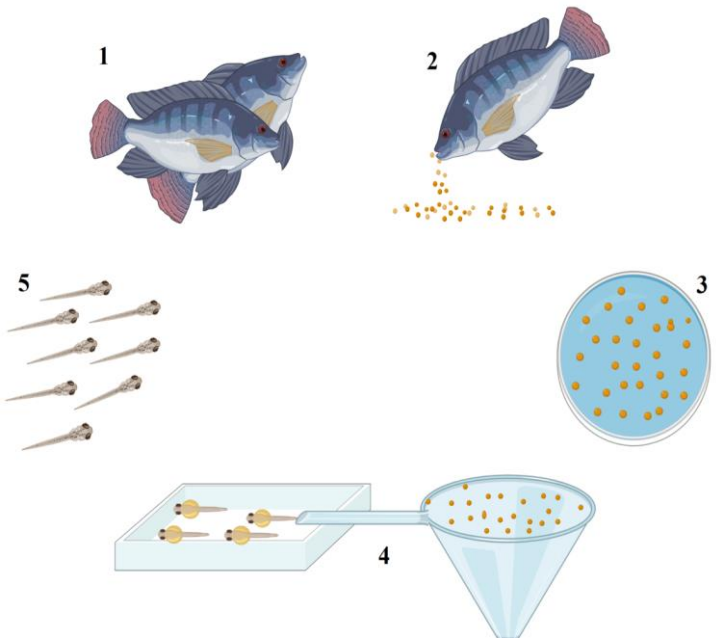

Figure 1. 1: The male attracts the female (pheromone) and induces spawning by pushing the female's abdomen with his mouth. The female responds by laying her eggs immediately. The male fertilizes the eggs with semen. 2: The female collects the eggs in her mouth and cleans them, releasing them and then collecting them again. The female incubates the eggs for 3 to 5 days in her mouth. 3: It is during this period that the eggs are captured and put to the incubator for hatching. The larvae hatch 1 to 4 days after collection, depending on the stage at which they were collected. 4: As the larvae hatch, they begin to swim on the surface of the water and fall into the larval catch tray. Directly after birth, the larvae feed on the reserves existing in the yolk sac and can be kept in the collection tray during this period or until the whole yolk sac is consumed. 5: After the absorption of the yolk sac, the larvae are transported from the collecting trays to nursery tanks, where the sexual inversion treatment with larvae of the same age will take place. SOURCE: Prepared by the author, 2022.

It is worth noting that, although tilapia is a fish of early sexual maturity and high fecundity, the larviculture stage requires a lot of attention as it associated with a high mortality rate (SENAR, 2017).

3. Sex inversion in tilapia farming with 17α-methyltestosterone

Early sexual maturity and high reproductive capacity of tilapia can become a production bottleneck if the animals start to reproduce in the rearing environment before reaching size and weight appropriate for commercialization. Consequently, overpopulations with reduced growth rate and deviations from standards can be observed in lots (Borges, 2009). The use of monosex male herds, a common management method in modern tilapia farming, is an efficient alternative that helps to minimize these problems (Rodrigues et al., 2013; Githukia et al., 2015).

The formation of these monosex herds occurs through sexual inversion of the larvae with 17α-methyltestosterone, a synthetic androgen, supplied with the feed in the first days of the animal's life (Pandian et al., 2003). The sex of tilapia is determined by XX genes for females and XY genes for males. However, environmental factors can interfere in the definition of physiological sex (Piferrer, 2001). During the embryonic period, the morphological characteristics of the germ cells are not yet defined. Thus, exogenous factors interfere with the definition of sex in oogonia or spermatogonia (Fostier et al., 1983).

The amount of hormone ingested by everyone during sexual differentiation determines the success of the inversion. It is important to provide animals with the correct dose of hormone, as excessive amounts can cause overdose or lead to the conversion (aromatization) of androgens to estrogens, and in the absence of androgens the hypothalamus is organized as a female. Insufficient dosage can result in intersex animals (El-greisy et al., 2012). The recommended dose is 60 mg of 17α-methyltestosterone per 1 Kg-1 of feed, previously diluted in 98% absolute ethanol, and the duration of supply is 28 days (Figure 2) (Popma et al., 1990).

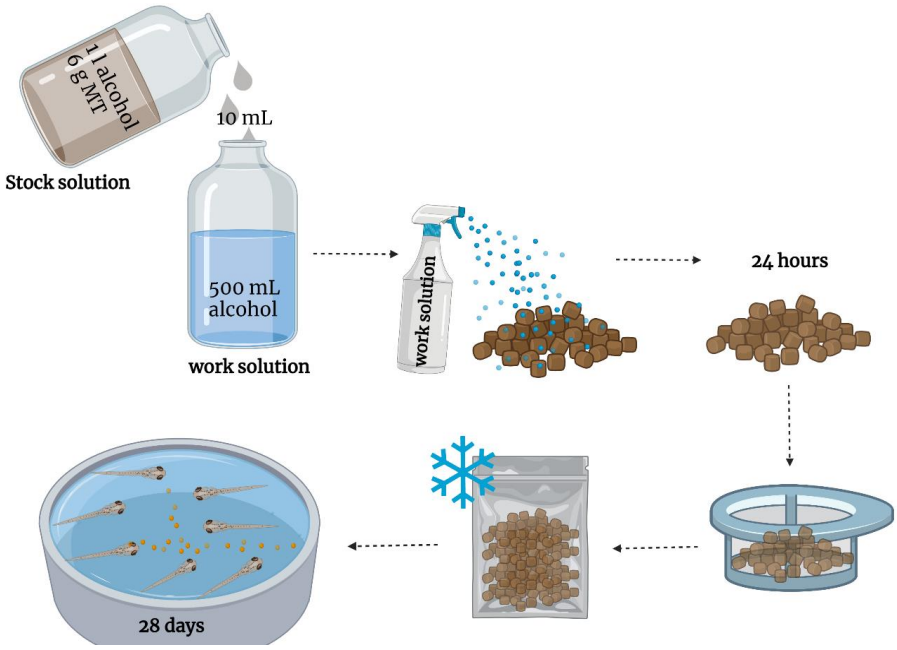

Figure 2. After the absorption of the yolk sac, the sexual inversion treatment begins with the recommended dose of 60 mg of 17α-methyltestosterone (MT) per 1 kg of feed. Preparation of the inversion feed is the following: Dissolve 6 g of 17α-methyltestosterone in 1 liter of ethyl alcohol. This solution is called stock solution and must be kept in a dark environment, avoiding high temperatures. The solution applied to 1 kg of feed must be prepared with 500 ml of ethyl alcohol and 10 ml of the stock solution (this solution is called the work solution) and applied homogeneously to the feed on a smooth surface in a ventilated, shaded, and protected place. The feed should remain for about 24 hours. During this period, the feed should be flipped a few times to allow the lower layers to dry. The feed is then sieved and placed in a plastic bag which should be stored inside a freezer. During preparation, it is necessary to wear gloves and masks to avoid contact with the hormone and the inhalation of gases released because of the evaporation of alcohol. SOURCE: Prepared by the author, 2022.

Inversion with 17α-methyltestosterone is considered a safe and efficient method if it is started soon after the larvae begin to consume exogenous feed (Rothbard et al., 1990; Curtis et al., 1991). After the inversion period, the inverted larvae are transported from the breeding unit to the fattening facilities.

4. Transportation

Fish transportation begins with capture and proceeds with loading, transportation, ending with unloading and storage (Iversen et al., 1998). These steps are inherently stressful and can trigger various physiological responses (Wendelaar-bonga, 1997). Regardless of the breeding phase, transportation represents a major source of stress for the fish, with the potential to increase the production of free radicals and activate the

antioxidant and immune defense system (Pakhira et al., 2015; Hoseini et al., 2019; Dawood et al., 2020).

The fingerlings can be transported in plastic bags (at least 150 microns thick to avoid the risk of tearing) or in isothermal boxes attached to trucks (associated with higher cost and lower mortality) (Oliveira et al., 2007). Prior to transportation, fish should be deprived of food for 24 hours to avoid excessive fecal production which may compromise the quality of water in the transportation bag (Pant et al., 2020). They must be transported during the coolest hours of the day, as high temperatures (30 °C) impair immune defenses and increase mortality (Elkatatny et al., 2020).

Transportation bags must be filled with one third of water and two thirds of pure oxygen injected under pressure (Oliveira et al., 2007). It is recommended to add 1 to 5 g of common salt for every 1 liter of water. Salt in water allows animals to maintain their vital functions in harmony and allows the osmotic gradient between water and fish plasma to equalize, reducing the diffusion of ions into the water (Marengoni et al., 2014). In addition to reducing stress, salt also stimulates mucus secretion in the gill epithelium and prevents the proliferation of fungi, parasites, and infections (Kubitza, 2011).

Upon arrival at the release site, the still sealed bags must be placed in the tank/pond so that the water temperature of the transport bag equalizes with that of the water in which the animals will be released. After the temperature is balanced, the transportation bag can be opened, and water from the tank is slowly mixed into the bag, followed by the careful release of the fish (Marengoni et al., 2014). In boxes, same level of care must be taken by slowly mixing the water from the boxes with that of the release site, and the fingerlings must be released through the discharge pipe of the box itself. If you suspect that there are pathogens in the transportation water, it is recommended not to dispense this water and not to mix it with that of the pond (Oliveira et al., 2007).

Although physiological responses to transportation-related stress appear to normalize after a certain period, depending on the intensity of the stress, chronic changes are possible that increase susceptibility to disease and induce mortality during the recovery period (Iversen et al., 1998; Obirikorang et al., 2020). Prior nutritional and immunological preparation of animals can alter their ability to withstand the transportation-related stress and improve their ability to recover without suffering much physiological damage.

5. Organic acids in fish nutrition.

The search for alternatives to improve the immunity and growth of animals is great, especially in intensive systems, where there is an increased risk of diseases (Segner et al., 2012). Dietary supplementation of bioactive compounds, such as organic acids and essential oils, is a promising alternative to modulate the immune and antioxidant response of aquatic animals, such as Nile tilapia (Ng et al., 2009; Abdel-Daim et al., 2020; Dawood et al., 2020; Jesus et al., 2021).

Any organic carboxylic acid, including fatty acids and amino acids (with an R-COOH general structure) is classed as organic acid. However, not all of these have a direct action on the intestinal microbiota. Organic acids that have antimicrobial activity and protect feed from microbial and fungal proliferation are short-chain acids (C1-C7) such as formic, acetic, propionic and butyl acids or carboxylic acids containing a hydroxyl group, such as lactic, malic, tartaric, and citric acids (Ricke, 2003; Mehdi et al., 2018). Organic acids can be added to water or feed, having great nutritional influence. They possess common characteristics of acidity, water solubility, and ninhydrin negativity (no primary or secondary amines) (Hajati, 2018).

Organic acids have direct and indirect modes of action: they act directly on the quality of the feed and gastrointestinal tract, their indirect action being on the modulation of the metabolome (Lückstädt, 2008). In the diet, they promote the reduction of pH, inhibit the growth of microorganisms, and increase shelf life (Partanen et al., 1999); in gastrointestinal tract, they can modulate gastrointestinal microbiota and thus improve digestion and absorption of nutrients (Partanen et al., 1999; Lückstädt, 2008). Finally, in metabolome, due to their bioactive compounds, they can modulate in a different way the expression and activity of several enzymes, and, therefore, of their metabolic products (Dibner et al., 2002). The response to dietary supplementation of these bioactive compounds, however, does not depend solely on the supplementation itself, but on the interaction with other factors, such as age, presence or absence of stressors and sex of the animal (Cetin et al., 2011; Upadhaya et al., 2016).

In fish, direct and indirect actions of organic acid have been described (Ringo et al., 2010; Hassaan et al., 2020), with an improvement of feed efficiency (Zhou et al., 2009; Elala et al., 2015), and growing parameters (Chen et al., 2017; Hassaan et al., 2018).

5.1 Direct action of organic acid on feed quality:

Organic acids are considered preservative agents used to protect food from fungal and microbial proliferation during storage, thus preventing its deterioration (Kum et al., 2010; Ng et al., 2017). Food preservation occurs by reducing the pH, and this reduction depends on the pKa (pH at which the acid is half dissociated) of the acid and the pH conditions of the gastrointestinal tract (Kin et al., 2015). Organic acids used as zootechnical additives have a pK between 3 and 5 and can be found in solid and liquid form (Dibner et al., 2002; Lückstädt, 2008). The lower the pK of the acid, the greater its tendency to ionize, that is, to reduce the pH (Ng et al., 2017).

5.2 Direct action of organic acid on the gastrointestinal tract:

The possible mode of action of organic acids in the gastrointestinal tract of fish includes 1: Selection and modulation of intestinal microbial activity; and 2: Reduction of gastric pH, which can improve the efficiency of digestive enzymes and increase solubility during the digestion process (Wet, 2005; Lückstädt, 2008).

1: In the gastrointestinal tract, organic acids act by inhibiting and balancing bacterial growth in the medium portion. Organic acids can change their form from non-dissociated to dissociated, depending on the pH of the medium (Partanen et al., 1999). Dissociation occurs specifically in the carboxyl group present in its structure, which releases protons capable of passively penetrating the membrane of pathogenic organisms through diffusion and thus modifying the electrochemical balance of the intracellular medium (Cherrington et al., 1991) (Figure 3). By altering the ionic concentration gradient, it compromises cytoplasmic pH balance, substrate transport and macromolecule synthesis, which can lead to bacterial death (Lückstädt, 2008). This antimicrobial action occurs mainly in gram-negative bacteria, which have a thinner layer of peptidoglycans that facilitates the entry of ions and protons (Dibner et al., 2002). Acidification also decreases the ability of gram-negative bacteria to adhere to the intestinal wall (Bellaver et al., 2004).

2: The dissociation of protons in the initial portion of the gastrointestinal tract acts on pepsinogen, facilitating its initial conversion to pepsin, and augmenting the unfolding of quaternary and tertiary structures of proteins, which improves digestion and results in greater use of feed nutrients (Lückstädt, 2008).

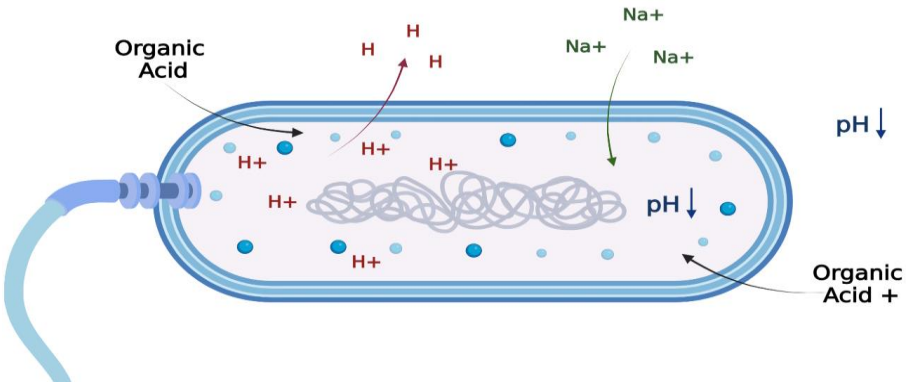

Figure 3. Organic acid enters the interior of the cell of gram-negative bacteria and dissociates, changing the internal pH. In response, the bacterium needs to pump out the protons and capture sodium ions to try to balance the low level of internal pH. The fact that it also reduces the pH outside the cell favors the entry of protonated organic acid, which is the active type. This process consumes a lot of energy, and the result is the death of the bacterial cell by energy depletion. SOURCE: Prepared by the author, 2022.

5.3 Indirect action of organic acid on the modulation of the metabolome:

In addition to having a direct effect on gram-negative bacteria, organic acids also provide a favorable medium for the multiplication of beneficial bacteria that can drive the absorption of nutrients from food (Ng et al., 2017). These bacteria, in turn, provide supplemental enzyme activity to the host, contributing to nutrition (Ray, 2012). In addition, organic acids can also have an energetic function, as observed in short-chain acids, and can be used in the Krebs cycle to generate ATP and provide a considerable amount of energy (Nelson et al., 2018). Citric and fumaric acids, for example, have the ability to provide approximately the same amount of energy (ATP) that is produced from glucose, and can be compared with glucose in terms of energy, playing an important role in the metabolic production of energy (Mroz, 2005; Freitag, 2007).

5.4 Benefits of organic acids in fish production:

Examples of some benefits of organic acids in fish production:

Citric acid: Is used in the acidification of diets, due to its high buffering capacity and flavor. It acts as an antimicrobial agent and stimulates fish feeding. As it is a strong chelator of calcium and phosphorus, it hydrolyzes the phytate, increases the efficiency of endogenous and exogenous phytases (providing an ideal pH in the intestine), and improves the bioavailability of minerals. It also can improve feed conversion in fish (Shah et al., 2015).

Formic Acid: Improves performance (weight gain, specific growth rate, and absolute weight gain) and muscle centesimal profile in fingerlings (Shafique et al., 2019).

Fumaric Acid: Effective to promote growth, improve intestinal morphometry, and decrease gram-negative bacteria in juveniles (Das Neves et al., 2022).

Sorbic Acid: Study with microencapsulated containing citric and sorbic acid, thymol, and vanillin, improve specific growth rate, feed conversion rate, increased protein, and lipid efficiency, thus improving growth and feed utilization (Pelusio et al., 2020).

6. Essential oils in fish nutrition.

Essential oils (EO) are natural multicomponent extracted from vegetable raw materials, with volatile, lipophilic, odorous, and liquid characteristics (Edris, 2007). The function of these plant-based secondary metabolites is believed to be the attraction of pollinators and evasion of pathogens thanks to their insecticidal, antiviral, antibacterial and antifungal effects (Bakkali et al. 2008). The composition of an EO can vary from 20 to 200 components, which are named according to their concentration in the composition, such as: main constituents (20 to 95%), secondary constituents (1 to 20%) and residual components (below 1%) (Bakkali et al., 2008).

They have antibacterial (Cunha et al., 2018), antioxidant, digestive (Farag et al., 2022), anthelmintic (Souza et al., 2020) and immunomodulatory properties in specific and non-specific immune defenses of fish (Elumalai et al., 2020). The antimicrobial action is the result of a series of events involving the entire bacterial cell, and due to structural differences in the cell wall, gram-positive bacteria are more susceptible to the effects of EO than gram-negative bacteria (Trombetta et al., 2005). The effect of EO on bacteria can include cell wall degradation, damage to the cytoplasmic membrane, damage to membrane proteins, decrease in proton motive force and ATP synthesis (Bouyahya et al. 2017). Carvacrol, menthol, thymol, geraniol, linalool, linalyl acetate, piperitone and citronellal are terpenoids that have antibacterial activity via functional group that acts on the bacterial outer membrane, thus altering the fluidity, permeability, and membrane protein, as well as periplasmic enzymes. At the same time, cinnamaldehyde, isoeugenol, eugenol, vanillin and safrole are phenylpropenoids have antimicrobial activity due to free hydroxyl group and normally act on the membrane, participating in the production of ATP, in the transport of ions and in the modification of the fatty acid and lipid profiles of bacteria (Nazzaro et al. 2013).

Dietary supplementation with EO in fish has been used mainly as means of prevention (Sutili et al., 2018). Their supply to the animals can be through water (a large amount is required) or in the feed (reduces waste when compared to application though water, however, it can affect palatability). An important point to be considered is the stability of the EO in the preparation of the feed, its storage and digestion as part of the diet, since changes in its structure can cause them to lose their effect (Sutili et al., 2018).

6.1 Example of some of the benefits of essential oils in fish

Examples of some benefits of essential oils in fish production:

Carvacrol: Melhora o crescimento e imunidade de peixes, e promissor para controlar infecções fúngicas emergentes (Mahboub et al., 2020).

Clove: Potente antioxidante através do aumento da atividade das enzimas antioxidantes, reduzindo a peroxidação lipídica. Efeito imunoestimulante ao induzir marcadores imunológicos em resposta a infecção (Abdelkhlek et al., 2020).

Thymol: Fornece aos animais benefícios através da supressão de radicais livres e constituintes nocivos pela interação com componentes biológicos. Os benefícios animais são: fatores anticancerígenos, antimicrobianos, antioxidantes, antivirais, anti-inflamatórios, imunomoduladores, equilíbrio da microbiota, aumento da digestão, metabolismo e absorção de nutrientes (Alagawany et al., 2021).

Vanillin: Uma mistura de ácido cítrico, ácido sorbico, timol e vanilina forma capazes de induzir uma reconfiguração funcional do microbioma intestinal e promover uma diminuição de vários fatores de inflamação e funções homeostáticas (Busti et al., 2020).

7. Microcapsules

Thinking about structural protection and aiming to improve the efficiency of EO and organic acids, coating technologies that allow the protection of these compounds and their release exclusively in the intestine of animals, such as nanotechnology, have been adopted. Nanotechnology systems include:

- Nanoemulsions: Dispersion of oil stabilized by surfactants (Anton et al. 2008).
- Nanocapsule or microcapsule: A thin layer of polymer surrounds an oily core with the compound dissolved inside (Figure 4) or compound adsorbed or dispersed in the polymer wall (Vauthier et al., 2009).
- Nanospheres: Spheres formed by a polymeric matrix where the compound can be

retained or adsorbed. There is no oil in the composition (Schaffazick et al. 2003).

Microencapsulation allows a slow and continuous release of components along the digestive tract, thus increasing luminal availability throughout the entire intestinal tract (Meunier et al., 2006; Silva et al., 2017). This technique also allows EO and organic acids to have better stability in the feed and facilitates its supply to animals (Meunier et al., 2007; Spanghero et al., 2009). The technique of microencapsulation of essential oil and organic acid has already been used in the feeding of Rainbow Trout (Pelusio et al., 2020; Huyben et al., 2021).

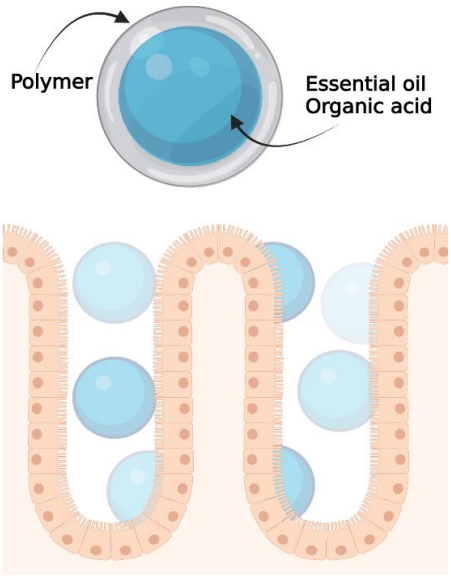

Figure 4. Illustrative scheme showing the structure of microencapsulated essential oil and organic acid in the intestine. Source: Prepared by the author.

8. Body growth in fish

The Nile tilapia Tilamax lineage was introduced in Brazil in 2005 through a partnership between the State University of Maringá and the WorldFish Center (Júnior et al., 2008) and is known for its high production performance and rusticity (Khaw et al., 2012). In genetic improvement programs, the growth rate is one of the main selection objectives (Rutten et al., 2004).

Fish growth is mediated by several pathways, such as the hypothalamic-pituitary axis through the growth hormone (GH), prolactin and somatotropin families, which are also involved in feeding behavior control, immune function, metabolism, and osmoregulation (Company et al., 2001; Kawauchi et al., 2006). Furthermore, animal growth is influenced by several physiological processes, such as food intake, digestion, absorption, assimilation, and excretion. Teleost fish, such as tilapia, have indeterminate

growth, with length and body mass constantly increasing. However, this growth occurs at a slow rate until reaching senescence stage or death (Kawauchi et al., 2006).

GH initiates many of its growth promoting actions by binding to GH receptors (GHR) and stimulating the synthesis and secretion of insulin-like growth factor (IGF) (Figure 5) (Reindl et al., 2011). In addition, GH stimulates the uptake of triglycerides (TG) in skeletal muscle, which can be stored as TG or broken down to release energy (Oscarsson et al., 1999; Khalfallah et al., 2001). When activated by GH, both IGFs (IGF-1 and IGF-2) and GHR act as strong regulators of animal growth, in addition to inducing an anabolic effect on protein and carbohydrate metabolism (Perez-Sanchez; Le-Bail, 1999; Amin et al., 2019). IGF-1 and IGF-2 are mainly produced in the liver, which is the main endocrine source of IGFs, and IGF-1 regulates differentiation, growth, and reproduction (Hossner et al., 1997).

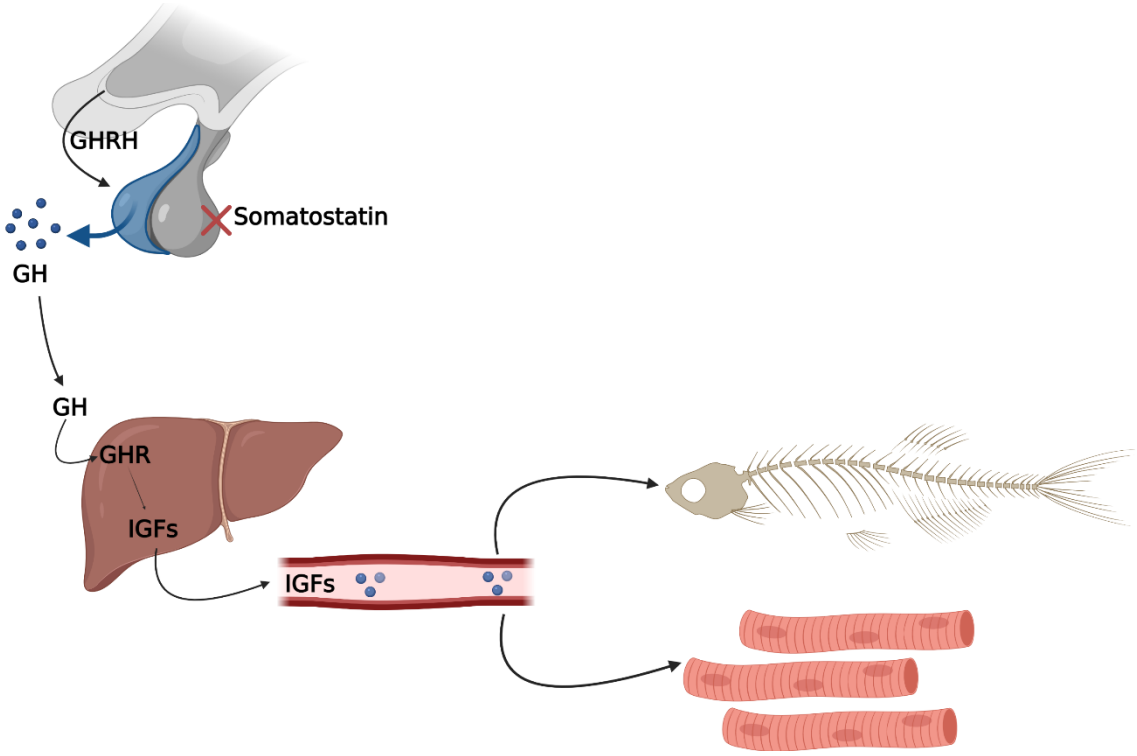

Figure 5. Growth hormone-releasing hormone (GHRH) stimulates GH secretion, while somatostatin (growth hormone-inhibiting hormone) exerts an inhibitory action. GH binds to a specific receptor (GHR) in tissues such as liver, skeletal muscle, cartilage, bone, and other tissues that secrete IGFs. The latter can act locally or enter the bloodstream, but for adequate growth, both circulating IGF (of liver origin) and IGFs produced in tissues are essential. Source: Prepared by the author.

In fish, muscle formation includes growth by hyperplasia (an increase in the number of cells) and hypertrophy (increase in cell size) (Mommsen, 2001). Skeletal muscle development is related to growth performance (Cai et al., 2018; Shi et al., 2017).

Hyperplastic growth of muscle (a process called myogenesis) requires the action of several proteins in the process of cell proliferation and differentiation. This, in turn, can occur through the differential expression of myogenic regulatory factors (*MRFs*) such as myoD, myogenin, myf5 and mrf4 (Hinits et al., 2009), acting on the activation and inhibition of other genes in the muscle differentiation pathway (Figure 6) (Bentzinger et al., 2012).

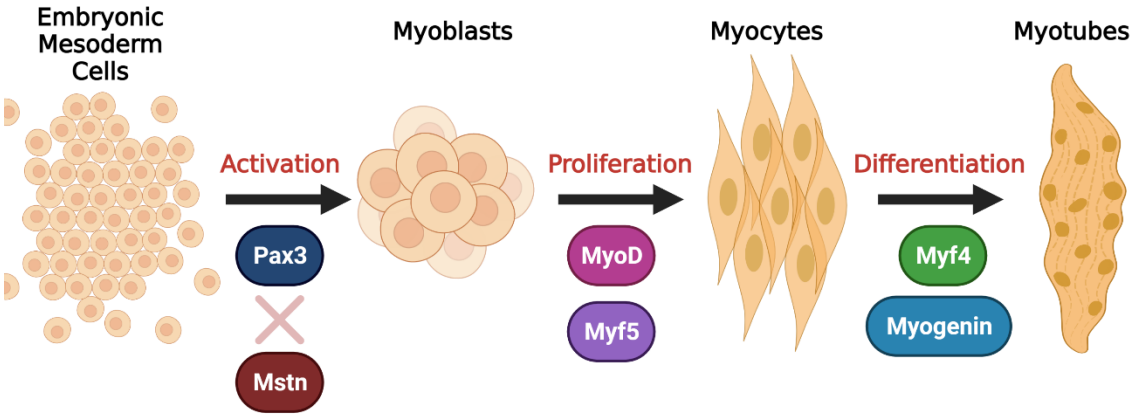

Figure 6. Myogenesis process: Recruitment of mesoderm cells through the action of the Pax3 gene, which is a transcription factor that initiates the activation of mesoderm cells and differentiates them into myoblasts. These proliferate and transform into muscle cells under the action of MyoD and Myf5, transforming into mycocytes. Through the action of myogenin and MRF4, they differentiate into myotubes, forming muscle tissue. The process of blocking myogenesis occurs through the action of myostatin (Mstn), which inhibits the expression of Pax3, reduces the expression of MyoD and blocks myogenesis. Source: Prepared by the author.

The *MyoD* gene regulates skeletal muscle differentiation and has a positive effect on fish growth (Alami-Durante et al., 2010; Shi et al., 2017). In contrast, myostatin (*Mstn*) is a negative regulator of skeletal muscle growth (Gabillard et al., 2013). Together, these pathways produce a dynamic balance of positive and negative signals that determine muscle development (Johnston et al., 2011).

The GH (Figure 7) binds to its GHR receptor on muscle fibers, induces phosphorylation and activates an enzyme called Janus Kinases (JAK 2) associated with the intracellular part of the receptor (Lanning et al., 2006). JAK 2 phosphorylates specific regions of the receptor, which will serve as an anchoring site for STAT (translational signals and activators of transcription) molecules, which translocate to the nucleus to induce the expression of several genes, including insulin-like growth factors (IGFs) (Duan et al., 2010). In addition, the GH also induces the signaling pathway of phosphatidylinositol 3-kinase (Pl3K) and mitogen-activated protein kinase (MAPK).

Pl3K activates Akt which, in turn, activates MyoD. However, if myostatin is present, it inhibits hyperplastic myofibrillar growth through SMAD phosphorylation and inhibition of PI3k/Akt. Akt acts by phosphorylating mTOR which activates the transcription of several genes including genes of the IGF system (Annunziata et al. 2011). MAPK interacts with mTOR, and these are both crucial for the regulation of important cellular functions in responses to mitotic stimuli (Robinson et al., 1997).

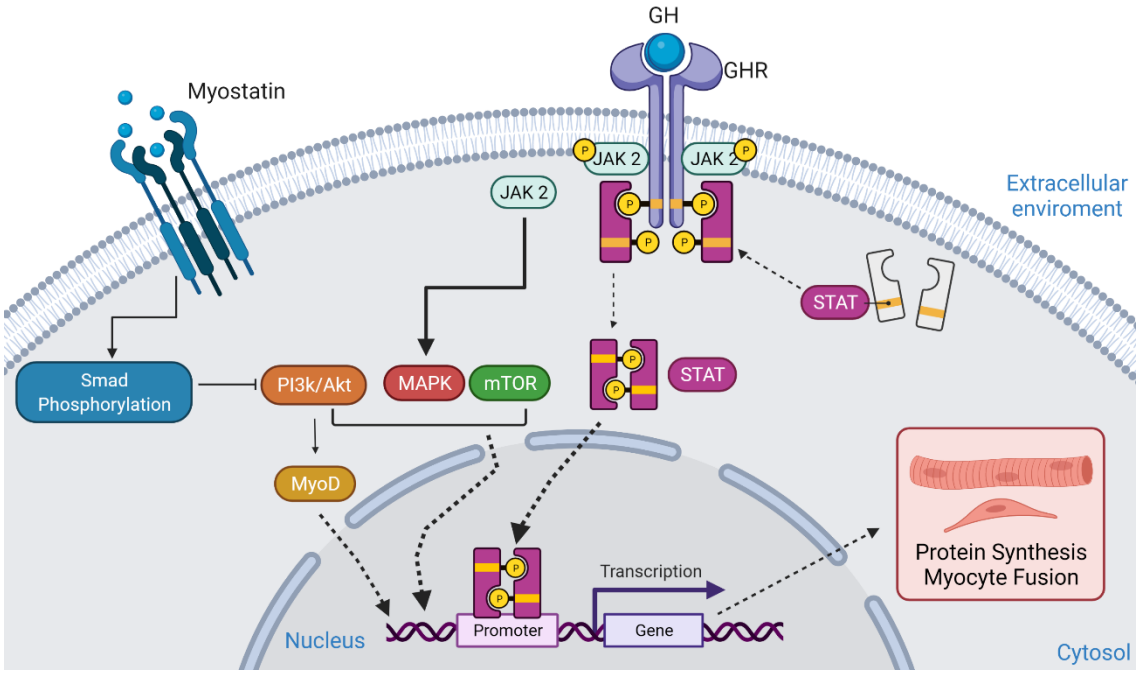

Figure 7. GH's action on muscle fibers: the GH binds to its receptor, activating the JAK 2/STAT pathway. Directly and indirectly, the GH acts on MyoD in a way which is dependent on IGF1 stimulation. Myostatin inhibits hyperplastic myofibrillar growth through SMAD phosphorylation and PI3k/Akt inhibition. The coordinated action of GH, GHR, GH-dependent IGF1 and MyoD activate nuclear transcription factors resulting in hypertrophic myofibrillar growth. Source: Prepared by the author.

9. Immune system

The immune system is responsible for protecting against and responding to pathogens in the environment in which the animal lives. Fish immunity can be divided into innate/non-specific and adaptive/acquired or specific (memory) immunity (Figure 8) (Iwama and Nakanishi, 1996). The innate defense mechanism is the first barrier that protects the animal from the environment. It acts quickly, building defense regardless of the recognition of the invader's structures, blocks their entry or eliminates them before they cause any damage to the animal (Fernández et al., 2016). Adaptive defense is more specific. It can recognize the pathogen and can trigger antibody-dependent (humoral

response) or cell-mediated responses, both with immunological memory (Magnadóttir, 2006).

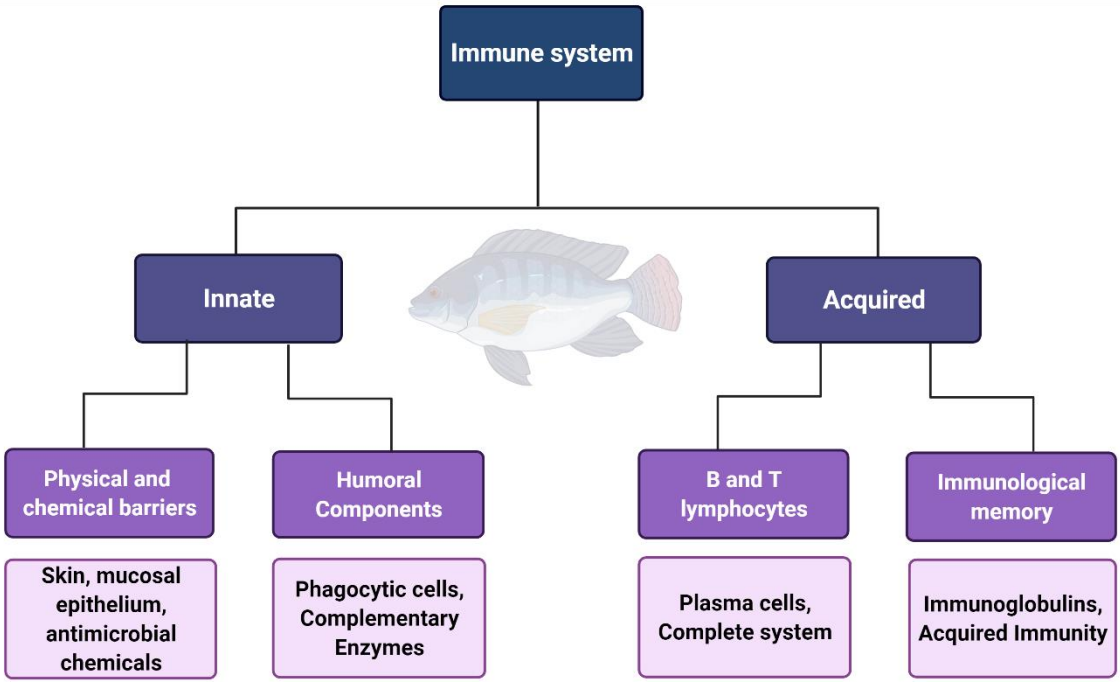

Figure 8. Fish immune system scheme divided into innate/non-specific and adaptive/acquired or specific immunity (memory). Source: Prepared by the author.

9.1 Fish immune system cells

Hematological evaluation is very important for the fishing industry, as it allows the detection of physical and chemical changes that occur in the animal organism (Adeyemo et al., 2009; Fazio, 2019). In addition to indicating the health status of fish, understanding the hematological parameters can also indicate responses related to nutritional status (Lim and Klesius, 2003), age (Jamalzadeh et al., 2009), sexual maturity (Vázquez and Guerrero, 2007), photoperiod (Leonardi and Klempau, 2003), water quality (Fazio et al., 2012), stress (Cnaani et al., 2004) and microbial infection plus parasitism (Jamalzadeh et al., 2009). In this way, blood tissue reflects information about the general metabolism and physiological state of animals that can affect growth and performance.

Fish blood is made up of three main types of cells: white blood cells or leukocytes (WBC); red blood cells or erythrocytes (RBC); and platelets or thrombocytes (PLT). Leukocytes participate in the cellular response and include the different types of white blood cells observed in the blood. Among these cells that make up the WBC are lymphocytes (LY), granulocytes (GR) (neutrophils, basophils, and eosinophils), monocytes/macrophages and cytotoxic cells (Tavares-dias and Moraes, 2004). LY and

GR participate in the defense of innate and adaptive immunity (Figure 9) (Mohr and Liew, 2007).

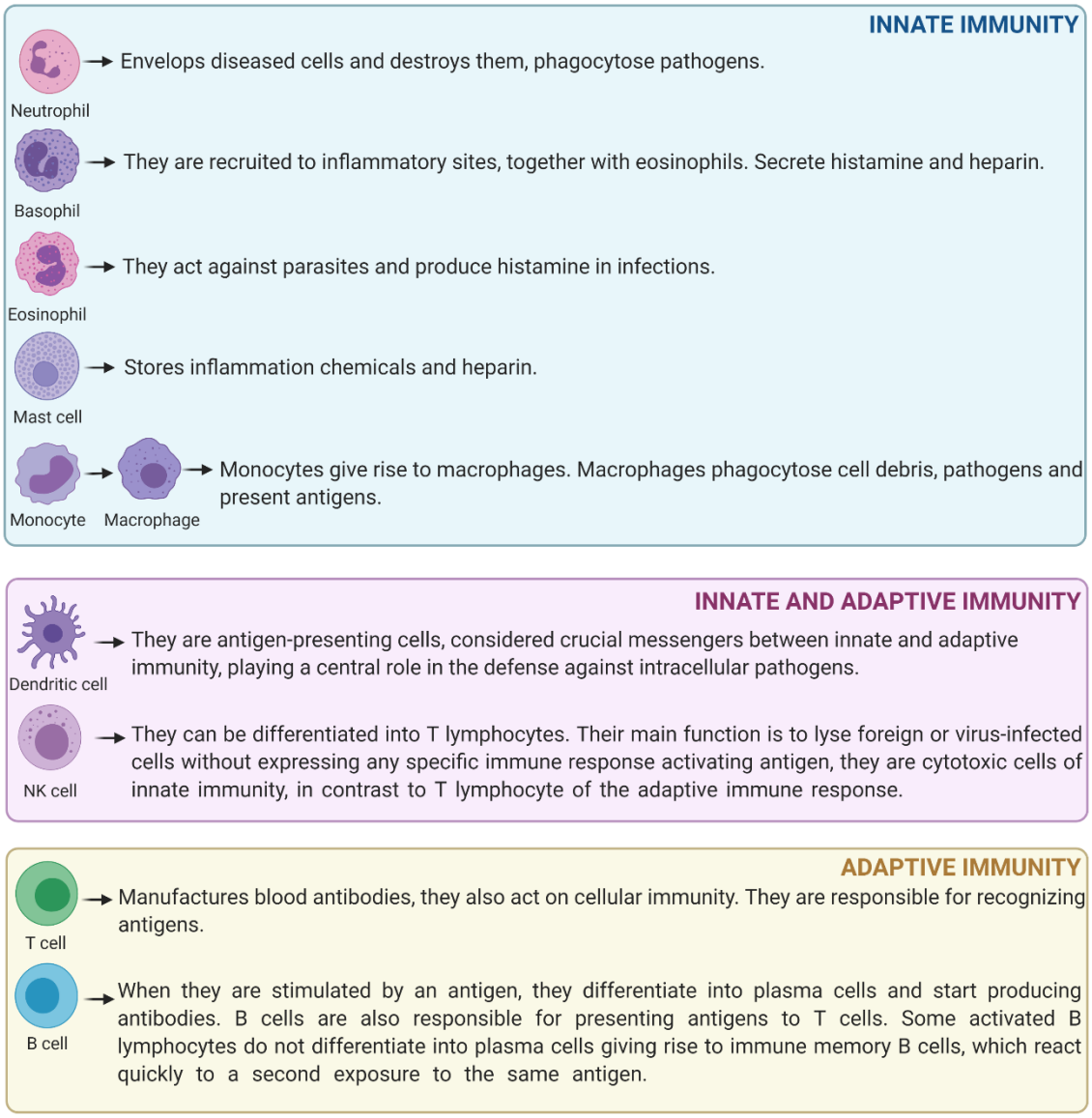

Figure 9. White blood cells that participate in innate/nonspecific and adaptive/acquired or specific (memory) immunity. Source: Prepared by the author.

9.2 Main blood parameters analyzed in fish

The hemogram is a set of analyzes divided into erythrogram, leukogram and thrombogram. Erythrogram: erythrocyte count, hematocrit determination, hemoglobin rate, mean corpuscular volume (MCV), mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration (MCHC); Leukogram: relative and absolute leukocyte count; Thrombogram: thrombocyte count (Figure 10) (Paiva et al., 2013).

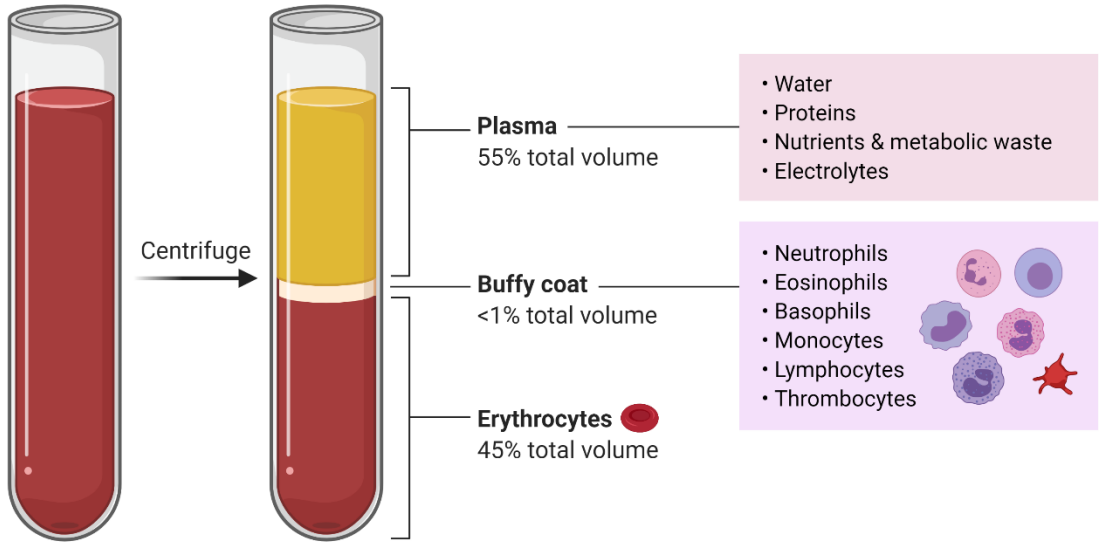

Figure 10. Blood after centrifugation. The first layer is plasma or serum, the middle layer is composed of leukocytes and thrombocytes and the lower red layer is composed of erythrocytes, which represent approximately 45% of the total blood volume. Source: Prepared by the author.

9.2.1 Erythrogram

- **Erythrocyte/Red blood cell (RBC) count**

RBC are the most abundant cells in the circulation and have the function of transporting gases to cells and tissues. However, other functions have been attributed to these cells, such as: interaction with the immune system (Morera and Mackenzie, 2011). Shen et al (2018) suggest that these cells are capable of functional responses in relation to viral infections. Due to the large number of elements in the blood, it needs to be diluted to be analyzed. For this, special solutions are used that do not change the morphology of the cells. Normally, globules are counted in the Neubauer chamber, using the following formula (Paiva et al., 2013): Number of erythrocytes = Number of cells counted X dilution X height between coverslip and chamber X number of squares counted.

- **Hematocrit (HTC) determination**

Hematocrit is the percentage of volume occupied by erythrocytes in the blood in relation to the number of leukocytes, thrombocytes, and plasma, that is, in the total blood volime. This analysis is performed with a heparinized microcapillary tubes filled 2/3 of its total volume, sealed (only at one end) and centrifuged in a microcentrifuge suitable for hematocrit. After centrifuging it is possible to see the three layers of the blood (Figure 10). For reading, a chart measuring hematocrit values is used (Goldenfarb et al., 1971)

Hemoglobin rate (HGB)

HGB, in addition to being the main protein of red blood cells, allows the transport of oxygen by the circulatory system and is an easy way to check the occurrence of anemia. To perform the analysis, whole blood is used, assing a solution with potassium ferricyanide and potassium cyanide (calculate correction factor by the standard curve of the solution). These components combine to produce a stable pigment, where the color intensity of the mixture is determined in a spectrophotometer or photoelectric colorimeter (Collier and Thome, 1994).

Hemoglobin rate = sample absorbance X correction factor (expressed in $g\ dL^{-1}$)

- **Mean corpuscular volume (MCV)**

MCV can indicate the state or size of erythrocytes and reflects abnormal or normal cell division during erythropoiesis (Rodgers and Young, 2018).

MCV = Hematocrit (HTC) / number of erythrocytes (RBC) X 10

- **Mean corpuscular hemoglobin (MCH)**

Establishes the amount of hemoglobin in each erythrocyte.

MCH = Hemoglobin rate (HGB) / number of erythrocytes (RBC) X 10

- **Mean corpuscular hemoglobin concentration (MCHC)**

Relates the concentration of hemoglobin by erythrocytes.

MCHC = Hemoglobin rate (HGB) / hematocrit (HCT) X 10

9.2.2 Leukogram

- **White blood cells or leukocytes (WBC)**

The WBC has the function of protecting the organism: if an infection develops, they attack and destroy the organism that is causing the problem. In this way, WBC can be an indicator of health in fish and are exclusively involved with immunity (Shen et al., 2018).

Leukocytes can be diluted and counted manually in a Neubauer chamber or in automated hematology counters (which normally already analyze all parts of the blood count). These devices count the total number of leukocytes and differentiate them. The individual count is the most useful value for interpreting changes in the animal organism. This information can be determined by evaluating a blood smear (Figure 11).

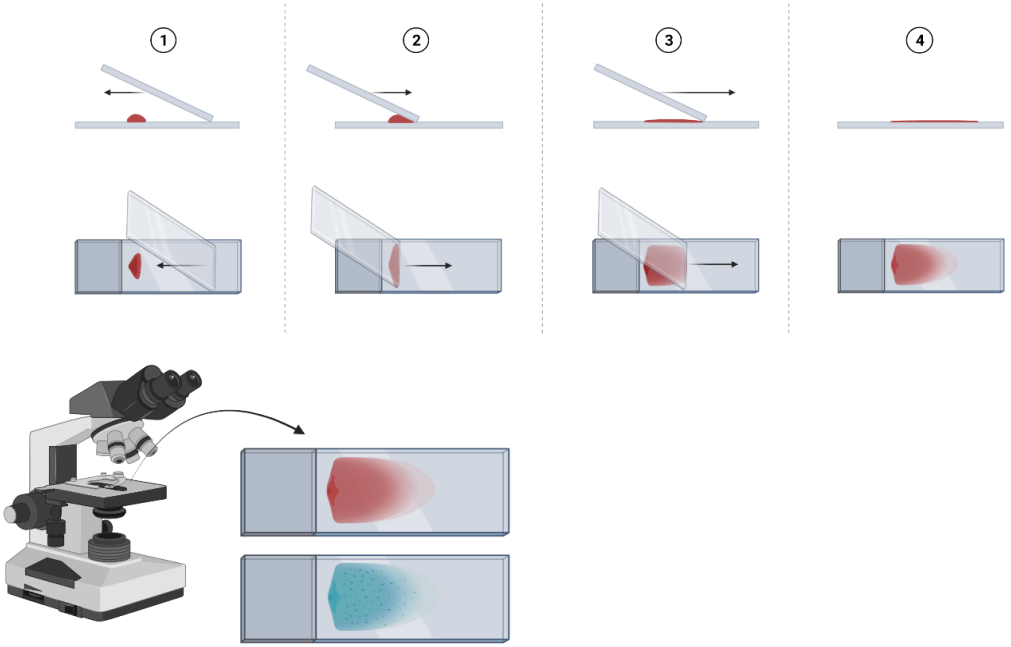

Figure 11. Blood smear for differential leukocyte count. A drop of blood is placed near the end of the first slide, with a second slide positioned at an angle of 45°C in front of the blood drop. In this way, it is brought back against the blood drop and is propelled to the end of the first blade. These slides are then stained with specific dyes and analyzed under an optical microscope. Source: Prepared by the author.

After the leukogram, different white cells can be observed, each with different functions and interpretations (Tizard, 2018):

CELL	INCREASE	DECREASE
Lymphocites (LY)	Viral infections, lymphocytic leukemia, and post-vaccination.	Elevated levels of glucocorticoids, disruption of lymphatic circulation, lymphosarcoma (tumor), lymphocyte hemoparasites.
Neutrophils	Inflammatory reactions, bacterial infections, injuries.	Serious infections and drug response.
Basophiles	Allergic reactions and hypersensitivity, presence of parasites.	Endogenous and exogenous glucocorticoids.
Eosinophils	Parasite infections, regulation of allergic reactions, inflammatory granulomas (fungi, foreign bodies), eosinophilic leukemia and neoplasms.	Constant stress and endogenous cortisol secretion.

Monocytes	Regulation of inflammatory responses, need for phagocytosis and tissue necrosis.	No stimulus.

9.2.3 Thrombogram

- **Platelets or thrombocytes (PLT)**

In addition to their importance in blood clotting, in fish they may play a role in the defense system, having a phagocytic action (Fazio, 2019). The thrombocyte count is performed by blood smear.

10. Indirect immune response in fish (oxidative stress).

In fish, just like in other animals, reactive oxygen species (ROS) are highly active moieties produced within the cell during cellular metabolism or under pathological conditions. The imbalance between production and extinction of these reactive substances through antioxidant mechanisms causes oxidative stress. To ensure this balance, animal's body has developed a defense mechanism involving enzymatic and non-enzymatic molecules. This mechanism is called antioxidant defense system (Wendelaar-bonga, 1997).

This system consists of the interaction of some enzymes, proteins, and low molecular weight molecules, in which the enzymes catalytically remove the reactive species, aided by other molecules participating in the system with temporary or final electron acceptors. Superoxide radical, for example, is dismutated by the enzyme superoxide dismutase into hydrogen peroxide, which, in turn, is degraded by the enzyme catalase or glutathione peroxidase (Figure 12) (IGHODARO and AKINLOYE, 2018).

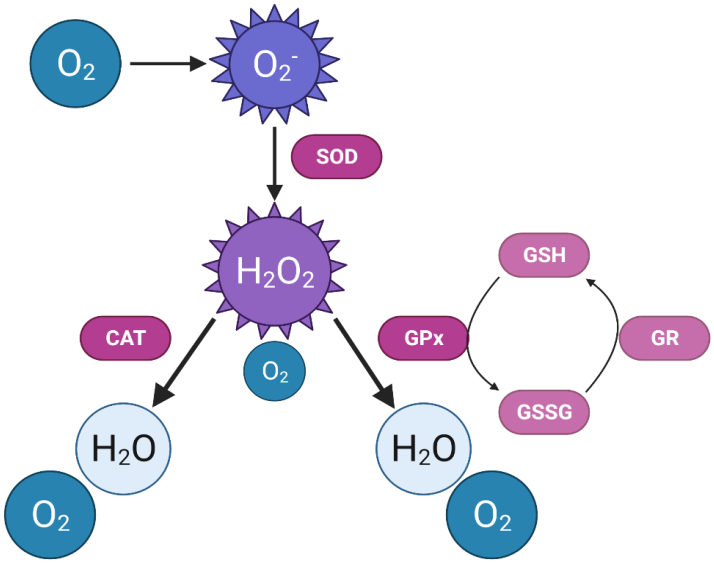

Figure 12. Superoxide anions ($O_2^{\cdot -}$) is formed in view of the reduction of O_2, and from this reactive species, all the others are generated. SOD is the first enzyme that attacks the superoxide radical. After the action of SOD (which converts the superoxide radical into hydrogen peroxide), CAT acts as a primary defense when H_2O_2 levels are higher. GPX also reduces H_2O_2 in a reaction dependent on glutathione (GSH), which is used as a reducing agent. For this, it is subjected to the action of glutathione peroxidase (GPX), which is transformed into glutathione disulfide (GSSG) to fulfill its role. Glutathione disulfide (GSSG) is reduced by glutathione reductase (GR) which regenerates glutathione (GSH). In this way, GSH can restart new reactions to reduce oxidant species. Source: Prepared by the author.

SOD activity in fish can be used to assess the animal's response to some stressor, and its increase is directly related to the animal's ability to defend itself against these factors (Ighodaro and Akinloye, 2018). Low hepatic SOD activity in fish, however, has been associated with both the absence of stressors and the saturation of the activity capacity of this enzyme (Almeida et al., 2022). In the same sense, other authors found lower SOD activity in animals subjected to stress factors, with an increase in SOD activity when some antioxidant is supplemented.

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In fish, catalase represents the primary route for elimination of hydrogen peroxide when it is in higher concentration, dependent on SOD, with the glutathione system as a second option (Ighodaro and Akinloye, 2018). Measurement of antioxidant enzymes has been performed to decipher the physiological events associated with this process, as the activity of these enzymes changes in response to oxidative stress (Abdalla, 2015).

Reactive oxygen species (ROS) can oxidize amino acid residues, break peptide bonds, and alter proteolysis rates (Galicia-moreno and Gutierrez-reyes, 2014). As a consequence of proteic oxidation, carbonyl groups are produced within protein molecules and can be evaluated as a direct indicator of damage (Silva et al., 2011; Rua et al., 2014).

Antioxidants are substances that can be produced by the organism itself or can be obtained by ingesting products of synthetic or natural origin (Augustyniak, 2010). Feeds with an adequate supply of antioxidants are essential to combat stress in the life cycle of fish and support the normalization of antioxidant capacity after transportation. When the primary antioxidant defense system is not sufficient to deal with ROS, an increase in the latter can cause damaged intracellular proteins (Birben et al., 2012).

Under these conditions, the HSP protein group comes into action, increasing in response to some stress situations to reduce the cellular damage caused (Luengo et al., 2019). In normal situations, the family distinguished by a molecular weight of 70 kDa (HSP70) act as chaperone proteins, providing the proper conformation of newly synthesized proteins (Kim et al., 2018). However, under physiological stress, HSP70s prevent the denaturation of other proteins (Zuanazzi et al., 2018). Thus, the expression of the HSP70 protein can be regulated by stress events, such as environmental and physiological stress, as well as non-stressful conditions (Morimoto, 1998; Molina et al., 2000).

In fish farming, the increase in *HSP70* is usually associated with the presence of environmental stressors (Goes et al., 2019; Gewaily et al., 2021; Lala et al., 2021; Abdel-Tawwab et al., 2021). In such situations, the regulatory role of *HSP70* in modulating growth can be suppressed, and the complex can be shifted to recognition and degradation of misfolded proteins; and thus, the greater the cellular damage, the greater the expression of these proteins (Figure 13) (Lindquist and Craig, 1988).

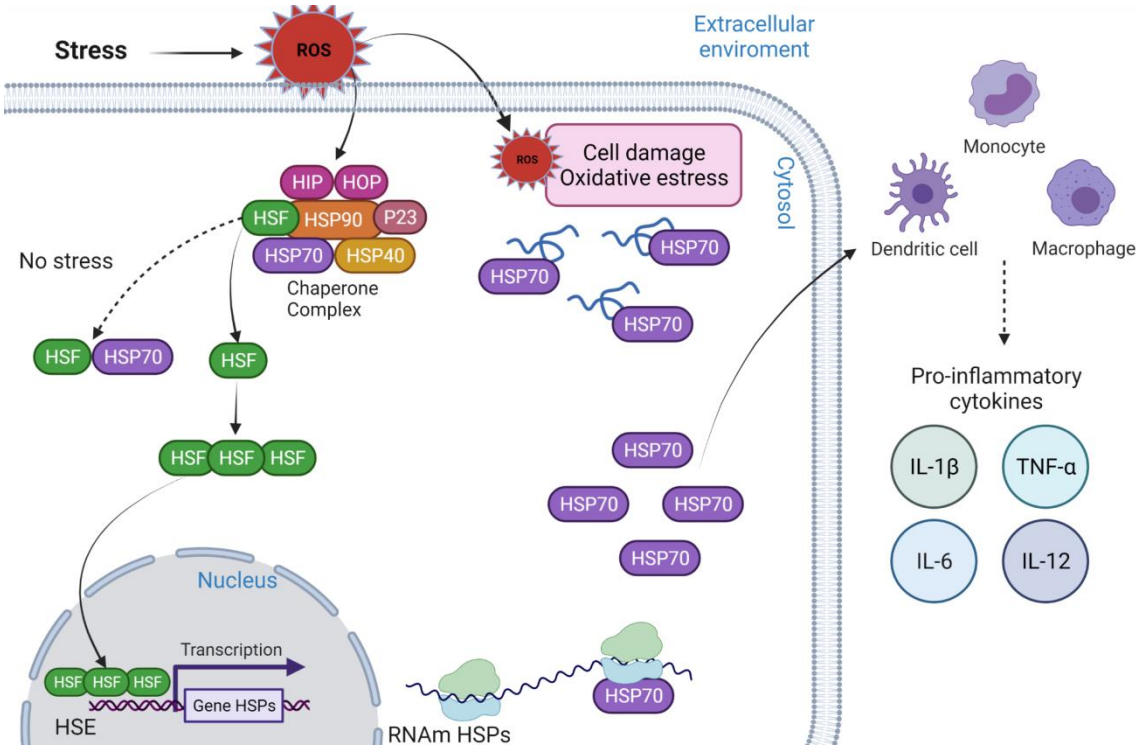

Figure 13. In a situation free from stress, HSP70 binds to the HSF regulatory protein (heat shock factor), which prevents HSF from becoming a trimer. In stressful conditions, though, stress increases the production of ROS, which activates the response of HSPs to maintain homeostasis. ROS can cause protein damage and causes HSP70 to act to degrade these denatured proteins. The dissociation of the HSP70-HSF complex allows the formation of the HSF trimer. The HSF trimer binds to the promoter region of the gene (HSE), promoting increased gene expression of HSPs, consequently increasing the production of HSP70. HSPs can induce immune cells to release pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12. Source: Prepared by the author.

11. Direct immune response to stress (IL and COX2)

The inflammatory response underlies a wide variety of mechanisms that induce inflammation, such as stress or tissue malfunction, which can be triggered by cellular damage, damage-associated molecular patterns (DAMPs), or by invading pathogens, molecular pathogen-associated molecular patterns (PAMPs) (Medzhitov, 2008). Through molecular pattern recognition receptors (PRRs) that are present on mast cells, monocytes and macrophages, DAMPs and PAMPs can bind and activate these cells (Walker and Sills, 2012). Macrophages stimulated by the inflammatory response release pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12 (Figure 14).

In situations of stress and injuries to the cell membrane, the enzyme phospholipase A2 is activated through pro-inflammatory cytokines, such as interleukin (IL) (Zhang, et al. 2019). This enzyme facilitates the degradation of phospholipids and subsequent production of arachidonic acid. The latter can be metabolized to leukotrienes and lipoxins

through the action of the lipoxygenase enzyme, or to prostaglandins, prostacyclins and thromboxanes, when metabolized by the cyclooxygenase enzyme (Cox) (Smith, 1989). One of the inflammatory mechanisms widely described involves the production of prostaglandins via COX, which culminates in vasodilation and increased tissue permeability, ending with the classic signs of inflation such as redness, pain, edema, and loss of function (Brown and Roberts, 2001).

The COX-1 subtype is considered a constitutive gene, while COX-2, commonly called prostaglandin G/H synthase 2 in tilapia, is amenable to induction and is often associated with pathophysiological processes such as inflammation induced by endotoxins and cytokines including IL-1 and TNF-α. (Lands, 1991; Tanabe and Tohnai, 2002; Gądek-Michalska et al., 2013). The increase in *COX 2* is directly associated with the inflammatory response (Hanana et al., 2021), and can cause several injuries, including DNA damage (Speed and Blair, 2011). The main inflammatory effect of IL-1 is the induction of COX-2 synthesis (Maier et al., 1990; Knott et al., 1994).

Acting together, all these mediators increase the inflammatory response. The cells involved in these responses are normally present in the tissues, in this way, the leukocytes go to the affected site marked by signaling (for example, chemokines and leukotrienes). Then, the leukocytes phagocytize the stressor agent and degrade the necrotic tissue, as the the purpose of this process is to resolve the inflammation and restart the recovery of the affected site (Abbas et al., 2008). Innate immune response molecules activate adaptive immunity, e.g. dendritic cells stimulate T cells to activate effector T cells, while B cells are also stimulated to produce antibodies (Abbas et al., 2008).

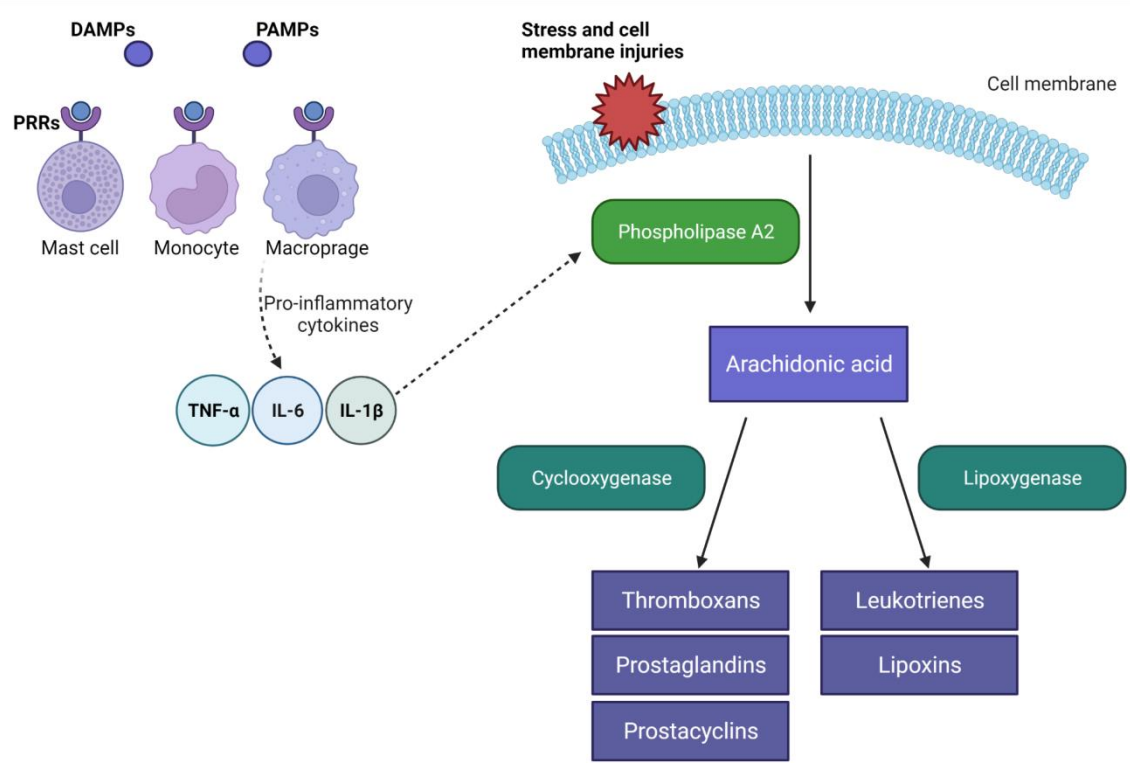

Figure 14. Signaling pathways, both IL-1B dependent and independent, involved in *COX 2* activation. Source: Prepared by the author.

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