ISABELA MARTINS DI CHIACCHIO

STUDY OF THE REPRODUCTIVE PERFORMANCE AND THE IMMUNOLOGICAL RESPONSE IN ZEBRAFISH (*Danio rerio*) FED WITH BEE POLLEN SUPPLEMENTED DIETS

ESTUDIO DEL RENDIMIENTO REPRODUCTIVO Y LA RESPUESTA INMUNITARIA EN PECES CEBRA (*Danio rerio*) ALIMENTADOS CON DIETAS SUPLEMENTADAS CON POLEN DE ABEJA

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To my parents, encouragers and deserving of all my recognition and gratitude in the face of any achievement, to my dear sisters and nephews, and to my great love, partner and best friend, Ricardo.

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“Let food be thy medicine, and let medicine be thy food.”

(Hippocrates)

ABSTRACT

Bee pollen is a natural product collected by bees from plants and stored in the hive mixed mainly with salivary enzymes. It is a rich source of various nutrients, so it can be an excellent dietary supplement. This product has been described with many beneficial properties, such as antimicrobial, immunostimulating and antioxidants effects, which reflects in possible therapeutic features for different pathological situations. As diet can be associated with animal performance and reproduction, microbiota modulation and potentially factor for cancer, this study aimed to analyze if dietary bee pollen addition could influence zebrafish parameters. The zebrafish *Danio rerio* is a teleost fish from tropical freshwater, widely used as an experimental model to study complex vertebrate biology using many genetic approaches. The identification of mechanisms involved in physiological responses of fish submitted to supplemental treatment with bee pollen can provide important information for the recommendation of this product in the diet of also other species. This work studied bee pollen addition to the zebrafish diet, focusing its effect on reproduction, growth parameters, intestinal modulation and response against tumor development. Adult zebrafish were fed with different diets, three times a day, using commercial feed, brine shrimp and bee pollen. The fish received the diets for 60 days and throughout this period were tested weekly for: total number of eggs, egg production per female, embryo viability rate (72hpf), larval survival rate after exposure to Spring Viramae of Carp virus, larval survival rate after exposure to *Salmonella enterica* serovar Typhimurium and analysis of neutrophil recruitment after tail wound. After this, fish from each treatment were analyzed for weight gain and increased length, and the intestines were collected to assess the intestinal microbiota through metagenomics analysis, which enables the identification of bacterial diversity between samples. Also, serum amyloid A (*saa*) transcript levels from abdominal organs and from separated intestines were analyzed, as the protein encoded by this gene has effects on immune cells and some intestinal bacteria strongly induce its production. After 120 days of diet, fish remaining from each of the treatments were evaluated for tumor growth after melanoma allotransplantation procedure, a very aggressive form of skin cancer. Our results show that bee pollen failed to improve egg production and embryo viability. Instead, the offspring of breeders fed with bee pollen diets showed longer survival upon virus exposure and higher neutrophil migration to wounds. These results indicate that bee pollen can influence vertical immunity through important mechanisms related to offspring immunity in the early stages. Bee pollen diet also revealed different gut microbial abundance at family, genus and species level compared with fish from control group; and, unexpectedly, fish fed with bee pollen showed higher tumor growth rate and larger tumor size. Although some studies attribute bee pollen with antitumor activities, mostly *in vitro* experiments, our results show that this link should be questioned. Due to its variable composition, the effects caused by bee pollen ingestion need deeper investigation before recommendation, as it can also vary between different species and physiological states.

Keywords: Fish; Immunology, Natural Products, Nutrition, Reproduction

RESUMEN

El polen de abeja es un producto natural recolectado de las plantas por las abejas y almacenado en la colmena mezclado principalmente con enzimas salivales. Es una fuente rica de diversos nutrientes, por lo que puede ser un excelente complemento alimenticio. Este producto ha sido descrito con múltiples propiedades beneficiosas, como efectos antimicrobianos, inmunoestimulantes y antioxidantes, lo que se refleja en posibles características terapéuticas para diferentes situaciones patológicas. Dado que la dieta puede asociarse con el rendimiento y la reproducción de los animales, la modulación de la microbiota y ser un factor potencial em el desarrollo del cáncer, este estudio tuvo como objetivo analizar si la adición de polen de abeja en la dieta podría influir en la reproducción e inmunidad del pez cebra. El *Danio rerio* es un pez teleósteo de agua dulce tropical, ampliamente utilizado como modelo experimental para estudiar la biología compleja de los vertebrados, ya que permite una fácil manipulación genética. La identificación de los mecanismos implicados en las respuestas fisiológicas de los peces sometidos a un tratamiento complementario con polen de abeja puede aportar información importante para la recomendación de este producto en la dieta. El presente trabajo tuvo como objetivo estudiar la adición de polen de abeja a la dieta del pez cebra, focalizando su efecto en la reproducción, los parámetros de crecimiento, la modulación intestinal y la respuesta frente al desarrollo tumoral. Los peces cebra adultos fueron alimentados con diferentes dietas, tres veces al día, utilizando alimento comercial, artemia y polen de abeja. Los peces recibieron las dietas durante 60 días y durante este período se analizaron semanalmente los siguientes parámetros reproductivos: número total de huevos, producción de huevos por hembra, tasa de viabilidad embrionária, tasa de supervivencia de larvas después de la exposición al virus de la viremia primaveral de la carpa, tasa de supervivencia larvária después de la exposición a *Salmonellae enterica* serovar Typhimurium y análisis del reclutamiento de neutrófilos en larvas después de la herida en la cola. Después de esto, los peces de cada tratamiento fueron analizados en busca de aumento de peso y longitude, y se recogieron los intestinos para evaluar la microbiota intestinal a través del análisis metagenómico, que permite identificar la diversidad bacteriana entre muestras. Además, se determino los niveles de mRNA del gen que cifra la proteína amiloide A (Saa) en los órganos abdominales de pez cebra y de intestinos separados, ya que esta proteína es producida en el intestino y el hígado y tiene efectos sobre las células inmunes y algunas bacterias inducen fuertemente su producción. Después de 120 días de dieta, se evaluó el crecimiento tumoral de los peces restantes de cada uno de los tratamientos después del procedimiento de alotrasplante con melanoma, una forma muy agresiva de cáncer de piel. Nuestros resultados muestran que la suplementación con polen de abeja no logró mejorar la producción de huevos y la viabilidad del embrión en los peces cebra reproductores. En cambio, la descendencia de los reproductores alimentados con dietas suplementadas con polen de abeja mostró una mayor supervivencia tras la exposición al virus y una mayor migración de neutrófilos a las heridas. Estos resultados indican que el polen de abeja puede influir en la inmunidad vertical a través de importantes mecanismos relacionados con la inmunidad de la descendencia en las primeras etapas de vida. La dieta de polen de abeja también reveló una abundancia microbiana intestinal diferente a nivel de familia, género y especie en comparación con los peces del grupo de control; e, inesperadamente, los peces alimentados con polen de abeja mostraron una mayor tasa de crecimiento tumoral y tumores de mayor tamaño. Aunque algunos estudios atribuyen al polen de abeja actividades antitumorales, principalmente experimentos *in vitro*, nuestros resultados muestran que este vínculo debería ser cuestionado. Debido a su composición variable, los efectos causados por la ingestión de polen de abejas necesitan una investigación más profunda antes de su recomendación, ya que también pueden variar entre diferentes especies y estados fisiológicos.

Palabras clave: Pez; Inmunología, Productos Naturales, Nutrición, Reproducción

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FIRST PART

1 INTRODUCTION

Apitherapy is a type of alternative medicine that uses products from bees which contain different natural agents and groups of chemical compounds with several beneficial activities. The pollen collected by bees has been used for a long time as a food supplement and also as an additive in cosmetics, foods and medicines (KOMOSINSKA-VASSEV et al., 2015). Its composition depends on the plant source, geographic origin, climatic conditions, soil type and bee activities (DA SILVA et al., 2014; NOGUEIRA et al., 2012).

Bee pollen consists of flower anthers, collected by forage bees and transported to hives, where it is agglutinated with salivary secretions and added nectar. It is rich in proteins, essential amino acids, fatty acids, minerals, enzymes and coenzymes, carbohydrates and flavonoids, carotenoids and phytosterols (LI et al., 2018a). The antioxidant activity of scavenging free radicals from bee pollen constituents (flavonoid glycosides, flavonoid clusters and phenolic acid derivatives) encourages its use as a medicine (LINSKENS; JORDE, 1997). Among other therapeutic properties, we can mention actions such as antifungal, antimicrobial, antiviral, anti-inflammatory, anticarcinogenic, immunostimulant, local analgesic and wound healing (KOMOSINSKA-VASSEV et al., 2015).

Currently, in addition to humans, bee pollen has also been used in animals such as chickens, mammals and fish, mainly to improve growth (ABBASS; EL-ASELY; KANDIEL, 2012; ATTIA; AL-HANOUN; BOVERA, 2011; EL-ASELY; ABBASS; AUSTIN, 2014; HAJKOVA; TOMAN; GALIK, 2014; WANG et al., 2007). The introduction of bee products in fish diets has also been shown to improve performance and immune status (ABD-EL-RHMAN, 2009; EL-ASELY; ABBASS; AUSTIN, 2014; MEURER et al., 2009). However, the use of bee pollen in the diet of animals in different physiological states has not been well studied and its effects on different species may vary and deserve to be analyzed in greater detail.

The zebrafish (*Danio rerio*), the model used in this study, is a freshwater teleost originating from Southeast Asia, belonging to the *Cyprinidae* family. This fish proved to be a very promising vertebrate for the development of scientific research in several areas, including basic mechanisms of development, physiology, genetics, toxicology, reproduction, cancer, stem cells, syndromes and diseases, immunology and infection, vision, regeneration, behavior,

among many others (KHAN et al., 2017; NOWIK et al., 2015; SIEBEL; BONAN; SILVA, 2015). Therefore, it is an excellent animal to be used also in the experiments of this thesis.

The development of this project foresees the understanding and clarification of the existing relationships between immunological responses, reproductive parameters and nutritional components of bee pollen, in order to establish favorable proposals for the well-being of the organism. The advantages of knowledge related to enriching diets with supplementation with bee pollen are mainly related to elucidating physiological responses and biological mechanisms related to the topic, integrating an important and current topic.

To increase the knowledge of bee pollen effects using the zebrafish as tool, we compared the effects of including this product in a standard diet on reproduction (eggs quality and offsprings' immunity throw maternal immunity transference), body growth, intestinal changes (microbiota and expression of serum amyloid A) and cancer (melanoma development). The mechanisms by which feed influence host immune system is highly related to microbiota modulation. Commensal microbes facilitate nutrient digestion and absorption but also exert critical influence in many substances production and molecular pathways inside the intestine and across the role body. Once the intestines are called the second brain and is orchestrated mostly by food ingested and microbiome, diet supplementations can change many body systems (LÓPEZ NADAL et al., 2020).

This work aimed to know better the effects that have not yet been reported about bee pollen addition to the diet. We understand that using this product can be more complex than it is actually addressed. Its great variety and variability in nutrient content makes it difficult to know what may or may not actually be beneficial in certain biological situations of different organisms. It is important to keep in mind that responsible recommendation of any food inclusion should only take place after careful studies. In this way, we contribute with our work to an important discussion about this very interesting bee product.

2 LITERATURE REVIEW

2.1 Bee products

The first evidences demonstrating bee products' acquisition by humans came from the rock paintings discovered in 1919 in Spain in the Cave Spider (Cuevas de la Araña) located by the River Cazunta near Valencia. The painting shows a person taking honey from wild bees and it is assumed that the painting was created in the years 8000–5000 before Christ (BC), at the Stone Age (Neolithic Age). The history of beekeeping dates to the ancient times and the products were not only highly valued products, but played a major role in the religious rites of almost all cults (NAYIK et al., 2014).

Bees' life is the subject of scientific interest and the use of bee products contributes to the development of apitherapy, a specific area of treatment. Biologically active substances of natural origin focus a great interest and this also applies to bee products. Bee products are multicomponent natural substances which includes: honey, bee pollen, and extracts derived from it, as bee bread, propolis, royal jelly, and bee venom (KIELISZEK et al., 2018). These products are described to have nutrients that participate to basic life reactions as has been related to increase the level of ATP and neutralize an effect of many toxic agents, increase immunity of an organism, improve the energy balance of tissues, participate in many stages of protein metabolism and in the synthesis of nucleic acids and also being essential to the proper functioning of the circulatory system of living organisms (BOBIŞ et al., 2010).

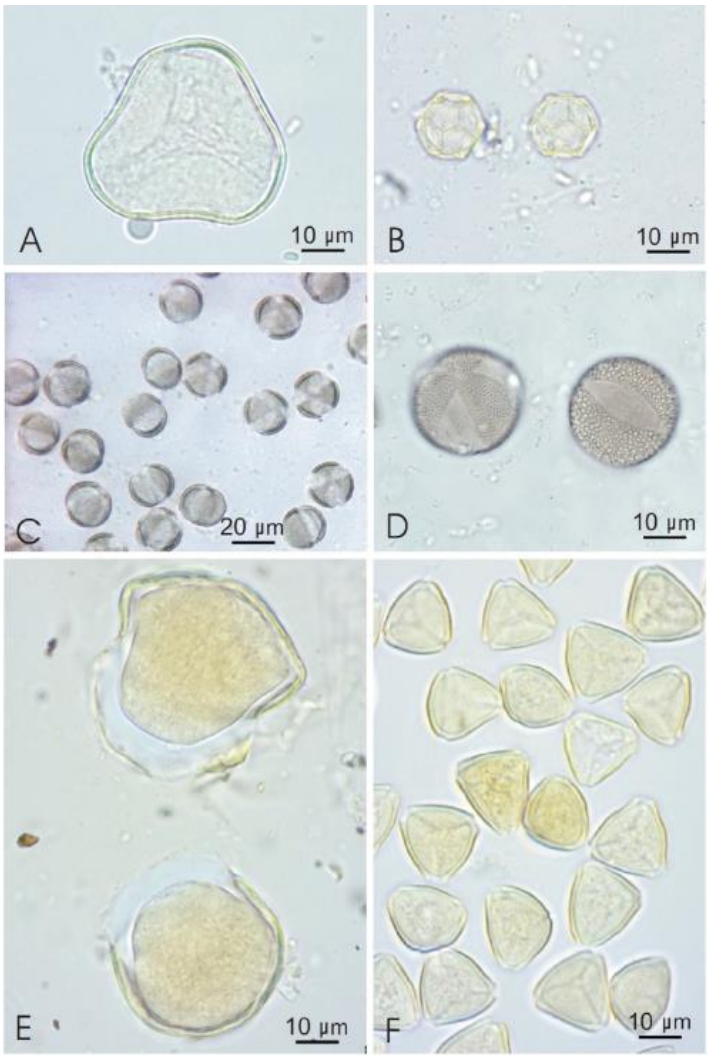
Among bee products, bee pollen has been gaining prominence. Bee pollen began to be used on a larger scale for human consumption after the Second World War, when the method of pollen traps was improved and easily accessible (CAMPOS et al., 2010). Bee pollen is often regarded as "a life-giving dust" or "the world's best food product" and also as "functional food" (ABDELNOUR et al., 2019; KIELISZEK et al., 2018). Functional foods are known as foods beyond their basic nutritional features, such as health-oriented products that positively influence the well-being and quality of life. This term underlines the positive correlation of the bioactive compounds present in these products along with health (MĂRGĂOAN et al., 2019).

2.1.1 Bee Pollen

Pollen is a powdery substance consisting of pollen grains, produced in the anthers of spermatophytes (seed plants) in various quantities. It plays an essential role in sexual

propagation, thus each pollen grain carries a variety of nutrients necessary for survival and fusion with a plant female gamete (DENISOW; DENISOW-PIETRZYK, 2016). Pollen grains can be as large as about 2.5–250 μm in diameter, with different shapes and colors, and usually spherical in shape (Figure 1).

Figure 1: Pollen grains of frequent monofloral bee pollen samples from Brazil.



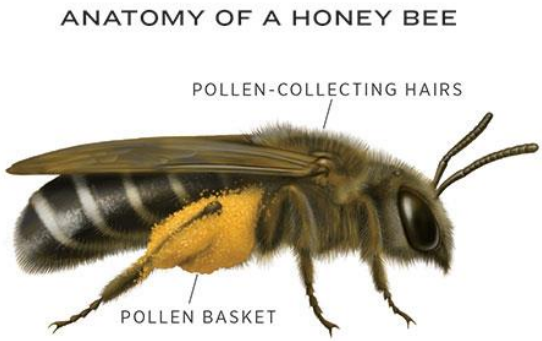
Legend: A =*Astrocaryum aculeatissimum*; B= *Alternanthera*; C and D =*Brassica rapa*; E=*Cocos nucifera*. F =*Myrcia*. Source: (DE-MELO et al., 2018b).

Pollen of anemophilous plants (grasses, ivy, sedges, rushes, olive, birch, alder, chestnut) contains allergens that may cause human pollinosis with severe allergy symptoms, e.g. harmful hay fever, skin rash, or asthma and may even be responsible for development of plant food allergy. In contrast, pollen of entomophilous plant species (insect-pollinated) is collected by

various species of honeybees (*Apis sp*.) and utilized as valuable food (DENISOW; DENISOW-PIETRZYK, 2016).

Worker honeybees during visits attract the hundreds to thousands of pollen grains using weak electrostatic field generated between flower (negatively charged) and bee body (positively charged) (CLARKE; MORLEY; ROBERT, 2017). The pollen grains are agglutinated using the several combs and hairs of bee's hind legs (figure 2) which are moistened with salivary secretions, enzymes, wax and nectar or honey to form a pellet (THAKUR; NANDA, 2020). The pollen mixture is transported in the pollen basket of the bee´s legs to the bee hive, where it is stored and used as food for all the developmental stages in the hive. From the moment in which the bees add their secretions to this pollen, it acquires certain peculiar characteristics which make it different from pollen collected by hand or that which is dispersed by wind (ARES et al., 2018).

Figure 2: Anatomy of a honey bee: pollen-collecting hairs and pollen basket.



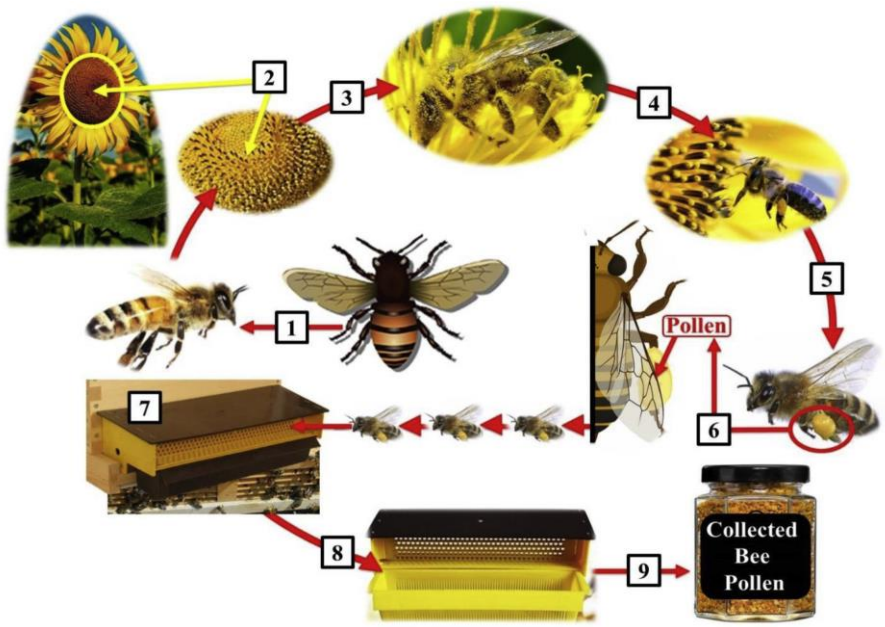
Source: (THE XERCES SOCIETY, 2016).

Bee pollen, also known as apicultural or bee-collected pollen can be harvested with the help of trap, fixed at the entrance of beehives. While returning home, the pollen is lost from the hind legs and collected in the collection tray of the trap (figure 3).

Climate, plant communities, and timing of floral resources differ significantly between regions and managing honey bee colonies across different regions changes throughout the year. Thus, beekeeper and bee activities follow what is called apicultural calendar usually divided into active and inactive times for the colony. The calendar also includes recommendations for major management like when to treat for parasites or pathogens and when to feed colonies or harvest honey. The management calendar should not be exhaustive and may change according

to the beekeeping activity performed. Bee pollen calendar orient beekeepers in the optimization of the product production and consequently the best period for pollen collection. It is also possible to make energy and protein supplementation of animals in periods of scarcity (DE CAMARGO et al., 2003).

Figure 3:The collection process of bee pollen by honeybees.



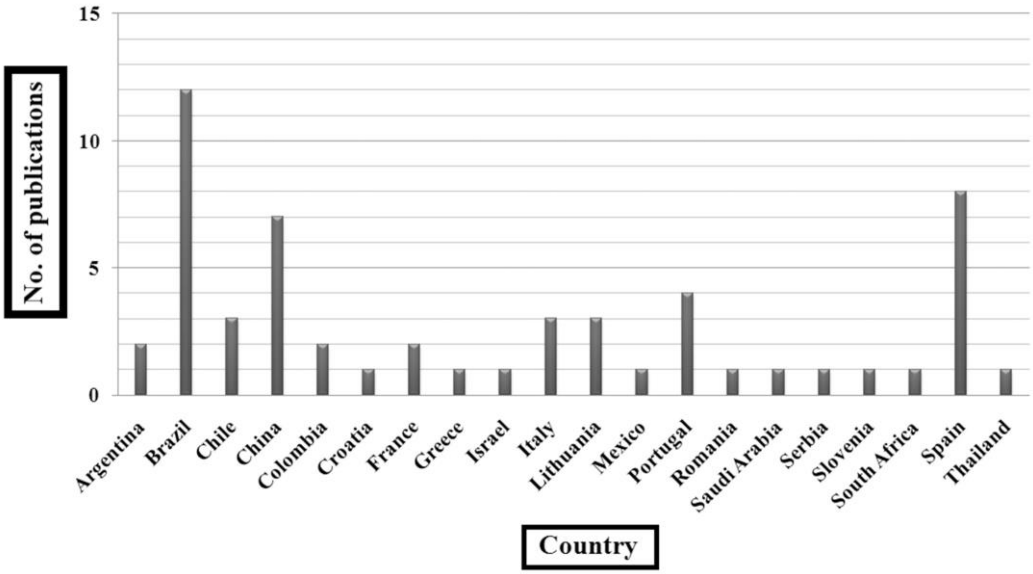
Source: (THAKUR; NANDA, 2020).

The plant sources of bee pollen strongly affect its nutritional, physicochemical, and functional properties (DA SILVA et al., 2014; NOGUEIRA et al., 2012; THAKUR; NA, 2018; YANG et al., 2013). The pollen pellets from unique botanical taxon or the ones having single predominant pollen (>90%) are considered as mono-floral. However, in case of inadequate flora surrounding the hive, the honeybee will visit the flower of other botanical sources and the pollen grains are mixed, resulting in the pellet known as multi-floral pollen (BARTH et al., 2010). The flower from plant species affects the color of pollen grain ranging from white, yellow to orange, red, green, gray and dark brown. Even after a similar plant source, pollen composition may vary due to seasonal and regional variations, influenced by the plant age, nutritional and environmental conditions (THAKUR; NANDA, 2020).

Over the past years, the rising interest in the extraction and determination of these beneficial bee pollen compounds has been demonstrated by the number of published research papers dealing with this issue and the large list of countries in which such studies were carried

out (figure 4). Curiously, among them, Brazil and Spain presented the greatest number of publications between the years 2011 and 2017, related to the analysis of bioactive compounds from bee pollen.

Figure 4: Summary of the number of publications per country related to the analysis of bioactive compounds from bee pollen.



Source: (ARES et al., 2018).

Bee pollen may contain up to about 250 different chemicals, including amino acids, vitamins, micro and macro elements, carbohydrates, nucleic acids, triglycerides, phospholipids and flavonoids (DA SILVA et al., 2014; NOGUEIRA et al., 2012). Therefore, the chemical compounds of this product show large variation between the minimum and maximum values, containing average of 54% (18–84%) carbohydrates, 21% (4–41%) proteins, 5% (0.4–14%) lipids, 9% (0.15–31%) fiber, 3% (0.50–8%) ash, 13 g/100 g (3–29 g/100 g) glucose, 15 g/100 g (5–34 g/100 g) fructose, 4 g/100 g (0.05–9 g/100 g) sucrose, 4951.61 mg/kg (3.06–13366.60 mg/ kg) potassium, 4157.86 mg/kg (234.40–9587.00 mg/kg) phosphorus, 1751.22 mg/kg (1.09–5752.19 mg/kg) calcium, 1246.99 mg/kg (44.00–4680.53 mg/kg) magnesium, 46.97 mg/kg (0.10–105.80 mg/kg) zinc, 197.41 mg/kg (2.60–1180.00 mg/kg) iron, and 30.59 mg GAE/g (0.69–213.20 mg GAE/g) total phenolic content (CAMPOS et al., 2008).

Some studies even reported that bee pollen intake is enough for human survival, as the vitamins from bee pollen contribute greatly to the nutrition and almost all the human essential amino acids and minerals are reported in bee pollen in good amounts (NOGUEIRA et al., 2012).

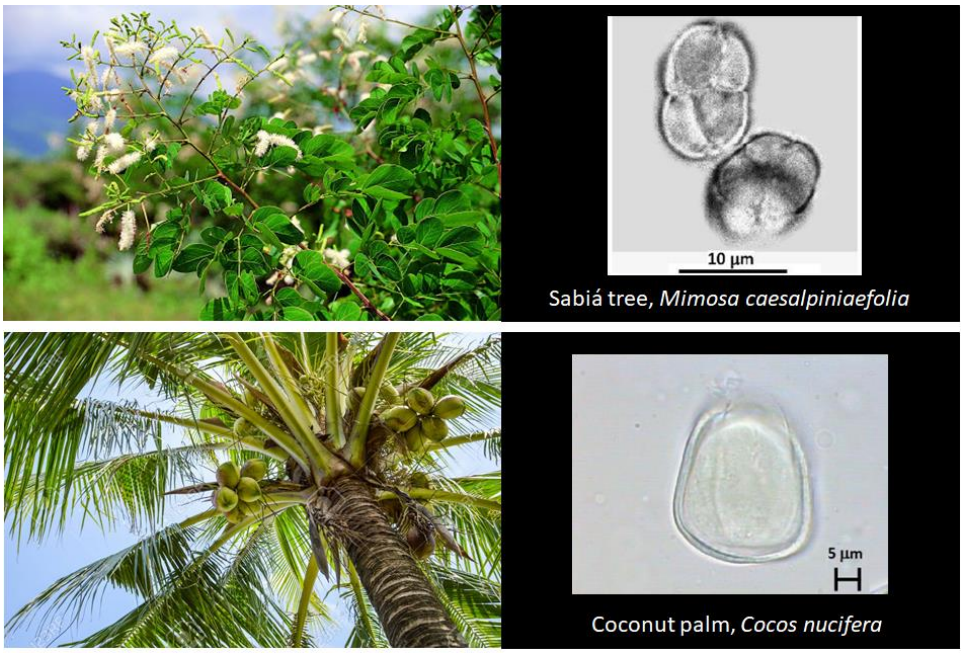
About 20% of bee pollen is composed of amino acids (10% of essential amino acids) and an average of 26% of reducing sugars, such as fructose and glucose (KOMOSINSKA-VASSEV et al., 2015). In this way, the high nutritional value of this product is verified.

Flavonoids, phenolic acids, fatty acids and phytosterols are indicated as the main responsible for the anti-inflammatory capacity of this product (KOMOSINSKA-VASSEV et al., 2015; MOSIĆ et al., 2019). The flavonoids compounds, termed secondary plant components, have also various beneficial impacts as pharmacological and physiological activities. These compounds are described to have diversified biological features such as antiaging, antioxidant, antimicrobial, antifungal, antiviral, antiinflammatory, anticarcinogenic, anti-atherosclerosis, cardioprotective, enhance the endothelial function and modulate the healing process of burns (ABDELNOUR et al., 2019; KOMOSINSKA-VASSEV et al., 2015).

It is also supposed that its regular intake has impacts on numerous medical disorders, such as anemia, depression, memory loss, intestinal and prostate problems, impotence, ageing, stress-related diseases and impaired immune functions, besides its antibiotic, antineoplastic and antidiarrheal properties (ABDELNOUR et al., 2019). Over the past few decades, the use of natural products was promoted to improve the performance and immunity of animals, promoting the animal growth, protecting the intestinal health and improving the animal products quality and safety (ATTIA et al., 2019). So far, generally, bee pollen is accepted to use in diets with no side effects.

An interesting study determined the physicochemical profile, as well as the phenolic profile and the antioxidant and antimicrobial capacities, of monofloral bee pollen samples collected in different locations in Brazil (DE-MELO et al., 2018a). All samples were classified as monofloral, since they had more than 90% of a unique pollen type. The results show the protein (10.6–33.9 g/100g), lipids (3.2–8.3 g/100g), ashes (2.6–3.8 g/100g), total phenolic (5.6–29.7 mg GAE/g), and total flavonoid (0.3–19.0 mg QE/g) values were variable, even between products with the same botanical origin. The profiles of each sample were distinct, and there was no pattern between monofloral products of the same pollen type. One of the bee pollen samples used in the study comes from the region Neópolis, Sergipe (composed by 96.3% of *Mimosa caesalpiniaefolia*, named Sabiá tree, and 3.7% of *Cocos nucifera*, the coconut palm – figure 5), as well as the bee pollen used for experiments in this thesis. Through the morphological and structural analysis of the pollen grain, it is possible to identify the botanical and geographical origin of the bee pollen.

Figure 5: Representative images of *Mimosa caesalpiniaefolia* and *Cocos nucifera*, as well as their corresponding pollen types.



Source: (The author, 2021).

In Brazil, there is a technical regulation for food quality, which contains the requirements for marketing products of animal origin. The normative instruction N3 of MAPA (Ministry of Agriculture, Livestock and Supply) describes the criteria for the commercialization of bee products and establish the identity and the minimum quality requirements that bee pollen must have. Among them, the physicochemical parameters required for bee pollen are: humidity - maximum 30% (fresh) or maximum 4% (dehydrated); ash - maximum 4%; lipids - minimal; proteins - minimum 8%; total sugars - 14.5% to 55.0%; crude fiber - minimum 2%. Thus, there is a standardization of what would be the appropriate levels of nutrients found in this product. Still, the nutrients content variability is very large, which makes difficult the evaluation of beneficial properties of bee pollen from different regions. Bee pollen is offered as complementary and alternative treatments for different diseases. In general, these treatments have not been proven effective and safe in clinical experiments. So, bee pollen has been regarded as a promising therapeutic and nutritional natural food supplement but more research and more experimental and clinical studies are required to verify its effectiveness (DENISOW; DENISOW-PIETRZYK, 2016).

2.2 Zebrafish: the animal model under study

Danio rerio (Hamilton, 1822), known as zebrafish (figure 6), is a small size teleost fish which belong to the Cyprinids family and lives in tropical fresh water (NÜSSLEIN-VOLHARD; DAHM, 2002). Native to streams in the southeastern Himalayas, including India, Pakistan, Bangladesh, Nepal and Myanmar, the name Danio is derived from the bangla “dhani”, which means “from the rice field” as it is commonly found in water columns in places where rice cultivation is practiced (ARUNACHALAM et al., 2013). Currently, this species is widely used as an experimental model in several areas of science, being equally valued when compared to rats and mice, with a similar prevalence in research programs (SILVEIRA; SCHNEIDER; HAMMES, 2012). In Brazil, the scientific community has been increasing its interest in using zebrafish as an animal model in experiments, reflecting on the amount of published works, mainly from the 1990s (GHENO et al., 2016).

Figure 6: Zebrafish (*Danio rerio).*

Source: (https://www.riometa.eu)

This species has its genome fully sequenced and several molecular markers already available, which makes it possible to work with vast genomic resources. When compared in their sequence, their genes show high similarity to those of humans and mice (BARBAZUK et al., 2000). In addition, due to its high capacity to absorb compounds added to water, its accelerated metabolism and its sensitivity to chemicals, zebrafish is favorable as a model for toxicological (YANG et al., 2009), genetic, teratological (BECKER; BECKER, 2008), pharmacological (BENCAN; SLEDGE; LEVIN, 2009; EGAN et al., 2009), neuro-behavioral studies (MATHUR; GUO, 2010; SISON; GERLAI, 2011) and to unravel mechanisms of

several human diseases and tests for new therapeutic agents (SILVEIRA; SCHNEIDER; HAMMES, 2012).

Despite being an ornamental species, zebrafish can also be used to study fish characteristics of economic interest, as for example commercial Brazilian fish species. Fish farming in Brazil has grown considerably, becoming an important economy sector (FAO, 2020). Studies of the immune system and their interaction with diet nutrients can contribute with nutrition implementations aiming healthy maintenance and decrease possible economic losses. Fighting diseases may increase the cost of production and decrease fish farming productivity. Therefore, keeping the immune system healthy is an interesting alternative and has been the focus of several research groups.

Scientific studies using zebrafish also demonstrate strong homology regarding mammalian lymphoid cells, including lymphocytes, monocytes, macrophages and neutrophils, in addition to great functional similarities (CROWHURST; LAYTON; LIESCHKE, 2002; DE JONG; ZON, 2005; ONNEBO; YOONG; WARD, 2004; SCHORPP et al., 2006; STACHURA; TRAVER, 2016). This fact makes this species an ideal model system to analyze leukocyte migration and inflammatory processes *in vivo*, emerging as a powerful organism to study the mechanisms of certain diseases. Experimental procedures involving zebrafish can be even less complicated and economically costly (SÁNCHEZ-VÁZQUEZ et al., 2011), making it an extremely interesting model for studies in many different areas.

In nature, zebrafish eating habits is considered generalist, as it consumes a wide variety of benthic and planktonic crustaceans, in addition to worms and insect larvae (SPENCE et al., 2008). The specific nutrient requirements (proteins / amino acids, lipids, carbohydrates, minerals and vitamins) for zebrafish are not yet clear and the requirements may also vary according to the frequency with which these animals are subjected to reproduction. In addition, requirements must be determined for each stage of life; larval, juvenile and adults, testing diet components effects on survival, growth, resistance to diseases / stress and reproduction (LAWRENCE, 2007).

Regarding the diet offer, there are two general approaches used in fish farming: feeding until satiety and feeding based on body weight (LAWRENCE, 2007). The first method is commonly used in zebrafish facilities, however, fish can be over-fed or under-fed, leading to reductions in water quality and / or growth depression, reproductive function and immune response. Feeding based on body weight involves feeding with a fixed percentage of fish body weight every day. In intensive farming systems, fish larvae are normally fed more frequently

throughout the day (from 50 to 300% of their body weight) compared to adult fish (1 to 10% of body weight). This second method represents the most efficient and scientifically sound method for studying zebrafish diets (LAWRENCE, 2007).

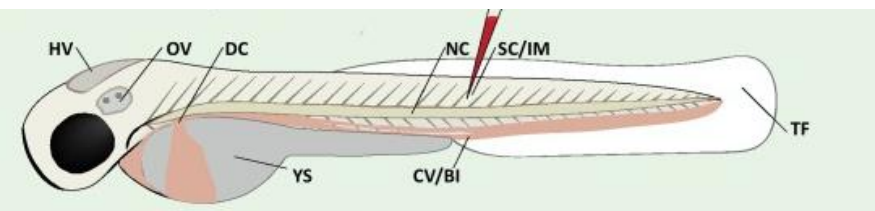
2.3 *Salmonella enterica* serovar Typhimurium (ST)

Salmonella is a gram-negative, non-spore-forming, rod-shaped and facultative anaerobic bacteria, which belong to the *Enterobacteriaceae* family. These microorganisms can range from around 0.7 to 1.5 μm in diameter and 2 to 5 μm in length. Infectious diseases, such as salmonellosis, are responsible for one-third of all mortality worldwide and have become a significant public health threat in both developed and developing countries (SOTOMAYOR et al., 2018).

It is a global food-borne pathogen that infects and replicates within macrophages of both humans and animals and cause an estimated 93.8 million salmonellosis infections and 155,000 deaths globally each year. Control of its infection is difficult due to the bacterium high tolerance to environmental stress, widespread distribution, multiple drug resistance, and adaptability. Moreover, continuous genetic re-assortment in *Salmonella*, leads to increased virulence and the emergence of resistance to multiple drugs, is of significant public health concern (BRANCHU; BAWN; KINGSLEY, 2018; CHEN et al., 2013).

Zebrafish is an animal model used in the study of inflammatory diseases and the infection caused by ST has already been well established (STOCKHAMMER et al., 2009). It is known that after infection by ST the activation of the innate immune response occurs in zebrafish embryos / larvae. There are different routes of infection depending on what is the purpose of the study. The yolk sac (YS) injection (figure 7) in larvae with 2 days' post-fertilization is used for systemic infection (BENARD et al., 2012) and will be implemented in our study.

Figure 7: Routes of larvae zebrafish injection



Legend: HV:Hindbrain, OV: Otic Vesicle, DC: Duct of Curvier, NC: Notochord, SC: Subcutaneous, IM: Intramuscular, YS : Yolk Sac, CV: Caudal Vein, BI: Blood Islands, TF: Tail Fin.
Source: (Adapted from TORRACA; MOSTOWY, 2018).

2.4 Spring Viremia of Carp Virus (SVCV)

The Spring Viremia of Carp Virus (SVCV) belongs to *Rhabdoviridae* family, species *Rhabdovirus carpio* (AHNE et al., 2002). This virus is responsible for the highly contagious spring viremia disease associated with hemorrhagic symptoms in cyprinids, especially in the common carp (*Cyprinus carpio*) (AHNE et al., 2002). The infection is highly lethal in young fish, mortality rates can range up to 90% (BAUDOUY; DANTON; MERLE, 1980) and, therefore, causes substantial economic losses for the aquaculture industry.

Currently, the disease is endemic in Europe, America and several Asian countries, where it causes significant morbidity and mortality in affected fish. SVCV infection is usually associated with exophthalmos; abdominal distension; petechial hemorrhage in skin, gills, eyes and internal organs; degeneration of the branchial lamellae; a swollen and coarse textured spleen; hepatic necrosis; enteritis; and pericarditis (ASHRAF et al., 2016).

After experimental infections reported in zebrafish, a model of waterborne infection was developed in 3 days after fertilization larvae (LÓPEZ-MUÑOZ et al., 2010). The procedure of challenging larvae by immersion considerably reduces animal handling and represents a more natural route of infection. In this previous work, authors show that zebrafish larvae are unable to develop a protective antiviral response to SVCV and this form of infection will also be implemented in our study.

2.5 Intestinal microbiota

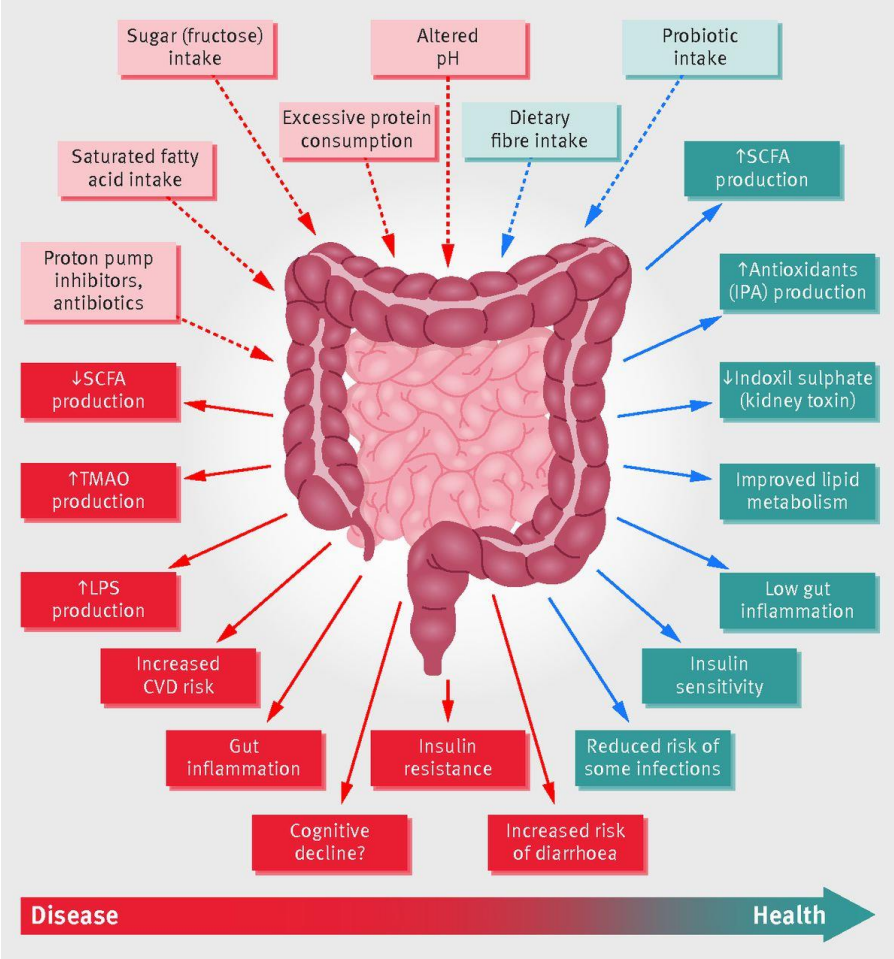
The intestinal microbiota is the set of microorganisms existing in the intestine (LOZUPONE et al., 2012), comprising a community with bacteria, archaea, eukarya, fungi and viruses. The intestinal microbiome concerns their genome. These microorganisms establish a mutualistic relationship with the host, in which both contribute and benefit (LEY et al., 2008) and can also be distributed in different parts of the body (such as skin, mouth, respiratory tract, large and small intestine), colonizing superficial or deep zones. Its distribution depends on a set of factors such as humidity, acidity, temperature and availability of nutrients (FIOCCHI; SOUZA, 2012).

The gastrointestinal tract is the site which hosts the largest number and diversity of microorganisms, and the intestinal microbiota can influence on homeostasis mechanisms. The development of the microbiota occurs in the first moments of the animals' life and will influence host's physiology throughout life in the maintenance of tissue balance (GONÇALVES, 2014).

It participates in food digestion, release of beneficial microbial products, metabolism of nutrients and toxins, production of vitamins (WILLEY, 2009), protection against pathogenic microorganisms and prevent the development of diseases (FIOCCHI; SOUZA, 2012). In addition, the intestinal microbiota also plays an important role in local and systemic immune responses (BELKAID; HAND, 2014).

There is a close and complex relationship, where survival and many essential host metabolic processes are carried out or facilitated by these microorganisms (QUESADA, 2019). The intestinal microbiota is characterized by its constant dynamism, and it can be affected by several internal and external factors. Through these interactions, the microbiota can affect host behaviors, fitness, phenotype and health (figure 8).

Figure 8:Schematic representation of the role of the gut microbiota in health and disease giving some examples of inputs and outputs.



Legend: CVD=cardiovascular disease; IPA=indolepropionic acid; LPS=lipopolysaccharide; SCFA=short chain fatty acids; TMAO=trimethylamine N-oxide
Source: (VALDES et al., 2018).

It is possible to modify health through food and measure the effects through microbes or metabolites. Fiber has been considered as a key nutrient for a healthy microbiome while debates have raged about sugar, fat and the adverse effects of drugs and processed food ingredients (VALDES et al., 2018). Given the current gaps in knowledge, more clinical evidences are necessary to assess changes in gut microbiota composition and in health outcomes.

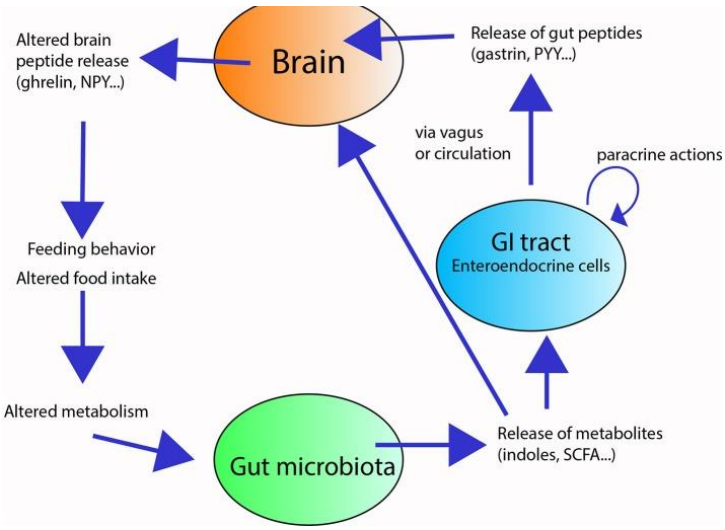
2.6 Intestinal microbiota in fish

Studies in different models suggest the intestinal microbiota in fish is mainly involved with food, digestion and metabolism; stress response; reproduction; development and immune responses (BUTT; VOLKOFF, 2019). Research carried out to date offers good understanding of these mechanisms capable to regulate fish metabolism providing improvements in aquaculture practices. However, there is still a long way to go in search of information that has not been fully clarified.

2.6.1 Food / digestion / metabolism

The intestinal microbiota influences the so-called brain-intestine axis. It is able to interact with neurotransmitters and influence their effects on gastrointestinal motility, hormone function and release and eating behavior (ZHANG; DAVIES, 2016). Some of its metabolites can act on enterocytes and regulate their intestinal barrier function, absorption capacity, nutrient uptake and storage, secretory activity and intestinal motility. In addition, the microbiota releases metabolites in response to substrates present in the lumen that stimulate enteroendocrine cells to release intestinal peptides acting both locally and in brain feeding centers, modifying eating behavior and energy homeostasis (Figure 9) (BUTT; VOLKOFF, 2019).

Figure 9: Overview of the gut-microbiota-brain axis in food and digestion.



Source: (BUTT; VOLKOFF, 2019).

The composition of the microbiota has been show to provide changes in the biosynthesis and metabolism of carbohydrates, amino acids and lipids pathways (NI et al., 2014) and fish fed with probiotics supplemented diets can show greater weight gain, feed efficiency and growth performance (YE et al., 2011). This may be attributed to increased food intake and improved digestibility of nutrients. However, some results may differ between species studied and variations in methodology and arrangement of nutrients in each diet.

2.6.2 Stress response

Stress response is mediated by various hormones and is the result of bidirectional communication between the brain and peripheral organs. Fish stress can be caused by several environmental factors (including low water quality, high particle levels, suboptimal photoperiod, oxygen levels, temperature), high population density, poor diet / malnutrition, inadequate transport and handling (BUTT; VOLKOFF, 2019). When stress occurs, the hypothalamus-pituitary-adrenal (HPA) axis releases hormones that stimulate adrenal glucocorticoid secretion to prepare the body to deal with stress. In fish, as well as in mammals, the microbiota affects the HPA axis, the stress response and behavior, especially anxiolytic and locomotor behaviors, which can also affect eating behavior and energy homeostasis. For example, in zebrafish, improving the microbiota (through pro and prebiotics) reduces anxiety behavior and decreases the stress response and cortisol levels (DJ et al., 2016; FORSATKAR et al., 2017).

2.6.3 Reproduction

Reproduction is also closely related to energy homeostasis, as it is expensive in terms of energy and can only be successfully performed when there is sufficient energy available (BUTT; VOLKOFF, 2019). Studies have shown that the intestinal microbiota can contribute to the development of gonads and subsequent host reproductive success. For example, when administered continuously from birth to sexual maturation, *Lactobacillus rhamnosus* altered the intestinal microbiota and accelerated zebrafish larval development, improving sexual growth and differentiation (CARNEVALI; MARADONNA; GIOACCHINI, 2017).

Female zebrafish treated with *L. rhamnosus* showed an increase in the number of vitellogenic follicles and higher gonadosomatic indexes (GSI), the calculation of the gonad mass as a proportion of the total body mass, higher number of oocytes and higher levels of reproductive hormone expression compared to control fish, improving the reproductive success. Likewise, in other species, supplementation of feed with probiotics increases the GSI, fertility and the production of fingerlings from breeding females and the length and weight of fingerlings (GHOSH; SINHA; SAHU, 2007; MEHDINEJAD; IMANPOUR; JAFARI, 2019). Although the mechanisms that mediate the actions of the intestinal microbiota in host reproduction are still poorly understood, it is possible that these mechanisms involve the regulation of food, absorption of nutrients and energy homeostasis.

2.6.4 Immune responses

A consequence of animals' coevolution with their microbiotas is the profound influence of these microorganisms on the immune system, both locally in the intestine and also systemically (ABBAS; LICHTMAN; PILLAI, 2011). The microbiota protects the host from colonization and proliferation of environmental pathogens by a process known as "resistance to colonization". Although the mechanisms behind this resistance are not entirely clear, it is suggested that commensal bacterial species compete with pathogens, produce and secrete antimicrobial peptides, and stimulate mucin expression, controlling undesirable bacteria (BUTT; VOLKOFF, 2019).

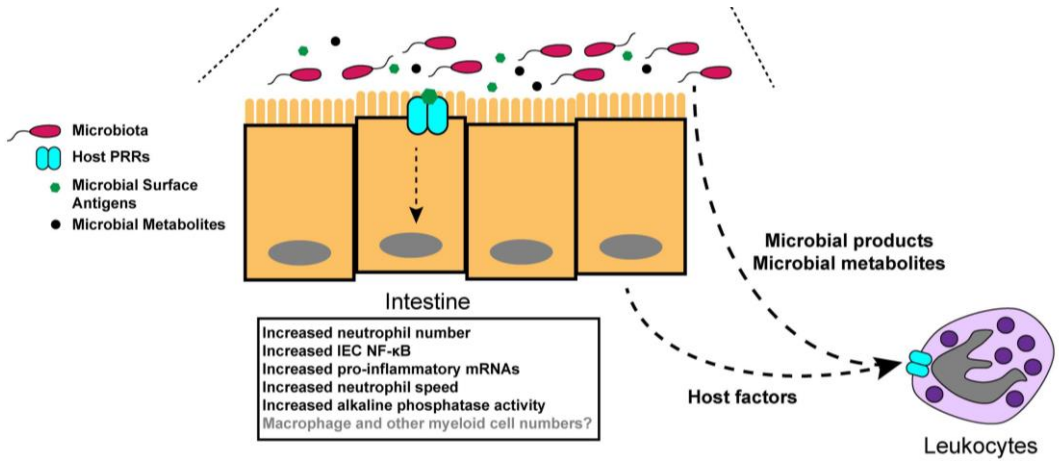
Commensal bacteria are also necessary for the proliferation and repair of the intestinal epithelial barrier after injuries (ABBAS; LICHTMAN; PILLAI, 2011). Any mucosal rupture can affect the intestinal balance and therefore lead to infections and activation of the Gut-Associated Lymphoid Tissue (GALT). The commensal microbiota associated with the mucosal

immune system has an important contribution to fish immunity as it also participates in GALT development and maturation (WANG et al., 2018).

Studies with germfree animals colonized with selected microbial communities have been essential to define microbiota-dependent changes in immune cell function and intestinal physiology during infections and diseases. In particular, zebrafish has emerged as a powerful vertebrate model organism with the ability to generate in vivo images, complete genetic approaches and easy methods to experimentally manipulate microbial communities (MURDOCH; RAWLS, 2019). Transcriptomic analyzes of the digestive tract of zebrafish larvae reveal many genes related to immune responses are regulated by the microbiota. In addition, colonization of the zebrafish intestine stimulates immune responses already observed in mammals, highlighting the importance of this relationship on evolutionary scales (RAWLS et al., 2006; RAWLS; SAMUEL; GORDON, 2004).

Neutrophil functions have been shown to be mediated by microbiota colonization, which can raise the inflammatory status of these cells in zebrafish during homeostasis (CLARKE, 2014). Neutrophils are professional phagocytes that promote microorganism's elimination and cellular debris through a variety of mechanisms (KRUGER et al., 2015). They are the most abundant circulating white blood cells and typically the first type of innate immune cell recruited to sites of injury or infection. The microbiota has shown to affect multiple aspects of neutrophil biology systemically and in distal tissues, and also promote intestinal infiltration of neutrophils in zebrafish larvae (Figure 10).

Figure 10: Several effects of the microbiota on the development and function of zebrafish innate immune system.

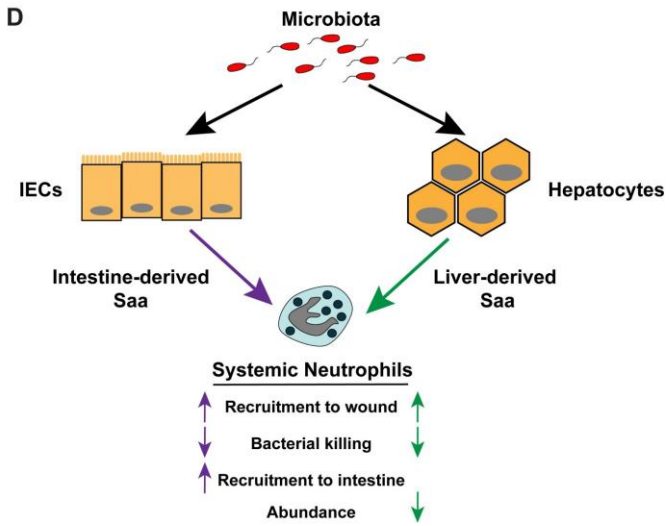


Legend: Germfree larvae colonization with microbiota stimulates inflammatory gene expression, especially neutrophil behavior and activity.
Source: (MURDOCH; RAWLS, 2019).

The molecular mechanisms underlying the intestinal microbiota influencing the development and function of myeloid cells are still unclear. A recent study identified an immune effector called Serum Amyloid A (Saa) as one of the most highly induced transcripts in digestive tissues after colonization of the microbiota in zebrafish larvae (MURDOCH et al., 2019). Saa is a protein produced and secreted in the intestine and liver with actions on neutrophils. The results of Saa's effects on neutrophils depend on the colonization of the microbiota, suggesting that this protein mediates the effects of the microbiota on the host's innate immunity.

Saa promotes the recruitment of neutrophils for peripheral wounds as shown in Figure 11. The analysis of isolated neutrophils revealed that Saa also reduces bactericidal activity and the expression of pro-inflammatory genes in a microbiota-dependent manner. These can be mechanisms developed by the host to limit an excessive activation of the innate immune system, through regulatory activities or even as mechanisms of immunological tolerance to the microbiota itself (MURDOCH et al., 2019).

Figure 11:The microbiota-induced Saa conditions neutrophils in vivo.



Legend: The microbiota induces the production of intestinal and hepatic Saa, leading to shared and distinct effects on neutrophils systemic functions
Source: (MURDOCH et al., 2019).

Although the microbiota and the host can have an extremely beneficial symbiotic relationship, defects in these complex regulations controlling homeostasis, or changes in an “ideal” microbiome, called dysbiosis, can also promote disorders. This close relationship risks

for several diseases, such as cancer (MURDOCH et al., 2019). Increasing evidence indicates a fundamental role of the microbiota in carcinogenesis, cardiovascular diseases, inflammatory bowel diseases, allergic diseases such as atopic asthma, behavioral disorders, diabetes, autoimmune diseases, among others (DURACK; LYNCH, 2019; RAZA et al., 2019; SCHWABE; JOBIN, 2013; SHREINER; KAO; YOUNG, 2015).

The interactions between the microbiota and the host's immune system are numerous, complex and bidirectional. The immune system must learn to tolerate the commensal microbiota and respond appropriately to pathogens, and in turn, the microbiota is essential to educate the immune system to function properly (SHREINER; KAO; YOUNG, 2015). Different factors that interfere in the composition of the bacteria present in the intestine, can modulate its functions and interferences in the organism, varying from a state of health to the predisposition to pathological states. It is not yet clear which changes in the microbiota associated with the disease are significant and the distinction between cause and effect is challenging.

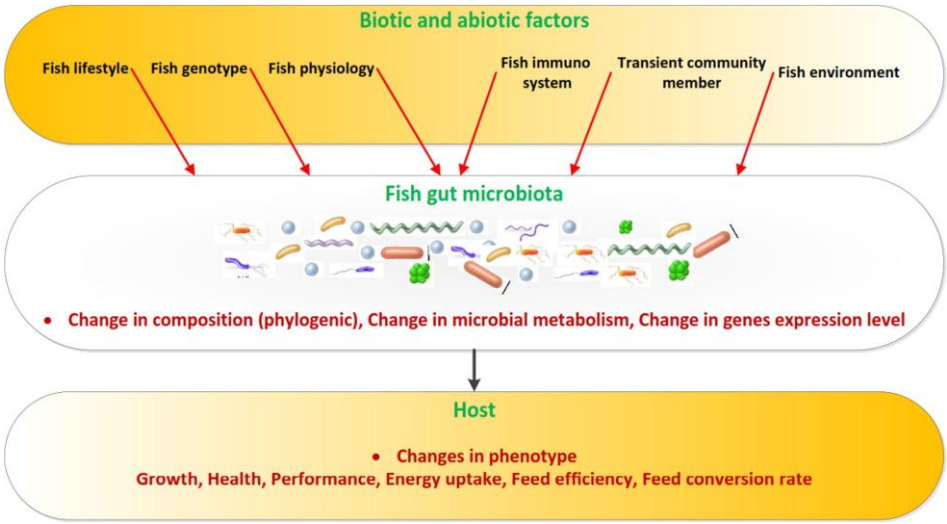
It is also noted that the disease state can lead to changes in the microbiota through several mechanisms, including changes in eating habits and bowel function, in addition to drugs administration such as antibiotics (SHREINER; KAO; YOUNG, 2015). Understanding these characteristics and their influences is extremely important in aquaculture when it comes to defining animal welfare, preventing and identifying problems (diagnosis and prognosis of diseases) or helping to find efficient treatments to reduce economic losses. It is clear that there is still a long way to go to better understand this complex network of factors that shape individuals' health status.

Studies on fish intestinal microbiota can help to improve well-being practices in aquaculture. However, when dealing with a group as diverse and broad as fish, many challenges can be encountered. Genetic and environmental influences, as well as a small number of species studied, indicate more research is necessary to understand particularities. Also, different types of experimental methods have been used, highlighting the need for appropriate standardization in microbiota description (VATSOS, 2017). Studying the intestinal microbiota in any species is extremely complex because many mechanisms of action, local and endocrine pathways, different physiological systems and molecules (hormones, metabolites) interact with each other. In addition, each microorganism within the microbiota may have different actions and the function of each one individually is still unknown (BUTT; VOLKOFF, 2019).

2.6.5 Factors that determine the composition of fish intestinal microbiota

Biotic factors (e.g., genotype, physiological status - mainly nutritional and immunological -, sex, sexual maturity, age) and abiotics (environmental factors such as water quality, temperature, light, diet) can affect fish intestinal microbiota and influence its composition and diversity, as well as its metabolic function and activity, affecting the behavior, feeding, growth, energy storage, stress response and animal´s general health (Figure 12) (GHANBARI; KNEIFEL; DOMIG, 2015).

Figure 12:A combination of biotic and abiotic factors affect the composition, function and metabolic activity of the fish gut microbiota.



Legend: Changes affect processes involved in growth, performance, energy storage and health in fish.
Source: (GHANBARI; KNEIFEL; DOMIG, 2015).

Among various factors, different eating habits / diets of fish species can greatly influence the structure and composition of the intestinal microbiota. In carnivores, bacterial diversity is generally less and progressively increases in omnivores, herbivores and planktophages. This diversity is related to the intestine size, as well as a more diversified microbiota that facilitates the fermentation processes of plant material in diet (BUTT; VOLKOFF, 2019). There are also greater abundances in some groups of bacteria associated with eating habits. For example, in carnivorous fish, the most abundant bacteria include *Clostridium*, *Cetobacterium* and *Halomonas*, in omnivorous fish include *Cetobacterium* and *Halomonas* and, in herbivorous fish, include *Clostridium*, *Citrobacter* and *Leptotrichia*. This trend was found in marine and freshwater fish, suggesting that the trophic level is probably one

of the most influential factors affecting the composition of the intestinal microbiota (EGERTON et al., 2018; LIU et al., 2016).

In addition, intestinal microbiota can also vary within species of the same trophic level. For example, in four species of herbivorous Asian carp (*Hypophthalmichthys molitrix*; *Hypophthalmichthys nobilis*; *Ctenopharyngodon idella*; and *Cyprinus carpio*) raised in the same environmental conditions, differences in relative abundance were observed in the phylum Firmicutes, known as cellulose degraders, probably due to specific diets of each species (LI et al., 2018b). In addition, changes in the composition of the fish's diet can result in changes in the microbiota and digestive tract as well. A study with Nile Tilapia (*Oreochromis niloticus*) after receiving a diet containing probiotic additive (*Bacillus cereus* and *Bacillus subtilis*) had induced intestinal colonization by these beneficial bacteria, in addition to promoting a higher percentage of survival, decreased intestinal mucosa peeling and favored the number of goblet cells increase in juveniles of the species (MELLO et al., 2013).

Interestingly, during fasting periods (short and long), morphological changes occur in the intestinal tract due to reduced nutrient uptake and a depletion of nutrients induces changes in the composition of the intestinal microbiota, decreasing microbial diversity and richness and favoring species that use a more diverse energy sources and are able to survive under limited nutrient conditions (XIA et al., 2014). This fact suggests that food frequency can also have consequences. Food when eaten in the diet can modulate the microbiota through nutrients bioavailability, which works as substrates for bacteria, but can also modulate by changing the conditions of the gastrointestinal tract, such as pH or release of specific digestion enzymes such as bile, favoring or impairing the development of certain bacteria (MERRIFIELD; RODILES, 2015).

2.7 Metagenomics studies

The development of genetic techniques allowed a metagenomic study, important to describe the microbial species diversity existing in the intestine, which would not be possible to detect through bacterial cultures because a large number is not yet cultivable (PREIDIS; VERSALOVIC, 2009). Metagenomics was first described in 1998 by Handelsman and Rodon and it aims to catalog all the genes from a community by the random sequencing of all DNA extracted from the sample (WANG et al., 2015). Firstly, the total DNA of all microorganisms is extracted from intestinal tissue or fecal samples. The comprehensive sequences are then analyzed to obtain either species profiles based on phylogenetic markers (SUNAGAWA et al.,

2013) or genomic profiles based on whole genomes (TRINGE et al., 2005). The information obtained from the sequence-based enables a more comprehensive understanding of the structure of microbial communities than ever before.

The microbial diversity evaluation, the identification of bacterial species and the performance of clusters by sequence comparison are possible due to the high conservation degree of the gene encoding the 16S rRNA in the Bacteria and Archaea domains (WOESE, 1987). Taxonomic attribution is possible due to the presence of 9 hypervariable regions (V1-V9) that contain sufficient sequence diversity to classify microorganisms. In addition, since the conserved regions flank these variable regions, PCR amplification using universal primers is possible. Each 16S rRNA amplification product and subsequent sequencing can be considered representative of a single bacterium within a mixed population. This approach allowed the characterization of bacterial communities without isolation or culture in a wide range of uses (PREIDIS; VERSALOVIC, 2009).

With the rapid development of advanced molecular technologies, it has been shown that the gut microbial ecosystem is far more complex than previously thought (ECKBURG et al., 2005). The detailed study of intestinal microbiota composition and its metabolic functions enables determine which microorganisms make it possible to keep the intestine healthy and which changes can lead to pathologies development (PREIDIS; VERSALOVIC, 2009). Metagenomics can not only identify the diversity of the gut microbiome, but can also reveal new genes and microbial pathways, and uncover functional dysbiosis. The application of metagenomics has huge potential in revealing the mechanisms and correlations between the intestinal microbiome and many diseases (WANG et al., 2015).

2.8 Cancer and its interaction with the immune system

The concept of cancer has undergone some changes in recent years, as it was initially considered to be just a set of altered cells in proliferation, and today cancer is better understood as a complex tissue in which there is a microenvironment, with interactions between cellular elements and molecular components, determinants in tumor progression (FIGUEIREDO, 2019). Thus, the understanding of the neoplastic event gains greater complexity, since the dynamics of tumor cells are now assessed as part of a real tissue (where there is vascularization, oxygenation, interstitial pressure and tissue necrosis). New components of this tumor niche are being identified, including elements of the immune system (ONUCHIC; CHAMMAS, 2010).

In the current oncogenesis, cancer cells undergo a sequence of mutations or genetic changes, a result of both intrinsic (inherited genetic mutations or random errors in DNA replication) and extrinsic factors, for example: damage and genetic instability induced by radiation, chemicals or microbial infections (TANNOCK et al., 2005). Studies show that non-cancer cells also play a significant role in several tumor development processes (ONUCHIC; CHAMMAS, 2010). Among these cells, those of the immune system and its products, as well as characteristics of the tumor inflammatory infiltrate, interfere with its development and progression. This indicates that not only does cancer constitute a microenvironment, but it is inserted in a macroenvironment - the organism - in which the immune cells migrate to the tumor and start composing its stroma and influencing its progression (FIGUEIREDO, 2019).

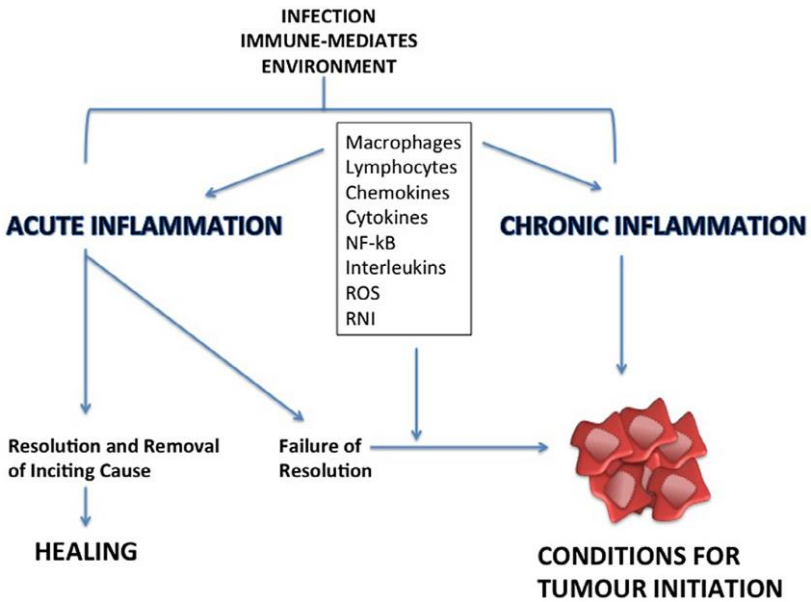
Several researches reveal an important association between inflammation and cancer, showing that chronic inflammation is one of the epigenetic factors that most contribute to the tumor appearance and progression (COUSSENS; WERB, 2002). Acute inflammation is a fast and self-limiting process, but it can evolve to a chronic status, which can be responsible for other diseases onset (ZHAO et al., 2017). The body's inflammatory response causes cellular changes and immune responses that result in the damaged tissue repair and cell proliferation (growth) at the site of the injured tissue. The inflammation can become chronic if the cause of the acute inflammation persists or if certain negative control mechanisms tasked with stopping the process fail. When these inflammatory responses become chronic, it can result in cell mutation and proliferation, often creating an environment conducive to the onset and especially the development of cancer (SINGH et al., 2019). Several signaling pathways are the main contributors to the creation of epigenetic changes outside the cell, activating these internal mutations. Chronic inflammation has been associated with several stages involved in tumorigenesis, including cell transformation, promotion, survival, proliferation, invasion, angiogenesis and metastasis (SINGH et al., 2019).

Currently, it is known that inflammation can play a fundamental role in cancer, from the beginning of the transformed phenotype to its metastatic spread. However, inflammation and cancer have a profound and ambiguous relationship. Inflammation (especially chronic) has protumorigenic effects, however inflammatory cells also mediate an immune response against the tumor and immunosuppression is known to increase the risk of certain tumors (SHALAPOUR; KARIN, 2015). More recent works address molecular and cellular activities that link inflammation and cancer and two types of pathways have been identified: an intrinsic and an extrinsic (AGGARWAL; VIJAYALEKSHMI; SUNG, 2009). Intrinsically, genetic

changes that causes neoplasms initiate the expression of inflammation-related stimuli that guide the construction of an inflammatory microenvironment. Intrinsic inflammation, induced by cancer, can be triggered by mutations of different causes and contribute to malignant progression through the recruitment and activation of inflammatory cells. In the extrinsic route, previous inflammatory conditions are what facilitate the onset and development of cancer. Inflammation extrinsic to the tumor is caused by many factors, including bacterial and viral infections, autoimmune diseases, obesity, smoking, intoxications and excessive alcohol consumption (SINGH et al., 2019).

In chronic inflammatory diseases, the dominant presence of leukocytes in the injured tissue is characterized (TANG; WANG, 2016) and many of these cells release toxins and substances that can be harmful to invading agents, but also to the tissues of the organism itself. Consequently, chronic inflammation is almost always accompanied by tissue destruction. Under infection or persistent injury, it drives the transformation of cancer precursor cells producing substances capable of inducing DNA damage and genomic instability (Figure 13). In a positive feedback loop, DNA damage can also lead to inflammation, supporting tumor progression (RAPOSO et al., 2015).

Figure 13: Change from acute to chronic inflammation and condition for tumor progression.



Source: (RAPOSO et al., 2015).

The main limiting step in the development of cancers is the progression of premalignant lesions, many of which may exist in an inactive state for years before they actually become malignant tumors. This stage can be controlled by intrinsic inflammation (triggered by the tumor) and also by extrinsic inflammation and can be attenuated by immunity called antitumor or immune surveillance, essential to maintain numbness (SHALAPOUR; KARIN, 2015). This makes this topic extremely complex and ambiguous. The perception of cancer as just a set of proliferating cells has been shown to be incomplete and reductionist, with the concept of TME (tumor microenvironment) emerging from new genetic, biochemical and molecular studies.

2.8.1 Tumor microenvironment (TME)

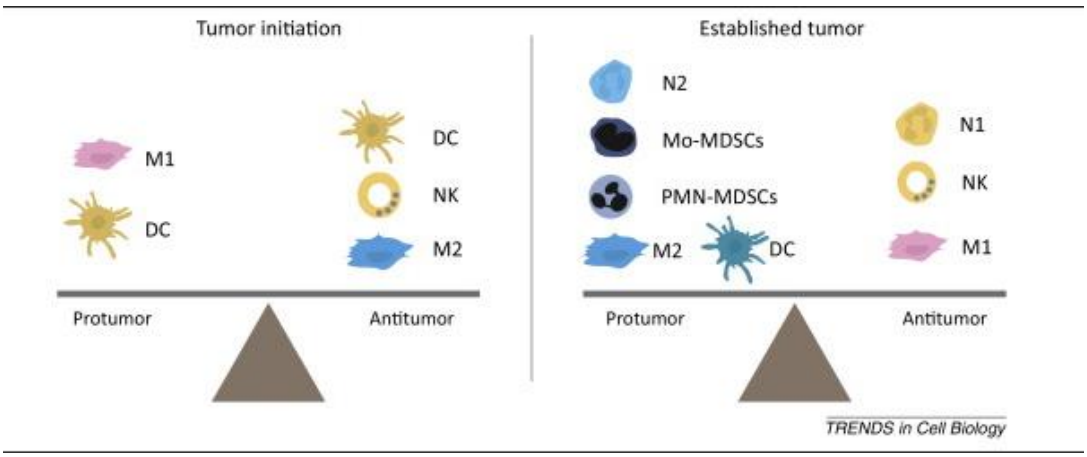
The tumor microenvironment is represented by neoplastic cells and non-neoplastic elements of the tumor, such as fibroblasts, immunoinflammatory cells, cells that make up the blood vessels and all the signaling molecules (positive and negative) produced, which reflect a powerful communication network active in the tumor sites. TME is therefore defined as a biologically complex tissue that exhibits important distortions of the original tissue homeostasis, in which non-neoplastic cells (which do not have unregulated proliferation rates or increased genetic instability) are reprogrammed to act in accordance with this new tissue dynamics, dictated mainly by neoplastic cells (ONUCHIC; CHAMMAS, 2010). MAT contains, in addition to neoplastic cells and the surrounding stroma (fibroblasts, endothelial cells, pericytes and extracellular matrix proteins), innate immune cells, including macrophages, neutrophils, mast cells, suppressor cells derived from the myeloid lineage, dendritic cells, NK cells (natural killer) and adaptive immune cells (T and B lymphocytes) (FIGUEIREDO, 2019).

The inflammatory process is considered a fundamental component of MAT, as it is part of the important communication network that characterizes it. Different types of cells, influenced by the immunoinflammatory process, interact (in an autocrine and paracrine way) to control tumor growth. Inflammation associated with MAT acts as a mediator between neoplastic and stromal cells, through the production of cytokines, growth factors and remodeling enzymes of the extracellular matrix, creating a multidirectional system that interferes with the development of the tumor (FIGUEIREDO, 2019). In addition, the tumor can direct the behavior of inflammation, both promoting its growth and stimulating the host's resistance to antitumor immunity (LIU; LIN; ZHOU, 2015). While acute and transient inflammation is a control and repair factor for tissue damage, the inflammation associated with

the tumor is of the chronic, non-resolving type, which promotes tumor progression (SUAREZ-CARMONA et al., 2017).

There is a system of multidirectional influence that has brought about, scientifically, a new definition of cancer, now understood as a complex tissue society, in which the majority of the members cooperate to facilitate the growth of the neoplasia, to convert to immune resistance and to favoring metastatic dissemination (FIGUEIREDO, 2019). Immune cells have the ability to tune into the inflammatory response and play a key role in cancer-related inflammation. Understanding what distinguishes pro-tumor immune cells from their antitumor counterparts and the ability to therapeutically tune the inflammatory response are crucial in the fight against cancer (Figure 14).

Figure 14: Balance of the immune system.



Legend: The pro-tumor versus anti-tumor role of different innate immune cells may differ depending on the context of the tumor, that is, in the "initiation of the tumor" or in the "established tumor", making it difficult to differentiate between what is a friend and an enemy.
Source: (HAGERLING; CASBON; WERB, 2015).

2.8.2 Anti-tumor immunity

Myeloid and lymphoid cells infiltrated in tumors can promote or inhibit the development of cancer, depending on the nature of the interaction between the cancer and the immune system. Innate and adaptive immune cells can and usually recognize tumor-specific antigens and molecular patterns and actively destroy transformed cells. The deregulation of the cytokine / chemokine profile that persists at the sites of inflammation is what results in the development of cancer and many other pathologies (PESIC; GRETEN, 2016; RAPOSO et al., 2015; SHALAPOUR; KARIN, 2015; TANG; WANG, 2016). Historically, the immune system was

first described as a mechanism for eliminating tumor cells. The concept of immunological surveillance argues that the immune system is able to recognize and eliminate developing tumors, thus being able to prevent the appearance of clinically apparent tumors, even in the absence of therapeutic intervention (ZITVOGEL; TESNIERE; KROEMER, 2006).

The theory supports the hypothesis that tumor cells express neoantigens (tumor specific antigens) that could activate an immunity called antitumor, which, in some cases, could lead to the rejection of early neoplasms (SHALAPOUR; KARIN, 2015). This theory gained prominence with the development of therapy using immunological checkpoint inhibitors, in which it was found that reactivation of cytotoxic T lymphocytes (by blocking their negative signaling) could cause rejection and elimination of tumors. The response to the signal of presentation of antigens in these cells is regulated by a series of coreceptors, which recognize ligands present on the surface of tumor cells. These co-receptors can induce both positive (stimulatory) and negative (inhibitory) intracellular signaling cascades, modulating T cell activities related to proliferation, cytokine secretion and cell lysis. These immune system molecules, which can stimulate and inhibit signals, are known as immunological checkpoints (FIGUEIREDO, 2019).

Among these molecules, PD-L1 stands out, which has been identified in the cells of several solid tumors, such as lung carcinoma, breast carcinoma, glioblastoma, mouth carcinoma and gastric carcinoma, being related to the immune escape of cells tumoral. In tumor cells, overexpression of PD-L1 is associated with the emergence of more aggressive clones (GONÇALVES, 2017). Tumors are able to escape destruction by the immune system by producing these proteins on the surface of their cells that act as a key to identify a lock, the PD-1 molecule present in T cells of the immune system. The key-lock connection leads to the blocking of T cells and the maintenance of tumor cells. The therapeutic block of this protein is used in immunotherapy for melanomas, for example (LANDSBERG et al., 2012). Such therapy is called the T cell checkpoint antagonist.

In the current scenery, the intestinal microbiota is also receiving significant attention, due to its influence on a number of diseases, including cancer. In the past decade, it has been substantial progress in understanding the development of cancer and the influence that the microbiota has on host-related processes. The interference of these microorganisms in the response to cancer treatment has become increasingly apparent, with evidence suggesting that modulation of the intestinal microbiota can affect responses to different forms of therapies (SCHWABE; JOBIN, 2013).

In recent years, a crucial role has been demonstrated in mediating the activation of the immune system for responses to chemotherapeutic agents (IIDA et al., 2013; VIAUD et al., 2013). Studies have shown that the intestinal microbiome can influence anti-tumor immune responses through innate and adaptive immunity (SIVAN et al., 2015; T et al., 2007) and that therapeutic responses can be improved through its modulation (GOPALAKRISHNAN et al., 2018; IIDA et al., 2013; VIAUD et al., 2013). With this, a practical knowledge of the intestinal microbiota becomes vital as we move into an era that precision medicine is needed.

2.8.3 Anti-inflammatory agents and cancer

Anti-inflammatory agents can be used as effective adjuvants in conventional cancer therapies. Clinical and preclinical studies suggest that the combined use of anti-inflammatory agents and conventional therapies can improve the patient's prognosis (RAYBURN; EZELL; ZHANG, 2009). Non-steroidal anti-inflammatory drugs (NSAIDs) are selective or non-selective inhibitors of COX-1/2, which are widely prescribed to decrease pain, reduce fever and inflammation. NSAIDs inhibit cyclooxygenase enzymes and angiogenesis. The COX2 / PGE pathway is involved in multicancer processes, including carcinogenesis, proliferation and metastatic spread. Although these molecular pathways are not clearly elucidated, it is known that within the tumor microenvironment, COX-2 can also be produced and amplified, promoting the progression of the tumor to advanced metastatic states (RAPOSO et al., 2015). Thus, NSAIDs inhibit COX2, which its abnormally high expression is seen in multi-cancers. The obvious treatment for cancer-related inflammation would be the use of NSAIDs, which are already used as a therapeutic option in the treatment of patients with colorectal cancer (DIN et al., 2010) and prostate cancer (LIU et al., 2014).

Although NSAIDs are effective anti-inflammatory agents, other agents (specific COX-2 inhibitors) were designed to generate more active compounds with less gastrointestinal toxicity (for example celecoxib). Corticosteroids, most commonly used to prevent or decrease the side effects of chemotherapy and radiation, also demonstrated anti-cancer activity when used alone or in combination with chemotherapeutic agents (RAYBURN; EZELL; ZHANG, 2009). In addition, there are numerous other anticancer approaches that seek to modify the host's immune response, decreasing the absence of a tumor immune response or decreasing the tumor's inflammatory microenvironment. These strategies use receptor agonists or antagonists present in immune cells, antibodies or other agents that can decrease the expression or activity

of pro-inflammatory molecules or their receptors or by treatment with specific cytokines or chemokines (RAYBURN; EZELL; ZHANG, 2009).

Natural products have also been shown to prevent or decrease inflammation through different mechanisms, including inhibition of NF-kB, COX-1 and -2 signaling, as well as decreased VEGF and iNOS (AGGARWAL; SHISHODIA, 2006). Many agents exert antiproliferative, pro-apoptotic or cell cycle inhibitory activities. Pre-clinical studies suggest that many compounds derived from natural products have potent activity against cancer cells or xenotransplanted tumors and that they can prevent the carcinogenesis or metastasis of existing tumors (STRIMPAKOS; SHARMA, 2008). Therefore, it is not surprising that natural products are now also being used for cancer prevention and / or therapy and as adjuvants to conventional therapies. The combination of these natural anti-inflammatory compounds with conventional therapies can provide interesting effects for cancer patients (RAYBURN; EZELL; ZHANG, 2009).

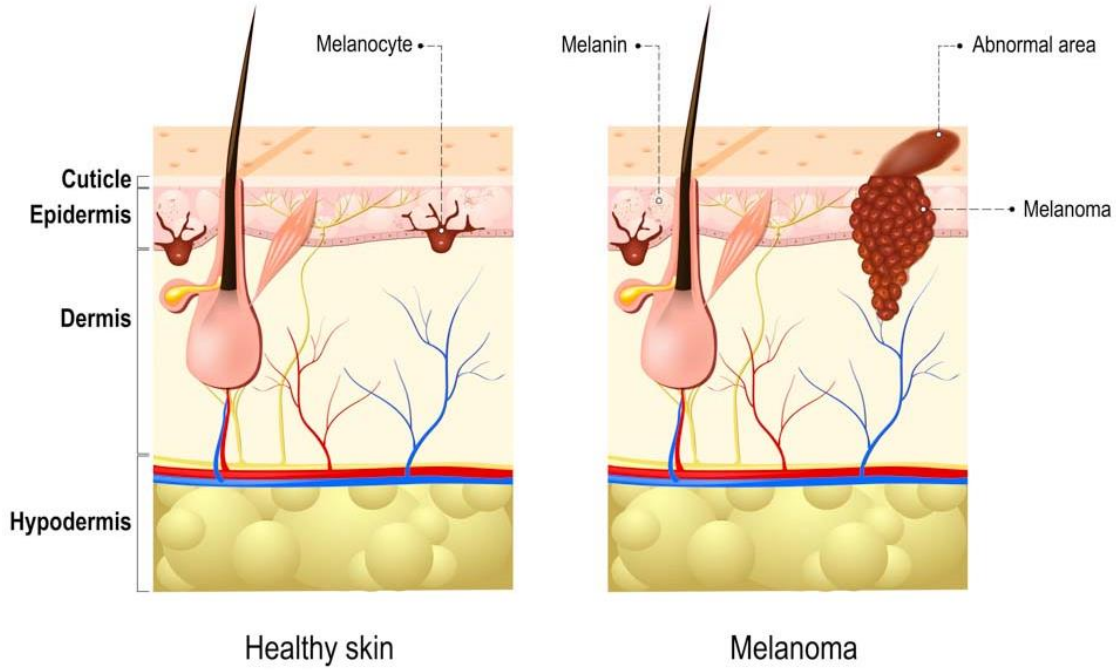
Cancer research has focused for many years on tumor cells alone; however, it is clear that the entire tumor must be considered as a system under constant selection pressure. The cancer-related complexity that evolves in a tumor microenvironment deserves attention and should be the focus of future research. As tumors develop, immune and inflammatory antitumor and pro-tumorigenic mechanisms coexist, but if the tumor is not eradicated, protumorigenic inflammation predominates. The key mediators of inflammation generally have dual roles that depend on the context, leaving a number of specific clinical challenges and the need to design therapeutic strategies based on this new understanding of the concept of cancer.

2.9 Skin Cutaneous Melanoma: aggressive and malignant skin cancer

Cutaneous melanoma (CM) originates from melanocytes, whose main function is to protect keratinocytes from UV-induced DNA damage (WELLBROCK; AROZARENA, 2016). The malignant transformation of melanocytes generates this fatal form of skin cancer with a complex multigenic etiology that becomes extremely difficult to treat after metastasis (Figure 15). It was estimated that 287,700 new cases of melanoma and 60,700 deaths of melanomas occurred worldwide in 2018 (FERLAY et al., 2019). Patients with metastatic melanoma have a shorter long-term survival time and survival outcomes can vary widely among patients even within the same stage due to the biological heterogeneity of melanoma (ZHANG et al., 2020). Currently, melanoma is very common in the Western world. Although incidence rates are decreasing for most cancers, they are constantly increasing for melanoma worldwide (VAN

ROOIJEN; FAZIO; ZON, 2017) mainly due prolonged exposure to the sun and, consequently, to UV (SCHADENDORF et al., 2015).

Figure 15: Health skin and melanoma.



Source: (VOCATURO; ZUMPANO; VELTRI, 2019).

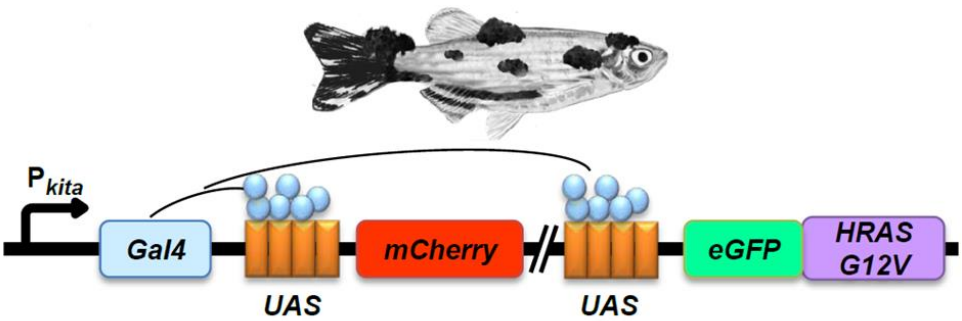
Due to their genetic heterogeneity, scientists around the world in recent years are looking to develop effective therapies. At present, the methods commonly used in the treatment of melanoma include surgical resection, chemotherapy and immunotherapy (ZHANG et al., 2020). Only a few patients with advanced melanoma have a persistent response to surgical resection and chemotherapy. In addition, the combination of chemotherapy drugs may improve drug resistance (CHAUBE et al., 2015; MOHAMMAD et al., 2014). However, because of the molecular heterogeneity, not all the melanoma patients responded well to the treatments (ZHANG et al., 2020).

The development of animal models has allowed a better understanding of melanoma pathomechanisms. In particular, the zebrafish can be used as an excellent tool (BOOTORABI et al., 2017). The injection of melanoma cells into zebrafish enables the study of tumor cell spread, melanoma progression and the phenotype switching toward metastatic behavior (BOOTORABI et al., 2017). Furthermore, it has already been shown that there is a conservation of the molecular mechanisms of carcinogenesis between zebrafish and humans,

since the expression of human oncogenes is capable of transforming zebrafish cells (MIONE; TREDE, 2010).

Zebrafish are valuable for studying the biology of melanocytes as they also share a high degree of conservation in underlying molecular mechanisms of these cells with the human species (MORT; JACKSON; ELIZABETH PATTON, 2015). Currently, it is known that transgenic zebrafish lines that express oncogenes triggered by specific promoters can be generated (BOOTORABI et al., 2017). The zebrafish melanoma model (Figure 16) used for this thesis is the kita:Gal4;eGFP-HRAS_G12V (GÓMEZ-ABENZA et al., 2019). This model expresses the oncogenic human HRAS_GV12 gene driven by the kita melanocyte cell-specific promoter and develops melanoma at 1-3 months of age, resulting from hyperproliferation of embryonic melanocytes 3 days after fertilization.

Figure 16:Schematic diagram of the SKCM model line in zebrafish.



Source: (Adapted from GÓMEZ-ABENZA et al., 2019).

Thus, the development of zebrafish models contributes to fill the gap between in vitro and in vivo studies, especially those that cannot use mammalian models for rapid preclinical studies. This improves science and facilitates new discoveries and treatments.

3 HYPOTHESIS

Supplementing zebrafish (*Danio rerio*) diet with bee pollen improves growth, reproductive performance and the immunological status of adult fish and their offspring.

4 OBJECTIVES

4.1 General Objective

The aim of this work was to analyze the effects of bee pollen on zebrafish (*Danio rerio*) diets through evaluation of fish reproductive, growth and immunological performance.

4.2 Specific Objectives

a) Evaluate whether bee pollen supplementation alters zebrafish parameters related to embryos and larvae quality

b) Evaluate immune response in larvae derived from zebrafish breeders fed pollen

c) Evaluate whether bee pollen addition in zebrafish diet could influence growth parameters

d) Evaluate bee pollen supplementation effects on adult zebrafish intestinal tract, in particular, in the microbiota

e) Evaluate whether bee pollen supplementation affects in vivo cancer development, once it has been described with anti-carcinogenic properties

5 FINAL CONSIDERATIONS

The present thesis demonstrated that the addition of bee pollen to the zebrafish (*Danio rerio*) diet does not alter its growth or improve its reproductive performance when compared to a control diet based on commercial feed and brine shrimp. However, the inclusion of bee pollen was able to alter immune responses of fish offspring that received the supplementation, favoring the survival of larvae after a viral challenge and increasing the migration of neutrophils in the wound site. Furthermore, adult animals that were supplemented with bee pollen in the diet had significant changes in the abundance of several intestinal microorganisms compared to non-supplemented animals, which may possibly be related to the unfavorable tumor development of cutaneous melanoma after allotransplantation. The animals that received the bee food supplement showed higher tumor growth rate. Although widely described as a beneficial food for performance and health, more attention should be given to studies regarding the use of bee pollen by different species in different physiological states.

[Español]

La presente tesis demostró que la adición de polen de abeja a la dieta de peces cebra (*Danio rerio*) no altera su crecimiento ni mejora su desempeño reproductivo en comparación con una dieta control basada en polvos comerciales y artemia. Sin embargo, la inclusión de polen de abeja pudo alterar las respuestas inmunes de las crías de peces que recibieron la suplementación, favoreciendo la supervivencia de las larvas ante un desafío viral y aumentando la migración de neutrófilos en el sitio de herida. Además, los animales adultos que fueron suplementados con polen de abeja en la dieta tuvieron cambios significativos en la abundancia de varios microorganismos intestinales en comparación con los animales no suplementados, lo que posiblemente puede estar relacionado con el desarrollo tumoral desfavorable del melanoma cutáneo después del alotrasplante. Los animales que ingirieron el complemento alimenticio para abejas mostraron una mayor tasa de crecimiento tumoral. Aunque se describe ampliamente como un alimento beneficioso para el rendimiento y la salud de humanos y animales, se debe prestar más atención a los estudios sobre el uso del polen de abeja por diferentes especies en diferentes estados fisiológicos.

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SECOND PART – SCIENTIFIC ARTICLES

MANUSCRIPT 1 – BEE POLLEN AS A DIETARY SUPPLEMENT FOR FISH: EFFECT ON THE REPRODUCTIVE PERFORMANCE OF ZEBRAFISH AND THE IMMUNOLOGICAL RESPONSE OF THEIR OFFSPRING

Manuscript submitted to *Fish and Shellfish Immunology*

Bee pollen as a dietary supplement for fish: Effect on the reproductive performance of zebrafish and the immunological response of their offspring

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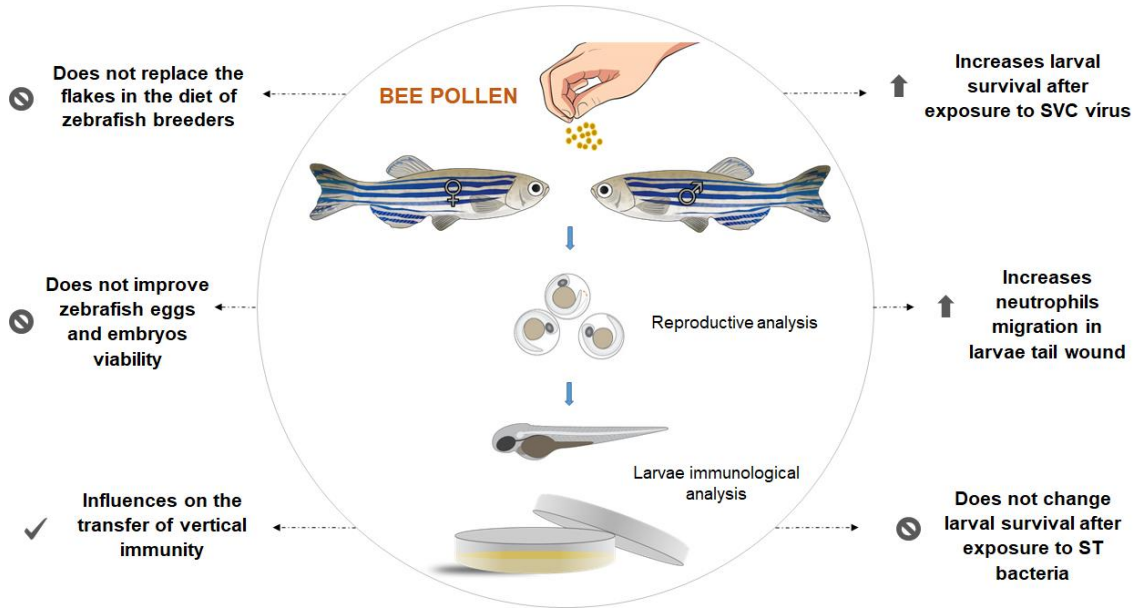
Abstract

Bee pollen, a natural resource collected by bees, is rich in many nutrients, therefore it may represent a useful dietary supplement. Different uses of bee pollen are proposed due to its beneficial health properties, which includes the capacity to improve animal performance and promote immunostimulation. Animal nutrition can directly affect adults and their offspring, and larval stage is a critical moment for fish due to high mortality related to immune challenges. Thus, the present study attempted to evaluate the effects of adding bee pollen to a zebrafish diet, specifically, analyzing the effects on reproduction and immunity transference to descendants. Zebrafish adults received control diets based on commercial flakes and live food *Artemia* sp. nauplii or bee pollen-supplemented diets, administered three times a day, at the same time. The animals received the diets over 60 d, and throughout this period, they were tested for: egg production per female, total number of eggs, embryo viability rate, larval survival rate after exposure to spring viremia of carp virus and to *Salmonella enterica* serovar Typhimurium, and larval neutrophil recruitment after tail wounding. Bee pollen supplementation failed to improve egg production and embryo viability, and was unable to substitute flakes in zebrafish breeders. Instead, the offspring of breeders fed with bee pollen supplemented diets showed longer survival upon virus exposure and higher neutrophil migration to wounds. These results indicate that bee pollen can influence vertical immunity through important mechanisms related to offspring immunity in the early stages, when larval immune system is not fully developed.

Keywords: Bee products; Immunology; Maternal immunity transference; Natural products; Nutrition; Reproduction.

Graphical abstract



Abbreviations

BW, body weight; hpf, hours post-fertilization; SVCV, spring viremia of carp virus; dpf, days post-fertilization; TCID, tissue culture infectious dose; ST, *Salmonella enterica* serovar Typhimurium; LB, Luria Bertani; PBS, phosphate-buffered saline; mpw, minutes post-wounding; IgM, immunoglobulin M; Ifn, interferon;

1. Introduction

Apicultural products have long been used in phytotherapy, as well as in diets, for their positive effects, and currently, bee products are gaining prominence due to their bioactive compounds, which are associated with beneficial health properties [1,2]. Bee pollen, in particular, is attracting increasing attention as a functional food due to its high content of essential amino acids, antioxidants, vitamins, enzymes and lipids [3]. Bee pollen is collected by honeybees (*Apis* sp.) and is a combination of primarily floral pollen with some nectar or honey, enzymes, wax and bee secretion. The pollen mixture is stored and used as food for all developmental stages in the hive [4], and analysis of different samples has indicated that bee pollen possesses good antioxidant activity and promising anti-inflammatory activity [5].

Bee products in fish diets have previously been described to provide an improvement in performance and immune status [6–8]. However, the use of bee pollen to improve reproduction and immunity in fish has not been thoroughly characterized. Although zebrafish, *Danio rerio*, is an ornamental species (Hamilton, 1822), it can also be utilized to study the characteristics of economically interest fish, and can be a model species for research on other animals and humans. At present, various commercial diets are used in research laboratories; in many cases, supplementation with live food (paramecium, rotifers and brine shrimp) is included during different stages of zebrafish development [9]. Brine shrimp, *Artemia* sp., nauplii are highly recommended for zebrafish diets and are essential for good development and reproductive performance [10].

Despite zebrafish widespread use, many diets used for the species are developed for commercial aquaculture or ornamental fish species, and the qualitative and quantitative composition of species-specific nutrients is unknown [11]. At present, there

are still no fully defined diets for zebrafish and no specific standardized nutritional requirements. Nutritional requirements may also vary according to each stage of life; larval, juvenile and adults, and there is a need to test the effects of diet components effects on survival, growth, resistance to diseases / stress and reproduction [12]. The frequency with which these animals are subjected to reproduction is also relevant.

Proper nutrition is highly important for reproductive success, as it facilitates more viable offspring and larger larvae [13]. Specific nutrients and food ingredients, or their lack, may alter fish physiology, behavior and/or molecular pathways [9], and some studies suggest that diet nutrient content provided to zebrafish adults can also influence the development and health of their descendants [14–16]. Little is known about the defense system in fish larvae, as they lack a developed immune system, and the exact role played by maternal immunity in the transmission of innate and adaptive immunity and the reaction of fish larvae to different environmental stressors have not been fully elucidated [17,18]. To date, no studies describing the effects of bee pollen supplementation on zebrafish breeders and the consequences on the health of their offspring have been reported.

The identification of mechanisms governing the biological activity of zebrafish fed with bee pollen supplemented diets can provide relevant information for the recommendation of this product in the diet of this and other species. The present work has a pioneering proposal and our goal was study the effect of a bee pollen-supplemented diet on the reproductive and immunological status of zebrafish determining parameters related to embryo and larval quality.

2. Materials and methods

2.1. Ethics statements

The experiments performed comply with the Guidelines of the European Union Council (Directive 2010/63/EU) and the Spanish RD 53/2013. Experiments and procedures were performed as approved by the Bioethics Committees of the University of Murcia (approval number 395/2017) and approved by the Ethical Research in Animal Use Committee (CEUA) of the Federal University of Lavras (approval number #001/18).

2.2. Zebrafish husbandry

Zebrafish (*Danio rerio* H. Cypriniformes, Cyprinidae) obtained from the Immunology, Inflammation and Cancer Laboratory at the Department of Histology and Cell Biology (University of Murcia, Spain), were maintained in 3-liter aquariums, in a water recirculation system with biological and mechanical filters, where each aquarium contained 1 male for each female. Animals were maintained in a 14/10 h light/dark cycle at 27 °C ± 1 °C, and water quality parameters were monitored daily.

2.3. Experimental diets

The experimental design was divided into groups with different types of diets, as outlined in **Table 1**. Adult wild-type zebrafish were fed three times a day, divided into 3 aquariums per treatment. All groups received different diets at the same time of day (9:00 am, 12:00 pm and 3:00 pm). The feed amount offered by individuals was 3% of BW (flakes and bee pollen), per meal, and the number of *Artemia* offered was 2000 per

individual per day (food protocol already established in the laboratory). Tropical Fish Flakes (Prodac, Italy) ware employed, which are also routinely utilized in the laboratory and are highly recommended for the species. According to the manufacturer, flake ingredients consist of cereals, fish, fish products, soy, yeast, crustaceans, algae, aloe vera and mineral and vitamin mixtures. The bee pollen samples used (obtained from the city of Neópolis, SE, Brazil) were crushed and sieved (0.5 mm) to enable ingestion by the animals. For brine shrimp (Inve Aquaculture, Thailand) hatching, cysts were subjected to the following protocol: incubation for 48 h with filtered marine water, at 28 °C under intense aeration until nauplii hatching followed by collection of nauplii after washing in fresh water immediately before being offered to the animals.

Table 1- Distribution of different types of diets by experimental group.

Groups	1st meal 9:00 h	2nd meal 12:00 h	3rd meal 15:00 h
1- Control	Flakes[1]	Flakes	Artemia[2]
2 - Pollen	Flakes	Bee pollen[3]	Artemia
3 - Pollen/Pollen	Bee pollen	Bee pollen	Artemia

[1]Tropical fish flakes (Prodac, Italy). [2]Brine shrimp (Inve Aquaculture, Thailand). [3]Neópolis, SE, Brazil.

The composition and proximate analysis of fish flakes and brine shrimp offered in the animals' basal diet are described in **Table 2** (data obtained from the manufacturers). Information not provided by the manufacturer was found in the literature [19].

Table 2 – Composition and proximate analysis of fish flakes and brine shrimp (data provided by manufacturers) offered in the animals' basal diet.

Composition	Proximate analysis (%)	
	Flakes	Brine shrimp
Crude protein	44.9	55.0
Ether extract	4.47	13.0
Ash	4.35	5.5
Fiber	2.14	6.8*

Moisture	7.73	68*
Carbohydrates	NI	13.22*

Values expressed for each 100 g of dry matter. NI = no information available. *Average values found in RIZK et al., 2018. Nutritional additives: vitamin A, 41.200 I.U./kg; vitamin D3, 3.000 I.U./kg; vitamin E, 297 mg/kg; vitamin C, 180 mg/kg.

The composition, proximate analysis and antioxidant capacity of bee pollen are also listed in **Table 3**, according to analyses performed at the Department of Food Sciences, University of Lavras, Brazil.

Table 3 – Composition, proximate analysis and antioxidant capacity of bee pollen.

Composition	**Proximate analysis (%)**
Moisture	14.56
Crude protein	17.57
Ether extract	5.14
Carbohydrates	60.38
Total sugar	50.41
Reducing sugars	24.78
Sucrose	25.64
Ashes[1]	3.02
Caloric value (kcal/100 g)	351.86
Antioxidant capacity	
Phenolic content	19.15
ABTS (μmol trolox/g)	3955.30

Values expressed for each 100 g of dry matter. *All values are in accordance with the Ministry of Agriculture, Cattle and Supplying (MAPA) normative instruction 3 (annex V) which addresses requirements for bee product commercialization. [1]Mineral analysis: N, 34.8 g/kg; P, 6.57 g/kg; K, 6.73 g/kg; Ca, 5.92 g/kg; Mg, 2.18 g/kg; S, 2.22 g/kg; B, 6.07 mg/kg; Cu, 11.69 mg/kg; Mn, 222.24 mg/kg; Zn, 64.40 mg/kg; Fe, 106.07.

2.4. Reproductive feature analysis

Throughout the diet period (42 d), 90 zebrafish, aged 11 months, were reproduced weekly to analyze spawning, embryo and larval quality. *Danio rerio* breeders from each treatment were separated on the day before breeding in small breeding tanks. The next day, in the morning the embryos were collected for analysis. Samples of 90 embryos per

treatment were maintained in Petri dishes containing egg water medium (30 embryos per plate) at 28 ºC. Egg water medium consists of a medium that allows zebrafish embryonic development standardization (200 mL of stock solution, 9800 mL of distilled water and 5 mL of methylene blue; stock solution composed of 0.1875 g of calcium carbonate, 1.875 g of sodium bicarbonate, 3 g of sea salt, and 1 liter of distilled water).

To determine zebrafish spawning and embryo quality throughout the diet period (42 d), egg production per female, total number of eggs and embryo viability (72 hpf) were analyzed weekly.

2.5. Larvae survival rate after spring viremia of carp virus (SVCV) exposure

responseTo evaluate the immune response of breeder offspring after receiving the various diets (21, 28 and 35 d after beginning the feeding regime), larval survival was evaluated after SVCV 56/70 infection. Groups of 30 zebrafish larvae were challenged 3 days after fertilization (dpf) in 5 mL of egg water containing ~ 10^8, 50% tissue culture infectious dose ($TCID_{50}$) / mL SVCV at 26 °C. Forty-eight hours later, the virus was diluted in 30 mL of egg water and larvae were monitored every 24 h over 5 d for survival curves [20].

2.6. Larval survival rate after *Salmonella enterica* serovar Typhimurium (ST) exposure

Based on previous results and to optimize the experiments, only two diet treatments were employed for subsequent analyses: Groups 1 and 2. Thus, the larval immune response (offspring obtained 21, 35 and 49 d after breeders started the feeding regime) was tested by evaluating larval survival after infection with ST strain 12023 (wild

type). ST was inoculated in 5 mL of Luria Bertani (LB; Condalab, Spain) culture medium and incubated overnight at 37 °C, and 250-300 rpm. The following morning, the inoculants were diluted 1/5 in the same medium with 0.3 M NaCl and incubated at 37 °C until reaching an optical density of 1.5 to 600 nm. Finally, the bacteria were diluted in sterile PBS for further experimentation. In the infection survival test, 70 zebrafish larvae (2 dpf) from each treatment were anesthetized in egg water medium containing 0.16 mg/mL of tricaine (Sigma Aldrich), and 10-50 ST bacteria per larva were microinjected into the yolk sac. The larvae were recovered in egg water at 28-29 °C and monitored for survival curves over 5 d [21].

2.7. Larval neutrophil recruitment analysis

To identify immune response differences in larvae derived from zebrafish breeders fed pollen diets (21 and 35 d after feeding started), neutrophil recruitment at the site of injury caused intentionally in the larval tail was analyzed [22]. Three dpf larvae were selected (30 larvae per treatment + 10 larvae as a control) and anesthetized in an embryo incubation medium (egg water) containing 0.16 mg/mL of tricaine (Sigma Aldrich). A complete transection of the tail tip was performed with a sterile disposable scalpel. After the procedure, larvae recovered from anesthesia in egg water medium at 28.5 °C. The larvae were euthanized with an anesthetic dose (1.2 mg/mL for at least 10 minutes) at 0, 90 and 360 minutes after tail injury and fixed in 4% formaldehyde solution. After applying the Sudan Black (Sigma Aldrich) staining protocol was applied and the total number of neutrophils that migrated to the wound site in each larva was individually counted using a light microscope. Neutrophils are known as one of the organism's first lines of defense, playing a fundamental role in host defense [23].

The experimental timeline is represented in **Figure 1.**

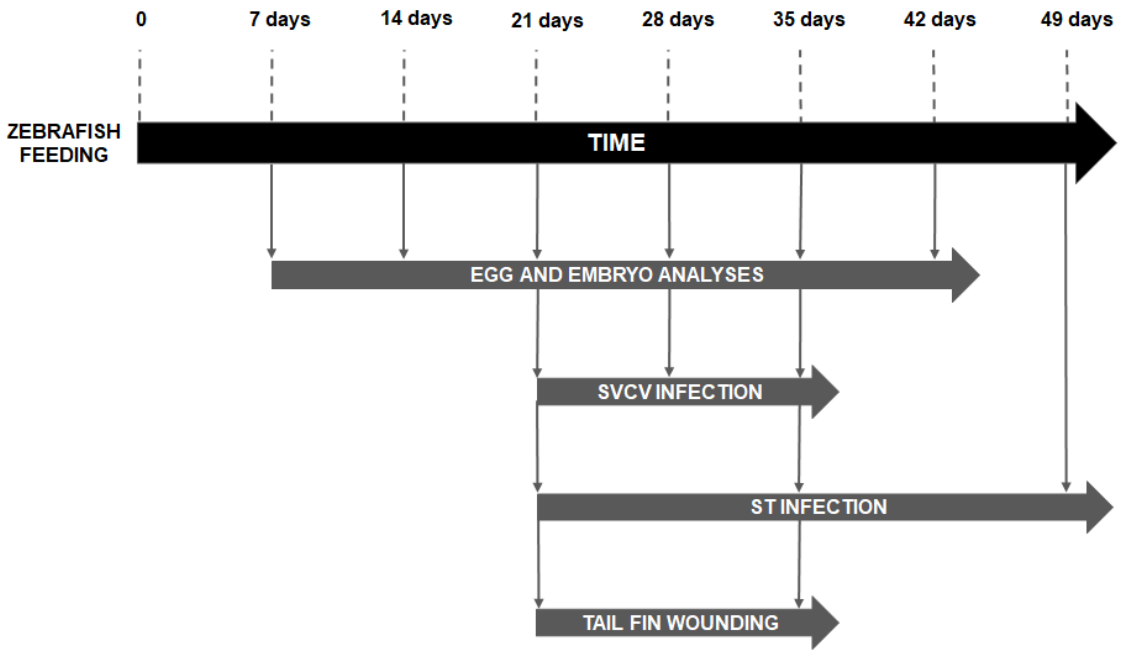


Figure 1: Experimental timeline showing all zebrafish analyses performed in our study and their corresponding times over the feeding period.

2.8. Statistical analysis

All data were analyzed for normality by the Shapiro-Wilk test. Using GraphPad Prism 7.01, one or two-way analysis of variance (ANOVA) and a Tukey multiple range test were used to determine differences among groups. The survival curves were analyzed using the log-rank (Mantel-Cox) test. Significance was defined as *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

3. Results

3.1. Reproductive features

Zebrafish spawning and embryo quality throughout the diet period are shown in **Figure 2a, b** and **c**. No differences were observed between the control diet and pollen-supplemented diet (mean of 122 ± 73 and 86 ± 36 eggs per female; 236 ± 99 and 193 ±

130 total eggs; 94 ± 8 and 88 ± 12 % viability, respectively) over the six weeks analyzed, but a significant difference ($p < 0.05$) between the control and pollen/pollen groups was observed for all parameters (mean of 73 ± 29 eggs per female; 192 ± 109 total eggs; 85 ± 11% of viability for pollen/pollen treatment), suggesting that total flake substitution for pollen in the diet is not recommended for breeders. No significant differences between pollen and pollen/pollen groups were identified ($p > 0.05$).

3.2 Offspring immune response

Larvae survival over 5 d after SVCV infection is presented in **Figure 3**. The virus infection did not affect ($p > 0.05$) larvae differently between treatments at 21 d, but after 28 and 35 d of diet administration, larval survival of the pollen group was longer ($p < 0.001$) compared with the two other groups. Therefore, although pollen treatment did not directly influence zebrafish reproduction, our results suggest an improvement in viral immunity of offspring.

The larval survival rate after ST infection (21, 35 and 49 d after the feeding regime started) is shown in **Figure 4**. No significant differences ($p > 0.05$) were observed between the control and pollen-supplemented diets over 5 d post-infection. Evidently, due to a mechanism that has not been elucidated, pollen treatment was not able to alter offspring bacterial immunity in the same way that it altered offspring viral immunity.

Larvae tested for neutrophil migration after tail wounding at 21 and 35 d after the diet started (cells were counted at the site of injury at 0, 90 and 360 minutes post-wounding (mpw)) are shown in **Figure 5**. The pollen-supplemented diet group presented significantly higher ($p < 0.05$) neutrophil migration at 360 mpw (mean of 15 ± 6 neutrophils at the site of injury per larvae) than did the offspring of fish fed the control

diet (mean of 11 ± 6 neutrophils at the site of injury per larvae) in both weeks of analyses. No changes were observed at initial times after larval tail wounding.

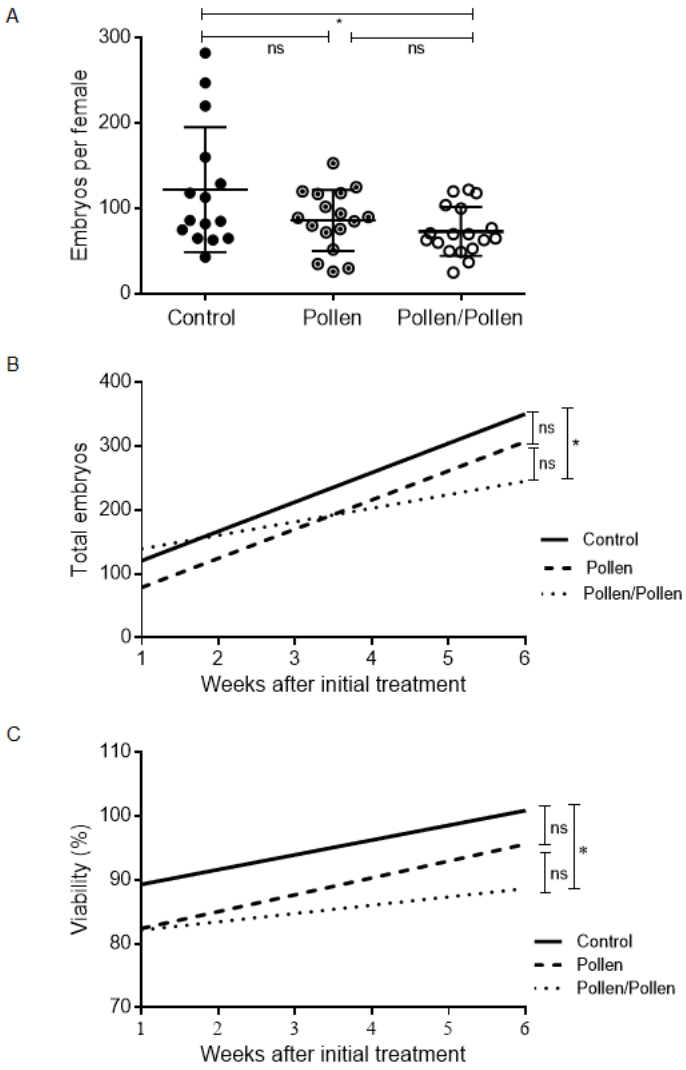
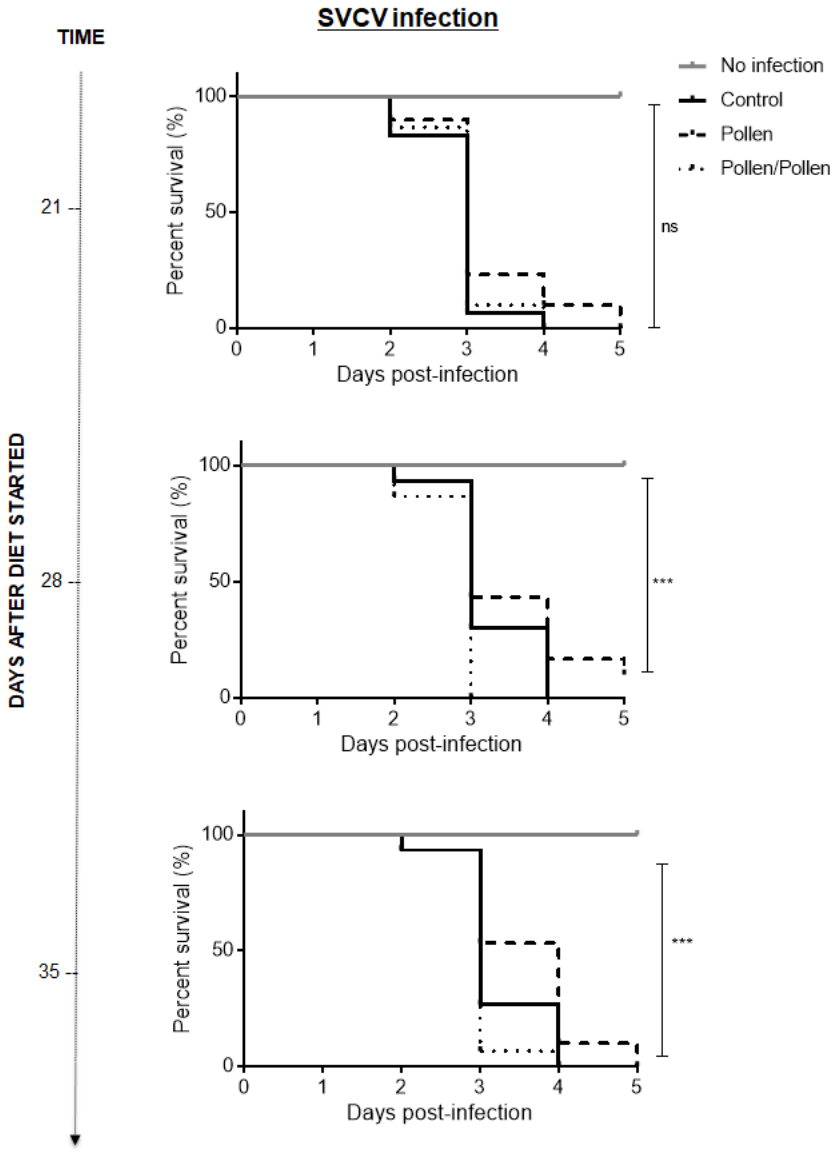


Fig. 2. Zebrafish reproduction features after bee pollen supplementation. Number of embryos per female (**a**), total embryo production (**b**) and 3 dpf larval viability (**c**) for different diet treatments throughout 6 weeks (control diet – black line; pollen diet – blue line; pollen/pollen diet – green line). Each dot represents a female fish. *p ≤ 0.05 according to one-way ANOVA and Tukey´s multiple range test.



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292

293 **Fig. 3. Zebrafish larvae (3 dpf) survival curve after SVCV infection.** Reproduction
294 took place 21, 28 and 35 d after the adult diet started, and offspring were evaluated
295 over 5 d post-infection (control diet – black line; pollen diet – blue line; pollen/pollen
296 diet – green line). ***$p \leq 0.001$ according to Kaplan-Meier Gehan-Breslow-Wilcoxon
297 and nonparametric log-rank tests.

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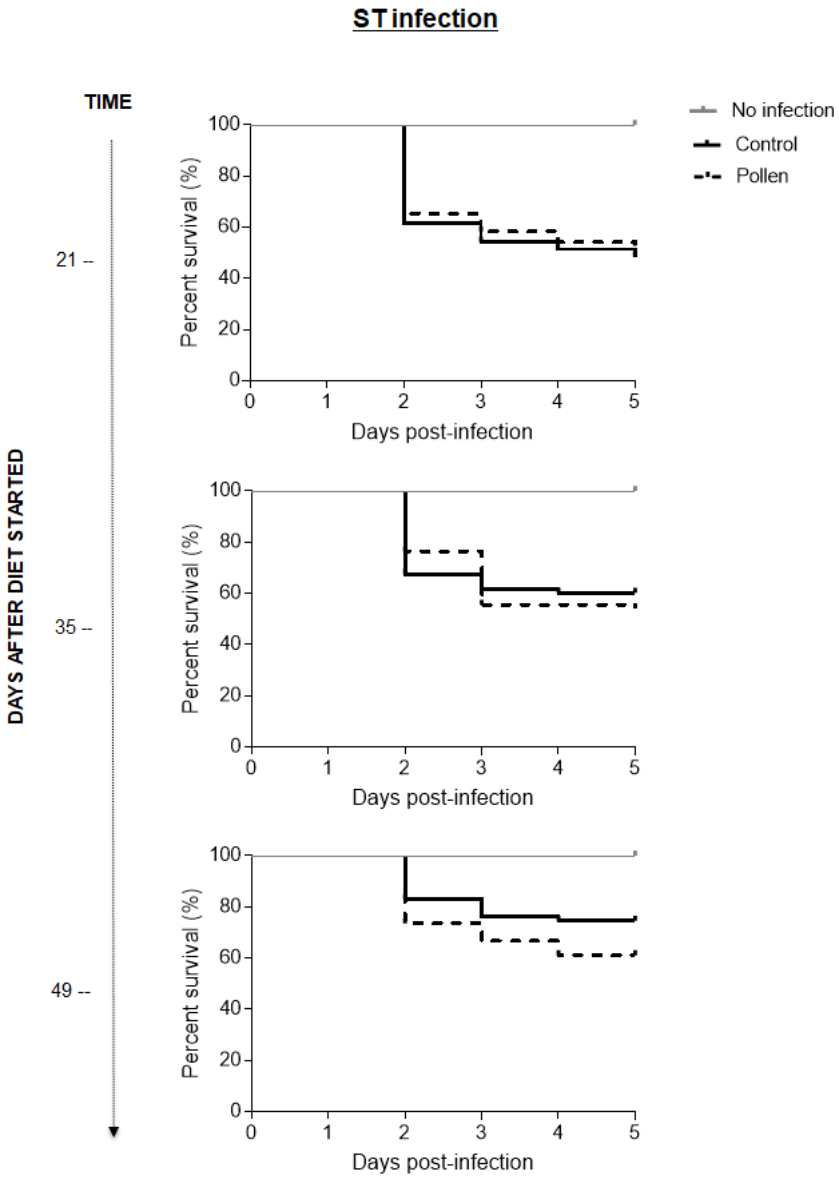
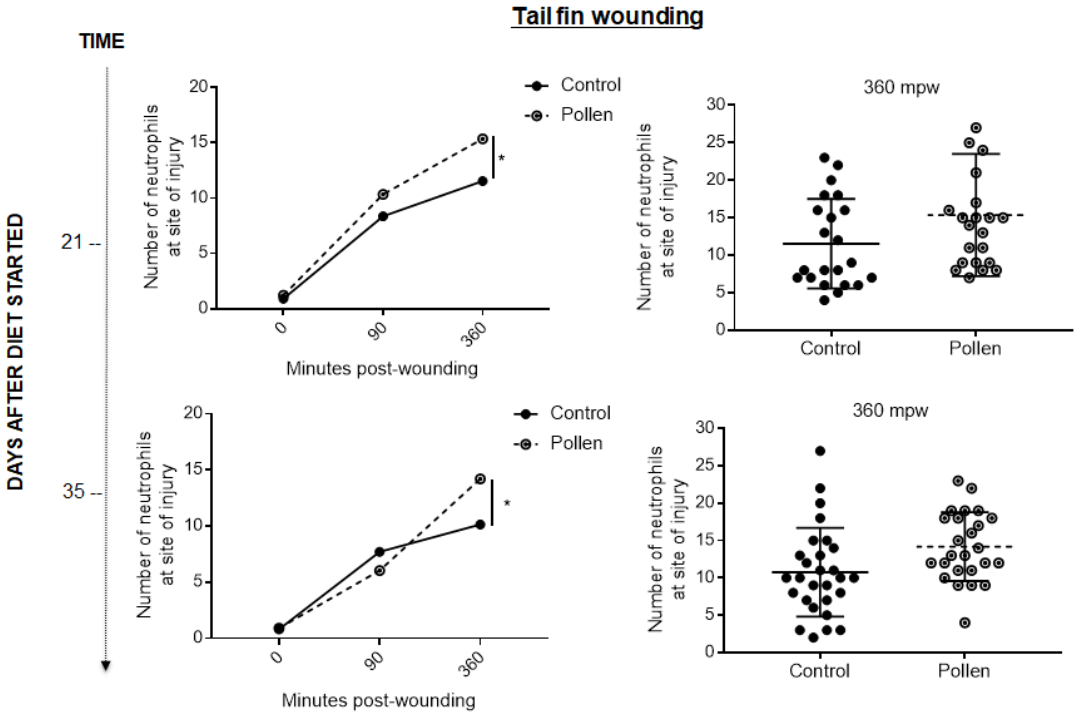


Fig. 4. Zebrafish larvae (2 dpf) survival curve after ST infection. Reproduction took place 21, 35 and 49 d after the adult diet started, and offspring were evaluated over 5 d after infection (control diet – black line; pollen diet – blue line). Kaplan-Meier Gehan-Breslow-Wilcoxon and nonparametric log-rank tests.



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312 **Fig. 5. Zebrafish larvae (3 dpf) neutrophil recruitment at the site of injury at 0,**
313 **90 and 360 minutes post-wounding (mpw) of the tail fin.** Reproduction took place
314 21 and 35 d after adult diet started and offspring were evaluated over 6 h post-
315 wounding (control diet – black spots and line; pollen diet – blue spots and line). *p
316 ≤ 0.05 according to ANOVA and Tukey's multiple comparison test.

317

4. Discussion

Proper nutrition is important for reproductive success. Zebrafish are continuous spawners with a short recrudescence period; therefore, there is a need for the rapid replacement of nutrients used in reproduction. Additionally, the quality of feed and feeding regime are essential aspects of gonadal development and fecundity [13]. To the best of our knowledge, this study is the first to employ bee pollen as a supplement in a zebrafish diet, and we characterized zebrafish spawning, embryo and larval quality throughout the diet period. Over a six-week period, no differences were observed between the control diet and pollen-supplemented diet regarding egg production per female, total number of eggs and embryo viability (72 hpf). Although bee pollen has been described as improving fertility, and reproductive health, and enhancing egg and sperm quality in some other species, such as rabbits, rats, swine and tilapia [24–28], there is still not enough scientific evidence to indicate its efficacy in animal reproduction.

Despite our findings in this study, the therapeutic activities of bee products related primarily to the presence of flavonoids that modulate steroid hormones (phytoestrogen activity) and consequently hormone-dependent ovarian activity have been suggested [29,30], as well as their capacity to interact with estrogen receptors-ß in the reproductive organs [31]. *In vitro* studies showed that bee pollen regulates the insulin-like growth factor-1 released by mammalian ovarian granulosa cells, which is important for the regulation of ovarian functions [27]. In rabbits, bee pollen feeding was observed to improve the conception rate [28]. Feeding pollen to Nile tilapia *Oreochromis niloticus* before restocking into breeding results in a higher rate of hatchability in females and fertilizing capacity in males (increased sperm count and sperm motility and decreased tail abnormalities), which is in keeping with the androgenic effect in fish [25]. These findings agree with the findings of previous studies, which indicate that bee pollen may induce a

remarkable improvement in sperm quality and an increase in the sperm count and testosterone level [24,32]. However, we believe that responses to pollen feeding can vary according to the species studied, physiological status, animal age, purpose of addition, animal trophic level, control-based diet, concentration offered and nutritional composition of each pollen.

A significant difference between the control and pollen/pollen diet groups was observed for the 3 features of egg production per female, total number of eggs and embryo viability; suggesting that flake nutrients are essential, and total flake substitution for pollen in the diet is not recommended for zebrafish breeders. In fact, there are still few studies addressing this topic, and some of them may have obtained conflicting results. Some authors have suggested that some substances present in bee natural products may cause an antagonistic effect on the hormone estrogen 17β-estradiol - E2, (antiestrogenic effect) [33–35]. The composition of bee pollen can be highly varied and depends on the plant's source, geographic origin, climatic conditions, soil type and bee activities [36,37]. Differing results reinforce the importance of conducting further studies on the topic to elucidate the effects of this product on animal reproduction.

Teleost fish eggs accommodate a distinctive yolk mass, which consists of various substances that serve as nutrients for embryonic and larval growth. Accordingly, acquisition of adequate yolk content is important for producing viable larvae [38,39]. Moreover, the production of fish larvae is often hampered by high mortality rates, and strategies to control the pathogen load and to identify immunoprophylactic measures must be devised to optimize fish larvae production and consequently improve the overall production of adult fish [17]. Maternal transfer of immunity in fish is affected by many elements, including environmental conditions experienced by brood fish, such as nutritional supply [18]. Both innate and adaptive immune-relevant factors are known to

be transferred from mother to offspring in fish, including: IgM, lysozymes, lectin, cathelicidin, complement components, yolk proteins, phosvitin and lipovitellin [18,40,41].

In the present study, the zebrafish offspring immune response was evaluated. Our results show a significant delay in the mortality of larvae (3 dpf) after viral (SVCV) infection 28 d after the pollen-supplemented diet in breeders was started. A previous study showed that zebrafish Ifnphi1 and Ifnphi2 increase the survival of embryos infected intravenously (i.v.) with SVCV [42]. Another study also showed that zebrafish larvae are unable to mount a protective antiviral response against waterborne infection by SVCV, but when a model overexpressing zebrafish Ifn in embryos was employed, the results showed that both groups I and II Ifn were able to significantly delay larval mortality and increase their resistance to SVCV [20]. One likely explanation for our findings is that breeders' pollen diet may have positively interfered with the Ifn pathway and altered the zebrafish larval response to viral infection.

In addition, notably, another study showed that although both groups I and II Ifn show strong *in vivo* antiviral activities in zebrafish, only group I Ifn was able to protect the fish against bacterial infection [43]. Our work identified no significant differences between the control and pollen-supplemented diet groups after ST infection, and apparently, due to a mechanism that has not been elucidated, pollen treatment was not able to alter offspring bacterial immunity in the same way as offspring viral immunity. In our opinion, the Ifn expression pathway should be investigated in future studies to elucidate the protection of zebrafish larvae against different pathogens after the ingestion of pollen by their progenitors.

Furthermore, a study in rabbits showed that adding pollen to the diet improved their reproductive performance, milk production and immune status with a consequent

positive effect on litter survival within the first 17 d after birth [44]. Bee pollen increased lymphocyte production in the spleen and induced greater phagocytic activity. The authors suggested that the immunological influence of bee pollen on doe rabbits and their offspring may be attributed to the high contents of macro- and micronutrients as well as protective agents and phytosterols [35]. In our study, we found that the pollen-supplemented diet group presented higher larval neutrophil migration at 360 mpw, in both weeks analyzed. Neutrophils are pivotal effector cells of innate immunity, and represent one of the organism's first lines of defense and their recruitment into peripheral tissues is indispensable for host defense [45]. Thus, neutrophils play a fundamental role at the beginning of larval life, in which they have not yet developed an adaptive immune system.

Some studies have reported the effects of bee pollen as an immunomodulatory product [46]. The relative weight of lymphoid organs is used as an indicator of immunity, and supplementation with bee pollen significantly increased the bursa of Fabricius and spleen index in chickens and broilers compared to the control group [47,48]. The beneficial effects of bee pollen on chicken health are supported by studies that showed early growth of the bursa and thymus, reduction of cloacal bursa degeneration and promotion of the splenic immune response, in broiler chicks [47–49]. Additionally, bee pollen in the diet was able to stimulate faster differentiation and proliferation of immune system cells in birds [35]. White blood cells and their differentiation are good signs of enhanced immune efficiency. Lymphocytes were significantly increased in rabbits fed pollen [50], and lymphocytes exhibited increased phagocytic activity and index and had a significantly higher antibody response than those of the control group in quail chicks [49]. Some authors suggest that this improvement in the immune response could be due to essential amino acids, fatty acids, vitamins and flavonoids as antioxidants, which may be important tools for the immune system [46]. Although immunostimulant properties

418 have been proposed for bee pollen in different species, to the best of our knowledge, the
419 vertical effect of bee pollen in modulating the immune of fish larvae response through the
420 breeder's diet has not been described previously.
421
422 **7. Conclusion**
423
424 The use of bee pollen as a dietary supplement did not directly affect zebrafish
425 reproductive performance but influenced immunological response of offspring. Due to
426 the variable composition and rich nutritional content of bee pollen, it may have
427 multifactorial and very complex effects.
428
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Declaration of competing interest

Authors declare that they have no conflict of interest.

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MANUSCRIPT 2 – BEE POLLEN IN ZEBRAFISH DIET AFFECTS INTESTINAL MICROBIOTA COMPOSITION AND SKIN CUTANEOUS MELANOMA DEVELOPMENT

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Bee pollen in zebrafish diet affects intestinal microbiota composition and skin cutaneous melanoma development

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1. ABSTRACT

Bee pollen, a natural product with high nutritional properties is recommended as dietary supplement due to immunostimulating functions including antioxidant, anti-inflammatory and anti-carcinogenic properties. Nevertheless, the effectiveness of such properties is still not well understood. As diet can be associated with animal performance, intestinal microbiota modulation and potentially factor for cancer, this study aimed to analyze if dietary bee pollen addition could influence growth parameters, gut microbial abundance and skin cutaneous melanoma development in zebrafish. Fish diets based on commercial flakes and live food *Artemia* were offered as control and compared with the same diet supplemented with bee pollen. After diet administration period, fish weight gain, increased length, intestinal bacteria metagenomics analysis, serum amyloid A gene expression and skin cutaneous melanoma transplantation assays were performed. We found that bee pollen affected intestinal microbiota composition and melanoma development. Differential abundance analyzed revealed higher abundance in the control group for *Aeromonadaceae* family, *Aeromonas* and *Pseudomonas* genus, *A. sobria*, *A. schubertii*, *A. jandaei* and *P. alcaligenes* species compared with pollen diet group. Pollen group presented higher abundance for *Chromobacterium* genus and for *Gemmobacter aquaticus*, *Flavobacterium succinicans* and *Bifidobacterium breve* compared with control group. Unexpectedly, fish fed with bee pollen showed higher tumor growth rate and larger tumor size than control group. This is the first study to report intestinal microbial changes and no protective cancer properties after bee pollen administration. Although additional experiments are required to uncover the mechanism orchestrating our tumor-promoting results, the attributed antitumor activity of bee pollen should be questioned.

Keywords: Alternative medicine; Apitherapy; Bee pollen; Cancer; Microbiome; Natural products, Nutrition.

54 **Graphical Abstract**

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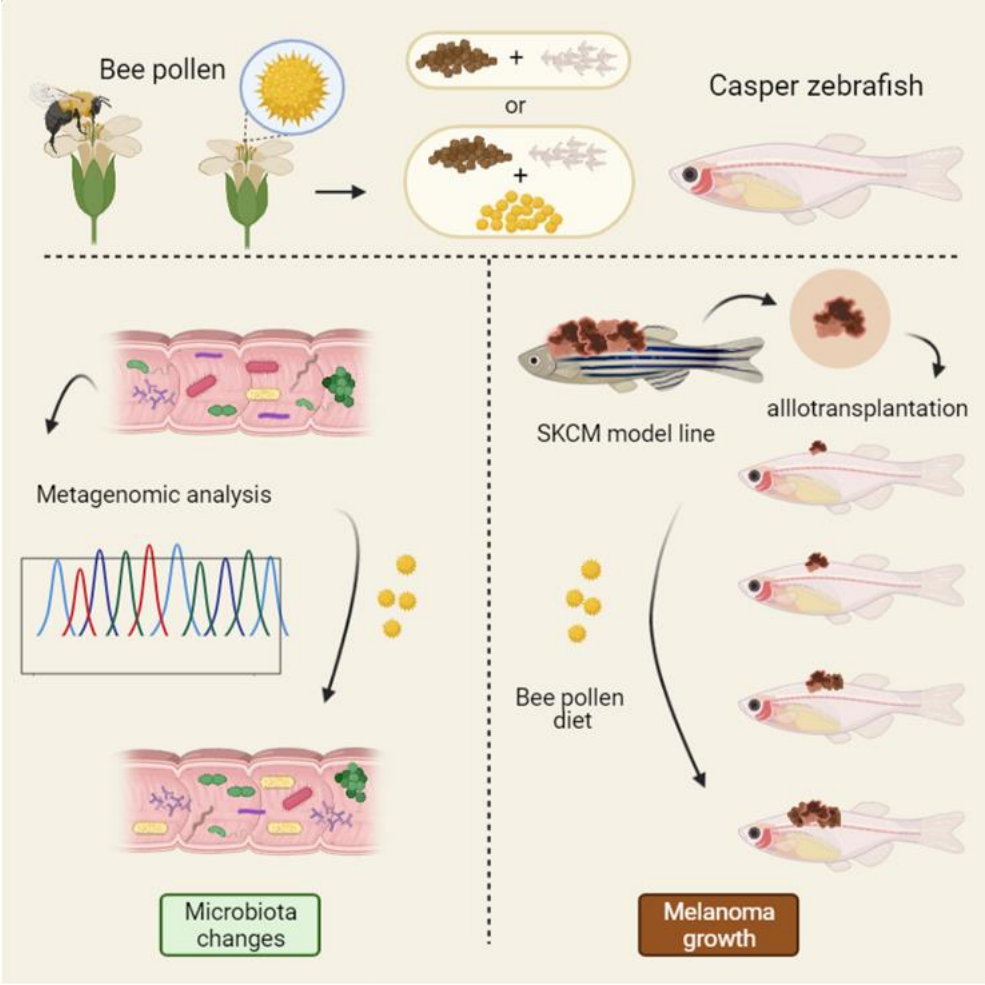


2. INTRODUCTION

Bee pollen is a natural food produced by bees to serve as a nutrient source for the colony development and maintenance. This product is particularly appreciated by consumers and used for therapeutic purposes due to its rich composition (Denisow and Denisow-Pietrzyk, 2016). In bee pollen, approximately 250 different substances can be found (Komosinska-Vassev et al., 2015), amongst them nutrients as carbohydrates, proteins, vitamins, minerals, and fatty acids as well as secondary metabolites as phenolic compounds. Thus, many biological properties are attributed to it, such as antioxidant, antibacterial, antifungal, anti-inflammatory, antiallergic, hepatoprotective, and antitumor potential (Abdella et al., 2009; Nogueira et al., 2012; Fatrcová-Šramková et al., 2013; Pascoal et al., 2014; De-Melo and de Almeida-Muradian, 2017). These pollen properties can vary depending on the origin and region of the plant, which directly affects its composition (De-Melo et al., 2018).

Bee pollen in animal's diet has been described especially related to the improvement in growth performance and immune status (Wang et al., 2007; Attia et al., 2011; Abbass et al., 2012; El-Asely et al., 2014; Hajkova et al., 2014). Besides, it is assumed that feed additives can alter intestinal microbiota, which in turn, interacts with the general host health, particularly affecting digestion, nutrients assimilation and modulation of the immune system (López Nadal et al., 2020). The intestinal microbiota can influence the development and function of immune cells, such myeloid lineages as neutrophils through immune effectors. Serum amyloid A (Saa), one of the most highly induced transcripts in digestive tissues following microbiota colonization, serves as a systemic signal to neutrophils to restrict aberrant activation, decreasing inflammatory tone and bacterial killing potential while simultaneously enhancing their ability to migrate to wounds (Sack, 2018; Murdoch et al., 2019).

To the best of our knowledge, the direct influence of dietary bee pollen through changes in the microbiota has never been studied. The detailed study of the intestinal microbiota composition and its metabolic functions allows determining which microorganisms make it possible to keep the intestine healthy and which changes can lead to pathologies development (Preidis and Versalovic, 2009). Intestinal microbiota generally maintains a constant relative pattern and altered bacterial abundance has been associated with complex diseases (Shreiner et al., 2015; Durack and Lynch, 2019). Dysbiosis of intestinal microbiota can be associated not only with intestinal but also with extra-intestinal diseases such as metabolic disorders (Rinninella et al., 2019). The identification of diet–microbiome associations may be particularly relevant for studying the downstream effects of diet on long latency chronic diseases such as cancer (Murphy, 2020). In this context, increasing evidence also indicates a fundamental role of the microbiota in carcinogenesis (Schwabe and Jobin, 2013; Mandal et al., 2015; Raza et al., 2019).

Several researches in cancer reveal that inflammation can play a key role from initiation of the transformed phenotype to metastatic spread. Chronic inflammation is considered one of the factors that most contribute to tumor appearance and progression (Coussens and Werb, 2002; Singh et al., 2019). In addition, the use of anti-inflammatory agents is shown to reduce tumor formation (Singh et al., 2019) and natural products are also being used for cancer prevention or therapy and as adjuvants to conventional therapies (Aggarwal and Shishodia, 2006; Strimpakos and Sharma, 2008; Rayburn et al., 2009). Bee pollen has been described with both anti-inflammatory and anti-carcinogenic properties (Furusawa et al., 1995; Abdella et al., 2009; Denisow and Denisow-Pietrzyk, 2016; Uçar et al., 2016; Wan Omar et al., 2016; Kieliszek et al., 2018; Li et al., 2018), but many studies are still based on *in vitro* experiments.

Skin cutaneous melanoma (SKCM) is the most aggressive type of skin cancer, with an increasing number of cases worldwide, potential for early metastasis and a high mortality rate (Siegel et al., 2020). Nowadays, skin cancers are attributed to chronically injured, non-healing wounds, scars or ulcers (Tang and Wang, 2016). It has recently emerged that factors beyond tumor genomics also influence cancer development and therapeutic responses, including host factors such as diet and the gastrointestinal (gut) microbiome (Garrett, 2015; Segre, 2015; Drewes et al., 2016; Elinav et al., 2019). Diet may be one of the few ubiquitous and potentially modifiable risk factors for cancer, but despite the large global evidence base, the divergence in results are disappointingly common in this field (Murphy, 2020). Also, a recent study indicated that a favorable gut microbiome (high diversity and abundance of some specific bacteria) may modulate responses to immunotherapy in melanoma patients, enhancing systemic and antitumor immune responses in the periphery and in the tumor microenvironment (Gopalakrishnan et al., 2018).

Given the unique advantages of the zebrafish model for molecular genetic analysis and *in vivo* imaging, together with the diverse set of research tools currently available, we believe it is a favorable model for study new therapeutic agents and mechanisms by which feed influences host. To date, there is no concrete and in-depth evidence on bee pollen prebiotic and antitumor effect. The present study aimed investigate if bee pollen addition in diet could influence zebrafish parameters. Fish diets based on commercial flakes and live food *Artemia* were offered as control and compared with the same diet supplemented with bee pollen and after diet administration period, fish weight gain, increased length, intestinal bacteria metagenomics analysis, serum amyloid A gene expression and skin cutaneous melanoma development after allotransplantation assays were performed.

3. MATERIALS AND METHODS

3.1. Ethics statements

The experiments performed comply with the Guidelines of the European Union Council (Directive 2010/63/EU) and the Spanish RD 53/2013. Experiments and procedures were performed as approved by the Consejería de Agua, Agricultura, Ganadería y Pesca de la CARM (authorization number #A13180602) and the Ethical Research in Animal Use Committee (CEUA) of Federal University of Lavras (approval number #001/18).

3.2. Zebrafish husbandry

Zebrafish (*Danio rerio* H. Cypriniformes, Cyprinidae) were obtained from the Zebrafish International Resource Center (ZIRC, Oregon, USA) and mated, staged, raised and processed as described in the zebrafish handbook (Westerfield, 2007). Zebrafish fertilized eggs were obtained from natural spawning of wild type and transgenic fish held at our facilities following standard husbandry practices. Animals were maintained in a 12 h light/dark cycle at 28 °C. *Tg(kita:GalTA4,UAS:mCherry)*^{hzm1} zebrafish were crossed with *Tg(UAS:eGFP-H-RAS_G12V)*^{io6} line (Santoriello et al., 2010) to express oncogenic human HRAS_G12 V driven by the melanocyte cell-specific promoter *kita*. The transparent *roy*$^{a9/a9}$; *nacre*$^{w2/w2}$ (casper) (White et al., 2008) of 4–8 month old were previously described.

3.3. Experimental diets

The experimental design was divided into groups with 2 different types of diets, according to **Table 1**. Adult zebrafish were fed three times a day, divided into 3

aquariums per treatment. All groups received different diets at the same time (9:00 am, 12:00 pm and 3:00 pm). The feed amount offered by individual was 3% of body weight (flakes and bee pollen), per meal, and the number of brine shrimp *Artemia* nauplii (48 hours nauplii) offered was 2,000 per individual per day (food protocol already established in the laboratory).

Table 1: Distribution of diets by experimental group.

	1st meal	**2nd meal**	**3rd meal**
Groups	**9:00 h**	**12:00 h**	**15:00 h**
1- Control	Flakes[1]	Flakes	Artemia[2]
2 - Pollen	Flakes	Bee pollen[3]	Artemia

[1]Tropical Fish Flakes (Prodac, Italy): cereals, fish and fish products, soy, yeast, crustaceans, algae, aloe vera and mineral and vitamin mixture. [2]Brine shrimp (Inve Aquaculture, Thailand). [3]Neópolis, SE, Brazil.

Composition and proximate analysis of fish flakes and brine shrimp offered in the animals' basal diet are described in **Table 2** (data obtained from the manufacturers). Information not provided by the manufacturer was found in the literature (Rizk et al., 2018).

Table 2 – Composition and proximate analysis of fish flakes and brine shrimp (data provided by manufacturers) offered in the animals' basal diet.

Composition	Proximate analysis (%)	
	Flakes	Brine shrimp
Crude protein	44.9	55.0
Ether extract	4.47	13.0
Ash	4.35	5.5
Fiber	2.14	6.8*
Moisture	7.73	68*
Carbohydrates	NI	13.22*

Values expressed for each 100g of dry matter. NI = no information available. *Average values by Rizk et al., 2018). Nutritional additives: vitamin A, 41.200 I.U./kg; vitamin D3, 3.000 I.U./kg; vitamin E, 297 mg/kg; vitamin C, 180 mg/kg.

Composition, proximate analysis and antioxidant capacity of bee pollen are also listed in **Table 3**, according to analyzes performed at the Department of Food Sciences, University of Lavras, Brazil.

Table 3 – Composition, proximate analysis and antioxidant capacity of bee pollen.

Composition	**Proximate analysis (%)**
Moisture	14.56
Crude protein	17.57
Ether extract	5.14
Carbohydrates	60.38
Total sugar	50.41
Reducing sugars	24.78
Sacarose	25.64
Ashes[a]	3.02
Caloric value (kcal/100g)	351.86
Antioxidant capacity	
Phenolic content	19.15
ABTS (μmol trolox/g)	3955.30

Values expressed for each 100g of dry matter. All values are in accordance with the Ministry of Agriculture, Cattle and Supplying (MAPA) normative instruction 3 (annex V) which addresses requirements for bee products commercialization in Brazil. [a]Mineral analysis: N, 34.8 g/kg; P, 6.57 g/kg; K, 6.73 g/kg; Ca, 5.92 g/kg; Mg, 2.18 g/kg; S, 2.22 g/kg; B, 6.07 mg/kg; Cu, 11.69 mg/kg; Mn, 222.24 mg/kg; Zn, 64.40 mg/kg; Fe, 106.07.

3.4. Increased length and weight gain

After 60 days of feeding with control and pollen-based diets, fish from each treatment were anesthetized in buffered 0.16 mg/mL trincaine (Sigma Aldrich) for growth parameters measurements. The growth parameters were determined according to following formula:

Mean weight gain (WG) = Mean final weight - Mean initial weight

Increased length (IL) = Mean final length – Mean initial length

3.5. Sample collection and genomic DNA extraction

Fish from each treatment (n=3) were transferred into well cleaned separate tanks and after 24 h of starvation period were anesthetized and euthanized according the European Union Council and IUAC protocol (tricaine overdose: 1.2 mg / ml; Sigma Aldrich). Then, their intestines were removed, quickly frozen in liquid nitrogen inside 1.5 mL tubes containing 500µl of RNAlater ™ stabilization solution (Invitrogen, Thermo Fisher) and subsequently preserved at -80°C until DNA extraction and samples preparation. The bacterial genomic DNA was extracted using a PureFood GMO and Authentication kit (Maxwell® RSC, Promega, USA) following the manufacture's protocol.

3.6. Intestinal microbiota assessment through metagenomics analysis

The intestinal microbial composition of animals (n=3) fed with 2 different diets was determined by sequencing 16S rRNA gene. The "Ion 16S Metagenomics Kit" (Ion Torrent) used includes primers to amplify variable regions V2, V4 and V8 in a single tube with ~ 250 base pair (bp), ~ 288 bp and ~ 295 amplicons bp, respectively, and in a second tube, a multiplex PCR reaction directed to variable regions V3, V6, V7 and V9 with ~ 215 bp, ~ 260 bp and ~ 209 bp, respectively. The primers are designed to capture> 80% sequences found in Greengenes database with 100% identity (BARB et al., 2016). For 16S rRNA PCR amplification, maximum DNA amount (6 µl) was used following conditions indicated in the protocol (25 cycles). PCR products were verified by 2% agarose gel electrophoresis, purified with AMPure XP Beads (Beckman Coulter), quantified with "Qubit dsDNA HS Assay" kit (Invitrogen) using 50 ng of total amplicons to generate "Ion" libraries Plus Fragment Library Kit "(Ion Torrent). The model was prepared using the Ion OneTouch ™ 2 system and the "Ion PGM ™ Template Hi-Q view

OT2 400" kit (Ion Torrent). The sequencing was performed using the "Ion PGM ™ Sequencing Hi-Q view 400" kit (Ion Torrent) in the Ion PGM ™ system. Samples with microbial identification were analyzed at family, genus and species level.

3.7. Analysis of Serum Amyloid A (*saa*) transcript levels

Animals from each treatment had their total RNA extracted from whole zebrafish abdominal organs (n=5) and different zebrafish intestines (n=5) using TRIzol reagent (Invitrogen), and then purified with Mini Kit total RNA purification system (Ambion) and treated with DNase I, amplification grade (1 U/μg RNA; Thermo Fisher Scientific). The SuperScript IV RNase Reverse Transcriptase (Thermo Fisher Scientific) was used to synthesize first-strand cDNA with oligo$(dT)_{18}$ primer from 1 μg of total RNA at 50°C for 50 min. Real-time PCR was performed with a QuantStudio 5 (Thermo Fisher Scientific) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C, 1 min 60°C and 15 s at 95°C. For each mRNA quantified, gene transcription was normalized in relation to the ribosomal protein S11 (*rps11*) housekeeping gene by Pfaffl method (Pfaffl, 2001). The primers used were zfSaa F: 5'-CGCAGAGGCAATTCAGAT-3' and zfSaa R: 5'-CAGGCCTTTAAGTCTGTATTTGTTG-3'. Each PCR was performed with triplicate samples.

3.7. SKCM transplant

The zebrafish Casper line (n = 22) fed with different diets (120 days) were used as recipients for melanoma transplantation (SKCM). Zebrafish *kita:Gal4*; *eGFP-HRAS-G12V*, which express human oncogenic HRAS in melanocytes and spontaneously

develop SKCM, were used as tumor donors (n = 2) for allotransplantation assays. All the next procedures were developed according to a previous study (Gómez-Abenza et al., 2019). Briefly, primary melanoma tumors were excised from adult zebrafish once they had reached between 3-5 mm in diameter and right after the procedure individuals were euthanized with an overdose of tricaine (1.2 mg / ml). The tumor was excised with scalpel and razor blade, placed in 2 ml of disaggregation media, composed by DMEM/F12 (Life Technologies), penicillin/streptomycin (Life Technologies) and 0.075 mg/ml of Liberase (Roche). After manually disaggregation with a clean razor blade and incubation at room temperature for 30 min, 5 ml of wash media, composed by DMEM/F12, penicillin/streptomycin, and 15% heat-inactivated fetal bovine serum (FBS, Life Technologies), was added to the tumor slurry and manually disaggregated. Next, the tumor cells suspensions were passed through a 40 μm filter (BD) into a clean 50 ml tube. An additional 5 ml of wash media was added to the initial tumor slurry that was filtered again. This procedure was repeated twice. Cell numbers were calculated with a hemocytometer and the tubes of resuspended cells were centrifuged at 800 g for 5 min at 4ºC. The pellet of tumor cells was resuspended in the appropriate volume of PBS containing 5% FBS and kept on ice prior to transplantation (Dang et al., 2016).

After fasting 48 hours, adult zebrafish used as transplant recipients, were immunosuppressed to prevent rejection of the donor material. Thus, the recipients were anesthetized, as previously described, and treated with 30 gray (Gy) of split dose sub-lethal X-irradiation (YXLON SMART 200E, 200 kV, 4.5 mA) two days before the transplantation. Then the immunosuppressed fish were maintained in carefully clean fish water with conditions preventing any infections onset and, consequently, preventing recipients' deaths. The animals were anesthetized with a double protocol, according to studies using longer anesthetic protocols (up to 40 min) (Dang et al., 2016). Briefly,

anesthesia was first induced by tricaine (Sigma-Aldrich) and then the fish were transferred to tricaine/isoflurane solution (dilution in ethanol, 1:9). Anesthetized fish (10-20 per tumor) were placed dorsal side up on a damp sponge and injections were performed using a 10 μl beveled, 26S-guaged Hamilton syringe, needle positioned midline and ahead to the dorsal fin. Three-hundred thousand cells resuspended in PBS were injected into the dorsal subcutaneous cavity. The syringe was washed in 70% ethanol and rinsed with PBS between uses.

Following transplantation, fish were placed into recovery tanks and weekly evaluated for melanoma formation. Photographs from adult transplantation assays were obtained at 1, 2, 3 and 4 weeks post injection (wpi). Zebrafish were anesthetized, placed in a dish of fish water, and photographed using a mounted camera (Nikon D3100 with a Nikon AF-S Micro Lens). The pigmented tumor size was represented by the number of pigmented pixels (Adobe Photoshop CS5).

3.8. Statistical analysis

All data were analyzed for normality by the Shapiro-Wilk test. Data (except metagenomics) were analyzed using *GraphPad Prism* 7.01 by one or two-way analysis of variance (ANOVA) and a Tukey or Sidak post-test for multiple comparisons evidencing differences between groups. The survival curves were analyzed using the log-rank (Mantel-Cox) test. Statistical significance was defined as *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Data from IonReporter program were analyzed using R Core Team 2019 to find statistically significant differences (differential abundance) in taxa composition between different diets. Thus, the abundance data were normalized by dividing the abundance value by the total number of sample readings and multiplied by 100,000 to guarantee

301 values greater than 1 or 0 in the absence of a taxon in the sample. Finally, data were
302 converted to phyloseq (McMurdie and Holmes, 2013) to generate the diversity graphs
303 and converted to DESeq2 (Love et al., 2014) to perform the differential abundance
304 statistical test. DESeq performs a differential analysis based on the negative binomial
305 distribution.
306

4. RESULTS

4.1. Bee pollen inclusion in diet presented similar growth parameters as control

Zebrafish growth parameters after the feed regime period (60 days) is shown in **Figure 1**. No significant differences ($p > 0.05$) were found between control diet and pollen supplemented diet for both measurements: increased length (**Fig. 1A)** and mean weight gain (**Fig. 1B**). Fish from the group fed with control diet had a mean growth of 0.43 ± 0.06 cm and 0.10 ± 0.012 g and fish from the group fed with pollen diet achieved a mean growth of 0.47 ± 0.12 cm and 0.09 ± 0.005 g.

4.2. Bee pollen diet induced gut microbial changes

Metagenomics analyses from zebrafish gut microbiome after control and pollen diets are shown in **Figures 2-5**. The PCA plot (**Fig. 2A**) and dendrogram (**Fig. 2B**) showed a closely related microbial community within each sample. The dendrogram analysis also supported the PCA plot clustering by showing the robustness of the differences between control and pollen supplemented diet samples.

Abundance data (quantitative values obtained from operational taxonomic unit, OTU) for each diet group were compared. OTUs were taxonomically grouped and differential abundance analyzed at the family, genus and species levels revealed that the microbiome of pollen supplemented group showed significantly altered abundance compared to the control diet fish. Stacked column bar graph illustrate the distribution and abundances of bacterial communities in zebrafish samples (control diet - $C_{1\text{-}3;}$ pollen supplemented diet - $P_{1\text{-}3}$). Each bacterial taxon was represented by different color (**Fig. 3, 4 and 5**).

At the family level, control diet group presented significantly higher abundance ($p < 0.001$) for *Aeromonadaceae* compared to pollen diet group (**Fig. 3**). At the genus level, control diet group presented significantly higher abundance for *Aeromonas* ($p < 0.001$) and *Pseudomonas* ($p < 0.05$) compared with pollen diet group, while pollen diet group presented higher abundance for *Chromobacterium* ($p < 0.05$) compared with control fish (**Fig. 4**). At the species level, control diet group presented significantly higher abundance ($p < 0.001$) for *Aeromonas sobria* ($p < 0.001$), *A. schubertii* ($p < 0.001$), *A. jandaei* ($p < 0.01$) and *Pseudomonas alcaligenes* ($p < 0.05$) compared to pollen diet group, while pollen group presented higher abundance for *Gemmobacter aquaticus* ($p < 0.05$), *Flavobacterium succinicans* ($p < 0.01$) and *Bifidobacterium breve* ($p < 0.05$) compared to control group (**Fig. 5**).

4.3. Similar transcript levels of *saa* gene for bee pollen and control fed fish

saa gene mRNA levels in zebrafish abdominal organs and also for separated intestines is shown in **Figure 6**. Our results revealed no differences ($p > 0.05$) in this protein expression in both cases (**Fig. 6A** and **B**).

4.4. Bee pollen diet induced higher tumor growth after SKCM transplant

Zebrafish SKCM allotransplantation process and tumor cell proliferation and dissemination *in vivo* assays are described by **Figure 7-10**. **Figure 7A** shows a schematic diagram of *kita:Gal4;eGFP-HRAS-G12V* and representative images of whole fish and nodular tail tumor (1 and 2) used as melanoma donors in our study are shown in **Figure 7B**. Analyzing separately tumor 1 and 2 transplantation for different diet groups, pigmented tumors engrafted were scored during 4 weeks for tumor size and in the first and second weeks of analysis there was find no significantly differences ($p > 0.05$)

between the treatments (**Figure 8A and B**). At the third week of analysis, zebrafish fed with bee pollen developed tumors with significant ($p < 0.05$) larger tumor size (mean of 35225 pixels for tumor 1 and 31348 pixels for tumor 2) compared with zebrafish fed with control diet (mean of 19083 pixels for tumor 1 and 23020 pixels for tumor 2). At the fourth week, bee pollen group developed tumors with significant ($p < 0.05$) larger size compared to control only for tumor 1 (mean of 50511 pixels for pollen group and 24434 pixels for control group), while tumor 2 presented no difference ($p > 0.05$) between treatments (mean of 36326 pixels for bee pollen group and 25871 pixels for control group). Representative images of Tumor 1 and 2 engraftment and tumor size average from week 1 to 4 post-transplantation are presented in **Figure 8A** and **B**.

Figure 9 shows tumor 1 and 2 analyzed together and both showed a similar pattern. At the first and second weeks, no differences ($p > 0.05$) were observed between the 2 treatments. From the third week of analysis, zebrafish fed with bee pollen developed tumors with larger ($p < 0.01$) tumor size (mean of 33157 pixels in the third week, 42774 pixels in the fourth week) compared to no pollen-fed fish (mean of 20045 pixels at third week, 25152 pixels at fourth week). Melanoma recipients fed with pollen and transplanted with SKCMs (tumor 1+2) also presented tumors with higher ($p < 0.01$) growth rate (166% at the third week, 243% at the fourth week) than those recipients fed with control diet (91% in the third week, 140% in the fourth week) (**Fig. 10A** and **B**). In relation to recipient survival curve, no significant differences ($p > 0.05$) were observed between diet groups during the 4 weeks analyzed (**Fig. 10C**).

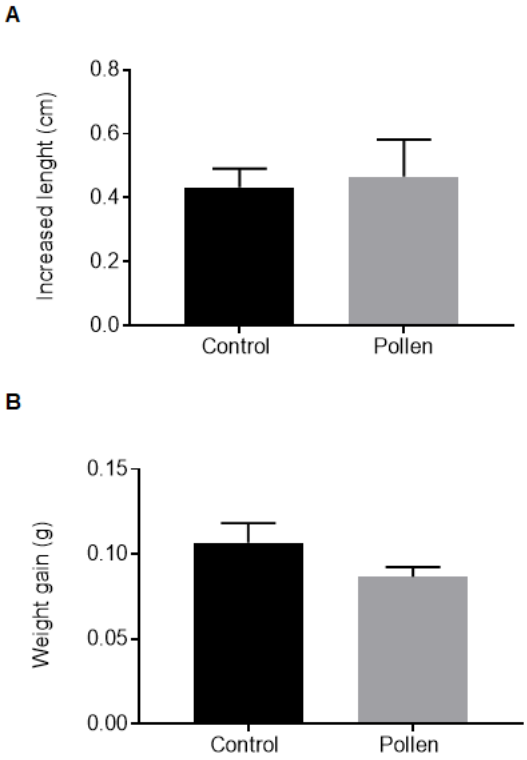
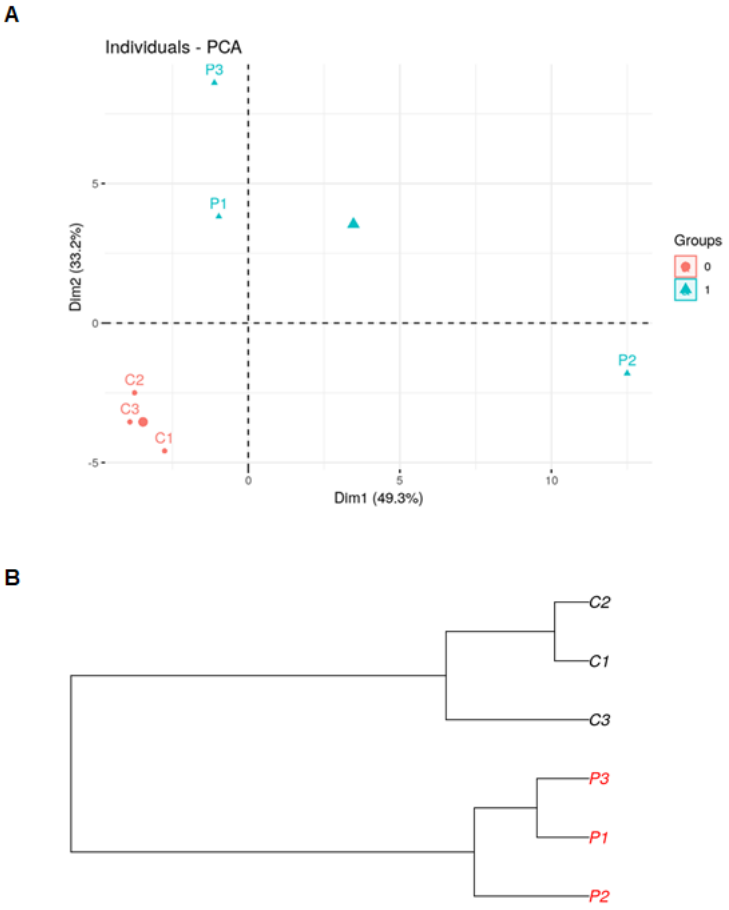


Figure 1. Growth parameters of adult zebrafish after feeding with control diet (black bar) vs. pollen diet (gray bar). A) Increased length (cm). B) Mean weight gain (g). ANOVA and Tukey's Multiple Comparison Test. The data are shown as mean + SEM (n=24).



383

384 **Figure 2**. **Relationship between the composition of the gut bacterial communities in**

385 **zebrafish fed with control diet (C_{1-3}) and pollen supplemented diet (P_{1-3}). A)** Principal

386 Component Analysis (PCA) plot. **B)** Dendrogram. Generated by R Core Team 2019.

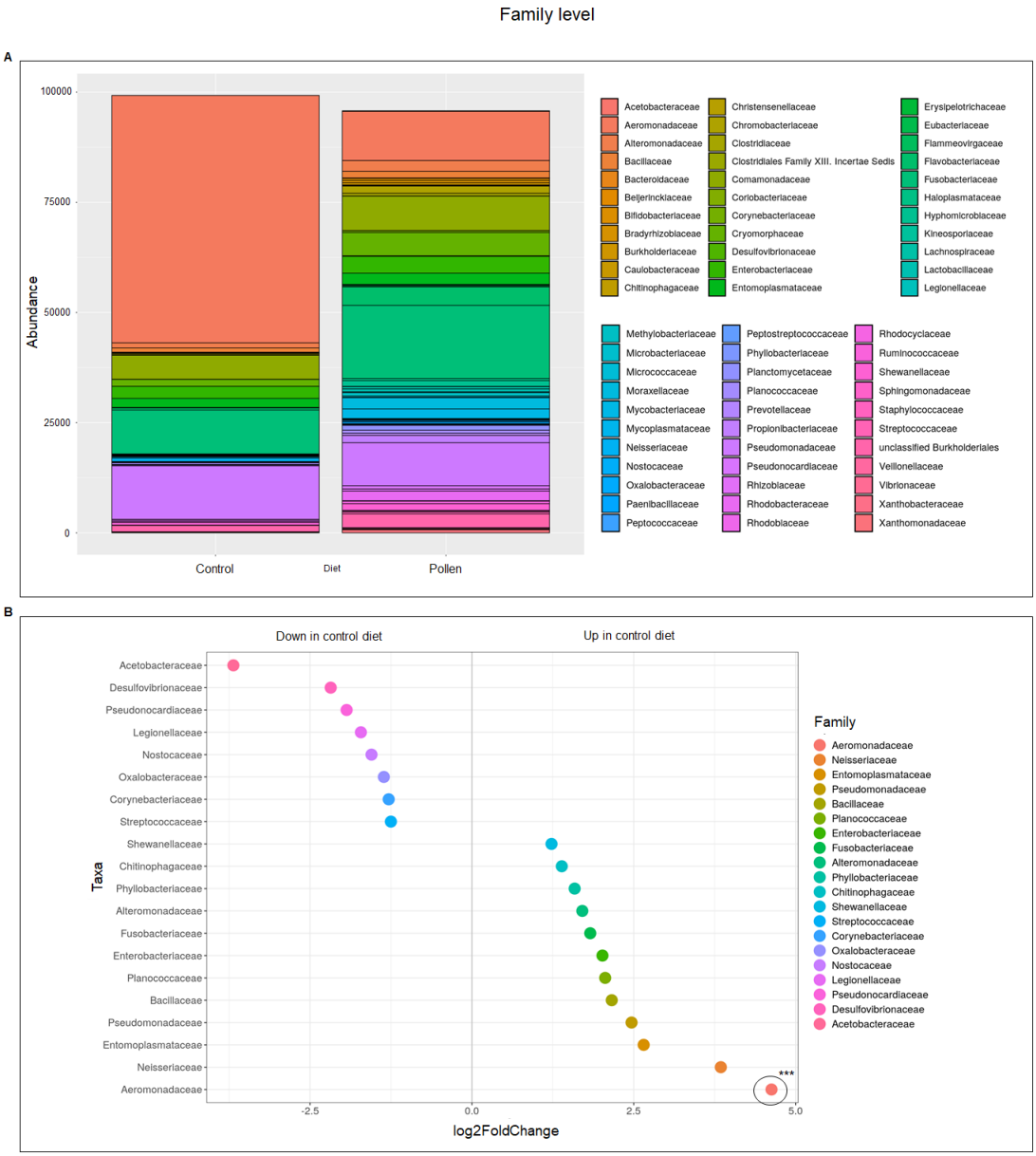
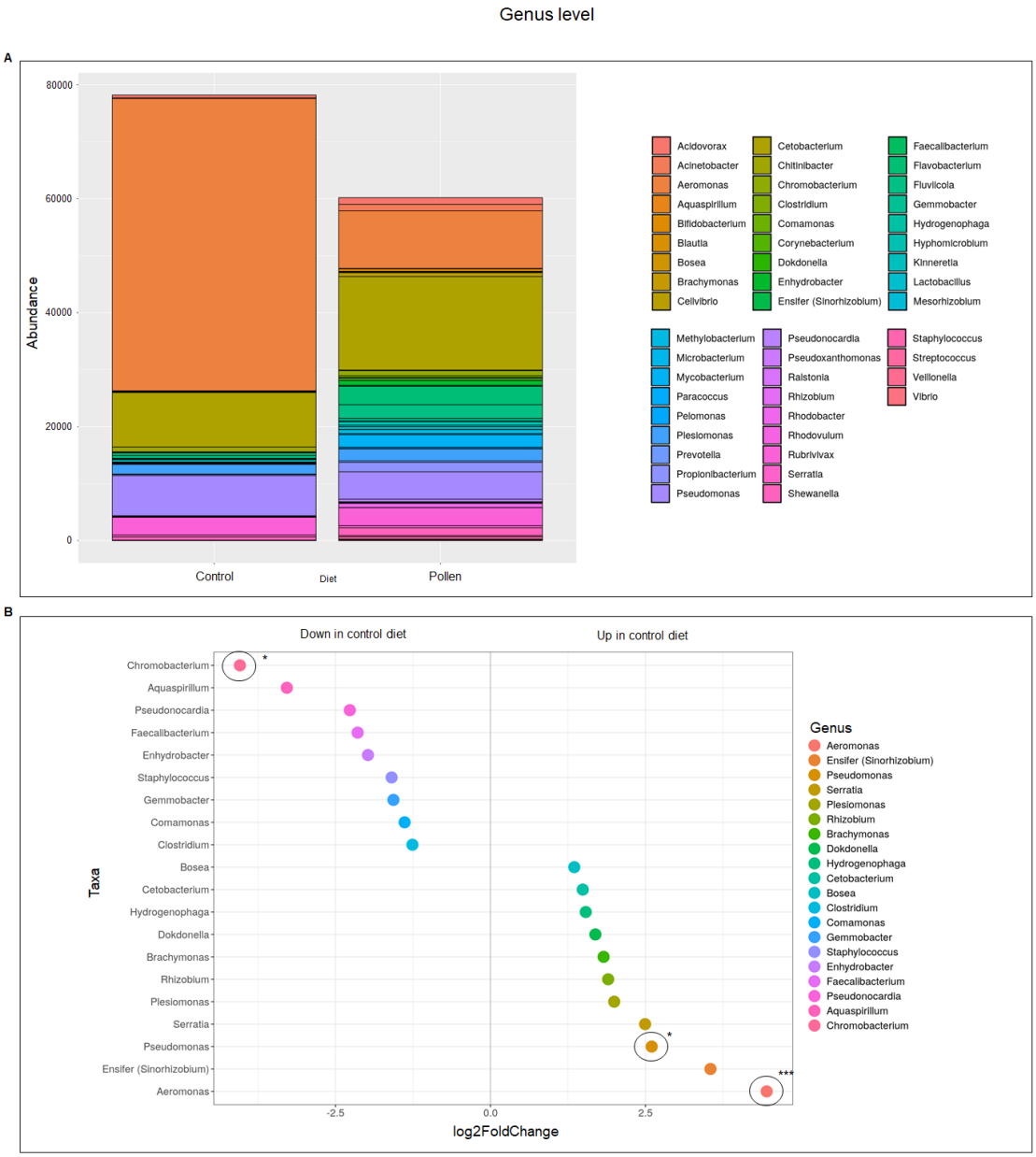
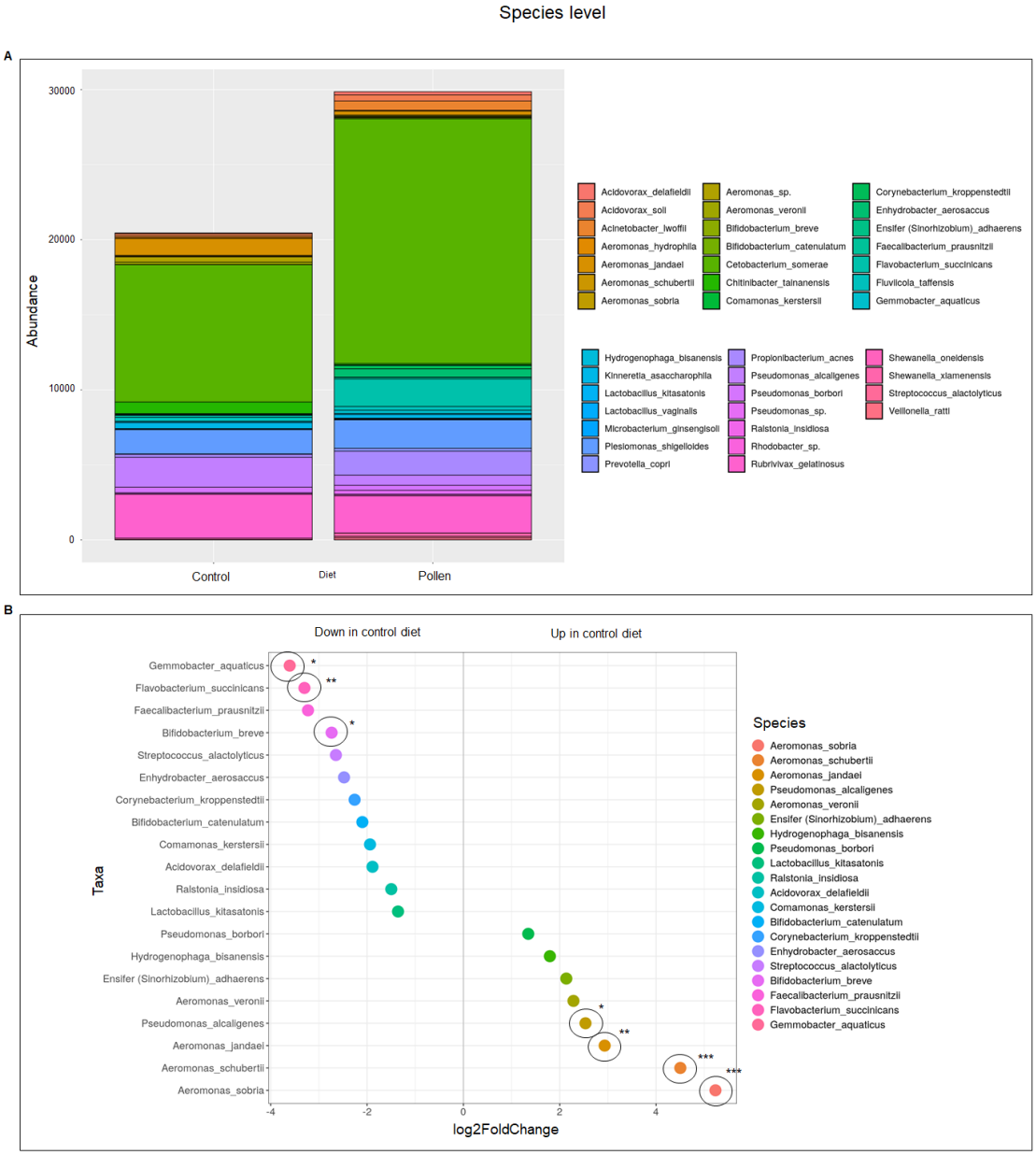


Figure 3. **Bacterial communities at family level. A**) Stacked column bar graph showing the distribution and abundances of bacteria in zebrafish fed with control diet and pollen supplemented diet. (**B**) Dot plot graph showing significantly different abundant OTUs (***$q < 0.001$), where OTUs are grouped by color family along the y-axis. The x-axis indicates the log2 fold-change in control diet compared to pollen diet.



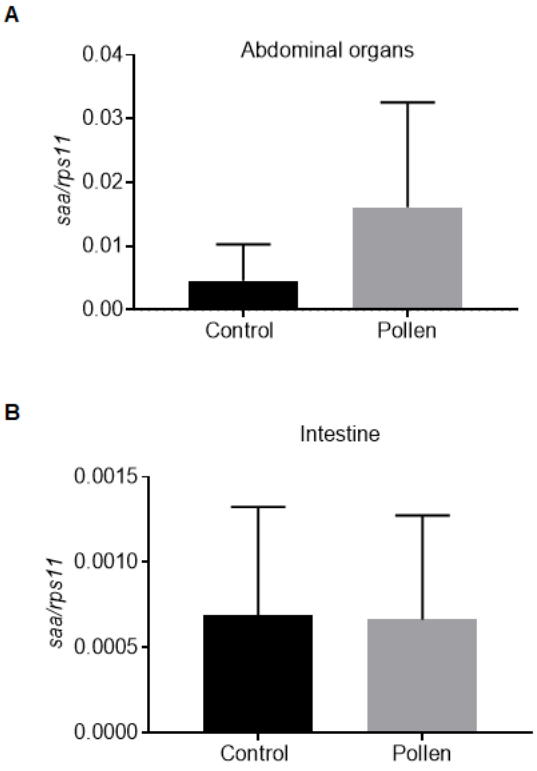
393

394 **Figure 4**. **Bacterial communities at genus level. A**) Stacked column bar graph showing
395 the distribution and abundances of bacteria in zebrafish fed with control diet and pollen
396 supplemented diet. **B**) Dot plot graph showing significantly different abundant OTUs
397 (*q<0.05; ***q< 0.001), where OTUs are grouped by color along the y-axis. The x-axis
398 indicates the log2 fold-change in control diet compared to pollen diet.



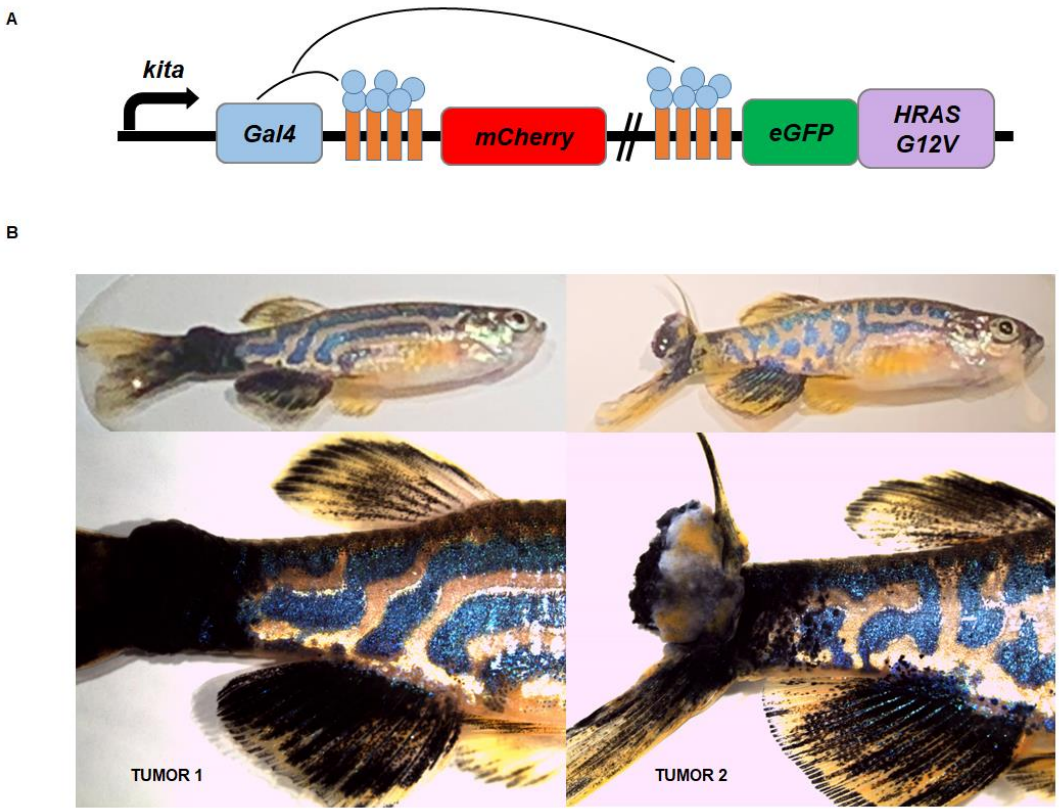
399

400 **Figure 5. Bacterial communities at species level. A)** Stacked column bar graph showing
401 the distribution and abundances of bacteria in zebrafish gut fed with control diet and
402 pollen supplemented diet. **B)** Dot plot graph showing significantly different abundant
403 OTUs (*q<0.05; **q<0.01; ***q< 0.001), where OTUs are grouped by color along the y-
404 axis. The x-axis indicates the log2 fold-change in control diet compared to pollen diet.



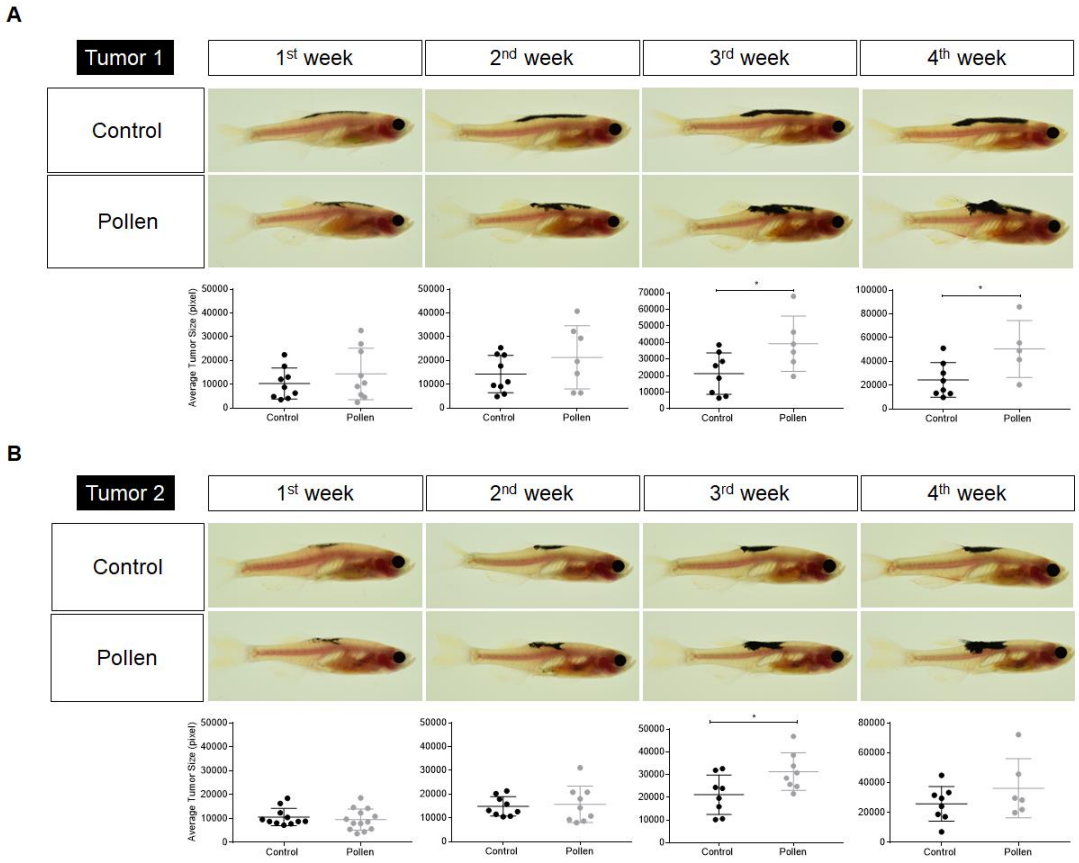
405

406 **Figure 6. *saa* mRNA levels in adult zebrafish after diet treatments. A)** Abdominal
407 organs. **B)** Intestine. ANOVA and Sidak's Multiple Comparison Test. The data are shown
408 as mean + SEM (n=5).



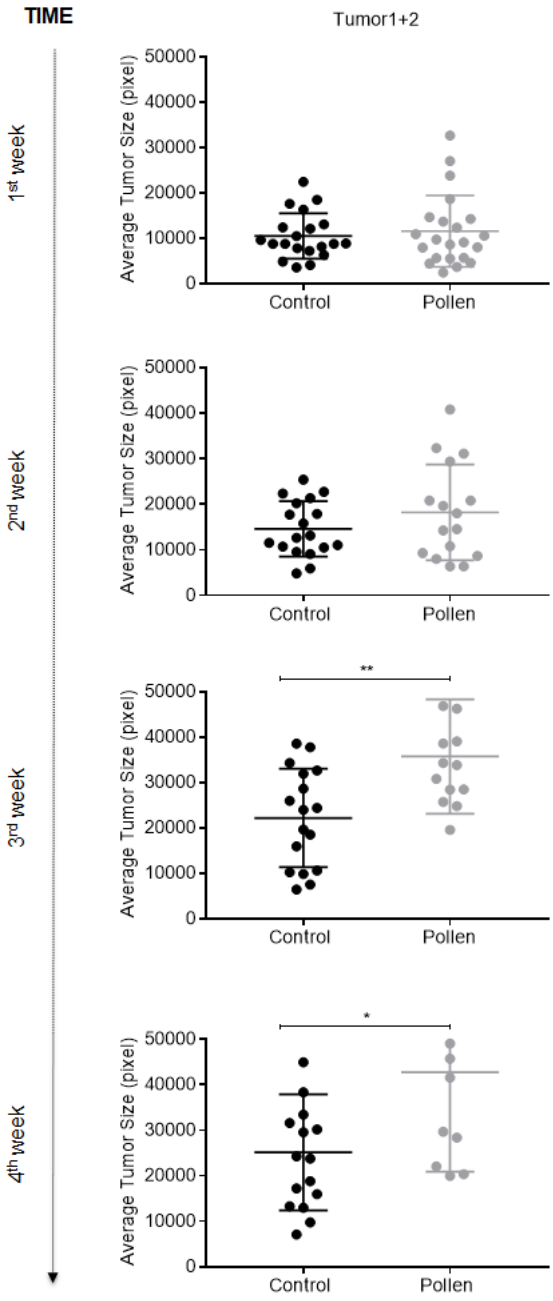
409

410 **Figure 7. Animals used as tumor donors for transplantation. A**) Schematic diagram
411 of SKCM model line in zebrafish. *Tg (kita:GalTA4,UAS:mCherry)*^{hzm1} zebrafish was
412 crossed with *Tg (UAS:eGFP-H-RAS_G12V)*^{io6} line to express oncogenic human
413 HRAS_G12V driven by the melanocyte cell-specific promoter *kita*. **B**) Representative
414 images of *kita:Gal4;eGFP-HRAS-G12V* whole fish and nodular tail tumor (1 and 2) used
415 in our study (biopsied and disaggregated for posterior allotransplantation).



416

417 **Figure 8**. **Tumors representative images and average tumor size (pixels) from 1 to 4**
418 **weeks' post-transplant. A)** Tumor 1. **B)** Tumor 2. Each dot corresponds to a recipient-
419 transplanted fish and the mean ± SEM is also shown. *p < 0.05 according to unpaired
420 Student t test.

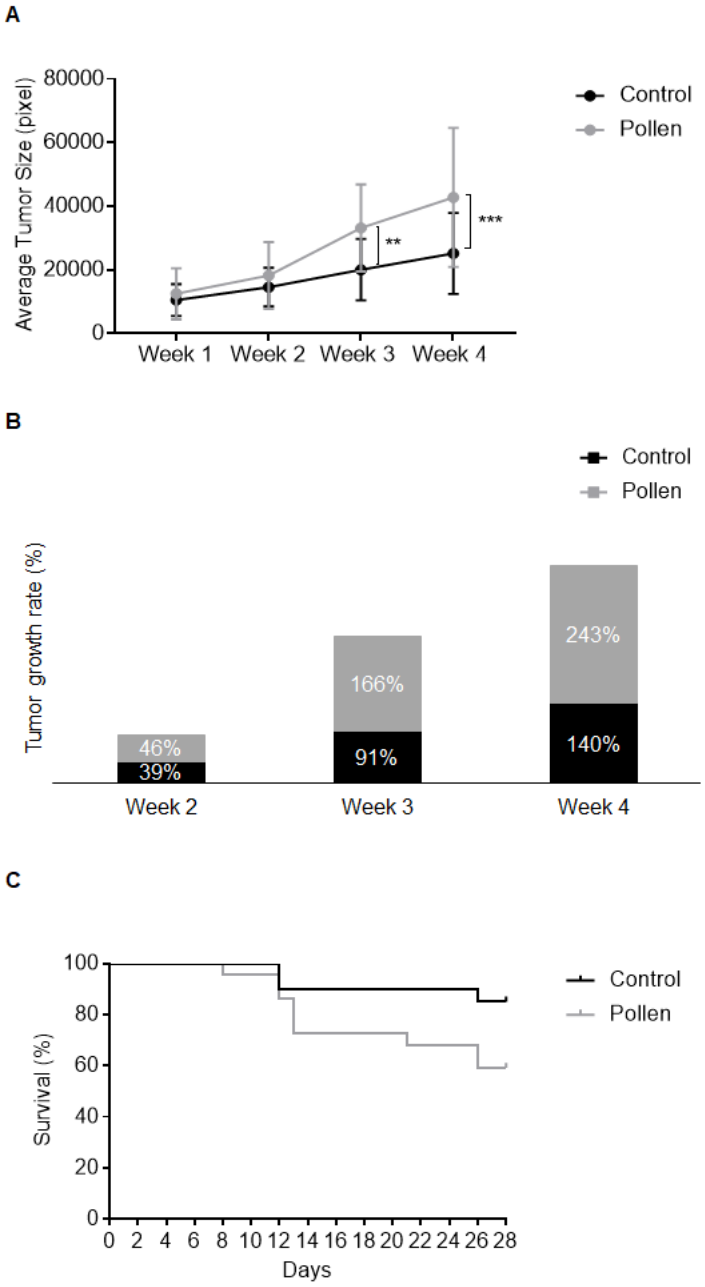


421

422 **Figure 9**. **Average tumor size.** Average tumor (1+2) size (pixels) from 1 to 4 weeks'

423 post-transplant. Each dot corresponds to a recipient-transplanted fish and the mean ± SEM

424 is also shown. *$p < 0.05$, **$p<0.01$ according to unpaired Student t test.



425

426 **Figure 10. Adult casper zebrafish fed with control diet (black color) vs. pollen diet**
427 **(gray color) over 4 weeks after melanoma allotransplant. A)** Average tumor (1+2)
428 size (pixels). **p < 0.01; ***p < 0.001 according to ANOVA and Sidak's Multiple
429 Comparison Test. **B)** Tumor growth rate (%). **C)** Survival curve (%). Kaplan-Meier
430 Gehan-Breslow-Wilcoxon and nonparametric Log-rank Test.

431

5.DISCUSSION

We here describe effects of bee pollen administration that have never been reported or that contradict many works in the literature on other species.

Our results do not show any significant effect of dietary bee pollen in growth performance in zebrafish. Nevertheless, supplementing diets with bee pollen has been reported to improved growth parameters in other species, as calves (Tu et al., 2015), rabbits (Attia et al., 2011; Zeedan et al., 2017), and also in fish Nile tilapia *Oreochromis niloticus* (Abbass et al., 2012; El-Asely et al., 2014). Additionally, studies with rats suggested increased intestinal absorptive capacity and nutrient usability in bee pollen fed animals (Wang et al., 2007; Hajková et al., 2013). Improvements in growth characteristics (length and weight gain) of bee pollen fed animals may be attributed to its components, like vitamins, minerals and enzymes or coenzymes, which may enhance digestion and assimilation of nutrients (Xu et al., 2009). However, we believe that responses to pollen feeding can vary according to the species studied, the control-based diet, the concentration offered and the nutritional composition of each pollen. The addition of pollen in the diet has also demonstrated effects on rat's intestine mucosal surface, causing a slight increase in epithelial layer of the small intestine and significantly increased the epithelium volume and decreased the connective tissue volume (Hajkova et al., 2014). These results may be related to positive changes found in other studies for growth parameters, but they can also indicate important changes in the animals' digestive tract and consequences in other structures, such as the microbiota. Thus, we hypothesized bee pollen could cause changes in zebrafish intestinal microorganisms.

Gut microbiota may vary according to the intestine anatomical regions, which changes in terms of physiology, pH and oxygen tension, digesta flow rates, substrate

availability, and host secretions (Flint et al., 2012; Valdes et al., 2018). Generally, fecal samples are accepted for microbiome investigations, but tissue biopsy containing multiple regions of the gastrointestinal tract has demonstrated to achieve a more comprehensive and appropriate representation of the microbial communities contributing to gut tissue health (Huse et al., 2014; Bashir et al., 2016; Koo et al., 2017). In accordance, we have sampled the entire zebrafish gut tissue in our study. To the best of our knowledge, this is the first study reporting the effects of bee pollen feeding on zebrafish intestinal microbiota.

Phenolic compounds, especially flavonoids, present in the wall of pollen grains are the main substances related to biological and therapeutic activities (Denisow and Denisow-Pietrzyk, 2016). These substances were shown to have an important influence on some specific bacteria in bee's intestinal microbiota, as *Bifidobacterium asteroides*, increasing the production of several metabolites (juvenile hormone derivatives and prostaglandins) that have key functions in immunity and physiology of these animals (Kešnerová et al., 2017). There is almost no information about bee pollen influencing the intestinal microbiome in other species but, interestingly, *Lactobacillus* and *Bifidobacterium*, widespread used as probiotics for humans and animals, have been isolated from bee pollen samples (Vásquez and Olofsson, 2009; Anderson et al., 2013; Asama et al., 2015).

In our study, we found that bee pollen affected intestinal microbiota composition with differential abundance at family, genus and species levels. Gut microbiota plays a central role in the regulation of multiple host metabolic pathways, such as homeostasis and immunostasis (Merrifield and Rodiles, 2015). However, little is known about the function of individual gut bacterium in zebrafish. There is a shared so-called core gut microbiota, found in different zebrafish facilities, dominated by members of the

Proteobacteria phylum (genera *Aeromonas* and *Shewanella*) followed by Fusobacteria or Firmicutes (class *Bacilli*), Actinobacteria and Bacteroidetes phyla (Roeselers et al., 2011). However, diet also plays a vital role in determining the composition of the resident gut microbes (Mandal et al., 2015). Although microbiota composition is relatively stable, permanent changes in terms of diversity and/or abundance of the community (dysbiosis) may occur due to dietary and environmental alterations (Blumberg and Powrie, 2012).

In the present study, pollen diet group presented significantly lower abundance at family level for *Aeromonadaceae* and at genus level for *Aeromonas* and *Pseudomonas.* *Aeromonas* and *Pseudomonas* spp. are genus commonly found in aquatic environments (Mena and Gerba, 2009; Gonçalves Pessoa et al., 2019). Some studies described *Aeromonas* spp as the only group of bacteria that are present throughout the zebrafish life cycle, suggesting the existence of this bacteria in the core microbiota with important colonization resistance functionality. They seem to play important roles in immune defense, gut cell growth, and inducing the transcription of important genes (Rawls et al., 2006; Stephens et al., 2016; Burns and Guillemin, 2017). It is known that the genus *Aeromonas* sp. also secretes an immunomodulatory protein called AimA that prevents the recruitment of excessive intestinal neutrophils (Rolig et al., 2018). In addition, both genus can be of great economical and medical importance, since members of this genus are distributed in freshwater and in association with aquatic animals are sometimes known to cause a diverse spectrum of diseases (Sugita et al., 1995).

Notwithstanding, at species level, we have identified *A. sobria*, *A. schubertii*, *A. jandaei*, and *P. alcaligenes* with significantly lower abundance at pollen diet group. Although they can be isolated from fish intestinal tracts, these *Aeromonas* species have also been described as animals and human's pathogens, associated with gastrointestinal problems, wound infections, septicemia, enterotoxin production and represent an

important economic problem in aquaculture (Igbinosa et al., 2012; Liu and Li, 2012; Beaz-Hidalgo and Figueras, 2013; Yu et al., 2015). *P. alcaligenes* has been also isolated as pathogen in fish causing hemorrhagic disease (Xu et al., 2015). Studies are still necessary to elucidate the role of each individual bacterium in the microbiota, as well as the effects of the complex interaction between different microorganisms to achieve a beneficial balance.

Pollen diet group presented significantly higher abundance at genus level for *Chromobacterium*. Species of the genus *Chromobacterium* have been described with probiotic effects. For example, *Chromobacterium violaceum,* which produce violacein, a violet pigment that possesses functions such as antibacterial, antiviral, antifungal, and antioxidant activities, was shown to have an impact in the mammalian gut microbiome (Pauer et al., 2018). Changes in rat's microbial diversity were found after orally violacein administration, modulating specially components of Firmicutes and Actinobacteria phyla. In fact, studies have demonstrated violacein immunomodulatory potential, and yet antitumor activity (Durán et al., 2016). Also, *Chromobacterium aquaticum* isolated from lake water samples and administered as a probiotic feed supplement, could enhance nutrient metabolism and growth performance, as well as could modulate innate immunity against *A. hydrophila* and *S. iniae* in zebrafish (Yi et al., 2019). The probiotic produced extracellular enzymes (protease and xylanase) and a bacteriocin-like substance, which exhibited tolerance to extreme pH and high-temperature conditions and broad-spectrum bactericidal activity against pathogens.

At species level, higher abundance for *Gemmobacter aquaticus, Flavobacterium succinicans* and *Bifidobacterium breve* were found in our study for bee pollen group. Although little is known about *G. aquaticus* and *F. succinicans*, *Bifidobacterium breve* has been described as effective probiotic bacteria. For example, it is widely used by

humans, especially in pediatric areas, since it has antimicrobial activity against human pathogens and immuno-stimulating abilities (Cionci et al., 2018; Cukrowska et al., 2020). Also, an interesting study showed that oral administration of commensal *Bifidobacterium* as probiotic promoted antitumor immunity (improving the function of dendritic cells and consequently increased infiltration of effector T-tumor cells) and controlled the growth of melanoma in mice, indicating that the composition of commensal microbial can also influence spontaneous anti-tumor immunity, as well as responses to immunotherapy. Oral administration of the probiotic improved tumor control to the same degree as specific antibody therapy for the tumor programmed cell death protein 1 ligand (PD-L1) and in a treatment with both combined, tumor outgrowth were almost abolished (Sivan et al., 2015). In mice, *Bifidobacterium breve* was shown to effectively induce the Regenerating islet-derived III (REGIII; one class of antimicrobials protein expressed in the intestine) production via the MyD88-Ticam1 pathway, demonstrating that this probiotic may enhance the mucosal barrier and protect the host from infection and inflammation (Natividad et al., 2013).

Serum amyloid A (Saa) analysis from zebrafish abdominal organs and from separated intestines were performed in our study to see if pollen in diet could modulate the transcription of this protein. Serum amyloid A is a conserved secreted protein produced in the intestine and liver and with described effects on immune cells as neutrophils. The microbiota is able to induce the gene encoding Saa expression in the zebrafish intestine and, microorganism's diversity can lead to varied levels of Saa protein; these factors could facilitate specific effects on host innate immune system (Murdoch et al., 2019). Some authors described some bacteria, such as *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Escherichia coli*, to strongly induce Saa transcriptions, while others such as *Shewanella* sp. and *Staphylococcus* sp. failed to modulate the same gene

(Rawls et al., 2006). Our results for *saa* gene analysis revealed no differences in its transcript levels. It is assumed that a complex interaction of different microorganisms in the digestive tract stimulates the more potent expression of proteins and immune markers compared to individual strains, indicating that may be necessary a combination of specific microorganisms to alter the mRNA levels of this gene.

A unique optimal gut microbiota composition does not exist since it is different for everyone. However, a healthy host–microorganism balance must be respected in order to optimally perform metabolic and immune functions and prevent disease development (Rinninella et al., 2019). There is a close mutualistic relationship between gut microbiota variations and diseases, including extra-intestinal diseases such as metabolic disorders (Rinninella et al., 2019). With this in mind, we have decided to study if pollen supplementation in diet, together with the changes in the intestinal microbiota found, could influence cancer development. Thus, SKCM allotransplantation assay was performed in Casper zebrafish to directly visualize tumor cell proliferation and dissemination *in vivo* over time.

Bee pollen has been linked to anti-carcinogenic properties (Denisow and Denisow-Pietrzyk, 2016; Kieliszek et al., 2018; Li et al., 2018) but there is still no full evidence for this attribution. Studies have shown bee pollen with greater or lesser antimutagenic properties in different types of cancer (Furusawa et al., 1995; Abdella et al., 2009; Uçar et al., 2016; Wan Omar et al., 2016). These activities may be derived from its antioxidant properties (mainly suppression of oxygen reactive species formation) (Denisow and Denisow-Pietrzyk, 2016), its ability to induce apoptosis and stimulate secretion of tumor necrosis factor-alpha (Wu and Lou, 2007; Komosinska-Vassev et al., 2015), cytotoxic activity on cells (Pascoal et al., 2014), and by simply enhancing and strengthening the immune system (Wang et al., 2013). Thus, in accordance with results

582 obtained mostly in cell cultures, it has been suggested that bee pollen extracts containing
583 different types of compounds, especially phenolic acids and flavonoids (e.g. kaempferol,
584 apigenin), help to control cell growth (Denisow and Denisow-Pietrzyk, 2016).

585 Nowadays, skin cancers are attributed to chronically injured, non-healing wounds,
586 scars or ulcers (Tang and Wang, 2016). Some studies suggest that bee pollen may also
587 affect the wound healing process of burn wounds (Olczyk et al., 2016). In this context,
588 we hypothesized whether it could have a beneficial effect on melanoma development. In
589 our study, bee pollen supplementation in zebrafish diet had no protective properties
590 against SKCM. Pre-clinical studies suggest that many compounds derived from natural
591 products have potent activity against cancer cells or xenotransplanted tumors and that
592 they can prevent the carcinogenesis or metastasis of existing tumors (Strimpakos and
593 Sharma, 2008). Instead, we observed a stimulating growth effect. A study proposed that
594 patients with a favorable gut microbiome enhance systemic and antitumor immune
595 responses and, by contrast, patients with an unfavorable gut microbiome have impaired
596 systemic and antitumor immune responses (Gopalakrishnan et al., 2018). Regarding our
597 results, it is possible that changes in the microbiota found in pollen group may have
598 interfered with tumor progression; or even the pollen composition, with a high level of
599 carbohydrates and sugars, could interfere negatively in the response to tumor
600 development. Some studies propose that higher levels of blood glucose and insulin are
601 cancer risk factors. Insulin has been shown to stimulate cell division, supporting the
602 growth and spread of cancer cells and making them more difficult to eliminate (Denley
603 et al., 2007; Rose and Vona-Davis, 2012; Paoli et al., 2013). In addition, higher levels of
604 insulin and blood glucose can contribute to inflammation, lead to the growth of abnormal
605 cells and possibly contribute to cancer (Paoli et al., 2013). The bee pollen used in our

study was composed by 60% of carbohydrates, amongst them, 50% of total sugar, which could have affected both microbiota composition and response to cancer.

Due to its variable composition, the effects caused by bee pollen ingestion cannot be simply generalized. There is a large amount of different substances, which can interfere individually and even with complex interactions between them. Studies with bee substances is challenging and deserves greater attention in future researches. In conclusion, bee pollen as dietary supplement did not affect zebrafish weight gain, increased length or serum amyloid A gene expression, but changed intestinal microbiota composition and had a stimulant effect on SKCM development.

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DECLARATION OF COMPETING INTEREST

Authors declare no competing interests.

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