Universidade de Aveiro Departamento de Química
2017

Marguerita Evangelho da Rosa

Produção e purificação de anticorpos IgY com propriedades antimicrobianas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Industrial e Ambiental, realizada sob a orientação científica da Doutora Mara Guadalupe Freire Martins, Investigadora Coordenadora no Departamento de Química, CICECO, da Universidade de Aveiro, e do Doutor Ricardo Simão Vieira Pires, Investigador Auxiliar do Centro de Neurociências e Biologia Celular, da Universidade de Coimbra.

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Aos meus pais Ida e Hugo e à minha chatinha e incansável irmã …

O júri

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Palavras-chave Aquacultura, *Vibrio anguillarum*, determinantes genéticos de virulência, resistência a antibióticos, anticorpos, imunoglobulina Y (IgY).

Resumo

A Aquacultura tem recebido especial destaque nos últimos anos como alternativa às atividades de pesca tradicional, atualmente restritas pelos limites legais de captura. Neste sentido, têm-se desenvolvido novas técnicas de modo a aumentar o lucro e o rendimento das atividades associadas à aquacultura. No entanto, a sobre-exploração de espécies, poluição, surgimento de doenças e o aumento de microorganismos resistentes a antibióticos, surgem como consequências deste desenvolvimento. *Vibrio anguillarum* é uma bactéria Gram-negativa que causa infeções em peixes de sistemas de aquacultura e que origina perdas económicas significativas. Estas infeções são normalmente tratadas com recurso a agentes antimicrobianos, tais como antibióticos. No entanto, a prevalência de bactérias resistentes a estes compostos destaca a necessidade crucial de desenvolvimento de estratégias terapêuticas alternativas. O uso de anticorpos, nomeadamente a imunoglobulina Y (IgY) produzidas em aves poedeiras e purificado a partir de gemas de ovos é uma abordagem promissora para o controlo de infeções por *V. anguillarum* na aquacultura. O atual trabalho centrou-se no desenvolvimento de anticorpos IgY específicos contra os determinantes de virulência associados a *V. anguillarum* como uma estratégia antimicrobiana capaz de melhorar a produtividade dos sistemas de aquacultura. Neste são apresentados resultados da produção, purificação e caracterização de anticorpos IgY de galinha e codorniz contra antigénios (extratos celulares, frações da membrana externa e péptidos do canal TolC) de *V. anguillarum*. Aves hiperimunes foram produzidas com sucesso para cada antigénio e foram purificadas as respetivas frações específicas de IgY (> 95% de pureza). Por fim, estudou-se o potencial antimicrobiano dos anticorpos anti-extrato celular de *V. anguillarum* por ensaios de crescimento bacteriano que revelaram um efeito bacteriostático promissor com 50% de inibição. Em suma, e face aos resultados obtidos, os anticorpos podem ser usados como agentes antimicrobianos alternativos para combater e prevenir infeções por *V. anguillarum* em sistemas de aquacultura.

Keywords Aquaculture, *Vibrio anguillarum*, antibiotic resistance, virulenece determinants, antibodies, immunoglobulin Y (IgY).

Abstract Aquaculture has received remarkable attention in recent years as an alternative to traditional fishing activities, currently restricted by fishing quotas. New techniques have therefore been developed to increase production and profit of aquaculture activities. However, over-exploitation, pollution, appearance of infectious diseases and antimicrobial resistance, have emerged as concerning consequences of such development. *Vibrio anguillarum* is a Gram-negative bacterium causing fish infections in aquaculture systems and leading to significant economic losses. These infections are usually treated with antibiotics; however, the prevalence of bacteria resistant to such drugs urges the development of alternative therapeutic strategies. The use of antibodies, namely avian Immunoglobulin Y (IgY) purified from bird egg yolks, is a promising approach for the control of *V. anguillarum* infections in aquaculture. The current work focused on the development of specific IgY antibodies against virulence determinants associated to *V. anguillarum*, envisaging an antimicrobial strategy capable of improving the productivity of aquaculture systems. In this work, the production, purification and characterization of chicken and quail IgY antibodies against *V. anguillarum* antigens are presented. Whole-cell extracts, outer-membrane fractions and outer-membrane TolC channel peptides were used as antigens in independent protocols to elicit target-specific *V. anguillarum* antibodies. Hyperimmune birds were successfully generated for each antigen and respective target-specific IgY fractions were purified (>95% purity) from selected bird eggs for downstream studies. Finally, the antimicrobial potential of anti-whole-cell *V. anguillarum* antibodies was studied by bacterial growth assays, revealing a promising bacteriostatic effect, with 50% of bacterial growth inhibition. In summary, and according to the results obtained, such antibodies can be used as alternative antimicrobial agents to prevent and combat infections by *V. anguillarum* in aquaculture systems.

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List of symbols and abbreviations

AMPs Antimicrobial peptides

BSA Bovine serum albumin

CFA Complete Freund's Adjuvant

CFU Colony forming units

ELISA Enzyme-linked immunosorbent assay

FAO Food and Agriculture Organization of the United Nations

Fc Fragment crystallizable

flaA| flaD | flaE | flagellin A | flagellin D | flagellin E

HAMA Human anti-mouse antibodies

HK Heat-killed *V. anguillarum* extract

HRP Horseradish peroxidase

IFA Incomplete freund's adjuvant

IgG Immunoglobulin G

IgY Immunoglobulin Y

IMT Inner membrane transporter

IPTG Isopropyl β-D-1-thiogalactopyranoside

ISO International Organization for Standardization

kbp kilo base pair

LB Lysogeny broth

LD_{50} Fifty percent lethal dose

LPSs Lipopolysaccharides

Mbp Mega base pairs

MFP Membrane fusion protein

MP	Membrane preparation
MW	Molecular weight
OMF	Outer membrane factor
ON	Overnight
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with 0.05% Tween 20
PEG	Polyethyleneglycol
Phyre2	Protein Homology/analogy Recognition Engine version 2.0
PL	17000 g centrifugation pellet
PNK	T4 Polynucleotide kinase
RT	Room temperature
RTX	Repeat in toxin
SEC	Size exclusion chromatography
SLIC	Sequence and ligation independent cloning
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TBS-T	Tris buffered saline with (1:1000) Tween 20
TCBS	Thiosulfate-citrate-bile salts-sucrose agar
TISS	Type I secretion system
TMB	3,3’,5,5’-Tetramethylbenzidine
TSB	Tryptic soy broth
WB	Western Blot
WSPF	Water soluble protein fraction

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1. Introduction

1.1.Scopes and objectives

The main objective of the present work comprises the production of Immunoglobulin Y (IgY) specific antibodies against the bacterium *Vibrio anguillarum*, which causes major economic losses in aquaculture, mostly associated to fish death. This antibody is important since there is an emergent problem related with antibiotic resistance in fish farming that cannot be solved with regular antimicrobial agents. As an effective alternative, IgY can solve the infection caused by *V. anguillarum* (vibriosis) without creating resistance and without interfering with the genetics of the bacterium. Through the refinement of IgY production the resultant antibody should be able of targeting one or more of the most prominent virulence determinants of *V. anguillarum*, reducing the amount of deaths associated to vibriosis, as well as the number of antimicrobial agents used, increasing aquaculture profit without compromising the health of the animals or of the surrounding environment.

1.2. Aquaculture

1.2.1. Definition and worldwide fish production

Aquaculture, also known as fish farming or aquafarming, is referred to the activity of breeding, rearing, and harvesting of aquatic animals and plants in different types of water environments, such as ponds, rivers, lakes and ocean (1). Aquafarming comprises not only the hatchery of fish and shellfish in order to be sold to markets and consequently to consumers, but also to aquarium trade as ornamental fish. Plants can also be produced through this technique with the objective of being used as food, as well as pharmaceutical and biotechnological products. Usually this technique can be classified in two major types: marine and freshwater aquaculture. The first one includes the production of species that live in the ocean, such as oysters, clams, mussels, shrimp, salmon, seabass, seabream, among others and is normally performed in man-made systems that mimic ocean's condition or in the ocean itself with cages on the seafloor or suspended in the water column (1). On the other hand, freshwater aquaculture produces species that are natural from freshwater ecosystems, such as rivers and lakes, and is mainly performed in on-land manmade systems.

The global per capita fish consumption is growing each year. In the 60s this value was 9.9 kg and grew to a value of 14.4 kg in the 90s, reaching 19.7 kg in 2013 (2). Indeed,

aquaculture activities have been critical to fulfil the global fish consumption demands, providing an important alternative to traditional fishery that faces capture limitations imposed by legislations. According to the analysis of commercial fish stocks by Food and Agriculture Organization of the United Nations (FAO), the share of the stocks within biologically sustainable levels decreased from 90% in 1974 to 68.6% in 2013, meaning that 31.4% of fish stocks were biologically unsustainable, i.e. overfished (2). Thus, aquaculture is crucial in order to decrease the levels of overfished animals. According to some studies, in 2010, aquafarming supplied 47% of world food fish production for human consumption (3), and in 2014 the contribution of aquaculture to human consumption surpassed the wild-caught fish contribution for the first time (2). In the same year, the total amount obtained from all the species were 73.8 million tonnes, of which 49.8 million tonnes of finfish, 16.1 million tonnes of molluscs, 6.9 million tonnes of crustaceans and 7.3 million tonnes of other aquatics animals (2).

In the last few years the Aquaculture sector gained more prominence, being considered the fastest growing animal food producing sector in the world (4), with total sales weight and number of employees increasing each year (Figure 1).

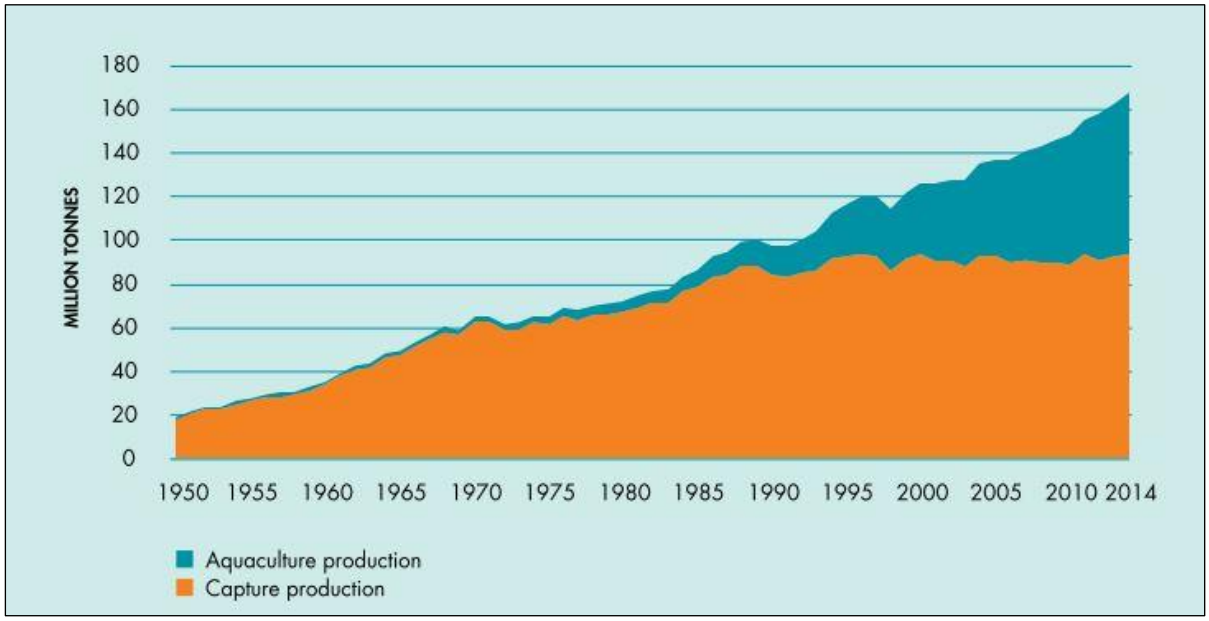


Figure 1. World capture fisheries and aquaculture production: 1950-2014 (2).

Analysing the situation of aquaculture in Portugal, in 2011, the total sales weight increased about 22% when compared to the previous three years during which this value had been constant. This increase consequently cause a growth in the total value of sales, increasing up to 57 million euros.

Portugal's aquaculture is based in familiar businesses, normally associated to bivalve's production, representing a total number of 1,453 enterprises and around 2,316 employees (4). Although the small scale of this activity, it continues to be quite important to boost economy and can be considered as an alternative to the reduction of fishing quota (4).

1.2.2. Problems associated to Aquaculture: over-exploitation, diseases and resistance to antibiotics

In order to cope with the market demands on aquaculture products and to improve their profit, some companies do not follow the guidelines of environmental and societal sustainability and put in danger animals, plants and also the surrounding environment. Indeed, major concerns resulting from the intensification of aquaculture activities include: 1) pollution with nutrient wastes and harmful chemicals; 2) depletion and salinization of potable water and agricultural land; 3) escape of non-native species; 4) spread of infectious diseases to native species and 5) overuse of antimicrobial agents that lead to the development of bacterial resistance (3).

The rise of aquaculture implies cultivation of a wide variety of animals, contemplating higher (e.g., salmon and sea brass) and lower trophic level animals (e.g., clams and oysters), and also an improvement in the cultivation methods, using more efficient techniques. However, the sustainability of each culture is not the same. Higher trophic level fishes normally need wild fish resource, since fishmeal and fish oil are still considered the most nutritious and easily digestible ingredients for farmed-fish (2). Thus, it is not surprising that in the last few years it was verified an increase in the amount of forage fish used as fishmeal, associated to cultivation higher trophic level animals that are more profitable (3,5).

Overfishing is an actual problem that puts in risk a broad range of ecosystems. Therefore, efforts have been made in order to reduce the amount of fished animals, including the 1) use fish by-products (e.g., heads, tails, bones, etc) that are normally discarded by the food industry as feed alternatives to farmed carnivorous species; and 2) the replacement of fishmeal by other sources of proteins (e.g., soy protein) and fish oil by vegetable oils (3). Together these solutions will not only contribute to improve the sustainability of aquaculture but also the economic profitability.

An additional problem associated to aquaculture is the stress that animals are submitted to, and despite the new techniques and methodologies for improved animal welfare within aquaculture, this remains a critical issue. Stress-prone conditions, such as frequent handling of

the animals, overfeeding, grading, transport, water temperature variation, insufficient water renewal or removal of injured and dead fish, are responsible for most of the infectious diseases in aquaculture as result of an opportunistic invasion of stressed fish by pathogens (e.g., bacteria, fungi, virus) which normally co-exist with the host (6). Nevertheless, the progression of diseases and their clinical signs do not depend only on external conditions, but from the overall interaction between the surrounding environment (pollution, stress, etc.), the pathogen and the host (e.g., species, age, disease stage) (7).

To reduce the economic losses in fish farming resulting from infection diseases, antimicrobial agents are widely applied, both to treat and prevent the spread of diseases (prophylactic measure). Antibiotics play a critical role in this process, since they are normally supplemented in the animal's diet (food or water); however, these end up in the environment through solid wastes (organic matter), such as uneaten food or faeces (8). Due to the high stability of some antimicrobial agents, the contact between environmental and commensal bacteria and these compounds increases, allowing adaptation of the microorganisms and leading to antimicrobial resistance. Resistance genes can be transferred intra or interspecies if anchorage in mobile genetic elements, creating multi drug resistant bacteria (9,10). On the other hand, antibiotic resistance can also be transferred through sequential mutations in chromosomal genes (11). The resistant bacteria are harder to eradicate, since the normal antimicrobial agents do not work, which consequently originates the unbalance of the aquatic ecology system, contamination of the natural environment, animals and human's intoxication (6). This way, there is the need of a strict legislation that can diminish the misuse of antimicrobial agents and consequent pollution, as well as alternative strategies that can help fighting multiresistant bacteria. For example, in Europe, the use of antibiotics to promote growth of the animals is forbidden, and these compounds are only used to medical purposes (12).

Antimicrobial compounds are not the only source of pollution related to aquaculture systems since there is many chemicals that are released in the surrounding environment and can compromise it, such as stabilizers, pigments, antifoulants, disinfectants and chemotherapeutics (8). All these compounds and excess of organic matter contribute negatively to the environment, polluting habitats with spare of nutrients, such as nitrogen and phosphorous that lead to eutrophication, acidification of the waters and oxygen depletion, all contributing to an unbalanced ecosystem and to the loss of biodiversity (2). Nevertheless, the amount of pollution depends directly on the culture system characteristics and on the cultivated species, as well as on feed quality and waste management.

1.3. Vibriosis: The case of *Vibrio anguillarum*

One of the diseases that has significant impact in aquaculture systems, causing enormous economic losses, is vibriosis. This disease is caused by bacteria within the *Vibrionaceae* family, being the following species the ones that are more prominent: *Vibrio anguillarum*, *Vibrio ordalii*, *Vibrio salmonicida* and *Vibrio vulnificus biotype 2*.

V. anguillarum is a bacterium that is normally present in gut microbiota of fish larvae because they make part of the surrounding water's microflora. Like *V. anguillarum*, other pathogenic bacteria live inside healthy fishes without showing signs of disease. However, it is not clear the type of interaction between the bacteria and the host, as it could be just an asymptomatic carrier state of the disease cycle, a preliminary colonisation step prior to pathogenesis, or even commensalism (13). The infection can be triggered by a change in the normal function of the animal or by the introduction of a stress factor, such as a rise on the temperature of water, low dissolved oxygen, etc. These stress factors make animals more susceptible to this type of infections, especially in aquaculture systems, however, in natural environments there are still some repercussions in fish death.

V. anguillarum affects more than 50 fresh and salt-water fish species, including salmon, rainbow trout, turbot, sea bass, sea bream, cod, eel, ayu and some types of bivalve molluscs and crustaceans, all of these representing very profitable species cultivated in aquaculture (14).

Vibriosis is characterized by a deadly haemorrhagic septicaemic disease, being the most prominent external signs: weight loss (anorexia), anaemia (pale gills), lethargy (tiredness, lack of energy), red spots on the ventral and lateral zones, ascites (accumulation of fluids in the tissues or body cavities, especially at the abdomen), inflamed and dark skin lesions that can ulcerate and bleed and even exophthalmia, that is characterized by opaque, bloody and inflamed eye balls that result in outer protrusion of the eye ball (pop-eye).

Despite these external signs, some of them are only noticeable in a later state of infection. Thus, several animals could die without showing any signs, especially in young fishes or if infection is acute, spreading rapidly the infection along the host (15). Thus, in the absence of external signs, the disease is harder to detect, being only detectable through molecular analysis or through autopsy of the animals. One of the inner signs of the disease is the high concentration of *V. anguillarum* in the blood and haematopoietic tissues, such as the spleen that becomes swollen and ulcerated (necrosis). However, not only these tissues are affected, but gastrointestinal tract suffers as well, since one of the possible ways of entrance of the pathogen into the host organism is through its mouth. The progression of the infection also

depends on the host organism's pH, since their increase in the posterior gastrointestinal tract and rectum intensifies the infection in these areas, so intestines become distended and filled with a clear and viscous liquid (14). There are also other factors that interfere with *V. anguillarum* progression, such as health conditions of the animal, age and immune system, and even the surrounding environment (e.g., water quality). As previously described, if the infected animal does not show external signs it is hard to confirm if the fish is actually sick, making almost impossible the prediction of the animal's lifetime. In this context, aquaculture companies normally apply antimicrobial agents as a prophylactic measure, which can initiate an antibiotic resistance problem.

1.3.1. General features of *Vibrio anguillarum*

V. anguillarum, also known as *Listonella anguillarum*, is a gram-negative bacterium, comma-shaped rod and polarly flagellated. As previously mentioned, it belongs to the *Vibrionaceae* family, Proteobacteria group and Gamma subdivision. According to its metabolism and life cycle, this bacterium is non-spore forming, facultative anaerobic and halophilic (14). *V. anguillarum* needs between 1.5-2% of sodium chloride (NaCl) in order to grow, registering optimal growth rates between 25-30ºC, forming round-shaped and cream-coloured colonies, but it does not tolerate temperatures close to 37ºC.

This bacterium can be classified in two biotypes, in agreement with the genetic and biochemical properties: (biotype 1) *V. anguillarum* that causes acute haemorrhagic septicaemia and the second biotype (biotype 2) designated *Vibrio ordalii* that causes a moderate infection, originating an irregular dispersion within the host's tissues and consequently forming biofilms (16). *V. anguillarum* biotype 1 can also be organized in 23 O serotypes according to their surface antigens, however only three of them are associated with vibriosis. Being the first two, O1 and O2, associated specifically to the death of farmed and wild fishes, and the O3 serotype prevalent in animals, such as eel and ayu. The rest of the serotypes are not causative agents of vibriosis, so they are considered environmental strains since only in rare cases are detected in fishes affected with this disease (7). On the other hand, no serotypes are associated to *V. ordalii* biotype since it is antigenically homogeneous (Figure 2). Some *V. anguillarum* O1 serotypes harbour pJM1, a 65 kbp (kilo base pairs) plasmid, that encodes for proteins related to the metal iron transport system, best described below. The rest of the O1 serotypes, as well as O2 and O3 serotypes, are normally plasmid-less (17).

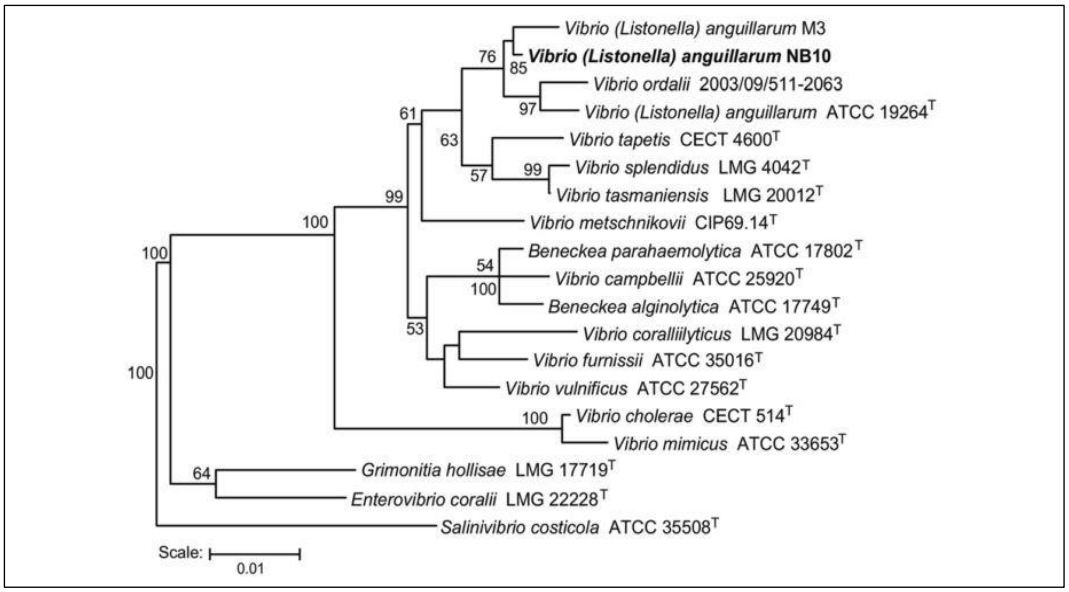


Figure 2. Phylogenetic tree of 16S RNA sequences from *Vibrio* samples (18).

The genome of *V. anguillarum* is about 4.2 Mbp (Mega base pairs) and is constituted by 2 chromosomes, of 3.0 and 1.2 Mbp, respectively. In the first chromosome are allocated the housekeeping genes and other essential genes (17). In both chromosomes are present mobile genetic called prophages, that can integrate themselves in the genome of the bacterium and play an important role in horizontal gene transfer (18). Nevertheless, there are also other types of mobile genetic elements found in the bacteria genome, such as integrons, that have the ability of capture genes cassettes, and harbour any gene of interest (e.g., antibiotic resistance gene), contributing to a higher rate of genetic modification and evolution. Although all these characteristics, there are some that vary between serotypes or even between strains. This variation can be related with the different location of the microorganism and different host, because the organisms need to adapt in order to survive and multiply (18).

1.3.1.1. Virulence determinants of *V. anguillarum*

As previously described the most virulent serotype of *V. anguillarum* is the O1, due to the presence of the pJM1 plasmid, even though some details of the infection process still remain unknown or poorly explained (19). Derome et al. (20) reviewed the different phases of infection caused by *V. anguillarum*. The first step is the recognition of the host cells (skin or gut mucosa components) by the pathogen, that unleashes a chemotaxis phenomenon that drives the bacteria in direction to the host cells. This movement is mostly performed due to flagellins, proteins that are present in the surface of *V. anguillarum* and that contribute to the bacteria virulence. Their importance was proved through a study performed by McGee et al. (21) that showed that

mutations in flagellin genes, such as flaA (flagellin A), flaD (flagellin D) and flaE (flagellin E), decrease virulence associated to the bacterium. Additionally, there are other mediators in this phenomenon including a cytoplasmic methyl transferase that is essential for the chemotaxis signals, and if mutated leads to a decrease in the *V. anguillarum* virulence (14). But this virulence alteration was only registered if bacteria were not injected intraperitoneally, showing that chemotactic motility was only needed for pathogen invasion of the host cells, since it was no longer required for posterior phases of infection.

After the recognition of the host cells, the pathogens adhere to the epithelial cells of the host, using adherence factors, outer membrane proteins, extracellular polysaccharides and glycoproteins (14). Rodkhum et al. (22) performed a study that identified eighteen genes associated to adherence and colonization of bacteria, thirteen of them were also associated to the flagellum.

Subsequently to the adhesion step the bacteria needs to cross the fish integument, that is likewise associated to flaA and plays a key role at this phase, however still not fully understood (21). After the previous step, the degradation of epithelial mucus (host tissues) is performed by an extracellular metalloprotease, EmpA, which acts as a mucinase. This protein is excreted as a 46 kDa proenzyme (pro-EmpA), that is posteriorly submitted to cleavage, removing a 10 kDa peptide (23). This process is performed by the secreted protease, Epp, that cleaves pro-EmpA into the mature active form of EmpA. This maturation is not fully understood as well, since it is detected in the extracellular environment remanescent pro-EmpA, suggesting that this form is also important to *V. anguillarum* virulence. Varina et al. (23) demonstrated that extracellular proteases are important factors in the virulence of *V. anguillarum*, are highly regulated and respond to environmental factors. After the invasion of the cells, the bacteria start to spread through the host body, penetrating and colonizing multiple fish organs, reaching the blood vessels, causing haemolysis and liberating the intracellular heme. This phenomenon is caused by hemolysins, that are coded by four distinct genes, namely vah1, vah2, vah3, vah4 and vah5. Rodkhum et al. (24) tested the virulence of each of the hemolysins, knocking-out each of the genes, and posterior submitting each of the resultant mutants to the haemolytic activity assays. These assays proved that each mutant is less virulent than the ones in the normal organism, since each of the four hemolysins mutants registered residual haemolytic activity, suggesting that the virulence of *V. anguillarum* needs the four proteins contribution. However, not all mutants registered the same value since vah4 mutant exhibited weakest virulence, suggesting that it has a prominent role in this phase of infection (24).

V. anguillarum is also gifted with another hemolysin gene cluster that is called rtxACHBDE, responsible for the expression of a repeat in toxin (RTX). This cluster is composed of rtxA gene that encodes the RTX toxin, rtxC for the RTX toxin activating protein, rtxH for a conserved protein, and rtxBDE for the RTX ABC transporter (14). This type of toxins belongs to the family of pore forming exotoxins, included in the gram-negative type I secretion system (TISS) (Figure 3) (14). Gram-negative bacteria such as *V. anguillarum* are gifted with a TISS, a tripartite system that plays an important role in exporting toxic compounds (e.g., antibacterial drugs) and secreting proteins or other molecules that can promote bacterial virulence. The molecules need to cross the inner and outer membranes and even the periplasm, so the efflux system (TISS) is composed of three parts: an inner membrane transporter (IMT), the outer membrane factor (OMF), and a periplasmic membrane fusion protein (MFP) (25). The IMT component belongs to the ABC (ATP binding cassette) transporter family, since the T1SS gets their energy through the ATP hydrolysis. The two other components: OMF and MFP are porin-like protein, being the MFP the connection between the OMF and ABC. The OMF protein that is commonly used in TISS is the multi-purpose TolC, that is involved not only in multidrug efflux but in other important cellular processes. In *V. anguillarum* TolC is still poorly studied, however, in other gram-negative bacteria such as *Escherichia coli* (*E. coli*) the inactivation of TolC increased the susceptibility of bacteria to broad spectrum antimicrobial agents, such as antibiotics, detergents, dyes, organic solvents, and others (26).

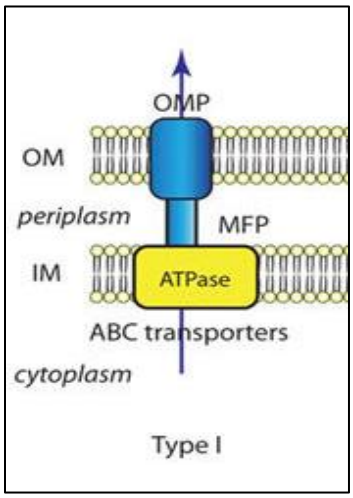


Figure 3. Schematic representation of a T1SS (Adapted from Tseng T et al. (2009)) (56).

The last stage of infection is the capture of iron ions. This element is not freely available in the host and the bacteria need them to grow. Thus, bacteria need an efficient iron acquisition system. Fishes have a non-specific defence mechanism that uses proteins such as transferrin, lactoferrin and ferritin, that binds to iron, making it inaccessible for potential pathogenic

microorganisms (14). According to the differences between the 3 serotypes of *V. anguillarum* (biotype 1), there are different iron acquisition systems. In the O1 serotype this capture is performed by a siderophore-anguibactin system encoded in the pJM1 plasmid, being siderophore a small and high-affinity iron chelating complex that acts together with a anguibactin protein. This plasmid harbours genes related to iron transport (fatA, B, C) and biosynthesis of anguibactin (angR, B/G, H, N, and M), centring its action on anguibactin synthesis and transport of the ferric–anguibactin complex (27). After anguibactin production, this protein is secreted and binds to the existent iron outside the bacterium, forming the ferric–anguibactin complex that is posteriorly transported to the bacterium's cytosol. This transportation to the cytosol is made through the outer membrane receptor FatA (receptor for ferric-anguibactin complex) and lipoprotein FatB. FatA binds to the complex, allowing its transference to the bacterium's periplasm, and FatB delivers the complex from the periplasm to the cytosol. Therefore, pJM1 is important to sequester iron and posteriorly to the O1 serotype virulence. A bacterium without this plasmid or with an angR mutated increases the respective LD_{50} values (number of microorganisms that will kill 50% of the animals tested). LD_{50} values of a wild type *V. anguillarum* range between $1.0 \times 10^2 – 1.5 \times 10^3$ and a *V. anguillarum* in the previous referred conditions is about 2×10^8 (27). Thus, it is needed a higher number of cells in order to kill 50% of the tested animals.

O2 and O3 serotypes have different systems since they do not have the pJM1 plasmid. In the O2 serotype there is a siderophore chromosomally encode called vanchrobactin, and in the O3 serotype the iron acquisition system is also gifted with iron-regulated outer membrane proteins (14).

Although the lipopolysaccharides (LPSs) are not directly implied in *V. anguillarum* infection mechanism, they are also responsible for its virulence. LPSs normally trigger an immune response in the host, however some gram-negative bacteria can surpass this system (14). LPSs are classified according to the length of the polysaccharide side chain, being smooth strains the ones that have a longer polysaccharide side chain, called O antigen, and that provide an additional protection against the complement system in the host. On the other hand, the rough strains that do not contain the O antigen are more susceptible to the immune system of the host. This protection from the O antigen comes from the steric hindrance phenomenon resulting from a conformational change due to assembling of atoms. However, the O antigen is also involved in anguibactin-mediated iron uptake, since an insertion in the O antigen operon induces an accentuated decrease in the outer membrane receptor for fatA (iron-anguibactin

transport), which consecutively results in an iron uptake deficiency. Thus, O antigen is necessary to keep fatA in place and to allow the iron uptake system to work properly (14).

1.3.2. Therapeutic and prophylactic strategies against vibriosis

Vibriosis is normally treated using drug therapies, such as antimicrobial agents, mostly antibiotics. However, due to the misuse of these compounds, phenomena such as food contamination and antibiotic resistance tend to happen. One of the challenges in vibriosis treatment is the fact that *V. anguillarum* infection process starts with an asymptomatic stage, where the infection spreads without showing external signs. Therefore, it is hard to predict if the animal is sick before it shows some external signals. This challenge becomes even harder because bacteria normally inhabit fish's gut, so it may not infect the host during a long time, until a stress factor (alterations in the environment or in the animal itself) is introduced and triggers the infection process. Due to these difficulties, antibiotics are applied in the water of aquaculture systems in a regular basis and in larger doses in order to prevent outbreaks and to improve animal growth (28). Nevertheless, this increases the probability of microorganisms to create resistance against the antimicrobial agents and the subsequent transmission of that resistance from the bacterial flora of fish to human microflora. Additionally, the presence of antibiotics tends to destabilise the balance of bacterial populations naturally present that are beneficial to animals, resulting in a lower protection from bacterial infections (28). In order to reduce the amount of antibiotics used in aquaculture and their negative impact, alternative approaches have been developed and implemented. These include immunostimulation through the use of antimicrobial peptides (AMPs), probiotics, vaccines and antibodies, such as chicken egg yolk immunoglobulins (IgY) (29).

The base of immunostimulation depends on the host immune system, and since *V. anguillarum* affects mainly fishes, their immune system is also important. Thus, the first barrier against pathogens in fishes can be divided in nonspecific defence mechanisms that do not require a prior contact with the antigen and specific defence mechanisms that activates the system with a delay between the recognition of the antigen and the subsequent activation (30). Fishes have an innate system that is composed of physical barriers, cellular and humoral components. Cell-mediated immunity does not involve antibodies, however bases their response against one antigen through the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes (white blood cells) and through the release of various cytokines (signal proteins (~5–20 kDa)). On the other hand, humoral immunity is intermediated by macromolecules that

are present in extracellular fluids, such as natural antibodies and antimicrobial peptides. Many factors can positively and negatively influence the innate immune system of fishes, such as stress factors that can inhibit the system and, on the other hand, immunostimulants that can activate it. An immunostimulant is considered an agent that boosts the nonspecific immune mechanisms when given alone, or the specific mechanisms when given with an antigen (30). Immunostimulants can activate the pattern recognition mechanism of the innate system, such as peptidoglycans of gram-negative and gram-positive bacteria, lipopolysaccharides (LPSs), as well as bacterial or synthetic oligo-dexanucleotides. However, the efficacy of some of them depends on the fish species, application method (e.g., in food, water, injections or a combination of methods), time and durability (31).

Other compounds that can be used to stimulate the immune system of fishes are AMPs, in fact, some animals already produce these compounds as a defence mechanism. Some AMPs act as antibacterial or bacteriostatic agents against several gram-negative and positive strains, binding to the pathogen's membranes or even destroying them (32). AMPs that enter to the host may modulate pathogen-responsive genes and consecutive protein expression to reduce the severity of the infection or enable the complete protection of the fish against the respective pathogen. They can also induce the adaptive immunity of the fish and reduce the brutality of the microorganism possible re-infection. On the other hand, AMPs can be used as adjuvants in vaccines in aquaculture, since they show excellent properties as a boost in less-immunogenic vaccines or protein antigens, or as a way of producing vaccines, inactivating the microorganisms that are going to integrate them (32).

Probiotics, "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance", can also be used in order to reinforce the immune system of fishes (33). These microorganisms can improve the fit of endogenous microflora, helping it resisting infections mainly in the gastrointestinal tract, where they are able of colonization (34). Probiotics have a higher multiplication rate comparatively to the expulsion/rejection rate, so they can adhere to the intestine and compete for nutrients with the existent pathogenic microorganisms. They can also modify the gastrointestinal microbiota through the secretion of antibacterial compounds (antibiotics, bacteriocins, siderophores, enzymes, hydrogen peroxide and organic acids) (34). In Aquaculture, probiotics are normally used as a food additive applied directly to the tanks or mixed with food, in order to promote the animal growth, inhibit pathogenic organisms, and improve the nutrients digestion, stress control, water quality and animal reproduction (34). These microorganisms can be used as a natural alternative to the use of antibiotics, since they can also act as a prophylactic measure.

Another advantage of the use of probiotics in aquaculture is the improvement of nutrient digestion, since they produce extracellular enzymes, such as proteases, amylases and lipases, and vitamins, essential fatty acids and amino acids that can help the host in digesting the food and consequently improve their growth (34). Water quality can also be improved through the application of probiotics in water, mainly due to the overpopulation in tanks and a higher amount of dispersed organic matter (34). Additionally, probiotics can help fishes to fight the stress that they are submitted to in aquaculture systems, and even improve the reproduction of the animals, enhancing their immune systems and consequently improving the quality of the aquaculture systems and the fishes' life. Besides all the advantages of probiotics, these microorganisms if applied without enough knowledge can have negative side infections. They can cause over stimulation of the immune system of the host, systemic infections, harmful metabolic functions and gene transfer between microorganisms. Moreover, due to the introduction of different bacteria in the host organism, some of them can contain virulence genes that might transfer to the natural microflora, causing problems in the future associated to antibiotic resistance or infections, since this transmission is uncontrollable and unpredictable. This technique also presents some obstacles since there is no legislation or control, and without any strait control their application can represent a threat not only to animals, but also to the surrounding environment, and ultimately to the human health (34).

Another alternative method to fight microbial infections in aquaculture is the IgY technology, detailed in the next chapter.

1.4. IgY Technology: an alternative strategy to combat microbial infections in Aquaculture

There is a crucial need for developing and identifying effective strategies to combat infections and antimicrobial resistance in aquaculture systems. One of the possible therapies that has been successfully applied to control infections mediated by microorganisms and viruses is immunotherapy, using avian IgY antibodies. This type of antibodies can be obtained in high quantities from bird's egg yolk, allowing the scalability and industrialization of the approach (35). This technique can provide specific antibodies against microorganisms or viruses through the immunization of laying birds (e.g., *Gallus gallus* and *Coturnix japonica*) with appropriate vaccines (e.g., microbial virulence factors). Furthermore, egg-yolk powder or other purification grades of yolk-derived IgY antibodies can be directly used as additives/supplements in aquaculture feed or water. IgY passive immunotherapies have shown

to allow effective neutralization and control of microbial or viral virulence towards farmed fishes (36). Ultimately, this alternative strategy will also be less prone to mediate long-term resistance of microorganisms unlike synthetic antimicrobials, thus will definitely play an important role in reducing such phenomenon. In order to become a viable strategy in aquaculture, IgY technology still faces important challenges, including cost-effective industrialization as well as targeted and eco-recovery techniques.

IgY is considered a promising alternative to antimicrobials and vaccines, mainly due to its broad spectrum and reduced side effects. Specific IgY allows a reduction in animal mortality, because it targets a specific antigen in the pathogen, contrarily to vaccines or even non-specific antibodies that act against several pathogens (29). Li et al. (29) also proved that vaccination only prevents infections, such as the one caused by *V. anguillarum*, contrarily to IgY that can also treat the infection. Through this study, it was proved that IgY can be used to control bacterial disease in aquaculture. It was also showed that specific IgY may suppress the inflammatory response through the inhibition of cytokines expression. This way, they were already successfully applied against a wide range of antigens, such as proteins, peptides, lipids, andcarbohydrate components from viruses, bacteria, fungi, plants and animals (37).

1.4.1. Avian immune system and antibodies diversity

Birds have a different immune system when compared to mammals. The primary lymphoid organs include the thymus and bursa of Fabricius and the secondary organs include the spleen, Harderian glands (gland found within the eye's orbit), bone marrow, conjunctival-associated lymphoid tissue, bronchial-associated lymphoid tissue and gut-associated lymphoid tissue (38).

In birds, the functional genes that encode for immunoglobulins are present in B lymphocytes (B cells) that are a type of white blood cells that mediate humoral immunity and secrets antibodies. After 15-17 days of the embryonic development of hen’s offsprings immature B-cells, that are produced in the bone marrow, travel to Bursa of Fabricius, where they maturate and become competent B-cells. During the process of maturation in the oviduct, one of each of V (variable), D (diversity) and J (joining) gene segments is selected randomly and used to encode the final antibody molecule. Nevertheless, unlike in mammals, birds present only a single functional VH (variable region of the heavy chain) and VL (variable region of the light chain) gene segment with additional 25 pseudo-V genes located upstream but unable to produce functional products due to the lack of transcription regulatory and signal-recognition

sequences. Due to the different gene organization, gene conversion is crucial to create diversity of avian immune repertoires, that is a non-reciprocal homologous recombination that occurs between an acceptor (functional) and donor (pseudo) genes. In addition, V-J flexible joining, somatic point mutation and even single or double nucleotide substitution also contribute to this variability process (39).

After the previous described maturation process, mature B-cells secrete IgY immunoglobulin molecules into the blood stream that provide the avian humoral immunity. IgY is the main immunoglobulin in birds as IgG (immunoglobulin G) is for mammalians, and it is present not only in the circulating blood (5-7 mg/mL of serum) but also in other organs (duodenal) or secretions of chickens (40). Although, IgY is predominant in the egg yolk (50-100 mg/egg yolk), because is transferred from the mother serum to the egg in order to promote an effective humoral immunity until the offspring reaches full maturation of their own immune system (40). This transference is mediated by specific receptors at the oocyte plasma membrane that recognize unique molecular features of IgY that are distinct from the other avian immunoglobulins, namely IgA and IgM, that remain in the egg-white fraction (41).

1.4.2. Structural and molecular features of IgY

Avian IgY is composed of two light chains (L) and two heavy chains (upsilon, υ) with molecular weights (MW) of approximately 22 kDa and 66 kDa, respectively, linked by a disulphide bridge. This results in an overall IgY with ~180 kDa, slightly larger than the mammalian IgG counterpart (~150 kDa). The avian IgY antibody has a higher MW due to the extra constant domain in the heavy chain, when compared to mammalian IgG (Figure 4). This extra domain leads to a more compact and rigid Fragment crystallizable region (Fc) in comparison with the IgG one and to an overall reduced flexibility and increased hydrophobicity of the molecule. This reduced flexibility can interfere with the precipitation of antigens, since in order to precipitate with IgY there is the need of a higher concentration of salt (37). Additionally, IgY presents 4 glycosylation sites, compared to IgG that have 2 sites.

IgY has an isoelectric point between 5.7 to 7.6 and loses its activity mostly by conformational modifications. This can happen at temperatures higher than 70ºC, although this (melting) transition temperature can be modulated through the application of carbohydrates and salts (42).

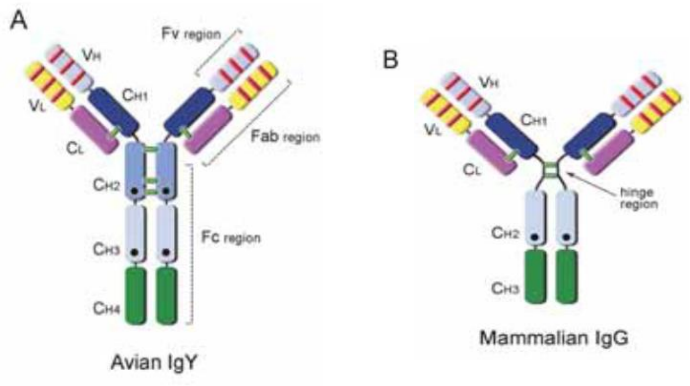


Figure 4. Structural differences between human immunoglobulins (IgG) and chicken immunoglobulins (IgY) (43).

(A) IgY: VH variable domain of heavy chain, VL variable domain of light chain, CL, CH1, CH2, CH3 and CH4, constant domains of heavy chain.

(B) IgG: VH, VL, CL, CH1, CH2 and CH3.

1.4.3. Advantages and Disadvantages of the IgY technology

The production of antibodies has traditionally been carried out using mammals, such as rabbits, goats and mice, which implies immunization of the animal followed by bleeding procedures (vein puncture of the jugular vein) to collect IgG antibodies. On the other hand, the use of chickens is a more promising alternative to the production of antibodies, because the collection of IgY is not only less labour intensive but also non-invasive and non-stressful towards the animals, when compared to blood sampling. In this case, only the collection of eggs is required with no bleeding involved. This technique gained more importance since the 90s due to the needs of improved animal welfare procedures and alternatives (43). Thus, this method is currently recommended by the European Center for Validation of Alternative Methods for Animal Experimentation (EURL-ECVAM) since 1996 (44) and by the Veterinary Office of the Swiss Government (Office Vétérinaire Fédéral) (45). Additionally, to the previous advantages, the costs implied in chickens raising is lower when compared to the costs of raising rabbits, furthermore, the antibodies yield is bigger, since a laying hen produces about 22.5 g of IgY during one year which is the equivalent production by 4.3 rabbits during the same time (41,45).

When comparing the antibodies production protocols using mammals or chickens, an immunization step is always needed, where the animals are vaccinated intramuscularly in the breast muscle with the antigen and an oil based adjuvant, to activate the non-specific immune

system and slowly release the antigen in the injection local. Eggs laid by immunized animals are then collected and processed to analyse the antigen-specific response. This response depends on several factors, including the nature of antigen (e.g., biological nature, dose, MW), type of adjuvant used (e.g., Freund, Aluminium oxide), application route (e.g., oral, intravenous, intramuscular, subcutaneous), chicken host (e.g., chicken strain, age) and immunisation schedule (39). In general, through chickens it is verified a reduction of the number of booster injections, since they tend to promote higher and long-lasting antigen-specific titers when compared to other animals. On the other hand, the purification step of chicken IgY implies the removal of lipids and lipoproteins from the egg yolk, that makes the technique a little more time consuming when compared to the serum recovering from blood (46).

IgY antibodies are more successful against mammal's antigens when compared to the immune response that IgG have against the same antigens, mainly due to the phylogenetic distance between the animal classes Mammalia and Aves. This means that with the application of IgY to mammalian antigens, a signal amplification can be obtained since the avian antibody binds more successfully to a higher number of epitopes on a mammalian protein when compared to the immune response obtained with IgG.

In immunological assays, bovine serum albumin solution (BSA) is usually used for blocking; however, since it can contain IgG antibodies as contaminants, these proteins may bind with the secondary anti-mammalian IgG antibody resulting in an increased unspecific background and consequently misleading results, as summarized in Figure 5. This problem can be solved using IgY as primary antibodies in order to avoid cross reactivity issues (41).

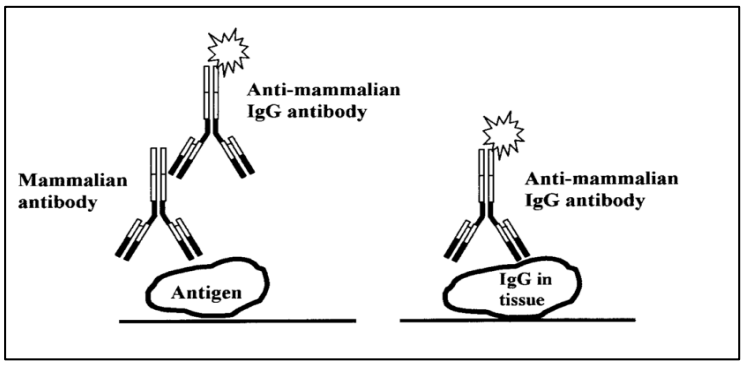


Figure 5. Cross reaction between IgG present in the sample or in the serum albumine solution and the anti-IgG antibody (41).

IgY can also be applied in the treatment of human diseases. IgY does not trigger an inflammatory response in the host and the compounds present in eggs are normally present in

human's diet, so the risk of allergic reactions is lower. Moreover, IgG contains an Fc region which binds to human Fc receptors found in blood cells. This interaction may cause an increase in the background of immune experiments, cell activation with modifications in the surface proteins expression and even an increase in cytokines production (41). In addition, bacterial Fc receptor components, such as *Staphylococcal* protein A and *Streptococcal* protein G, can bind to Fc parts of the mammalian IgG antibodies. On the other hand, the Fc portion of IgY does not react with mammalian or bacterial (protein A or G) Fc receptors. Additionally, IgY does not form immune complexes with the rheumatoid factor (RF), an immunoglobulin that binds to other immunoglobulins of other mammalian species (e.g., IgG), because it does not have Fc regions that correspond to the Fc binding sites existent in RF. Due to this absence, there is no complexes formation, which is considered an advantage, since they are associated with autoimmune diseases (e.g., rheumatoid arthritis) (39,41). This type of disorders is usually treated with mouse monoclonal antibodies, however some of these induce an allergic reaction in the patients through the production of human anti-mouse antibodies (HAMA). Nevertheless, HAMA may also be found in patients who have not been treated with antibodies. Therefore, the presence of these human antibodies can reduce the success of the treatment and create future problems when contacting with mouse antibodies. IgY avoids interference and disorders in immune tests since it does not react with RF or HAMA (41). The differences between IgG and IgY are summarized in Table 1, supporting the interest on avian antibodies as more prominent options to be applied in many areas, such as immunological tests, antibiotic alternatives and xenotransplantation (45). Nevertheless, as any other technology it has its disadvantages. These disadvantages are mostly related with the cost and time of the purification to obtain high quality IgY, as well as the lack of scale-up and ecological procedures, especially if the final application implies higher amounts. Therefore, the development of methods for the large-scale purification of IgY that allow high recovery and purity level is a crucial requirement (42).

Table 1. Comparison between rabbit IgG and chicken IgY (Adapted from Schade et al. (2005) (39).

Characteristics	Rabbit IgG	Chicken IgY
Antibody sampling	Invasive (bleeding)	Non-invasive (egg collection)
Antibody amount	200 mg/40 mL blood	50-100 mg/egg
Interference with mammalian immunoglobulins	Yes	No
Interference with HAMA	Yes	No
Interference with RF	Yes	No
Interference with complement system	Yes	No
Protein A/G binding	Yes	No

1.5. Work Purpose

IgY passive immunotherapies have already been successfully used in aquaculture systems (47). In this work, the objective is to refine and optimize IgY therapies against fish infections mediated by *V. anguillarum*, using the current knowledge on the cellular and molecular mechanisms of the bacterium virulence and thus rationalize a targeted approach based on avian IgY antibodies. This will include the identification of surface-exposed or secreted proteins components responsible for bacterial adhesion, colonization, proliferation or overall virulence. The neutralization or modulation of *V. anguillarum* virulence using IgY antibodies as biological therapeutic drugs will be then investigated.

In order to achieve the described goals, immunization protocols were performed using heat-killed *Vibrio anguillarum* in order to test the immune response of the chickens towards this pathogen. It was also evaluated the variability between different chicken batches, allowing to infer on the animals which are producing a higher amount of IgY antibodies. It was also performed immunization protocols with surface exposed antigens (membrane preparations) and even specific proteins, such as TolC protein, which allowed the production of specific antibodies against *V. anguillarum*, were also performed.

1.5.1. TolC channel from *Vibrio anguillarum*

Concerning the final goal of the production of a specific antibody it was decided to produce a protein in agreement with the epitope of TolC channel from *V. anguillarum.* TolC is a major outer membrane protein involved in *V. anguillarum* multidrug resistance and consequently

survival during infection, not only in Vibrio strains but also in other gram-negative bacteria. Thus, if TolC is universal among gram-negative bacteria it can be considered a possible target to the antibody treatment (48). Antibodies normally bind to the extracellular domain of the antigens, in this case, the protein such as the TolC one, that way the desired antigen should comprise the extracellular domain of the desired protein. Regarding this extracellular domain, it was produced a fusion protein constituted by the extracellular residues of the TolC channel and a carrier protein that could present those residues and increase the contact between the antigens and the immune system of the immunized animal. According to the available sequence of TolC *V. anguillarum* (UniProt ID: A0A191W654), this protein is 435 aminoacids long and has a MW of 47 kDa. However, this protein does not have a solved 3D structure yet.

2. Materials and Methods

2.1. *Vibrio anguillarum*

2.1.1. *Vibrio anguillarum* growth

Vibrio anguillarum PC 969.1 (O2 serotype) was provided by Dr. Benjamin Costas (CIIMAR Interdisciplinary Centre of Marine and Environmental Research of the University of Porto). This strain was grown overnight (ON) (16h-24h) in petri dishes with TCBS (Thiosulfate-citrate-bile salts-sucrose) agar medium, as shown in Figure 6, at 25ºC using the stove BINDER BD 115. In parallel, liquid cultures of *V. anguillarum* were also prepared using TSB (Tryptic soy broth) medium, where the cultures were grown at 25ºC ON in test tubes under 160 rpm agitation, using Infors Scotron AG CH-4103 BOTTMINGEN shaker.

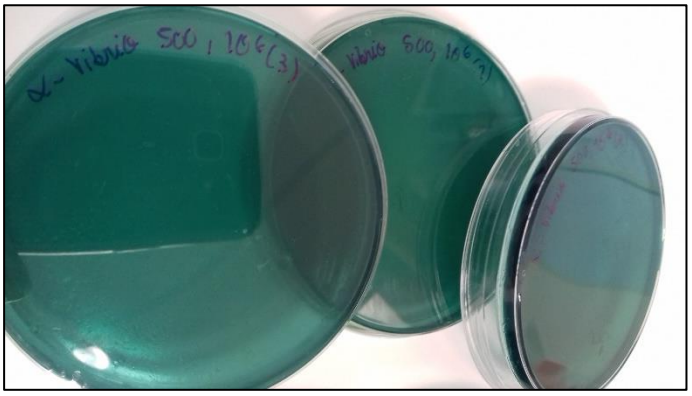


Figure 6. Picture of cutures of *V. anguillarum* plates in petri dishes with TCBS medium.

2.1.2. Bacterial Culture Media

2.1.2.1. TSB Medium

TSB (VWR Chemicals REF: 84675.0500) is a general medium used for cultivation of a wide variety of microorganisms, however to be more selective towards *Vibrio anguillarum* that is a halophilic bacterium, the medium was supplemented with 1.5% of NaCl (Sodium chloride), and further prepared with Milli-Q. 30.0 g of TSB mixture were ressuspended in 1L of water followed by NaCl supplementation. To keep sterile conditions, the mixture was autoclaved at 121ºC during 15 min. The TSB medium composition is summarized in Table 2.

Table 2. TSB medium composition [pH 7.3 ± 0.2 at 25ºC].

TSB medium composition (g/L)	
Pancreatic digest of casein	17.0
Papaic digest of soyabean meal	3.00
Sodium Chloride	5.00
Dextrose	2.50
Dibasic potassium phosphate	2.50

2.1.2.2. TCBS Agar solid medium

TCBS (VWR Chemicals REF: 84641.0500) is a solid medium for the selective isolation of *Vibrio parahaemolyticus* according to the ISO (International Organization for Standardization) Standard. This medium is prepared using Milli-Q water, in which 88.0 g of TCBS mixture were ressuspended in 1L of water. This medium cannot be autoclaved or even re-melted, therefore, boiling water was used to dissolve the powder. Subsequently, the mixture was poured into petri dishes that were stored at 4ºC. The composition of TCBS medium is given in Table 3.

Table 3. TSB medium composition [pH 7.3 ± 0.2 at 25ºC].

TCBS medium composition (g/L)	
Proteose peptone	10.0
Yeast extract	5.00
Sodium citrate	10.0
Sodium thiosulphate	10.0
Ox bile	8.00
Sucrose	20.0
Sodium Chloride	10.0
Ferric citrate	1.00
Thymol blue	0.040
Bromthymol blue	0.040
Agar	14.0

2.1.3. Glycerol Stocks

Bacterial glycerol stocks are important to make a longer-term storage of bacteria samples that can be used in future procedures, since glycerol acts as a cryoprotectant, avoiding bacterial viability loss. Glycerol stocks were prepared using ON liquid (10 mL) cultures, each obtained from of a single colony of *V. anguillarum* PC 969.1. Briefly, 150 µL of filtered glycerol were added to 850 µL of the culture medium and mixed by gently inversion of the stock tubes. The final glycerol concentration was 15% (v/v). The stocks were immediately flash-frozen in liquid nitrogen and stored at -80ºC until use to avoid loss of viability.

2.2. Antigen Preparation

2.2.1. Heat-killed *Vibrio anguillarum* procedure

A sample of heat-killed whole-cell *Vibrio anguillarum* (HK) was prepared to be used as antigen for bird immunization and subsequent antibody production. Bacterial cultures were killed by heat treatment, at 65ºC during 3 h, using the thermostat LAUDA ECOLINE STAREDITION. Subsequently, 100 µL of heat-killed culture were plated in petri dishes with TCBS medium and in parallel inoculated into liquid culture containing 10 mL of TSB medium. Both culture formats were grown for two days, at 25ºC to confirm the efficacy of the heat-killing procedure. The efficacy of the treatment was evaluated through the colour of the medium and the absence or presence of culture growth in petri dishes, since when the TCBS medium turns yellow it means that culture growth is happening (Figure 7A). In the case of test tubes, the opacity of the medium reveals culture growth and consequently a deficient heat killed procedure (Figure 7B).

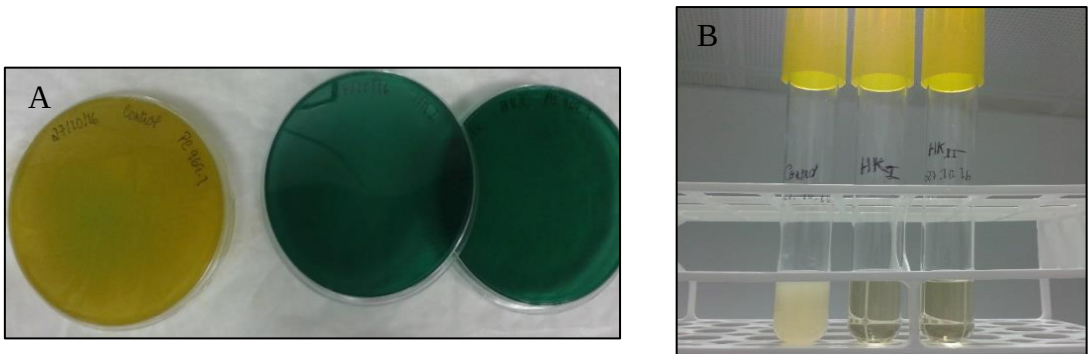


Figure 7. Heat killed procedure results.

A) Petri dishes containing a control culture of *V. anguillarum* (yellow) and two replicas of the culture submitted to the HK procedure (green).

B) Test tubes containing a control culture of *V. anguillarum* (left) and two replicas of the culture submitted to the HK procedure (right).

2.2.2. Membrane preparation procedure

In order to prepare membrane-enriched fractions from *V. anguillarum* bacterial cultures, to be further assayed as antigens for bird immunization, a Membrane Preparation (MP) protocol was used. ON grown cultures of *V. anguillarum* were submitted to the previously described HK procedure, and then centrifuged at 4200 rpm during 20 min using the Thermo Scientific Heraeus Megafuge 40R Centrifuge (TX-100 rotor). The resultant pellet was ressuspended in 40 mL of PBS (Phosphate-buffered saline) 1x and further submitted to sonication using BioBlock Scientific Vibracell™ 75041 sonicator with the S&M-220 tip, selecting 40% amplitude during 5 min. Cell lysates obtained by sonication were then submitted to centrifugation at 17000 g during 45 min, to separate all the insoluble fraction from the soluble one (Beckman Coulter Avanti Centrifuge J-26 XPI, 25.50 rotor). The resultant pellet (insoluble fraction) was then ressuspended in 2 mL of PBS 1x and used as an antigen (PL). The remaining supernatant was centrifuged at 35000 g during 45 min, using Beckman Coulter Optima TM L-100 XP Ultracentrifuge and the resultant pellet was kept at -20ºC to be used as an antigen.

2.2.3. Recombinant Protein Epitopes

2.2.3.1. Cloning TolC protein from *V. anguillarum* and VceC from *V. cholerae*

To clone the desired epitopes, it was used a technique called SLIC (Sequence and Ligation Independent Cloning) that allows an insertion of any nucleotide sequence into any other with defined junctions and a precise cloning with no changes in the amino acid sequence of the target protein. The first step of this procedure involves the production of reverse and

direct oligonucleotides (primers) in agreement with the sequence of the TolC *V. anguillarum* and VceC protein, is this case. Subsequently, these were phosphorylated using T4 Polynucleotide Kinase (PNK) for 60 min at 37ºC. The previous phosphorylation step was then interrupted through the PNK inactivation, at 65ºC, during 20 min. The following step is the linearization of the plasmid, the pCoofy plasmid, through PCR reaction (conditions given in Table 4.) with the mixture: 10 µL 5x phusion buffer, 1.5 µL forward primer (10 µM), 1.5 µL reverse primer (10 µM), 0.5 µL template (35 ng), 0.5 µL phusion, 1 µL dNTPmix and ddH_2O, in order to obtain a final volume of 50 µL.

Table 4. PCR conditions (pCoofy linearization).

Number of cycles	Temperature (ºC)	Time of each cycle (min)
1	98ºC	1 min
25	98ºC	0.5 min
	53ºC	0.5 min
	72ºC	9 min
1	72ºC	10 min

The PCR product was then treated with DpnI (1 h, 37ºC) in order to eliminate the template DNA. After this step, the enzyme was inactivated (20 min, 80ºC) and the resultant product was purified using a NZYGelpure kit. The cleaned product was used for ligation reaction at 16ºC, ON, with a mix of 2 µL of the PCR product, 2 µL 10 x buffer T4 DNA ligase, 1 µL DNA ligase and 5 µL H_2O). The resultant DNA construct was then transformed in DH5α cells (30 min on ice, 1 min at 42ºC and 3 min on ice), that were recovered in 1 mL LB (Lysogeny broth) medium at 37ºC for 1 h. Recovered cells were then centrifuged at 16 000 g for 1 min (eppendorf Centrifuge 5417R), 900 µL of the resultant supernatant were discarded and the remaining LB medium was used to ressuspend the bacterial pellet, that was then plated on a Petri dish with LB Agar/ kanamycin and incubated ON at 37ºC. After 16h, some isolated colonies from the plate were ressuspended in 20 µL of ddH2O and an analytical colony PCR assay was performed to select positive clones harboring the insert of interest; this was performed using the following mixture: 0.5 µL of the ressupended colony, 1 µL of the T7 elongator (10 µM), 1 µL of the reverse primer (the same primer used in the plasmid linearization step), 0.4 µL DNTPs, 0.25 µL of the Taq polymerase and 2 µL of the Taq buffer. The respective PCR conditions are summarized in Table 5.

Table 5. PCR conditions (colony PCR).

Number of cycles	Temperature (ºC)	Time of each cycle (min)
1	95ºC	1 min
30	95ºC	0.5 min
	52ºC	0.5 min
	72ºC	1.5 min
1	72ºC	5 min

The resultant colony PCR products were analysed using agarose gels (0.5 g agarose, 50 mL TAE (Tris-acetate-EDTA) 1x, 1.5 µL of ethidium bromide) ran for 25 min, 100 V, using BioRad Powerpac HC. Positive colonies containing the correspondent size DNA were selected and posteriorly grown in 10 mL of LB with 10 µL of kanamycin, 37ºC ON. After 16h, the cultures were processed using NZY Miniprep kit in order to prepare plasmid DNA samples for sequencing confirmation (GATC sequencing services).

2.2.3.2. Bacterial Transformation and Recombinant Protein Expression

After confirmation of positive constructs harbouring the inserts of interest (extracellular epitopes) by DNA sequencing, 1 µL of selected clones was used to transform competent *E. coli* BL21* DE3 cells for expression purposes. Competent cells were placed on ice (30 min), followed by a heat-shock of 1 min at 42 ºC and recover on ice for 3 min. After that, 1 mL of LB medium was added and the culture was placed in the Infors Scotron AG CH-4103 BOTTMINGEN incubator at 37ºC, 170 rpm, during 1 h for cell recovery. Cells were then centrifuged at 16000 g for 1 min (eppendorf Centrifuge 5417R), the resultant supernatant was partially discarded, leaving a minor volume of media for cell pellet resuspension and inoculation in plates with Agar, LB and kanamycin, incubated at 37ºC, ON. After 16h, grown colonies were scrapped from the plate and ressuspended in 5 mL of LB medium. 1 mL of this bacterial resuspension was used to inoculate 1 L of LB medium that was grown until reaching OD_{600} between 0.6 and 0.8. At this point, 1 mL of IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to induce recombinant protein expression and at the same time the temperature was lowered to 20ºC, 170 rpm (New Brunswick Scientific innova 44 Incubator shaker series) and the culture was grown. After 16h, the culture was centrifuged at 4000 g during 15 min, and the resultant pellet was ressuspended in a buffer containing 50 mM Tris-HCl pH 7.5 and 50 mM de KCl and frozen (-20ºC) until use.

2.2.3.3. Purification by HiTrap ionic exchange column

After the expression, it is needed a step to fraction the pellet (sample) in order to recover the desired protein, a step that can be achieved using the Hitrap column, that separates proteins according to their charge. However, the cellular pellet was first submitted to some procedures. After the thaw of the desired pellet, the sample was submitted to sonication using BioBlock Scientific Vibracell™ 75041 sonicator with the S&M-220 tip, selecting 40% amplitude during 5 min. Cell lysates obtained by sonication were then submitted to centrifugation at 17000 g during 30 min, to get a clearer supernatant (Beckman Coulter Avanti Centrifuge J-26 XPI, 25.50 rotor). The obtained supernatant was then loaded in Akta™ Pure Ge Healthcare Bio-Sciences [Unicorn 5.0] (HiTrap), where the elution was performed using an increased percentage of salt, with a gradual decrease of buffer A (50 mM Tris and 50 mM KCl) and an increase of buffer B (50 mM Tris + 1M KCl), being the final goal 100% of buffer B. This amount of salt was already optimized towards the carrier protein and the mAU peak was verified at around 70 mL, where the fractions with the higher absorbance were collected and posteriorly applied in an ATP column.

2.2.3.4. Purification by ATP immobilized column

Since *V. anguillarum* TolC recombinant epitope was expressed in fusion with a carrier protein which has affinity towards ATP (proprietary technology), the second purification step was performed using a ATP affinity column (Innova Biosciences ATP-Agarose High). As previously referred, peak elution fractions obtained from the previous Hitrap Q column were loaded onto the ATP-column and incubated at 4ºC, ON under agitation on Snijders ROCK ‘N ROLLER 34201E mixer. After about 16h, the column was washed three times using 50 mM Tris and 150 mM KCl buffer and protein elution was performed with the same buffer supplemented with 5 mM of ATP, the elution peaks were collected and concentrated by spin concentrator of MWCO (Molecular weight cut-off) 10 kDa.

2.3. Bird housing and immunization procedures

As previously referred laying hens need to be immunized with the antigen to produce the desired IgY antibodies. In the present work chickens (*Gallus gallus*) and Japonese quails (*Coturnix japonica*) were selected. For chickens, a typically immunization protocol used 1 experimental group with 2 animals each (pair of birds); to produce antibodies against *V. anguillarum* whole-cell extract one used two experimental groups of chicken with

approximately 1 year of age. In parallel two experimental groups of 8-12-week-old female quails were used for similar procedure. Moreover, 4 more trios of quails were subjected to an immunization protocol with specific antigens (MP, PL and TolC from *V. anguillarum* and VceC from *V. cholerae* epitopes). The animals were brought to the lab with different ages. It should be remarked that chickens take about 21 weeks to reach the adult age, while quails reach that sexual maturity within 8 weeks, and start laying eggs at 9-10 weeks, although small deviations may occur and depend on the animal's health and housing conditions (49).

Both bird species were kept under controlled conditions, namely supplied with water and food *ad libitum* and subjected to a light cycle of 16 h of light and 8 h dark (16L:8D) to maintain regular egg-laying cycle. Eggs were collected daily, labelled and stored at RT (room temperature) or 4ºC until processing. The avian facility is shown in Figure 8.

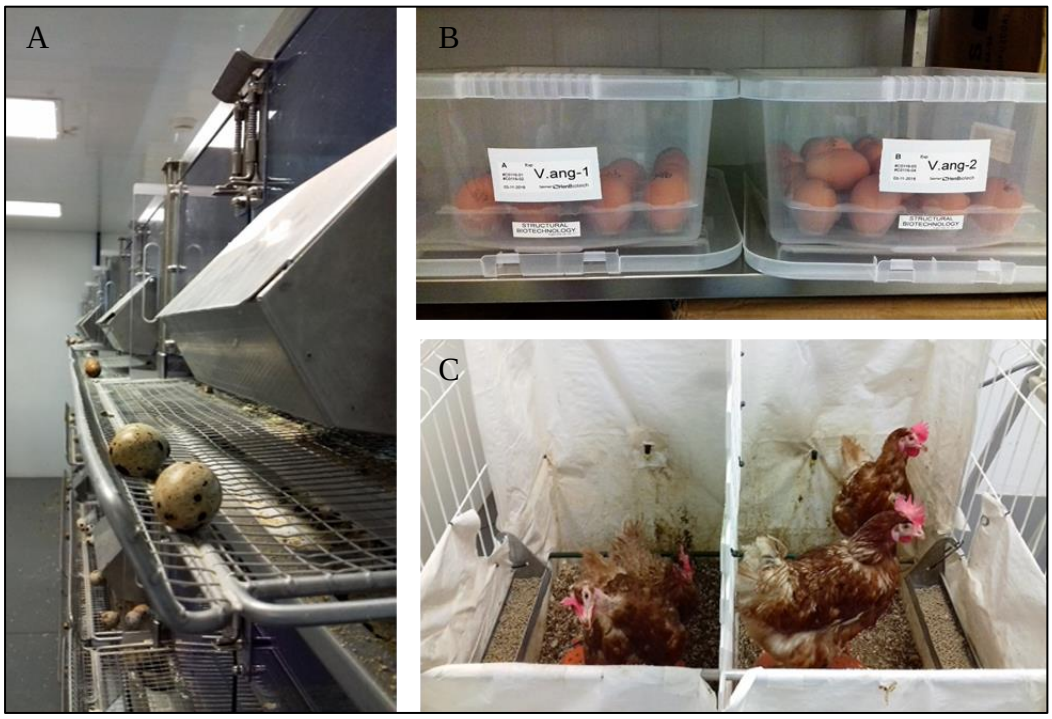


Figure 8. Avian Facility.

A) Quails cages ; B) Chicken eggs box; C) Chicken cages.

The immunization procedure involved four injection events, intramuscularly in the birds' breast muscle, one every 15 days (Day-0, Day-15, Day-30 and Day-45). Injectable emulsions were prepared by standard emulsification with emulsifying needles; briefly, the protein antigen was mixed with the adjuvant in a 1:1 (v/v) proportion. The amount of antigen and total volume of immunization was prepared according to the animals' weight. For Day-0/Day-15 emulsions it was used Complete Freund Adjuvant (CFA) and for the last two, Day-30/Day-45 Incomplete

Freund Adjuvant (IFA) was used. CFA includes dried mycobacteria (normally *Mycobacterium tuberculosis*) to boost the immune system of the chicken, unlike IFA that lacks such component.

2.3.1. Immunization of experimental birds

2.3.1.1. Heat-killed *Vibrio anguillarum* antigen

After *V. anguillarum* heat killing procedure, the obtained cell suspension was diluted with PBS 1x in an appropriate proportion in order to obtain a concentration of 10^8 CFU (Colony forming units)/mL, so that the final (1:1) dilution with the adjuvant provided a final concentration of 10^7 CFU/mL of immunization. The resultant cell suspension was used as an antigen, for which chickens were vaccinated each time with 200 µL or in the case of quails with 50 µL, of the prepared emulsion (29).

2.3.1.2. *V. anguillarum* Membrane preparation antigen

This protocol involved the preparation of two membrane fraction samples resultant from *V. anguillarum* cell processing, that were then used as separate antigens for antibody production. The first corresponded to the pellet of the 17000 g centrifugation of the *V. anguillarum* cell lysate (as described above) and the second to the pellet of the 35000 g ultra-centrifugation of the previous supernatant. The final total protein concentration of these samples, was adjusted to 0.66 mg/mL by dilution with PBS 1x, in order to allow injection of 100 µg of protein per immunization per bird.

2.3.1.3. *V. anguillarum* TolC and *V. cholerae* VceC recombinant epitope antigens

The preparation of the immunizations using as antigen the TolC epitope from *V. anguillarum* and the VceC epitope from *V. cholerae* proceeded the same way as the membrane preparation samples. Each bird was also immunized with 100 µg of protein per injection event.

2.4. IgY purification

To isolate and purify the antibodies, egg yolks need to be submitted to several processing steps. During the present work, different purification methods were used according to the final antibody application and purity desired. There are three main methods of IgY purification: 1) salt precipitation; 2) chromatography; and 3) ultrafiltration. The first method is normally

performed using ammonium or sodium sulphate salt, polyethylene glycol (PEG), caprylic acid and carrageenan (39). For chromatographic methods, affinity, ion exchange, hydrophobic interaction, thiophilic interaction and gel-filtration chromatography can all be used. Due to the characteristics of IgY, commercial affinity columns commonly used for antibody purification, namely Protein-A (*Staphylococcus* species) and Protein-G (*Streptococcus* species) columns cannot be used to purify this type of antibodies.

In this work, a combination of PEG precipitation and a gel-filtration chromatography was used to purify IgY antibodies Previously to these procedures the eggs were broken, the egg yolk and egg white were separated and the resultant egg yolk was rolled and busted in a towel paper. If the egg yolk pool was not used in the same day, it was kept at 4ºC with a 0.05% percentage of sodium azide (NaN_3), used as a preservative and antibacterial growth inhibitor. On the other hand, if the egg yolk pool was prepared in the same day, it was frozen (-20ºC), and posteriorly submitted to a slow thawing (RT), and then diluted with Milli-Q water in a (1:8) proportion. After this dilution, the samples were adjusted to pH values between (4.8-5), to facilitate the precipitation of lipoproteins, and frozen again. Subsequently to the thawing, the samples were centrifuged at 4000 g (Beckman Coulter Avanti Centrifuge J-26 XPI or Thermo Scientific Heraeus Megafuge 40R, depending on the volume of the sample) during 30 min and posteriorly vacuum filtered using 1.2 µm filters. The resulting sample is called Water soluble protein fraction (WSPF), that provides a percentage of purification inferior to 40%, which is normally not enough to the final application of the antibodies. Nevertheless, in this work, this sample was submitted to a gel filtration chromatography, using Superdex^{TM} 200 10/300 GL, GE Healthcare High Performance column, in order to demonstrate which chickens, produce more amount of IgY and thus would be more profitable to the desired production. Nevertheless, as previously referred, the percentage of purification obtained is not enough to the standard purification process and final objective, so the following step applied was PEG precipitation. This precipitation allowed a removal of insoluble lipids and lipoproteins from the egg yolk, since it has a high lipid content and allows achieving an IgY purification grade with values between 50% and 80% purity. Using chicken eggs the percentage of PEG 6000 used was 8.5%, however using quail eggs the percentage was lower, 7%. PEG was added to the WSPF samples and those were incubated at 4ºC, ON. Subsequently to the precipitation, the samples were centrifuged at 10 000g during 45 min (Beckman Coulter Avanti Centrifuge J-26 XPI) and then the resultant pellet was resuspended using PBS 1x. After this ressuspension the sample was filtered in 0.2 µm filter units FP 30/0.2 CA-S GE Healthcare Whatman ^{TM} and further loaded onto a size exclusion chromatography (SEC) column (Superdex ^{TM} 200 10/300

GL, GE Healthcare High Performance column). Final IgY peak elution fractions present ideally a purity grade between 90-95%. After this last step of purification, the antibodies were concentrated, using GE Healthcare Vivaspin 20 12 pack, 10 kDa MWCO and then used in posterior analysis.

The concentration of the IgY fraction in each step was measured at A_{280} using NanoDrop ND-100 Spectrophotometer, using the Lambert-Beer's law with the extinction coefficient of 1.33 for the IgY, so each measured concentration was then divided by 1.33 in order to give the exact amount of IgY in the sample (50).

2.5. Dot blot assays

Dot blot is a technique used to detecting proteins directly from the sample, since the mixture is not submitted to electrophoresis, as in northern, western (WB) or southern blot. This procedure was used to identify the antibodies present in the egg yolk pools (test samples). The Dot Blot assays imply some prior egg-yolk sample preparation. The first step implied the collection of 3 mL of the desired egg yolk pool (4-day yolk pool) to a 50 mL falcon, then 21 mL of a 20 mM citrate solution pH 4.8 were added in order to achieve a 1:8 diluted solution (v/v). After this, diluted samples were frozen at -20ºC for 3 h, at least, and posteriorly thawed at RT. In order to prepare the WSPFs, thawed samples were centrifuged at 4200 rpm (4122 g) using a Thermo Scientific Heraeus Megafuge 40R Centrifuge with the TX-100 rotor, and the supernatant was collected and placed in a clean Falcon, discarding the pellet. Collected supernatant was filtered using cellulose acetate membrane filter (1.2 μm) under vaccum filtration s. The previous collected supernatant was filtered using cellulose acetate membrane filter (1.2 μm) on the vacuum filter apparatus. To promote protein precipitation, the resultant WSPF was then submitted to a precipitation step with 8% PEG 6000 at RT, with agitation for 30 min. After this procedure, the precipitated protein was collected by centrifugation at 4200 rpm (4122 g) in the same centrifuge, during 20 min, at 4-6ºC. The supernatant was discharged, the resultant pellet ressuspended using 2 mL of PBS 1x, pH 7.4 and the protein concentration measured using a OD_{280} in the Nanodrop apparatus. The final test sample was analysed by SDS-PAGE gel, 12% of polyacrylamide, ran at 120 V (BioRad Powerpac Basic) for 5 min followed by 180 V until the loading dye reached the bottom of the gel.

For Dot Blot assay, 4 μL of the antigen were applied onto a nitrocellulose membrane (BioRad, 0.45 μm) in properly labelled spots; different dilutions of the antigen were used in order to obtain different intensities (e.g., 1:2, 1:15,1:10,1:100 v/v dilutions). The membrane

was dried at RT naturally and subsequently blocked, using 5% of milk in TBS-T (Tris buffered saline (TBS) with (1:1000) Tween 20) (ON, 4ºC), in a shaker. After blocking step, the membrane was cut into strips and placed in Falcon tubes with 6 mL of TBS-T plus 5% of milk and the antibody test samples were added at 1:2000 dilution (normalized total protein concentration) and incubated during 1 h, RT. Then the strips were washed three times with TBS-T, during 5 min each wash, shaking the containers at RT. After the wash step, the strips were incubated with the secondary antibody (Anti-chicken IgY HRP (Horseradish Peroxidase)-conjugated, Sigma) for 1 h, RT and then washed again with TBS-T. The strips were then incubated with ECL (Enhanced chemiluminescence) reagent (GE Heathcare) for 1 min, mixing in a 1:1 proportion the reagents in the kit. After that the excessive solution from the surface was removed and the membrane was revealed in VWR® Gel Documentation System Imager, VWR Imager CHEMI. The obtained image was then analysed according to the concentration of the test samples and the antigen.

2.6. ELISA protocol

ELISA (Enzyme-Linked Immunosorbent Assay) assays were performed in order to characterize and quantify the target-specific antibody titer in egg-yolk samples and/or antibody preparations. Two types of ELISA, using different coatings (antigens), were investigated. The first were performed using heat- killed *V. anguillarum* medium as an antigen for plate coating. Coating was performed by diluting 500 µL of the *V. anguillarum* heat-killed medium (10^{12} CFU /mL) in 9.5 mL of carbonate buffer pH 9.6 (ON, 4ºC), obtaining a final concentration of approximately 10^{10} CFU/mL and placing 50 µL in each well of a 96-well plate. The other type of ELISA was performed using as coating specific proteins, such as TolC *V. anguillarum* and VceC *V. cholerae*. In these assays, the plates were coated using a concentration of 0.5 µg/mL of the desired protein, placing 100 µL in each well. Despite the different coatings, the following procedure was similar for both ELISA assays.

After the coating step, the wells were washed three times using PBS-T (0.05% Tween 20), 200 µL/well, followed by antigen blocking using PBS-T with 3% BSA, ON at 4°C. After this blocking step, the samples with the antibodies (WSPF of each of the analysed immunization protocol) were diluted using PBS-T with 1% BSA from an initial sample with a 1:150 dilution and then incubated (50 µL/well) for 1 h at 37°C (incubator BOEKEL Scientific, Shake 'N Bake™). After incubation, the wells were washed again using PBS-T and then incubated with the secondary antibody (Anti-chicken IgY HRP-conjugated) diluted at 1:10000 with PBS-T

with 1% BSA (50 µL/well), for 30 min at 37°C followed by three wash steps with PBS-T. Posteriorly to the washing , 3,3',5,5'-Tetramethylbenzidine (TMB) reagent was added at RT for 30 min, that is the substrate of the secondary antibody and allows the production of a blue final product, reaction that is then stopped by the addition of a 0.5 M H_2SO_4 solution (transforms the colour of the solution in yellow). The colour intensity of the wells is proportional to the amount to specific antibody present in the sample, quantified by reading the absorbance values at 450 nm (A_{450}). A microplate spectrophotometer Bio-Tek Powerwave XS was used, and the data analysis was carried out using CurveExpert Professional 2.6..

2.7. Western Blot protocol

This type of assays was used as an analytical technique to detect specific binding of IgY to the antigen sample. The first step of this procedure involved a SDS-PAGE run using heat-killed *V. anguillarum* medium extract, TolC *V. anguillarum* or VceC *V. cholerae* proteins, 1 mm 12% polyacrylamide gel was used, according to the desired test. The samples were run at 120 V during 5 min and 180 V after, until the loading dye left the gel. The resultant gel was then transferred to a nitrocellulose membrane (BioRad, 0.45µm) using a TransBlot Turbo (Bio-Rad), 20 min at 25 V. After this transference, the membrane was then blocked using a solution with TBS-T supplemented with 5% of milk, ON at 4ºC. The blocked membrane was then incubated with primary antibodies at a dilution of 1:2000, 1:1000 or 1:500, with a solution of TBS-T supplemented with 5% of milk for 1 h at RT. The following step included three washes using TBS-T supplemented with 0.5% of milk and then a new incubation with a solution of TBS-T with 0.5% of milk and the conjugated secondary antibody 1:10000 (Anti-chicken IgY HRP) for 1 h at RT. After this step the membrane was washed again using TBS-T (0.1% Tween 20) and left for drying for a few seconds. The membrane was then incubated for 2 min using 200 µL of the developer solution. The acquisition of the signal was obtained using the ECL program in VWR® Gel Documentation System Imager, VWR Imager CHEMI.

2.8. Bacterial growth functional assays

The main objective of the present work was the production of avian IgY antibodies that could block/modulate the activity of *V. anguillarum*, ultimately reducing its proliferation. To test such capacity, the produced antibodies were incubated in different concentrations (200 µg/mL and 500 µg/mL) with *V. anguillarum* growing cultures, and bacterial growth profile was analysed over time. Briefly, the growth assays were performed in sterile 96 wells plates;

to each well it was added 180 µL of TSB medium supplemented with 1.5 % of NaCl, 20 µL of *V. anguillarum* bacterial culture (0.3 OD_{600}) and 20 µL of a solution with the desired antibody, at different concentrations. In order to test the real effectiveness of the desired antibodies, samples corresponding to different moments of the immunization protocol were prepared: 1) a purified preimmune antibody sample (SEC-grade purity), before the beginning of the protocol and 2) a hyperimmune antibody sample, when the production of the specific antibodies was higher, according to the ELISA results. The plate was incubated during at least 7 h and time course cultures samples were collected and the OD_{600} was measured (EnSpire Multimode Plate reader) at each hour in order to evaluate the bacterial growth profile in the different conditions.

3. Results and Discussion

3.1. Production of IgY antibodies against *V. anguillarum* whole-cell extracts

The first immunization protocol towards the final objective of producing antibodies against *V. anguillarum* comprised the use a heat-killed bacterial extract (HK protocol); two pairs of chickens and two pairs of quails were immunized with this antigen. This technique of using heat-killed bacteria extract is the most commonly used to produce antibacterial antibodies and was the most appropriate for implementation of standard protocols to work with chickens. It should be remarked that the current work was the first ever performed in the host laboratory with chicken models hosts.

3.1.1. Generation of hyperimmune chicken eggs against *V. anguillarum* whole-cell extracts and characterization of target-specific IgY antibodies

As previously referred, 4 chickens were immunized, both pairs immunized in the same days, as described in the Table 6. These animals were submitted to 8 vaccination moments, all of them with the same amount of heat-killed *V. anguillarum* medium 10^7 CFU/mL, the only difference was in the adjuvant used: in the first two immunizations CFA was used and in the others ones (boosts) IFA was used. The boost immunizations B4 to B7 were only administered after 89 days from the last vaccine, when the production of the antibodies against *V. anguillarum* had already decreased. Thus, the objective of these boosts was to re-exposure the immune system of the animal to the immunizing antigen and to consequently increase the production of antibodies that recognize it. The calendar was organized considering the first day of immunization as Day 0.

Table 6. Date of each of the immunization protocol for C0116-01/02 and C0116-03/04 pairs. (I=immunization, B=boost immunization).

Protocol days	0	14	28	43	132	146	161	175
Number of the immunization	I1	I2	B1	B2	B3	B4	B5	B6

The eggs from each pair were collected and processed in pools, each egg yolk pool representing 4 days of the immunization protocol. During the protocol, each bird was weighted in order to follow up their body weight variation and to evaluate if immunizations had any major influence in the animals' normal health. Figure 9 shows that the body weight of the bird

C0116-03 was steady during the 200 days of the immunization protocol, unlike for the other C0116-04, that presented a higher body weight variation during the same time. Another important parameter that was also monitored during the immunization protocol was the number of laid eggs per day per pair of birds. This is also an indicator of the animal's health, and importantly, if during the 4-day egg pool only one chicken had laid eggs, in the posterior analysis that will be noticeable, meaning that one might be evaluating the reactivity of a single chicken as opposed to the chicken pair. The egg-laying capacity allows to calculate the percentage of eggs laid in the experimental period, compared to the possible maximum of 2 eggs per day, per pair, in this case 382 eggs (191 eggs x 2 birds). With that in mind, the number of laid eggs in the pair C0116-03/04 during 191 days of protocol was 343, representing a percentage of approximately 89.9 %. This value corroborates the obtained graph, since during the protocol in most of the days both chickens had laid eggs. In agreement with the previous variable it was also measured the amount of egg yolk mass per pool (Appendix 6.1.), because if the animals were laying less eggs, this is reflected on the amount of egg yolk mass per pool. This variable is important so that the posterior analysis, such as the purification of IgY is performed taking in account the amount of the sample obtained. Analysing these variables, it is possible to conclude that the animals showed no sign of sickness during the period of the experiment. It is also important to mention that the previous referred variables were also analysed in the other pair of animals C0116-01/02 (Appendix 6.2.).

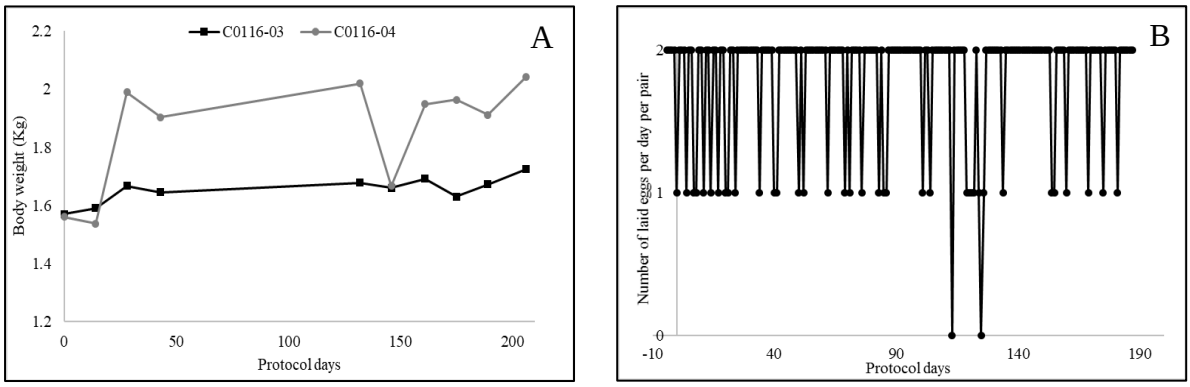


Figure 9. Chicken experimental C1016-03/04 pair monitoring during the immunization protocol.
A) Body weight monitoring; B) Egg-laying capacity.

In the beginning of the immunization protocol, a Dot Blot assay was performed for preliminary monitoring of the IgY reactivity against the heat-killed extract of *V. anguillarum* and *E. coli* (positive control). WSPFs from four pools from each pair of chickens (Pools 1,3,5 and 8) were used for that purpose. The samples comprised the first 27 days of the immunization

protocol. This approach allowed a quick check of the immune response of each pair of chickens while waiting for the complete 90-day immunization protocol samples (Appendix 6.3.).

To further characterize the reactivity of the produced antibodies against the desired antigen, an ELISA assay was performed, valuable to quantify the relative amount of specific antibody against heat-killed *V. anguillarum* extracts. Figure 10 shows the analysis of the 48 egg yolk pools collected along the 191 day-protocol. WSPF of each of the pools (1 to 48) from the C0116-03/04 pair were used according to description elsewhere. The results obtained, clearly show a higher optical density, meaning a higher amount of specific IgY at around Day 44-47 (Pool 13) up until Day 68-71 (Pool 18), when it starts to decrease again, as expected. Moreover, it is also noticeable that after the boosts B3-B6, the production of specific IgY starts again to increase. One would expect a more robust reactivity (higher titer) after the first period of immunization (I1-B2), since this is long enough to generate immunological memory and subsequently a more effective antigen response in the second boost period (B3-B6). Notably, this is not very clear in the C0116-03/04 pair, but is much more evident in C0116-01/02 pair (Appendix 6.2.), suggesting clear bird-pair variations and the need for individual titer monitoring for each experimental bird group.

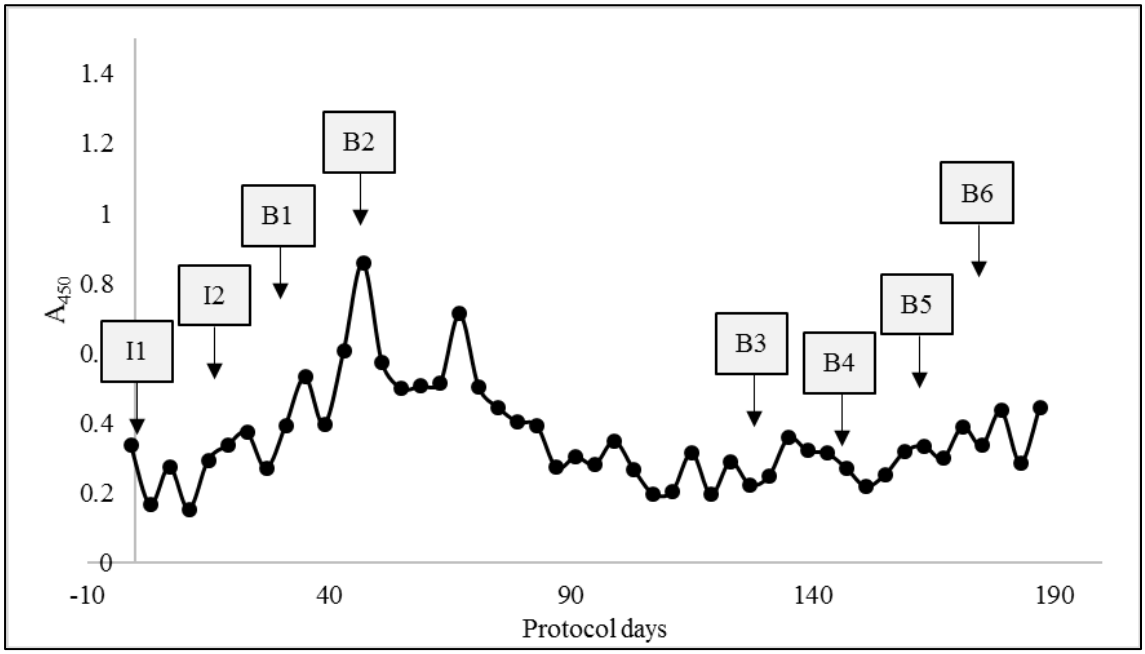


Figure 10. ELISA analysis of egg yolk IgY antibody titer (C0116-03/04, HK protocol) against heat-killed *V. anguillarum* extract.

Assay plates were coated with heat-killed *V. anguillarum* extract (10^{10} CFU/mL), incubated with WSPF of all the 48 pools (at 1:1600 dilution), the secondary antibody, chicken anti-IgY HRP-conjugate was used in a 1:10000 dilution and the absorbance was measured at 450 nm. Each arrow indicates a performed immunization (I) and boost (B).

At the end of the protocol, after the first period of immunization (I1-B2) two samples of antibodies were purified from chicken pair C0116-03/04: one in the beginning of the protocol -Pool 1 (comprising days -4 to -1), and the other one after the first four immunizations - Pool 24 (comprising days 88 to 91). The antibody samples were purified in agreement with a grade III. Figure 11 shows the SDS-PAGE analysis of three samples of the purification process of each antibody obtained from Pool 1 and Pool 24. During the purification procedure, the sample became enriched in the IgY fraction, clearly demonstrated by the stronger presence of Light and Heavy chain bands (approximately 22 KDa and 66 KDa, respectively); as expected, the PEG-step supernatant is composed of other contaminants.

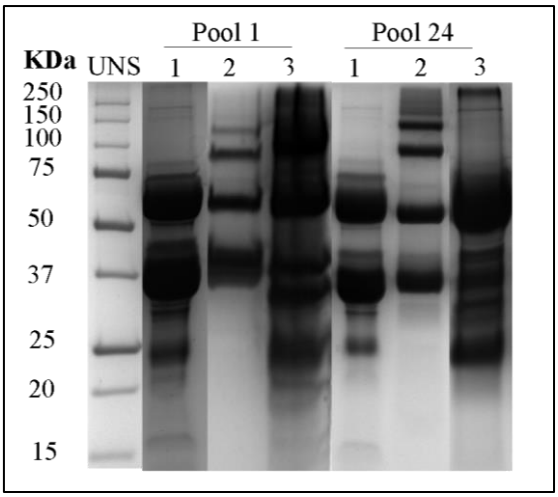


Figure 11. Analysis of IgY antibody (Pool 1 and Pool 24 from C0116-03/04) purity by SDS-PAGE.
UNS = protein marker, lane 1: WSPF, lane 2: supernatant of the PEG precipitation [S(PEG)], and lane 3: ressuspended pellet of the PEG precipitation step [R(PBS)]. All the samples were run on a 12% SDS-PAGE gel, using 20 µL of sample and 10 µL of loading buffer.

The last purification step was a size exclusion chromatography (SEC) run, 5 mL of the ressupended PEG pellet [R(PBS)] were injected on a preparative Superdex 200 26/600 column. Figure 12. shows the obtained elution profile of the sample injected; the first peak corresponds to proteins aggregates (~ 110mL), the second and major elution peak corresponds to the IgY (~ 160 mL) and the last one to the contaminants present in the sample. As shown in Figures 12 and 13, the combination of all the purification steps allows the isolation of the IgY fraction from the other constituents (contaminants and aggregates) of different egg-yolk pools. Of note, the IgY amount obtained from Pool 1 (preimmune) and Pool 24 (hyperimmune) of chicken pair C0116-03/04, is rather comparable, with both resulting in maximum absorbance reaching approximately 500 mAU.

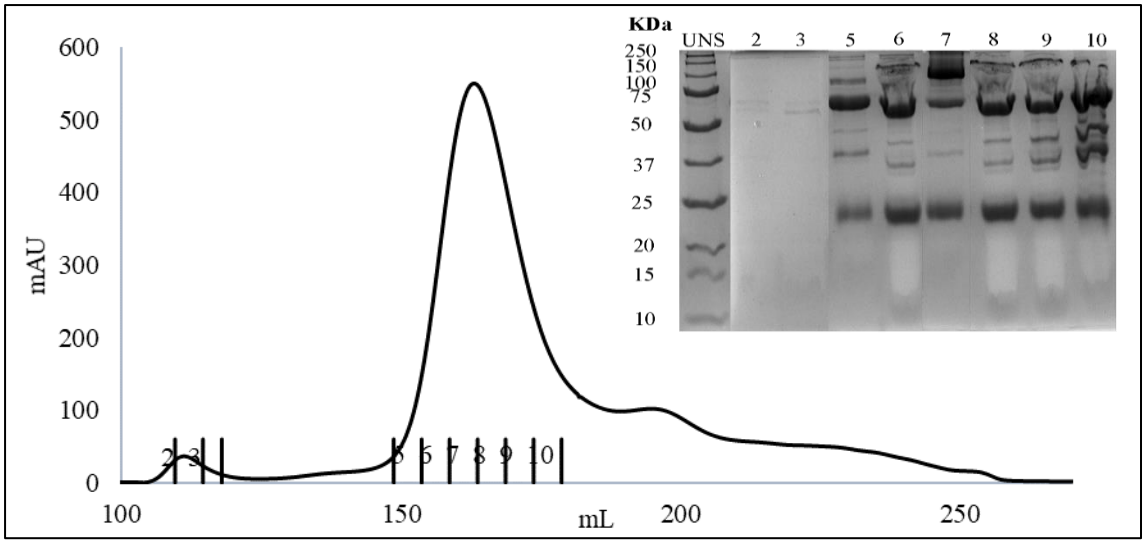


Figure 12. Size-exclusion chromatography elution profile during IgY fraction purification from egg-yolk Pool 1 of chicken C0116-03/04 pair.

SDS-PAGE 12% gel analysis of the corresponding elution fractions.

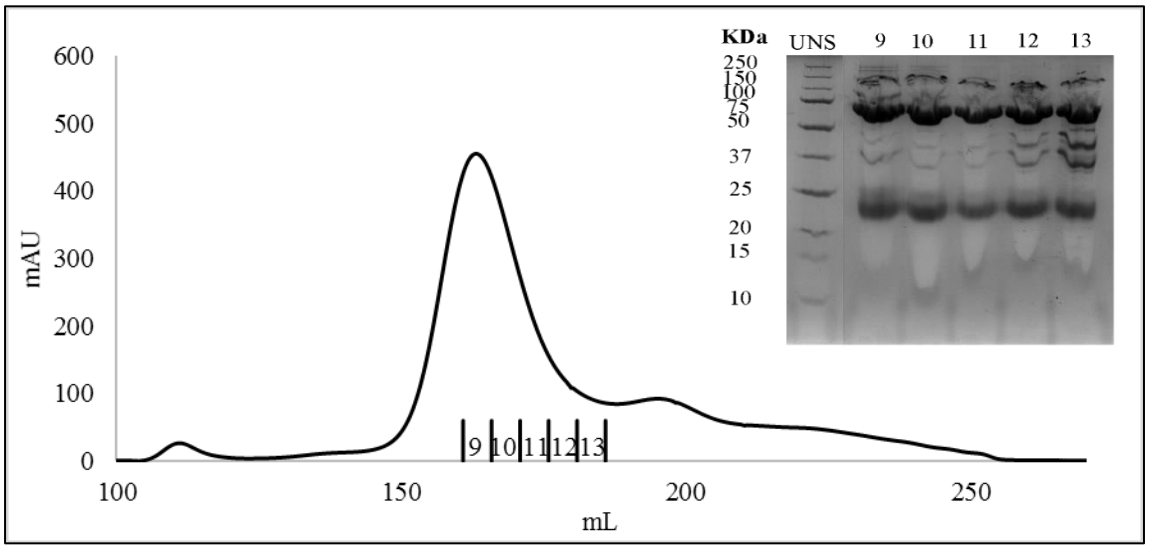


Figure 13. Size-exclusion chromatography elution profile during IgY fraction purification from egg-yolk Pool 24 of chicken C0116-03/04 pair.

SDS-PAGE 12% gel analysis of the corresponding elution fractions.

In order to elucidate the relative percentage of each component separated by SEC (aggregates, IgY and contaminants), the integration of the obtained peaks was used. The respective data are presented in Table 7.

Table 7. Percentages of aggregates, IgY and contaminants in each of the analyzed pools.

Sample	% aggregates	% IgY	% contaminants
Preimmune pool (Pool 1)	1.14	81.23	17.63
Hyperimmune pool (Pool 24)	0.68	78.67	20.65

In the results from Pool 1 the following percentages of aggregates, IgY and contaminants were respectively obtained 1.14%, 81.23% and 17.63%; and for Pool 24 these were 0.68%, 78.67% and 20.65%. After this step of purification, the collected peak fractions containing IgY antibodies from each of the Pools were concentrated; and the total protein concentration was measured, revealing values of 9.94 mg/mL (Pool 1, fractions 6,7,8 and 9) and 8.45 mg/mL (Pool 24, fractions 9,10 and 11). Although, the Pool 24 had a smaller percentage of IgY, that does not mean that the produced antibodies were less effective against *V. anguillarum*. Thus, to further characterize the produced antibodies, a WB using the WSPF of three pools as primary antibody was used: a preimmune one (Pool 1) and two hyperimmune (Pool 18 and 24) (Figure 14). All tested WSPF were normalized to a final (total) protein concentration of 1.5 mg/mL and were diluted at 1:2000. This assay revealed that the preimmune antibodies do not have any specificity toward the heat-killed *V. anguillarum* extract. On the other hand, the other tested pools displayed specificity towards protein bands in a defined MW window, ranging between 37 and 75 KDa. The Pool 18 showed a stronger reactivity towards the previous referred window than Pool 24, that corroborates the obtained titer in ELISA assay, where the OD_{450} is lower in Pool 24. This MW window might relate with the band that is always present in SDS-PAGE gels of different dilutions of the heat-killed *V. anguillarum* extract (Appendix 6.4.). This band has a MW (~40 KDa) typical of well described outer-membrane proteins of the bacterium (51).

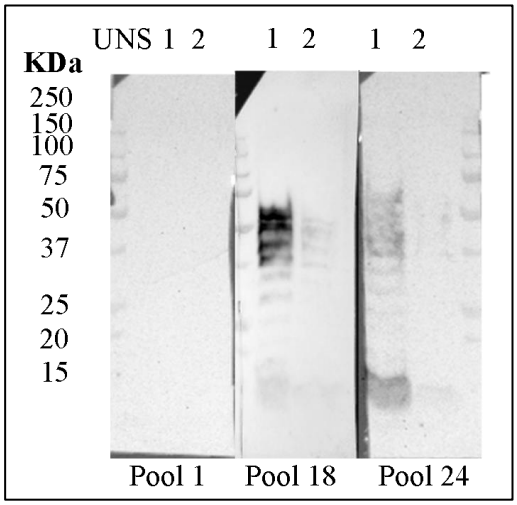


Figure 14. Western blot analysis performed with Pool 1,18 and 24 WSPF from chicken pair C0116-03/04 to evaluate anti-*V. anguillarum* reactivity

Lane 1: 10^{11} CFU/mL and lane 2: 10^{10} CFU/mL.The medium was separated on 12% SDS-PAGE and transferred to nitrocellulose membrane.The membrane was divided and probed with WSPFs of each pool as a primary antibody (total protein normalized to 1.5 mg/mL, dilution 1:2000).

To test the robustness and effectiveness of anti-*V. anguillarum* antibodies as functional inhibitors of bacterial proliferation, grade III purified antibodies from Pool 1 and Pool 24 were used in bacterial growth assays and their effects characterized. *V. anguillarum* cultures were grown for 7 h in the presence of different initial concentrations of anti-*V. anguillarum* antibodies; the OD_{600} was measured every hour and the results are shown in Figure 15. It is clear that hyperimmune antibodies (obtained from Pool 24) could reduce the OD_{600} of the bacteria medium in 50%, in both concentrations tested (200 and 500 µg/mL), unlike preimmune antibodies (obtained from Pool 1) that presented a rather similar bacterial growth profile as the control, with no supplemented antibody. This demonstrates that hyperimmune antibodies against *V. anguillarum* heat-killed extracts are capable of effective growth inhibition, thus likely capable of substituting or improving the effect of antimicrobial agents, including antibiotics.

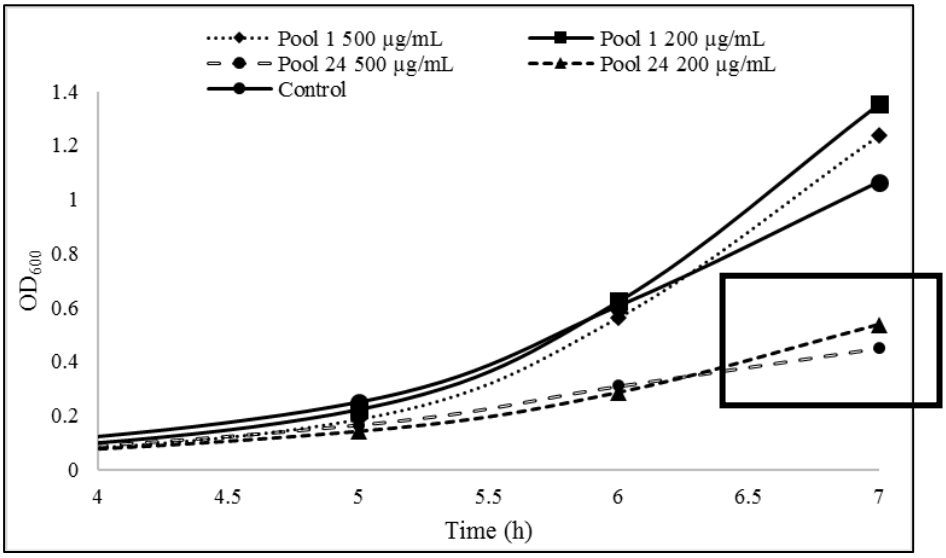


Figure 15. Bacterial growth inhibition by anti-*V. anguillarum* antibodies.

Pool 1 and 24 grade III purified anti-*V. anguillarum* antibodies were used in bacterial growth assays and revealed effective growth inhibition effects as shown by OD600 monitoring. The control curve refers to a nornal growth of a *V. anguilllarum* culture with no supplemented antibody.

3.1.2. Generation of hyperimmune quail eggs against *V. anguillarum* whole-cell extracts and characterization of target-specific IgY antibodies

Coturnix japonica (Japonese quail) was the other animal model used to produce anti whole-cell *V. anguillarum* antibodies. These birds are also used in the production of avian antibodies. Although they have a rapid maturation, approximately 6-8 weeks after hatching

they reach adulthood, the amount of IgY per yolk is significantly smaller because one quail egg has only about 3 mL of yolk, contrasting with the 15 mL of yolk in a chicken egg.

As previously referred, a total of four quails were immunized with heat-killed *V. anguillarum* extract, with an adequate amount to the body weight of the animals. In this protocol, the animals were submitted to 4 vaccination moments. However, all the variables, such as body weight, egg-laying capacity and egg yolk pool mass were monitored and the data are presented in Figure 16 and Appendix 6.6..

In general, no significant variation in the body weight of the experimental birds was observed during experimentation, thus reflecting adequate animal housing and maintenance, resulting in proper experimental conditions. Nevertheless, some exceptions were identified, for example for quail Q0816-55, for which after the second measurement its weight was reduced almost 100 g, as shown in figure 16. In contrast, the other quail from the same pair revealed an increase in body weight. As for the pair Q0816-58/59 the weight of the two animals remained relatively steady during the 90 days of protocol. Analysing the egg-laying capacity of each of the pairs and calculating the percentage of laid eggs, the Q0816-55/56 pair reached a percentage of 80.0% and the Q0816-58/59 pair a value of 85.4%.

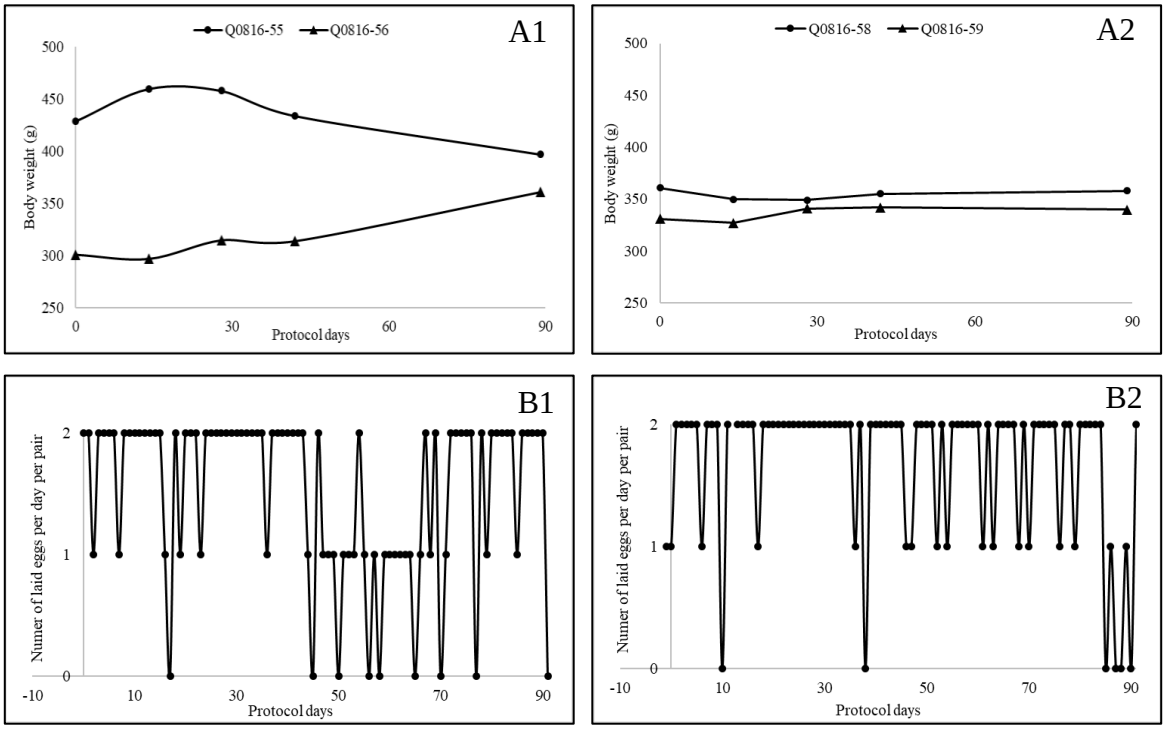


Figure 16. Quails experimental Q0816-55/56 and Q0816-58/89 pairs monitoring during the immunization protocol.

A1 and A2) Body weight monitoring of Q0816-55/56 and Q0816-58/89, respectively.

B1 and B2) Egg-laying capacity of Q0816-55/56 and Q0816-58/89, respectively.

Figure 17 shows the anti-*V. anguillarum* titer evolution profile along the 90-day immunization protocol, for both pairs of quails used. WSPF of each of the 4-day egg yolk pool (total of 24 pools) were prepared and used as primary antibody in ELISA assays. The result shows a similar global profile for both experimental groups, though maximum production of specific IgY against the heat-killed *V. anguillarum* extract was reached at different times in each of the protocols. In the Q0816-55/56 ELISA the maximum reactivity was obtained in the Pool 16 (Days 56-59), after the 4 immunizations performed, in contrast, the Q0816-58/59 reached the maximum before, at the Pool 11 (Days 36-39). Regardless of the different profiles in the middle of the protocol, in the end, the production decreases similarly, reaching the same A_{450}. These differences only prove that different animals have different responses to the same amount of antigen, and that variability between animals should always be considered in this type of protocols, especially if the animals have different ages, as demonstrated in the Appendix 6.7.. Additionally, it is shown the variability within the same animal, since for the same animal, different eggs from different days have an intrinsic different amount of IgY

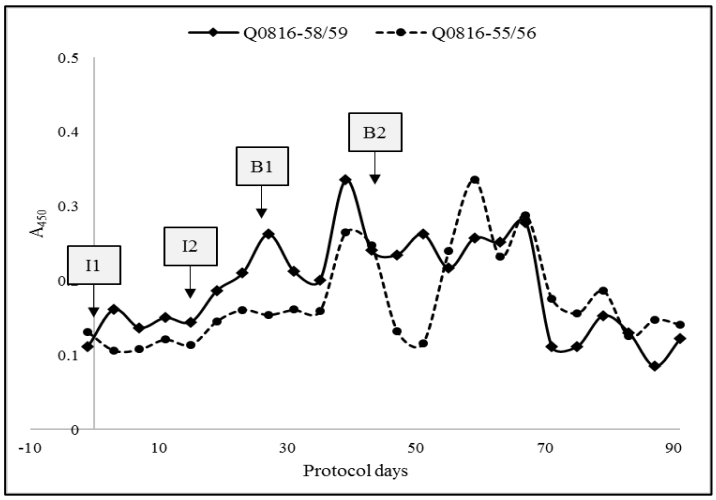


Figure 17. ELISA analysis of egg yolk IgY antibody titer (Q0816-55/56 and Q0816-58/59 , HK protocol) against heat-killed *V. anguillarum* extract.

Assay plates were coated with heat killed *V. anguillarum* extract (10^{10} CFU/mL), incubated with WSPF of all the 24 pools (1:1600 dilution), as secondary antibody, chicken anti-IgY HRP-conjugated was used at 1:10000 dilution and the absorbance was measured at 450 nm. Each arrow indicates a performed immunization (I)/boost (B).

The pools of antibodies for purification was chosen according to ELISA assay, so it was purified a sample of 50 mL from Pools 15-18 from both pairs. Figure 18 shows the SDS-PAGE gel analysis of different samples along the purification process of IgY antibodies from the mentioned egg yolk pools. The lane 2 is the pellet resultant from the centrifugation of the WSPF, that is composed of lipoproteins that precipitate at pH of 4.8-5 and thus, are excluded

from the WSPF. The lane 3 is the supernatant from PEG precipitation and that is also composed of other constituents besides IgY, also shown in lane 4, though this lane is also less concentrated.

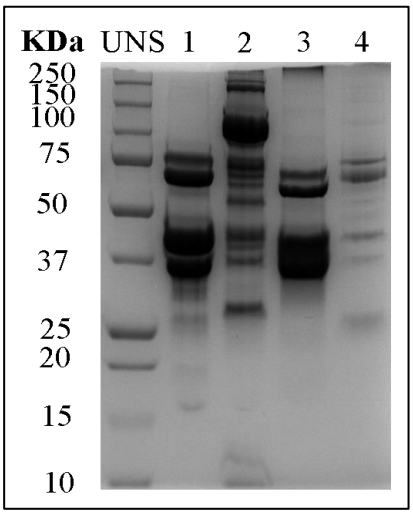


Figure 18. Analysis of IgY antibody (Pools 15-18 from Q0816-55/56 and Q0816-58/89) purity by SDS-PAGE 12%.

UNS= protein marker, lane 1: WSPF, lane 2: Pellet WSPF, lane 3: S(PEG), and lane 4: R(PBS). Samples in lane 1 and 3 were run using 10 µL of sample ,10 µL of loading buffer and 10 µL of water (1:2). On the other hand, the lanes 2 and 4, were run, using 5 µL of sample, 15 µL of water and 10 µL of loadind buffer (1:4).

After the ressuspending the pellet from the PEG precipitation step, the sample was further purified using a SEC step, similarly to described for chicken produced antibodies, represented in Figure 19.

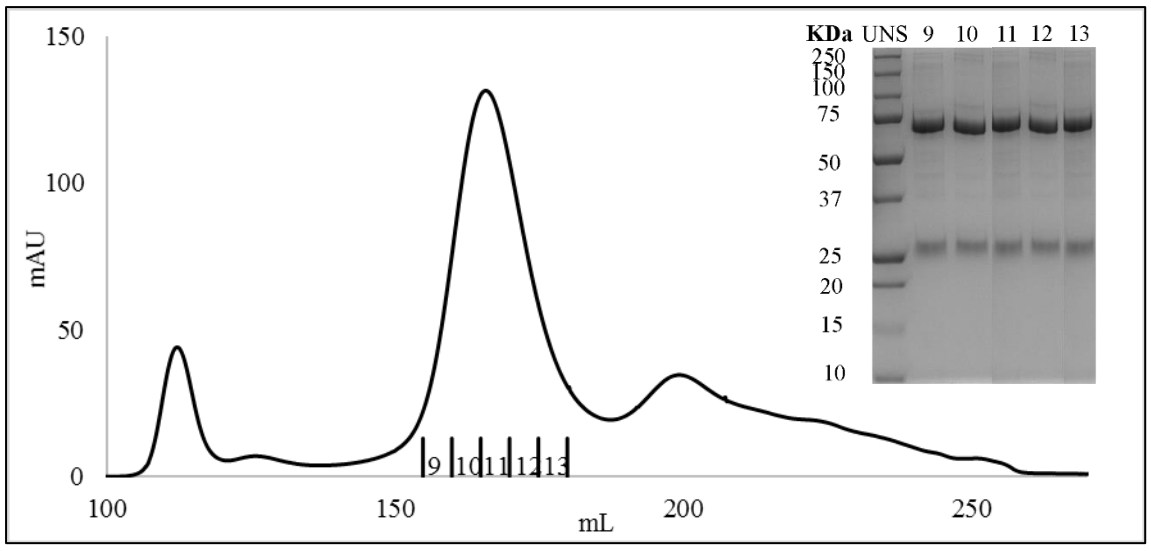


Figure 19. Representation of the SEC run of the Pools 15-18 from Q0816-55/56 and Q0816-58/59 pairs and SDS-PAGE of the correspondent IgY peak fractions.

All the fractions were run in the SDS-PAGE gel, using 20 µL and adding 10 µL of loading buffer.

When both SEC purifications (quail and chicken) are compared, it is clear that the intensity of the IgY peak is significantly smaller; that difference is due to the reduced amount of IgY in the injected sample from quail models. Nevertheless, as expected the size-exclusion profile is similar for both avian IgY enriched samples. Using SEC curve profiles, the relative amounts of aggregates, IgY and contaminants were calculated using peak curve integration, whose results are shown in Table 8.

Table 8. Percentages of the aggregates, IgY and contaminants in the Pools 15-18 of the pairs Q0816-55/56 and Q0816-58/89.

Sample	% aggregates	% IgY	% contaminants
Pools 15-18	12.71	73.28	14.01

The obtained percentages of aggregates, IgY and contaminants of the analysed pools were 12.71%, 73.28% and 14.01%, respectively. After this step of purification, the collected peak fractions containing IgY antibodies were concentrated and the total protein concentration was measured, obtaining a final value of 10.95 mg/mL(fractions 10,11 and 12).

To further characterize the produced antibodies, a WB was performed, using WSPF from the preimmune pool (pools 1 of Q0816-55/56 and Q0816-58/89 pairs) and the hyperimmune ones (pools 15-18), as primary antibodies (Figure 20) All tested WSPF were normalized to a final (total) protein concentration of 1.5 mg/mL and were diluted at 1:2000.

This assay revealed once more that the preimmune antibodies (pool 1) do not have any specificity towards the heat-killed bacterial extract. Nevertheless, the hyperimmune sample displayed a specificity towards protein bands in a defined MW window (37-75 KDa), such as the hyperimmune antibodies purified from chickens. As previously referred, this MW window might relate with the band present in the SDS-PAGE gels of different dilutions of the heat-killed *V. anguillarum* extract used as antigen (Appendix 6.4.).

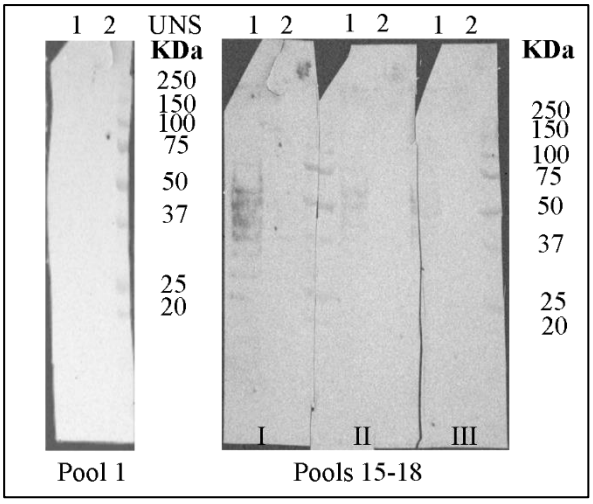


Figure 20. Western blot analysis performed with Pool 1 and Pools 15-18 WSPF from quails Q0816-55/56 and Q0816-58/89 pairs to evaluate anti-*V. anguillarum* reactivity.

Lane 1: 10^{11} CFU/mL and lane 2: 10^{10} CFU/mL.The medium was separated on 12% SDS-PAGE and transferred to nitrocellulose membrane.The membrane was divided and probed with WSPFs of each pool as primary antibody (total protein normalized to 1.5 mg/mL). Pool 1 was diluted in a proportion of 1:2000, pools 15-18 at 1:500 (I), 1:1000 (II) and 1:2000 (III).

3.2. Production of IgY antibodies against *Vibrionaceae* family specific antigens

3.2.1. IgY antibodies against *V. anguillarum* membrane fraction

3.2.1.1. Preparation of membrane fraction antigens

In addition to the first approach, where antibodies were produced against whole-cell *V. anguillarum* extract and with the aim of refining a targeting strategy, a membrane-enriched fraction antigen was also prepared and used for bird immunization. Figure 21 show a SDS-PAGE gel of samples collected along the purification protocol of the membrane-fraction antigen, used in the immunization of 6 quails. In fact, two types of membrane fraction antigens were prepared: 1) pellet of the 17000 g centrifugation (PL) and 2) final membrane preparation, referred as MP; both antigens were used in the immunization of 3 quails (1 trio of birds per antigen). On the SDS-PAGE gel both pellets are more complex in content when compared to the supernantants of the centrifugations that are enriched in a protein with about 35 KDa, reported in the literature as being a porin-like protein that is predominant in the bacterial outer-membrane portion (52). The obtained PL is instead enriched in mitochondrias and other inner cellular organelles.

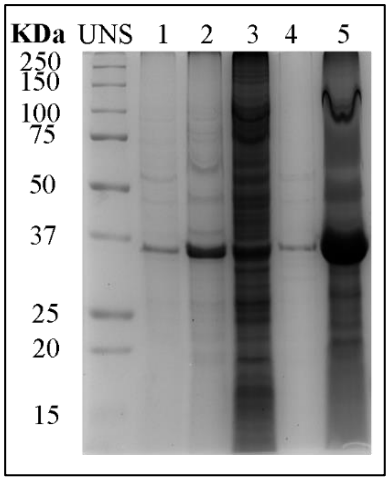


Figure 21. Analysis of the antigen purification process by SDS-PAGE 12%.

Lane 1: supernatant of 4200 rpm centrifugation (1:2) , lane 2: supernatant of 17000 g centrifugation (1:2), lane 3: pellet 17000 g centrifugation (1:4), lane 4: supernatant of 35000 g centrifugation (1:2) and lane 5 : pellet of the 35000 g centrifugation, MP (1:2).

3.2.1.2. Generation of hyperimmune quails eggs against *V. anguillarum* membrane fraction and characterization of target-specific IgY antibodies

After the purification of the antigens, both quail immunization protocols were performed, according to the scheme described for anti-whole-cell *V. anguillarum* extract (HK protocol): I1 (D0), I2 (D14),B1 (D28) and B2 (D42). In these protocols, using as antigen MP and PL, further referred as MP protocol and PL protocol, two groups of three animals were vaccinated: Q1016-07/08/09 and Q1016-10/11/12, respectively.

During the immunization protocol the body weight of each animal, and the trio egg-laying capacity were monitored - results presented in Figure 22. Body weight data analysis of trio Q1016-07/08/09 (MP protocol), shows that the animal Q1016-08 was the only that lost weight (~50 g) during the immunization protocol. This weight loss might be an indicator of some kind of illness, with no other visible signs in the animal nor a clear egg-laying capacity change in the trio; strikingly the animal was found dead at D97, thus revealing the importance of regular body weight monitoring in such experimental set-up. It should be noted that the body weight of the other two animals within the same protocol increased during the protocol, suggesting that neither the procedure or the antigen itself could have caused weight loss and subsequent death. On the trio of the PL protocol, the three animals increased their body weight, after D30. Nevertheless, on D42, two of them (Q1016-10/11) registered a maximum weight, followed by a weight loss, more pronounced in Q1016-11. Such weight variations reflect

changes in the egg-laying capacity of the animals, as observed in the egg-laying performance profile. Calculating the percentage of laid eggs of each trio during the 90-day immunization protocol, the animals of the MP and PL protocols reached a percentage of 81.9% and 72.1%, respectively. The egg yolk pool mass of each of the protocols was also measured and the respective results are given in the Appendix 6.8.

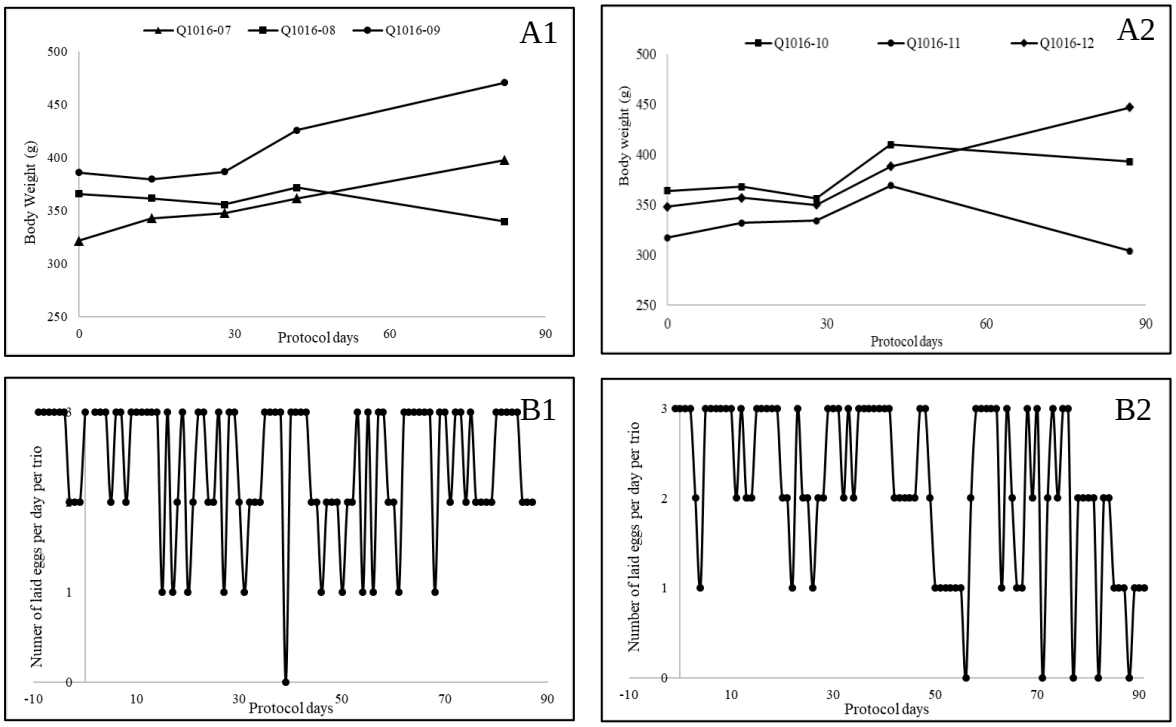


Figure 22. Quails experimental Q1016-07/08/09 (MP protocol) and Q1016-10/11/12 (PL protocol) trios monitoring during the immunization protocol.

A1 and A2) Body weight monitoring of Q1016-07/08/09 and Q1016-10/11/12, respectively.

B1 and B2) Egg-laying capacity of Q1016-07/08/09 and Q1016-10/11/12, respectively.

After the end of the protocol the titer target-specific antibodies was analysed by ELISA assays as shown in Figure 23. Comparing the obtained results with the ones for anti-whole-cell *V. anguillarum* extract described above, it is clear that the overall values of A_{450} are lower. This was indeed expected since the antigens used (MP and PL) correspond to only a defined fraction of the whole-cell extract; nevertheless, this ELISA signal reduction is not drastic, only about a 2- to 3-fold, indicating that MP and PL antigen still have an important contribution for the immunogenic response in quail model. On the other hand, when comparing MP and PL ELISA results, it is noticed that the MP protocol seems to have an ascendant and more consistent titer after four immunizations, when compared to the PL one. This suggests, that the MP antigen might be more robust to generate anti-*V. anguillarum* antibodies, though further replicas would

have to be done to confirm this, particularly since most of our results indicate bird intrinsic variability in immunoresponse.

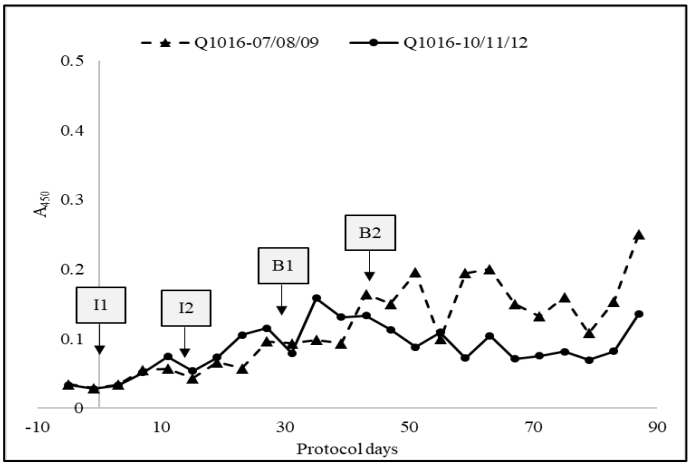


Figure 23. ELISA analysis of egg yolk IgY antibody titer (MP and PL protocols) against heat-killed *V. anguillarum* extract.

Assay plates were coated with heat killed *V. anguillarum* extract (10^{10} CFU/mL), incubated with WSPF of all the 24 pools (1:1600 dilution), as secondary antibody, chicken anti-IgY HRP-conjugated was used at 1:10000 dilution and the absorbance was measured at 450 nm. Each arrow indicates a performed immunization (I)/boost (B).

WSPF fractions from the egg-yolk Pool 1 and 24 of MP protocol were prepared and used as primary antibody to perform WB assays. It was expected that Pool 24 antibodies (hyperimmune fraction) would present different target specificity and thus a WB band profile distinct from that obtained with anti-whole-cell extracts. However, the results obtained (Figure 24.) show that Pool 24 antibodies recognize bands within the same molecular-weight window (37-75 KDa) as anti-whole-cell extract antibody. As previously referred, this window is related with the outer membrane portion content as described in the literature (52). Further studies, namely functional assays should be performed in order to characterize and understand if besides the similar WB reactivity profile, the antibodies produced with MP antigen are indeed more effective, for instance, in inhibiting bacterial growth and reducing proliferation of *V. anguillarum*.

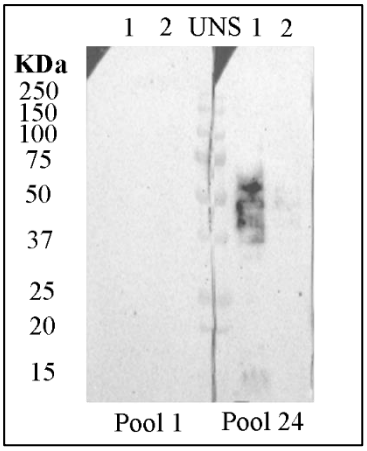


Figure 24. Western blot analysis of the antibodies from Pool 1 and Pool 24 from the MP protocol, Q0816-07/08/09 trio.

Lane 1: 10^{11} CFU/mL and lane 2: 10^{10} CFU/mL. The medium was separated on 12% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was divided in 2 and probed with each WSPF (1.5 mg/mL), using a dilution of 1:2000.

3.2.2. IgY antibodies against extracellular proteins of *Vibrionaceae* family

3.2.2.1. Production of recombinant extracellular epitopes of TolC *V. anguillarum* and VceC *V. cholerae* outer-membrane proteins

Based on previous work in the host laboratory demonstrating that *E. coli* TolC outer-membrane channel seems to be a promising target for immunotherapies with avian antibodies, herein same rational was followed to target the homologous outer-membrane channel in *V. anguillarum*. Since the crystal structure of TolC from *V. anguillarum* has not yet been reported in the literature, it was used the online bioinformatic tool Phyre 2.0 (Protein Homology/analogy Recognition Engine version 2.0), that allows to obtain a prediction for the tridimensional structure of a protein for which the primary sequence of residues is known. Phyre 2.0 uses such input protein sequence and runs a structural homology prediction based on already existing protein crystal structures of homologous proteins. The final result is a list of structural homology models, ordered by prediction confidence and primary sequence coverage that can be used to rationalize structure-based hypothesis.

Using *V. anguillarum* TolC protein sequence (UniProt entry A0A191W654), Phyre 2.0 modeled a number of tridimensional protein structures, with the first three identified in the Figure 25. Indeed, the first two were obtained using the *E. coli* TolC crystal structures respectively PDB ID: 1TQQ and PDB ID: 1EK9 as structural templates and the third one used

PDB ID: 5AZP corresponding to *Pseudomonas aeruginosa* TolC channel. For these three predictions, the prediction confidence (100 %) as well as the identity (46%,46% and 16%, respectively) were higher, supporting further use of the *V. anguillarum* TolC structure models for vaccine/antigenic epitope selection and rationalization.

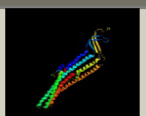
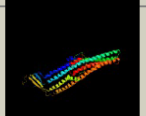
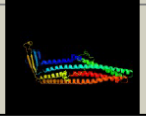
#	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
1	c1tqqC_	Alignment		100.0	46	**PDB header:**transport protein **Chain:** C: **PDB Molecule:**outer membrane protein tolc; **PDBTitle:** structure of tolc in complex with hexamminecobalt
2	d1ek9a_	Alignment		100.0	46	**Fold:**Outer membrane efflux proteins (OEP) **Superfamily:**Outer membrane efflux proteins (OEP) **Family:**Outer membrane efflux proteins (OEP)
3	c5azpA_	Alignment		100.0	16	**PDB header:**membrane protein **Chain:** A: **PDB Molecule:**multidrug efflux outer membrane protein oprn; **PDBTitle:** crystal structure of a membrane protein from pseudomonas aeruginosa

Figure 25. Three first results of Phyre 2.0 job for TolC *V. anguillarum* protein.

The region corresponding to *V. anguillarum* TolC extracellular epitope was rationalized according to the output structural homology models obtained with Phyre 2.0. The selected region corresponds to residues 279-290 of *V. anguillarum* TolC (M.W. ≈ 1.28 KDa) and was cloned using a Sequence and Ligation Independent Cloning (SLIC) method.

Figure 26 represents the 3D prediction structure of the TolC *V. anguillarum*, including the overall structure using the 3 monomers, aligned in order to form the outer membrane channel and the close up of the epitope that was cloned (shown in magenta colour).

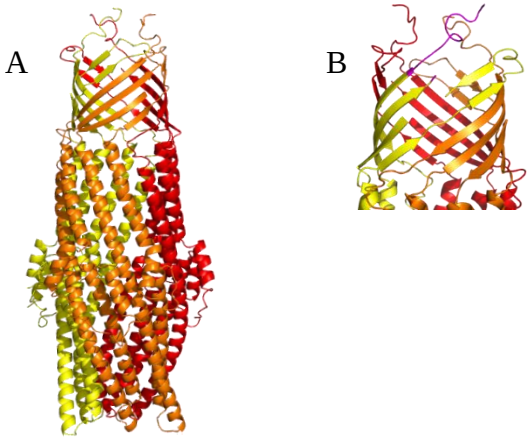


Figure 26. Representation of the prediction structure for TolC *V. anguillarum*.

A) Representation of the three monomers; B) . TolC *V. anguillarum* epitope (D279-D290) 3D scheme (magenta).

Vibrio cholerae VceC (UniProt Entry: Q9KS51) is the only TolC-like channel from *Vibrionaceae* family with a known 3D crystal structural (PDB code: 1YC9). As described for *V. anguillarum* TolC, the corresponding VceC extracellular epitope was identified and cloned using a similar approach, in order to obtain an equivalent recombinant immunogen for antibody production. The chosen protein sequence harboured residues 127 to 139, as depicted in Figure 27B.

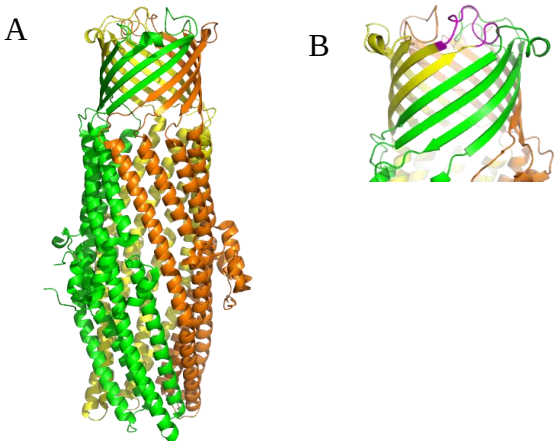


Figure 27. Representation of the 3D structure of VceC from *V. cholerae*.

A) Representation of the three monomers; B)VceC epitope (V127-G139) 3D scheme (magenta).

Concerning protein sequence identity, Clustal Omega software was used to run a protein sequence alignment (Figure A.14. , Appendix 6.9.) between the well characterize *E. coli* TolC and *V. anguillarum* TolC, as well as *V. cholerae* VceC. The results showed a sequence identity of 46.31% for *V. anguillarum* TolC, and 21.82% for *V. cholerae* VceC. This confirms a considerable protein sequence divergence between proteins that seem to fold into similar 3D structure.

As previously referred the sequence of the epitope used as antigen for immunizations was chosen according to the exposed area in the 3D structure. In TolC-like outer-membrane proteins, each monomer has 2 main extracellular loops, as depicted in Figure 28 for *E. coli* TolC. Strikingly, there seems to be a unique protein sequence for each of the loops in all three proteins (Figure 29); this supports the idea that one can customize specific antigens for each protein using these exposed regions, and particularly these extracellular loops.

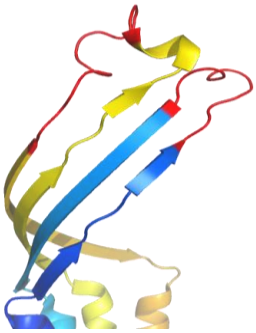


Figure 28. Representation of loops in one of the three monomers from TolC *E. coli* (red).

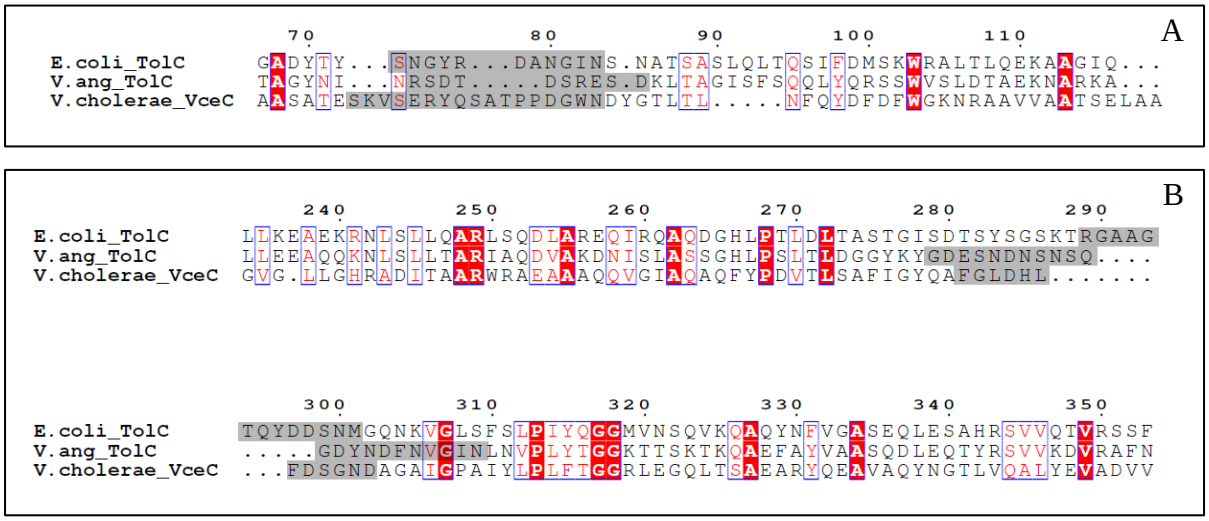


Figure 29. Partial protein sequence alignment of TolC-like outer-membrane proteins

Alignment was obtained in Clustal Omega (57) and ESPript 3.0 (58) was used for graphical representation. Gray highlighted area correspondent to loop 1 (A) and loop 2 (B).

Table 9 shows the loop correspondence as well as protein sequence of both *V. anguillarum* and *V. cholerae* epitopes chosen for recombinant production and subsequent immunization. Individual carrier-protein fusions were designed for each epitope resulting in different genetic constructs; all were prepared to code an additional short GS linker to guarantee minimal distance from the carrier protein C-terminal, thus proper presentation of the target-epitope for immuneresponse. It should be remarked that, the carrier-protein is still under proprietary development and is not further described in the present work.

Table 9. Sequence of the recombinant contruct of TolC *V. anguillarum* epitope.

Construct	Loop	Linker	Epitope sequence
TolC 279-290	2	-GS-	DESNDNSNSQGD
VceC 127-139	1	-GS-	VSERYQSATPPDG

The cloning strategy used a standard SLIC approach, where two primers (forward and reverse) were designed to code for half the epitope sequence and allow its genetic sequence

reconstruction in the plasmid. After PCR reaction with the referred primers, an agarose gel was run in order to confirm the amplification/linearization of each novel plasmid. Figure 30 represents the successful linearization in both processes, since each of the lanes has a band around 6 Kbp, that is the length of the plasmid.

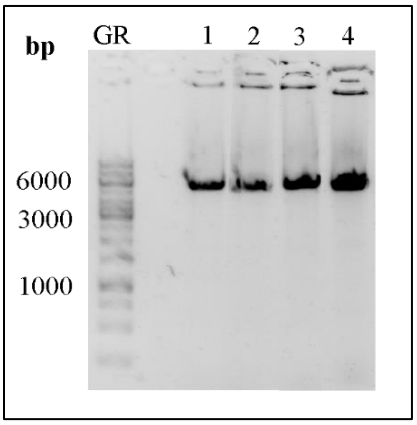


Figure 30. Plasmid linearization confirmation in agarose gel.

GR= Gene ruler™ 1kb DNA Ladder. Replicas of the linearization with the reverse and forward primer for VceC *V. cholerae* (V127-G139) epitope (Lane 1 and Lane 2) and with reverse and forward primer for TolC *V. anguillarum* (D279-D290) epitope (Lane 3 and 4). The 6 Kbp band represents the successful linearization of the plasmid in each procedure.

After the linearization step, plasmids were ligated and further transformed in competent DH5α cells to ensure a high copy number of the desired genetic information. DH5α cell colonies were obtained ON in petri dishes with LB agar and kanamycin. Since the plasmid has genetic information that codes for kanamycin resistance, if the plasmid was integrated in the cells, they would become resistant and would be able to grow in the plate. Thus, after the ON incubation, 7 single colonies of each of the plates (incubated with PCR replicas) were ressuspended and used in colony PCR (Figure 31). Colony PCR analysis was then performed in order to preliminary identify positive clones. This PCR was performed using as a forward primer, the T7 promoter that initiates the process before the carrier protein and as reverse primer, the same primer used in the linearization of the plasmid. If a band of about 700 bp appears, this means that the sequence of interest most likely had been successfully integrated in the plasmid construct. According to the agarose gels, at least one positive colony was obtained for each construct and the corresponding plasmid DNA was analysed by sequencing.

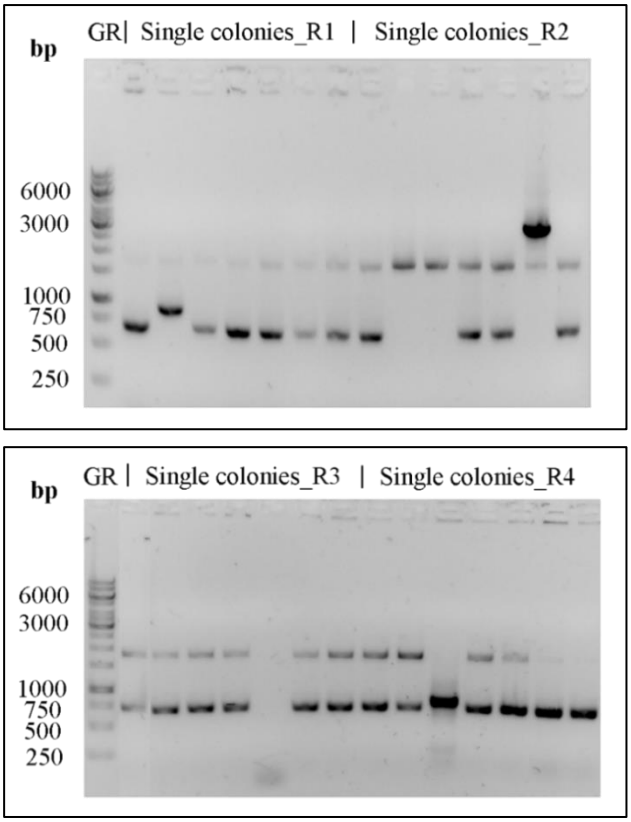


Figure 31. Results of colony PCR amplification with T7 primer.

GR= Gene ruler^{TM} 1kb DNA Ladder. R stands for replicas of the amplification process, using the reverse and forward primers for VceC epitope (R1 and R2) and TolC *V. anguillarum* (R3 and R4).

Positive constructs harbouring the genetic epitope sequence, were then used for expression in *E. coli* BL21* DE3 cells. During recombinant epitope expression, culture samples were collected and used to verify the presence of an over-expressed protein band, namely by comparing a collection time point before the induction of the expression (addition of the IPTG), T0 and other after the ON procedure, TON (Figure 32).

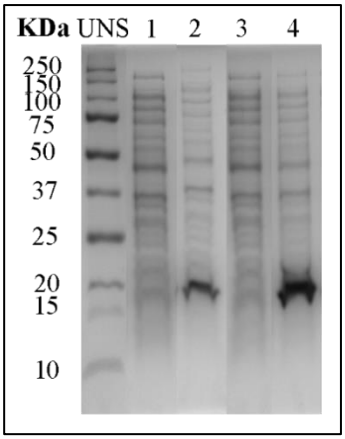


Figure 32. SDS-PAGE analysis of the expression of VceC epitope (V127-G139) and TolC epitope (279-290) in BL21* cells.

Lane 1 and lane 2: T0 (expression induction sample) and TON (after the ON expression sample) from VceC (V127-G139) expression. Lane 3 and lane 4:.T0 and TON from TolC (D279-D290) expression. Each sample was run using 20 µL, adding 10 µL of the loading buffer.

Figure 32 clearly shows the presence of such overexpressed bands (MW. aprox. 16 kDa) for both *V. anguillarum* TolC and *V. cholerae* VceC epitopes. The band has a MW that corresponds to the fusion: carrier protein plus the epitope.

The cellular pellet of the correspondent cultures was then sonicated, and posteriorly centrifuged, and the resultant supernatant was injected in a Hitrap column (anion exchange column). The amount of salt used in the elution was already optimized towards the carrier protein. The following figures represent the profiles of the elution of the cellular pellet in the Hitrap column (Figures 33 and 34) and the collected fractions SDS-PAGE gels (Figure 35 and 36).

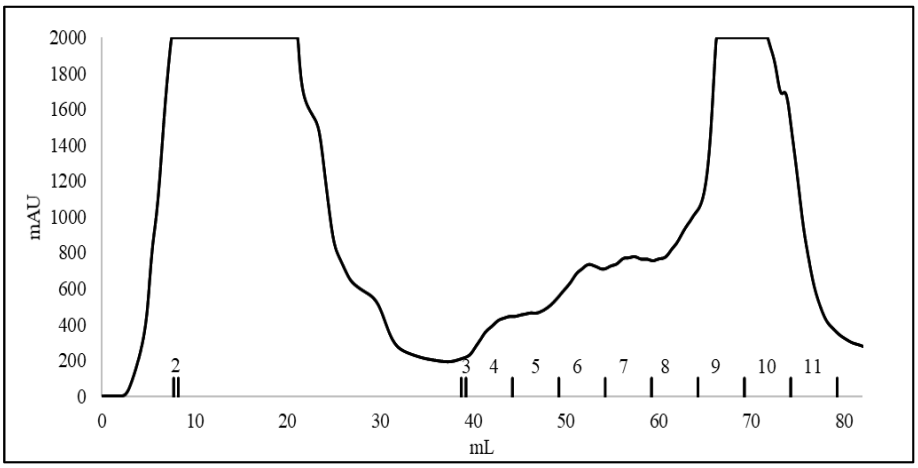


Figure 33. Hitrap ellution of the cellular pellet from the selected culture where it was express the TolC *V. anguillarum* epitope.

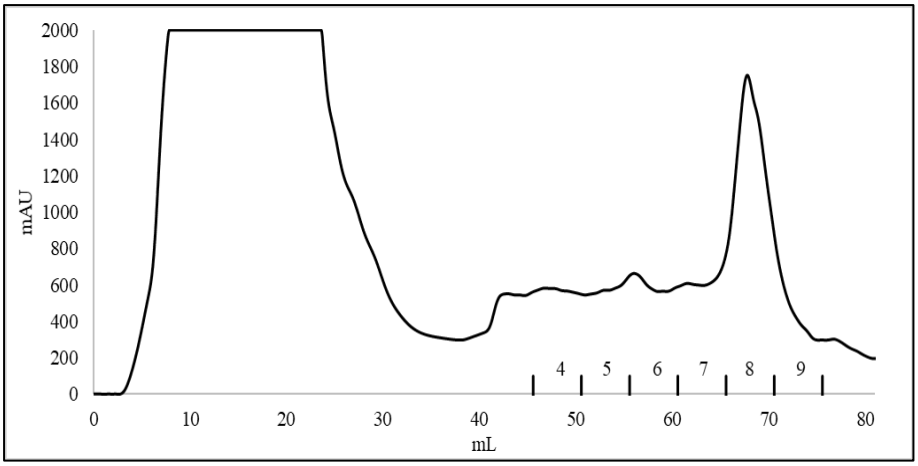


Figure 34. Hitrap ellution of the cellular pellet from the selected culture where it was express the VceC *V. cholerae* epitope.

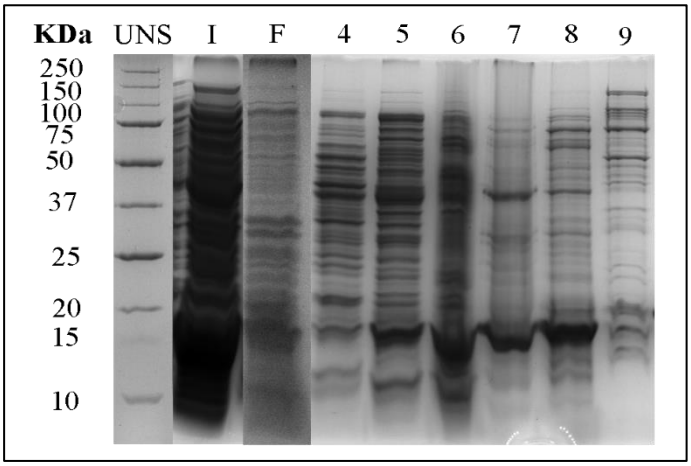


Figure 35. Analysis of the Hitrap fractions by SDS_PAGE 12% from the VceC *V. cholerae* epitope cellular pellet.

I = injected sample in the Hitrap column, F = column's flowthrough, and lane 4 to 9 are the correspont collected fractions. Each sample was run using 20 µL and adding 10 µL of loading buffer.

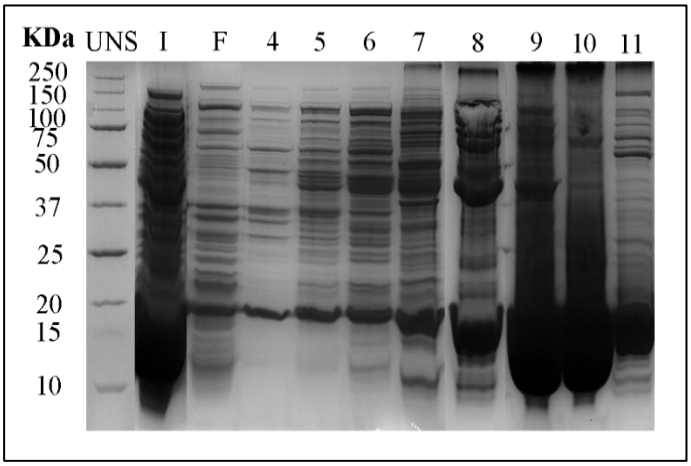


Figure 36. Analysis of the Hitrap fractions by SDS_PAGE 12% from the TolC *V. anguillarum* epitope cellular pellet.

I = injected sample in the Hitrap column, F = column's flowthrough, and lane 4 to 11 are the correspont collected fractions. Each sample was run using 20 µL and adding 10 µL of loading buffer.

In both ion exchange chromatography runs, a peak eluted at around 70 mL (fractions 9, 10 and 11 from TolC *V. anguillarum* epitope run and fractions 6, 7 and 8 from VceC *V. cholerae* epitope run) and was clearly enriched in a 16 kDa protein band corresponding to the recombinant fusion carrier: epitope, as observed by SDS-PAGE analysis. Such fractions were used for a subsequent purification step using an ATP column. Figures 37 and 38 show the SDS-PAGE analysis from samples along this purification step; as expected, both column supernatant (sample loaded ON for incubation with the resin) and the first wash (W1) contain a considerable amount of the recombinant epitope, since the column binding capacity is not able to immobilize the large amount of overexpressed protein. Nevertheless, the first and second elution samples (E1 and E2) are also enriched in recombinant epitope (~ 16KDa) with a very high purity grade, as desired. E1 and E2 samples were pooled and concentrated for further use in the immunization protocols.

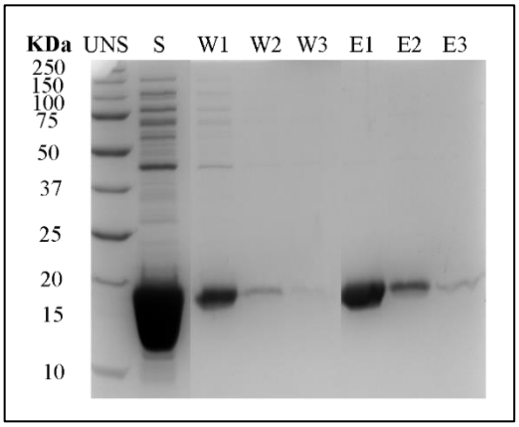


Figure 37. Analysis of the ATP column purification of TolC *V. anguillarum* epitope (D279-D290).
S = column's supernantant; W1, W2 , W3 = washes and E1, E2, E3 = elutions.

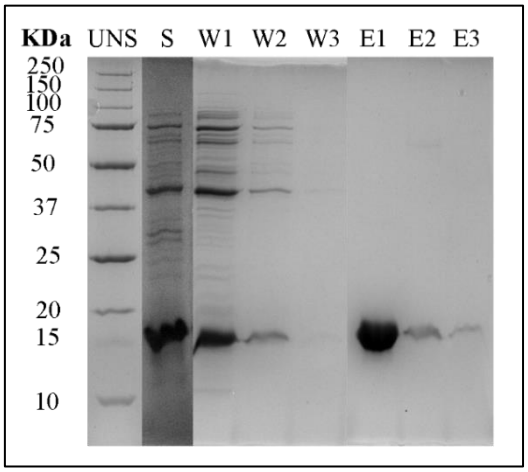


Figure 38. Analysis of the ATP column purification of VceC *V. cholerae* epitope (V127-G139).
S = column's supernantant; W1, W2 , W3 = washes and E1, E2, E3 = elutions.

3.2.2.2. Generation of hyperimmune quail eggs against *V. anguillarum* and *V. cholerae* extracellular epitopes and characterization of target-specific IgY antibodies

After preparation of TolC and VceC recombinant epitopes as described before, immunization protocols were performed. In both protocols, hereafter referred as TV (TolC *V. anguillarum* protocol) and VC (VceC *V. cholerae* epitope protocol), a trio of quails was used. During each experimental protocol the body weight of the animals, as well as their egg-laying capacity were registered and are shown in Figure 39. On the TV protocol, the immunizations were performed in D0(I1), D14(I2), D27(B1) and D42(B2) and on VC protocol on D0, D14, D28 and D42.

On the TV protocol, the three immunized animals were Q1016-28/29/30. During the protocol one of the animals (Q1016-30) was found dead during the experimental protocol (D28), but as the body weight of the remaining animals in both protocols kept increasing, it is very unlikely that the antigen nature was related with such event. On the VC protocol, all the three animals (Q1016-34/35/36) registered different behaviours: Q1016-34 registered a small increase, followed by a decrease in their body weight; Q1016-35 weight remained steady and Q1016-36 weight registered a decrease during the first 60 days of the protocol, showing signs of a recover after that. Concerning the egg-laying capacity, trio Q1016-28/29/30 suffered a decrease around the D30, since the Q1016-30 quail was found dead in D28; this resulted in a rather reduced percentage of laid eggs, about 62.3%, in comparison with VC trio that achieved 74.4%.

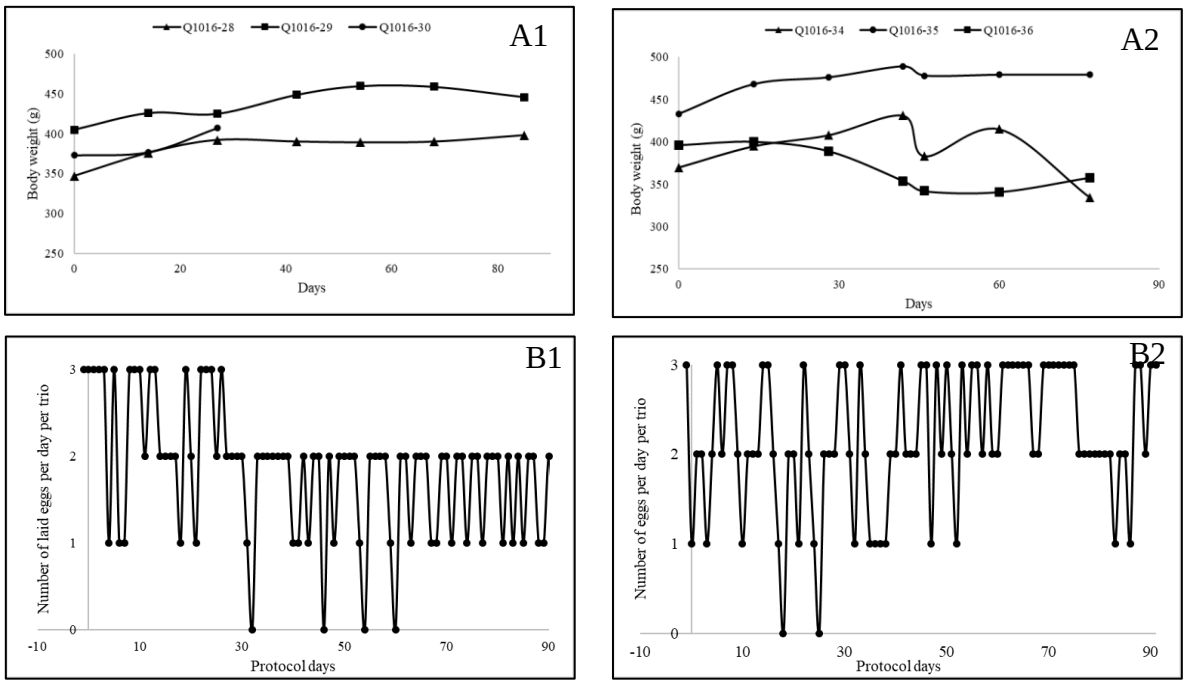


Figure 39. Quails experimental Q1016-28/29/30 (TV protocol) and Q1016-34/35/36 (VC protocol) trios monitoring during the immunization protocol.

A1 and A2) Body weight monitoring of Q1016-28/29/30 and Q1016-34/35/36, respectively.

B1 and B2) Egg-laying capacity of Q1016-28/29/30 and Q1016-34/35/36, respectively.

After analysing the variables described above, an ELISA assay was performed using as primary antibody the WSPF of each of the egg-yolk pools (1 to 24) for each protocol (TV and VC). For ELISA plate coating, each specific purified epitope used for immunization were adsorbed to the plate bottom; the plates were then used to run and analyse the reactivity of each 24 WSPF of the correspondent protocol, as shown in Figures 40 and 41.

The titer results for both TV and VC protocols reveal a typical immune-response profile with both anti-epitope signals increasing at around Pool 7 (D20) and maintaining a moderate/high titer until Pool 24 (D90). The titer evaluation is particularly important in order to evaluate a proper immune-response against the antigen of interest, but also in our particular work, was critical to identity a high titer window were anti-epitopes antibodies could be purified from. The data shows that TV protocol has a more consistent titer evolution, when compared to VC one, where the titer suffers a drastic reduction after boost B2; TV protocol reaches its maximum signal around Pool 8 (D27-D30) and VC protocol slightly later, at around Pool 11 (D39-D42). Interestingly, both high titers were registered even before the end of all planned immunizations events.

For analysis of VC protocol antibodies two different ELISA assays were performed in parallel. In one, plates were coated with VceC epitope (V127-G139) and in the other with TolC

epitope (D279-D290). This last one, was intended to quantify the background signal due to antibodies produced against the carrier protein, instead of against the epitope, and understand the contribution of the carrier for the reactivity. As expected, there was still some signal resulting from the presence of the carrier, though about 3 times less than the obtained when coating with VceC epitope; the similar titer profile observed, suggests a proportional increase when the correspondent epitope is present, besides just the carrier protein.

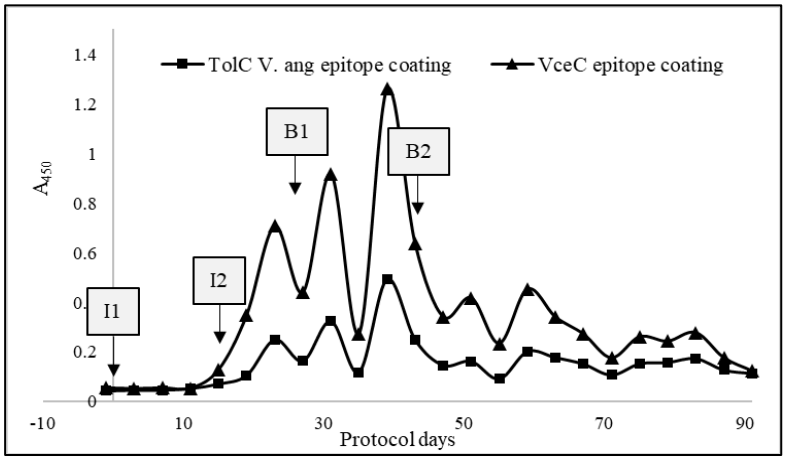


Figure 40. ELISA analysis of egg-yolk IgY antibody (VC protocol) titer against VceC *V. cholerae* epitope (V127-G139) and TolC epitope (D279-D290).

Assay plates were coated with VceC epitope (V127-G139) and TolC epitope (D279-D290) , incubated with WSPF of all the 24 pools (1:1600), the secondary antibody, chicken anti-IgY HRP conjugate was used in a 1:2000 dilution and the absorbance was measured at 450 nm. Each arrow indicates a performed immunization (I)/boost (B).

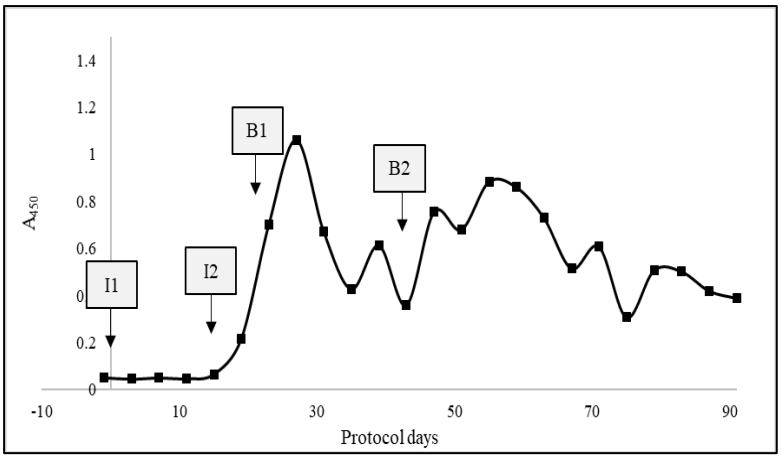


Figure 41. ELISA analysis of egg-yolk IgY antibody (TV protocol) titer against TolC epitope (D279-D290).

Assay plates were coated with TolC *V. anguillarum* epitope (D279-D290) , incubated with WSPF of all the 24 pools (at 1:1600 dilution), the secondary antibody, chicken anti-IgY HRP conjugate was used in a 1:2000 dilution and the absorbance was measured at 450 nm. Each arrow indicates a performed immunization (I)/boost (B).

Based on the ELISA results obtained for both TV and VC protocols, a high titer window was selected in order to proceed for purification of epitope-specific antibodies and their

subsequent characterization. The protocol window ranging from Pool 15 to Pool 18 (D55-D67) was chosen and used for both TV and VC for consistency reasons; at this point both protocols have already passed the 4 immunization/boost events and the titer seems steady. Thus, Pool 15-18 of each protocol was processed to WSPF (antibody sample) and the reactivity/specificity against TolC *V.anguillarum* epitope and VceC *V. cholerae* epitope was studied. Figure 42 shows a SDS-PAGE gel of the different antigen samples used for WB assays. Of note, *E. coli* TolC recombinant epitope, in fusion with the same carrier protein as used for the other epitopes, as well as its respective antibody (WSPF, anti-*E. coli* TolC epitope), were used as an additional control protein/antibody.

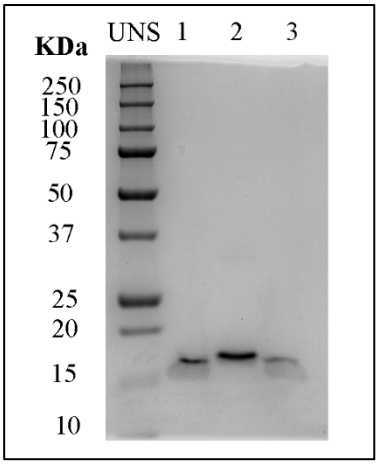


Figure 42. SDS-PAGE 12% gel with the different antigens used in the following WB.

Lane 1: VceC *V. cholerae* epitope (V127-G139) (0.5 µg), lane 2: TolC *E. coli* protein (5 µg) and lane 3: TolC *V. anguillarum* (D279-D290) (0.5 µg). All proteins run using 20 µL, adding 10 µL of the loading buffer.

The performed WB testing the purified WSPF from the two protocols using specific immunogens, TV and VC protocols. These assays clarified about the specificity of the antibodies, since within the membrane probed with WSPF from the VC protocol (1:1000), the signal was more intense on the lane with the VceC epitope, as expected, however the tested antibodies sample also detected the other two proteins, but with a less intense signal. The antibodies sample from a hyperimmune pools of a TolC *E. coli* protocol (existent in the lab) only detected the correspondent TolC *E. coli* protein, used as immunogen in the immunization protocol.

The results shown in Figure 43 demonstrate a clear specificity of each antibody generated, though with certain limitations as explained below. Within the membrane probed with anti-VceC antibodies (Figure 43, A1, left panel) a stronger signal was indeed observed in the lane containing the VceC epitope antigen, though additionally a very mild signal can be seen in the other two, containing *V. anguillarum* and *E. coli* epitopes. This is in agreement with

the ELISA results for VC protocol, demonstrating that a background signal can be found along the immune-response, given the fact that a common carrier protein is used in all recombinant epitopes studied. Nevertheless, and considering the reduced size of the epitope (1-2 kDa) in comparison with the carrier (16 kDa), the contribution of the epitope for the immune response, is still very significant leading to epitope-specific antibodies. Additionally, one should also keep in mind that each immunized animal can have a different immune response towards the same antigen; in this case, the carrier protein seems to have been more reactive in TV protocol, leading to less specific (high background) anti-TolC *V. anguillarum* antibodies, as explained below.

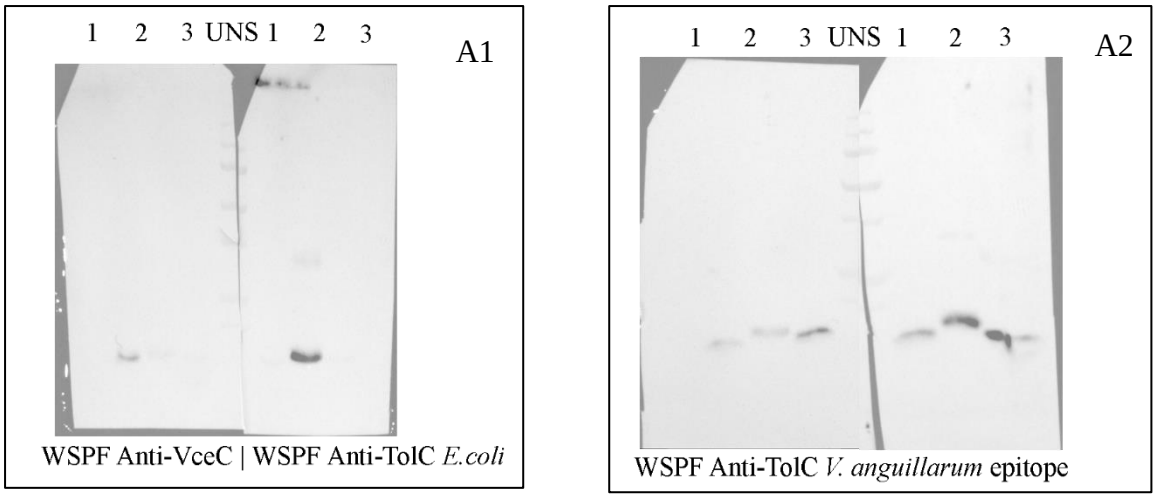


Figure 43. Western blot analysis performed with WSPF from hight titer pools of the TV, VC and *E. coli* TolC epitope protocol.

Lane 1:VceC *V. cholerae* epitope (V127-G139) (0.5 µg), lane 2: TolC E.coli protein (5µg) and lane 3: TolC *V. anguillarum* (D279-D290) (0.5 µg).The proteins were separated on SDS-PAGE 12% gel and transferred to a nitrocellulose membrane.The membrane was divided and probed with WSPF (1.5 mg/mL) (1:1000 dilution). Only right panel of A2 was probed using a dillution of 1:500 of high titer WSPF from TV protocol.

Similarly, to anti-VceC antibodies, anti-TolC *E. coli* antibodies (Figure 43, A1, right panel), previously produced and characterized in the laboratory, also show clear specificity against TolC *E. coli* antigen and only very low signal (or none) against the other two. In contrast, anti-TolC *V. anguillarum* antibodies (Figure 43, A2, right and left panel) presented a clear and unexpected cross-reactivity, with signal present in all the three antigens tested, indicating apparent low specificity. In Figure 43, A2, left panel the signal seems to be slightly more intense in the lane 3 antigen corresponding to *V. anguillarum*; nevertheless, the non-specific signal is much clearer than observed with the other two antibodies.

Finally, it is important to mention that the cross-reactivity/background described for these assays, only reflects the fact that the same carrier protein has been used. This does not imply that the epitope specificity is compromised and additional studies would need to be done to further characterize both specificity and functionality (e.g., antimicrobial activity) of each antibody produced.

4. Conclusion and Future Perspectives

In this work, immunization protocols were performed for two model hosts, namely chickens (*Gallus gallus*) and quails (*Coturnix japonica*), aiming at producing antibodies against bacterial pathogens, specifically *Vibrio anguillarum*, a gram-negative bacterium that infects fishes in aquaculture systems. The main objective of this work was to design strategies to produce avian antibodies (IgY) capable of effectively controlling the associated vibriosis. To this end, the work carried out involved the implementation of different protocols for the use of chickens as model hosts for antibody production, since the quail model had been effectively used in previous protocols in the laboratory. The implementation of several procedures was successfully achieved, including bird manipulation, housing, maintenance as well as bird immunization with recombinant antigens, subsequent antibody titer monitoring and antibody purification.

During this work, several important findings have been gathered. It was observed an increase of the IgY production with the life time of chicken, meaning that older chickens (> 1 year) produce, in a regular basis, a larger amount of IgY antibody per mL of egg yolk. This is an important aspect of the approach and may determine that future immunization protocols in the laboratory should tend to use older chickens to achieve larger IgY production. Furthermore, an intrinsic variability in what concerns immune reactivity of each bird against the same antigen has been observed, and thus larger amounts of IgY do not necessarily imply a higher amount of specific IgY. This was clearly demonstrated with the two experimental chicken pairs immunized with *V. anguillarum* whole-cell extracts (HK protocol) that presented significantly distinct reactivity profiles in ELISA. The antibody titer monitoring along the immunization procedures seems to be critical for the optimization of polyclonal IgY production in hen eggs.

Chickens were immunized to produce IgY antibodies against *V. anguillarum* whole-cell heat-killed extract, and showed relevant functional effects (bacteriostatic effect), supporting their use in therapeutic strategies against vibriosis. The production and functional characterization of IgY antibodies against *V. anguillarum* whole-cell extracts has been previously described in the literature (36). However, the purpose of this work was to establish Anti-*V. anguillarum* whole-cell extract approach as a starting point to support further refinement strategies based on virulence factor specific-targeting. By establishing both chicken egg yolk polyclonal IgY production methodologies against *V. anguillarum*, as well as by implementing bacterial growth assays with such gram-negative model, it is possible to achieve the basic setup for downstream development of different antibodies against *V. anguillarum*. In

this work, it was found that chicken antibodies against *V. anguillarum* whole-cell extracts present a robust bacteriostatic effect, resulting in 50% bacterial growth inhibition as reflected by OD_{600} reduction in growth assays. Additional experiments, including bacterial viability, aggregation and host infection assays after antibody incubation, need to be attempted in the future, particularly to explain and validate the promising bacteriostatic effect observed. Moreover, additional assays are needed to optimize the therapeutic delivery of the antibody in fishes, namely to overcome problems related to the antibody degradation in the water or in the digestive system of the fish. Quails were also model hosts to produce antibodies against *V. anguillarum* whole-cell extracts in order to perform a comparative evaluation of the antibody response within the two avian models. According to the ELISA results, the anti-whole-cell extract specific response seems to reach higher titers in chickens than in quails. However, it is also important to note that the amount of antigen immunized in quails, despite being appropriate to the animal's weight, was inferior when compared with the amount used in chicken immunizations, which can justify the difference in the obtained titers. However, WB analysis suggests that the specificity seems to be rather similar, with both chicken and quail antibodies presenting a similar protein band recognition profile (mostly around 37-75 KDa range) on *V. anguillarum* heat-killed medium samples. As mentioned, to further refine the antibody-based strategy against *V. anguillarum*, quails were immunized to produce antibodies against target immunogens. These were rationalized according to two different working hypotheses: 1) targeting surface-exposed outer-membrane enriched protein content, thus producing a standard membrane preparation (MP) as immunogen and 2) targeting TolC outer-membrane protein channel, responsible for *V. anguillarum* adaptation of adverse environments, thus producing recombinant target-specific protein immunogen.

Anti-MP antibodies results revealed a moderate reactivity on ELISA assays as expected, since this was performed using *V. anguillarum* whole-cell extract coated-plates for comparison reasons. Nevertheless, the specific response showed a clear increase along immunization procedure and interestingly the WB reactivity presents a similar profile to the anti-whole-cell *V. anguillarum* antibodies, suggesting that the MP content is enriched in highly immunogenic protein components. This is in accordance with the literature describing several outer-membrane proteins (OMP), in the MW range of 35-42 kDa, as main surface-exposed immunogenic factors (52).

The production of antibodies against *V. anguillarum* TolC outer-membrane protein channel, was motivated by previous work in the laboratory that showed promising results on immunotargeting the homologous *E. coli* TolC channel as an antimicrobial strategy (54). A

parallel strategy was therefore used to prepare recombinant immunogens to raise specific antibodies that could target *V. anguillarum* TolC outer-membrane channel. Moreover, immunogens of the homologous *V. cholerae* outer-membrane channel, VceC (55) were also prepared to allow a comparative analysis using different Vibrio family strains.

The obtained results clearly demonstrate that both *Vibrio* recombinant immunogens can induce target-specific antibody generation as demonstrated by ELISA and WB assays. Interestingly, the antibody titer obtained with VceC immunogen was considerably higher to that obtained with *V. anguillarum* TolC immunogen, suggesting that the latter might be less immunogenic than the former; even so, the assays should be repeated to exclude any possible effects derived from intrinsic changes in animal immunoreactivity. Other important observation relates to the fact that only the antibodies raised against *V. anguillarum* TolC immunogen, presented high cross-reactivity signal in WB assays, while antibodies against the respective outer membrane channels of *V. anguillarum*, *V. cholerae* and *E. coli*, are compared side-by-side. This could easily result from the fact that the same carrier protein was used for recombinant immunogen production and immunization and additionally some experimental bird groups are more likely to raise carrier-specific antibodies than others, resulting in cross-reaction and background signal; which seems to be the case for anti-TolC *V. anguillarum* antibodies.

Due to time constraints, it was not possible to evaluate the effect of the different quail-derived anti-*V. anguillarum* antibodies by functional assays, as the ones performed with the anti-whole-cell *V. anguillarum* extract. It is still necessary to optimize the bacterial growth assays in the presence of antibiotics and antibodies to properly study the effects of the anti-VceC and anti-TolC *V. anguillarum* as potential antimicrobial adjuvants.

The antibodies produced against *V. anguillarum* using different starting immunogens (Whole-cell extract, Membrane fraction, TolC outer-membrane channel) revealed promising target specificity, thus demonstrating that avian models are indeed very robust to produce antibodies for research, diagnostics and therapy of aquaculture microbial infections.

Concerning the choice of the best avian model host for IgY polyclonal antibody production, there are several aspects to take in consideration. During the present work both chickens and quails were model hosts for antibody production against *V. anguillarum*-derived immunogens. It can be concluded that the avian model host should be chosen in agreement with the final envisaged. Chicken eggs have an average egg-yolk volume of 15 mL; which is 5-fold more than for quails that have an average egg-yolk volume of 3 mL. Moreover, quails reach maturity 3 times faster than chickens, meaning that female egg-laying quails are ready

for immunization procedures in 6-8 weeks (after hatching), but chickens need on average 21-24 weeks. Therefore, chickens are the ultimate avian host when the scale-up production of IgY antibodies is envisioned, whereas quails can compete in fast experimental turn-over, ideal for antigen-specific customization and studies of multiple immunogens in parallel immunization procedures.

5. References

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6. Appendix

6.1.Egg yolk pool mass from chicken experimental pair C0116-03/04 (HK protocol)

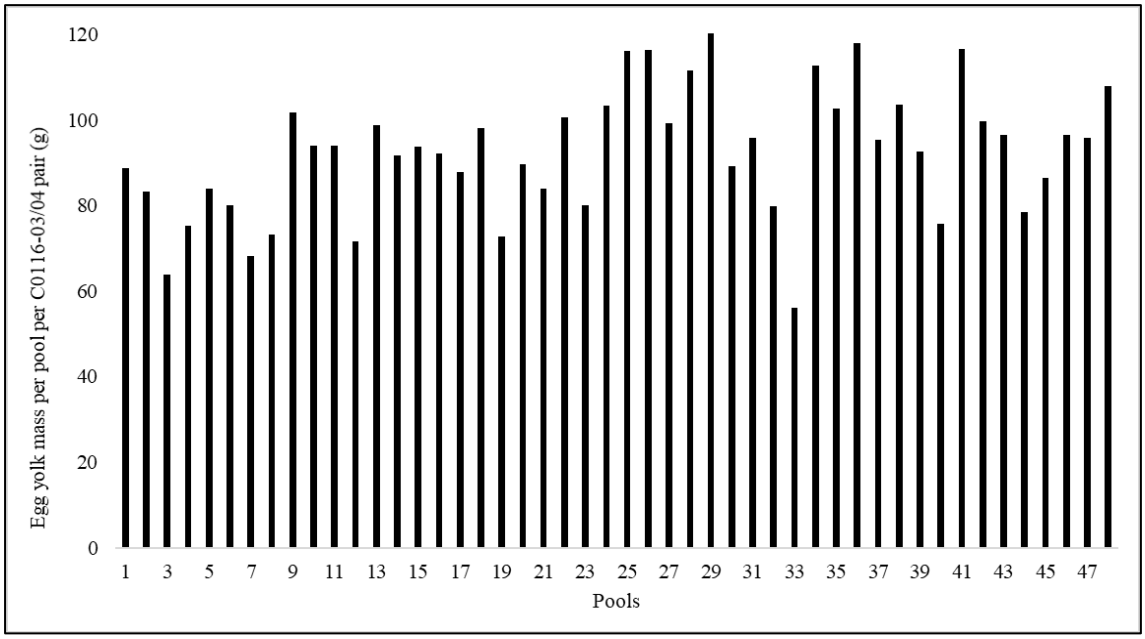

Figure A. 1. Egg yolk pool mass from the pair C0116-03/04, during the immunization protocol.

6.2. Immunization protocol variables from chicken experimental pair C0116-01/02 (HK protocol)

The chicken pair C0116-01/02 was immunized in the same days as the pair C0116-03/04, and the variables 1) body weight and 2) egg-laying capacity monitored during the immunization protocol, which are presented in Fig. A.2.. According to the egg-laying capacity the percentage of laid eggs was also calculated; the value obtained - 66.2% - is below the one obtained for the other chicken pair.

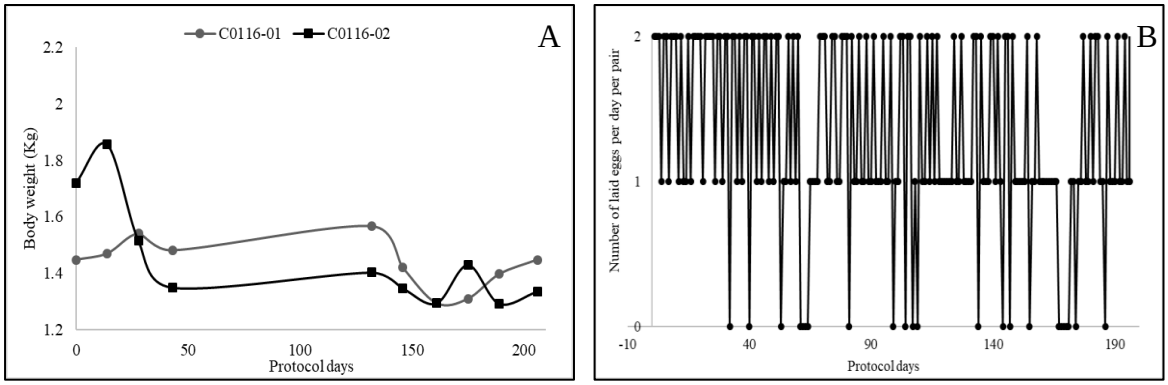

Figure A. 2. Chicken experimental C1016-01/02 pair monitoring during the immunization protocol.
A) Body weight monitoring; B) Egg-laying capacity.

The other followed up variables, such as Egg yolk pool mass and the production of specific IgY against the HK extract are presented in Figures A.3. and A.4., respectively.

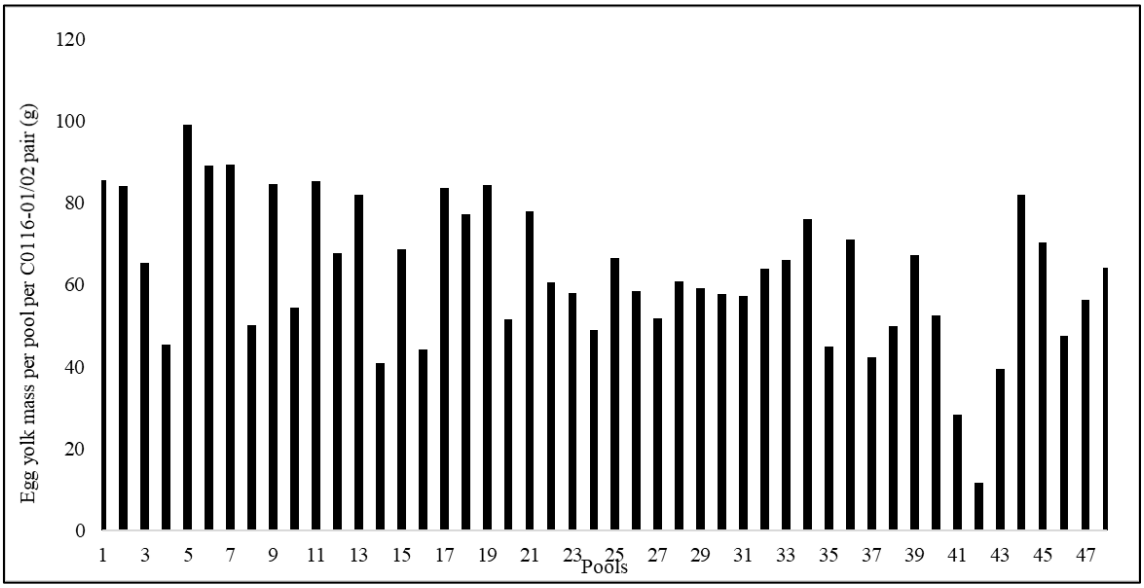

Figure A. 3. Egg yolk pool mass from the chicken experimental pair C0116-01/02, during the immunization protocol.

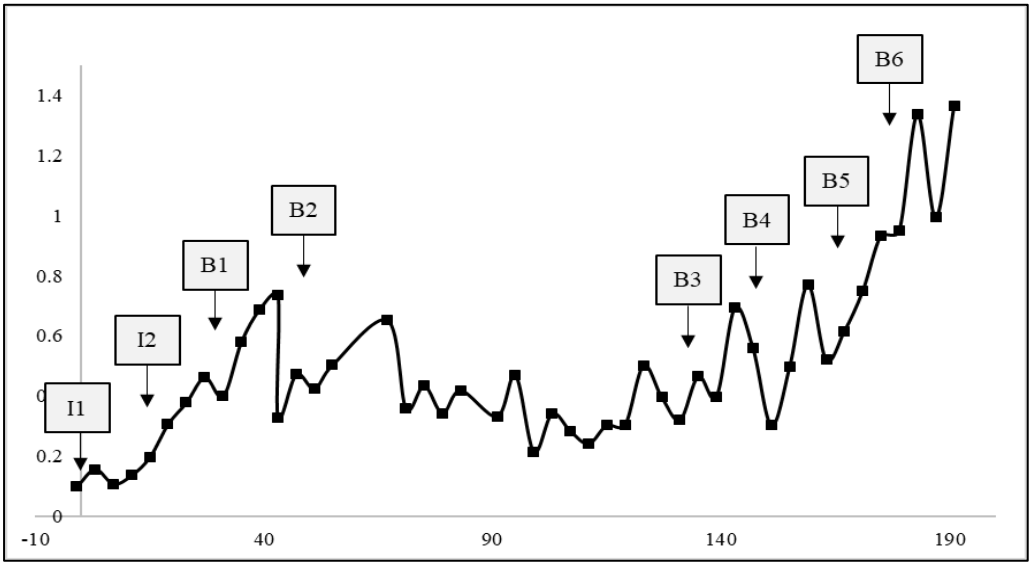

Figure A. 4. ELISA analysis of egg yolk IgY antibody (C0116-01/02) titer against HK *V. anguillarum* extract.

Assay plates were coated with heat killed *V. anguillarum* extract (10^{10} CFU/mL), incubated with WSPF of all the 48 pools (at 1:1600 dilution), as secondary antibody, chicken anti-IgY HRP-conjugated was used at 1:2000 dilution and the absorbance was measured at 450 nm. Each arrow indicates a performed immunization (I)/boost (B).

The obtained ELISA assay revealed a higher production of specific IgY using the produced antibodies of the C0116-01/02 pair, when comparing with the obtained results from C0116-03/04 pair.

6.3. Dot Blot assay

Dot Blot assay was carried out to verify any activity in the first egg yolk sample of the immunization protocol. Pool 1 comprises D-4 to D-1, Pool 3 from D 4 to D 7, Pool 5 from D 12 to D 15, and Pool 8 from D 24 to D 27. In this assay, two types of antigens were used, heat-killed *V. anguillarum* cell extract and heat-killed *E. coli* cell extract, being this last one a positive control test since the egg yolks normally contain IgY antibodies against this type of bacteria.

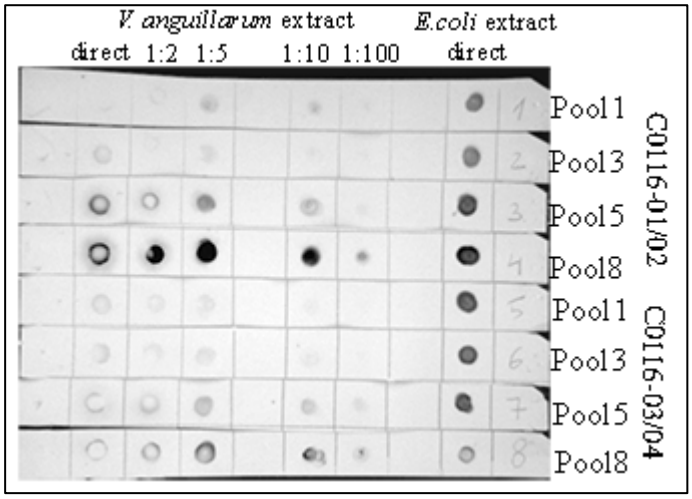

Figure A. 5. Monitoring anti-*V. anguillarum* reactivity in egg yolk samples.

Dot blot assay performed with WSPF of 4 pools from chicken pairs C0116-01/02 and C0116-03/04 against *V. anguillarum* and *E. coli* cells extracts. Different antigen dilutions were used

Figure A.5. clearly shows that all the WSPF from the different pools revealed an intense signal towards the *E. coli* extract, as expected. On the other hand, the signal against *V. anguillarum* extract was increasing with the pools, since the last tested pool (Pool 8) revealed an intense signal towards the direct extract. As expected, the signal decreased with the increasing of the dilution.

With this test, it is also clear that there is variability between the obtained signals within the analysed pools from the different pairs of animals (C0116-01/02 and C0116-03/04). These differences highlight the native variability on the immune response of each bird (thus experimental bird group) against the same antigen.

6.4. SDS-PAGE gel of the HK *V. anguillarum* extract

Figure A.6. represents a SDS-PAGE gel, where different dilutions of the same heat-killed *V. anguillarum* cell extract were run. This gel was important in order to choose from the different dilutions, two samples that could be used in the WB assays. Accordingly, lane 3 and 6 were chosen, these having around 10^{11} CFU/mL and 10^{10} CFU/mL, respectively.

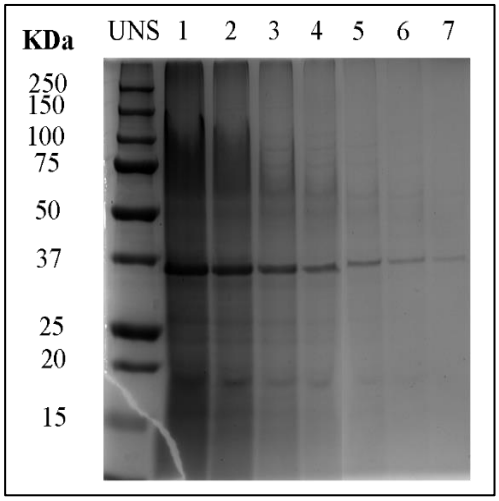

Figure A. 6. SDS-PAGE gel with the different dillutions of the heat-killed *V. anguillarum* medium used in WB:

Lane 1: Original extract, lane 2 to 7: consecutive 2-fold dilutions from the original extract.

6.5. Replicas of functional assays: bacterial growth assays

The bacterial growth functional assays with the purified (Grade III) antibodies from the HK protocol of C0116-03/04 chicken pair were performed in duplicate, in order to obtain more robust results. Thus, Figure A.7. represents the bacterial growth curves in medium with different concentrations of the two antibodies.

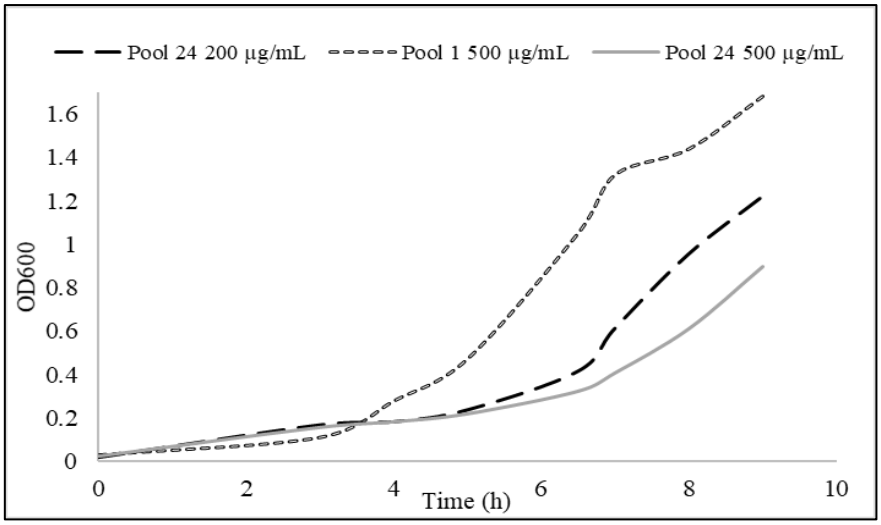

Figure A. 7. Replica of the functional assays of the grade III purified antibodies (Pool 1 and 24), using different final concentrations of the antibody.

The control curve refers to a nornal growth of a *V. anguilllarum* culture without antibodies.

6.6. Egg yolk pool mass from quail experimental pairs: Q0816-55/56 and Q0816-58/59 (HK protocol)

The Figures A.8. and A.9. represent the measured egg yolk mass of each of the pools from the Q0816-55/56 and Q0816-58/59 quail pairs from the HK protocol, respectively.

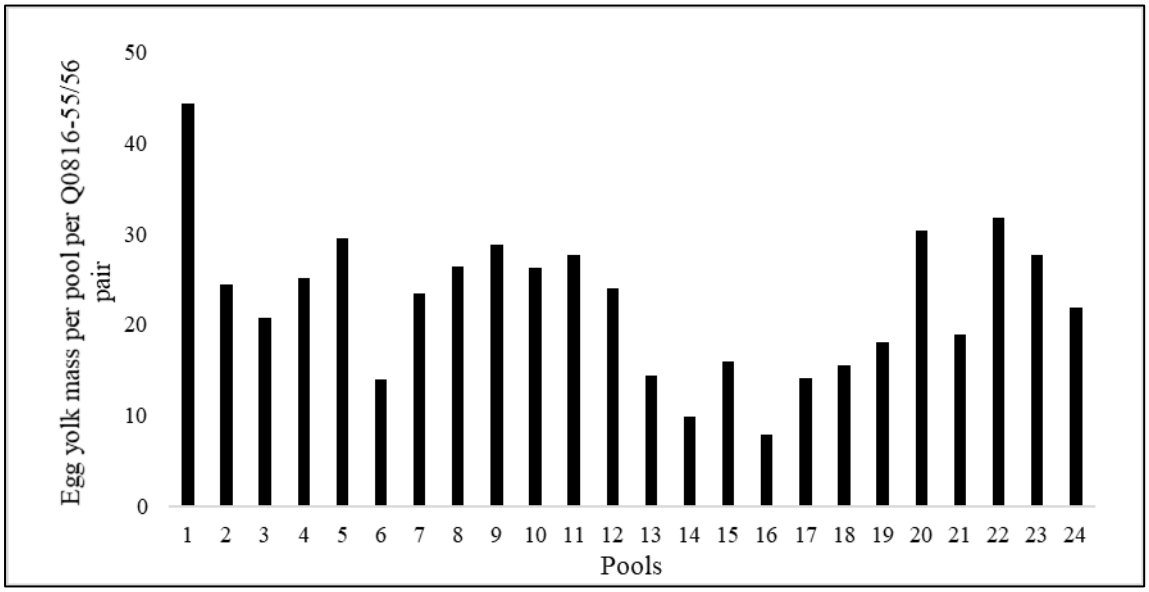

Figure A. 8. Egg yolk pool mass from the Q0816-55/56 pair, during the immunization protocol.

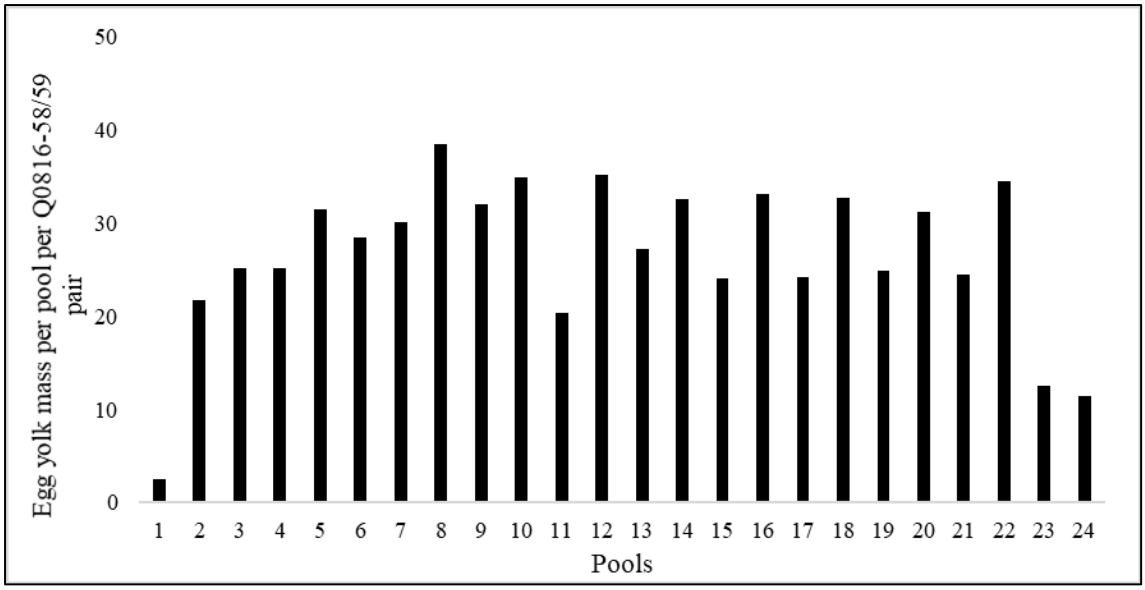

Figure A. 9. Egg yolk pool mass from the Q0816-58/59 pair, during the immunization protocol

6.7. Comparison of the IgY quantities in different animals

According to Pauly et al. (2009) using chickens as hosts to the production of antibodies is advantageous due to the high yield obtained, although the egg production decreases along time (53). To test this fact, the WSPF of the egg yolks from three single eggs from two different chickens were analysed separately using SEC. The two chosen animals had different ages, where the oldest one had approximately one year (Old) and the other one about 6 months (New).

The analysed eggs from the Old chicken were designated Old I, Old II and Old III and are individual eggs from consecutive days. In Figure A.10., SEC runs from the WSPF of the three previous eggs from the Old chicken are shown. This gel filtration presents 5 peaks; the two first peaks (elution volume 2 mL and 5 mL) are simply the result of consecutive runs, since they elute before the void volume of the column used (aprox. 8 mL), where aggregates typically run. No aggregates were observed, thus indicating that the WSPF content seems very stable. The third peak around an elution volume of 12 mL is the IgY elution peak, and the last two represent the additional contaminants present in each of the samples.

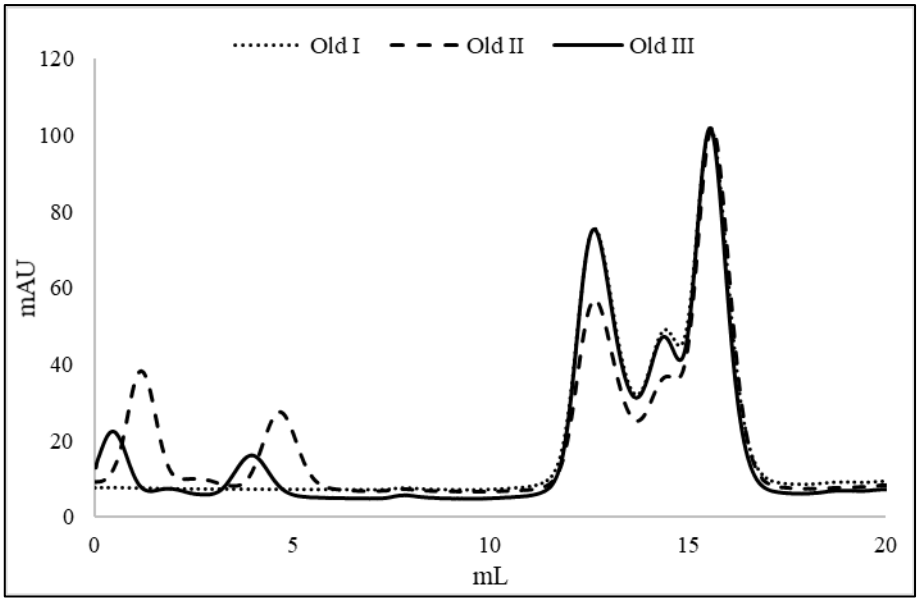

Figure A. 10. Size exclusion chromatography profiles of egg-yolk WSPF obtained from older chickens. For relative comparison purposes, the absorbance of all graphs was normalized considering Old III data (that presented a higher IgY peak) as reference. The relative % of IgY was then calculated (Table A.1.). Old I, II, III represent indiviual egg yolks and thus correponding WSPF.

Eggs from younger chickens (designated as New I, New II and New III) were analysed following the same approach. In Figure A.11., SEC runs from the WSPF of the three eggs from such chicken are shown.

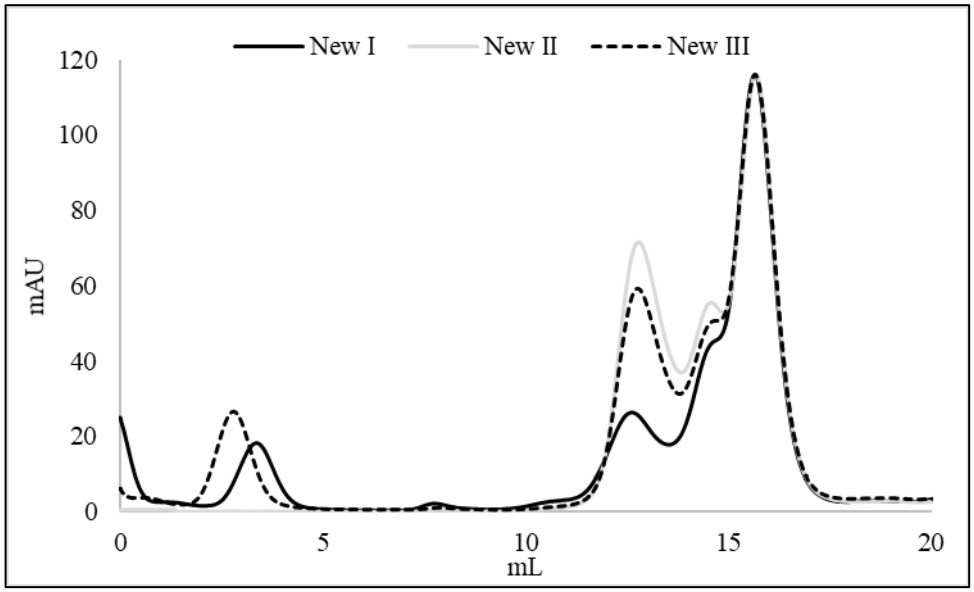

Figure A. 11. Size exclusion chromatography profiles of egg-yolk WSPF obtained from younger chickens. For relative comparison purposes, the absorbance of all graphs was normalized considering New II data (that presented a higher IgY peak) as reference. The relative % of IgY was then calculated (Table A.1.). New I, II, III represent indiviual egg yolks and thus correponding WSPF.

In order to compare the percentage of IgY with the previous analysed samples, it was also calculated the integral of each group of the peaks (IgY and contaminants) to know what percentage of IgY was included in the sample of the WSPF of each of the analysed eggs (Table A.1.).

Table A. 1. Results of the integration of each Old and New SEC runs: relative amounts (%) of IgY and contaminants.

Sample	% IgY	% contaminants
Old I	40.35	59.65
Old II	30.69	69.31
Old III	37.35	62.65
Mean (Old)	36.13	63.87
New I	12.99	87.01
New II	30.96	69.04
New III	28.64	71.36
Mean (New)	24.20	75.80

According to the obtained results of Pauly et al. (2009), older chickens produce larger amounts of IgY per egg. Indeed, the obtained results in this work are in agreement with this, since older chicken had an average percentage of IgY of about 36.13% and younger chicken about 24.20%. On the other hand, the percentage of contaminants was inferior in the WSPF of the eggs from older chicken, namely 63.87% against 75.80% in the younger ones. So, the final decision of using older or newer animals for the immunization protocols depends on the balance between the basal (natural) amount of IgY in the egg yolk and the laying capacity of the animal, because if the animal produces a greater amount of IgY but lays fewer eggs per month, the final obtained IgY in the end of the immunization protocol might be smaller than using an animal that although produces less amount of IgY lays eggs almost every day.

6.8. Egg yolk pool mass from quail experimental trios Q1016-07/08/09 and Q1016 10/11/12 (MP and PL protocols)

Figures A.12. and A.13. represent the measured egg yolk mass of each of the pools from the Q1016-07/08/09 and Q1016-10/11/12 quail trios from the MP and PL protocol, respectively.

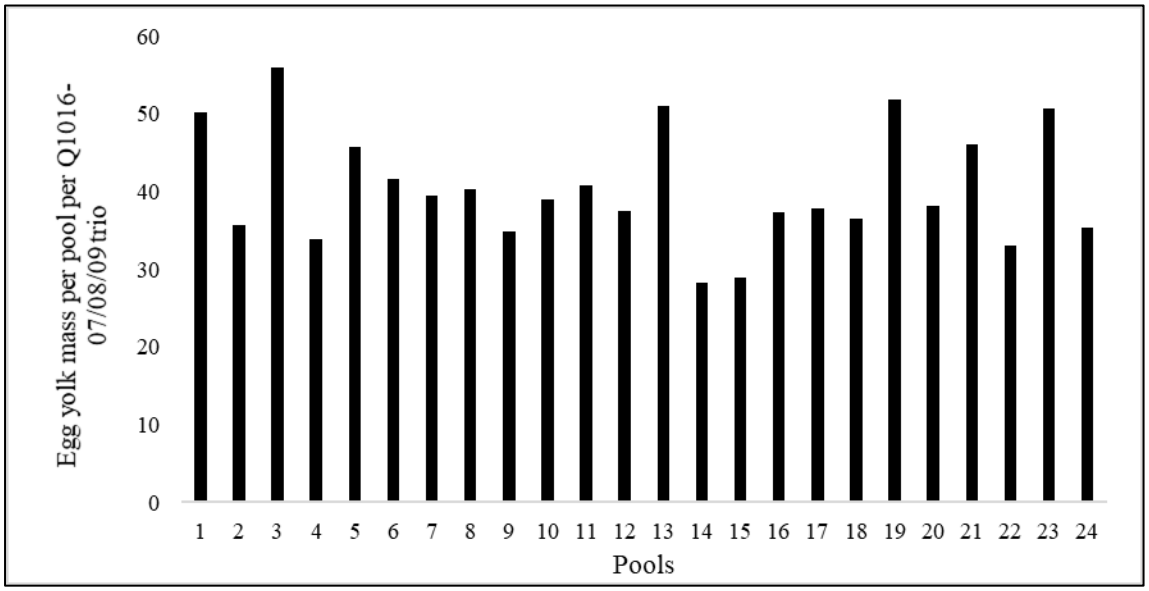

Figure A. 12. Egg yolk pool mass from the Q1016-07/08/09 trio (MP protocol), during the immunization protocol.

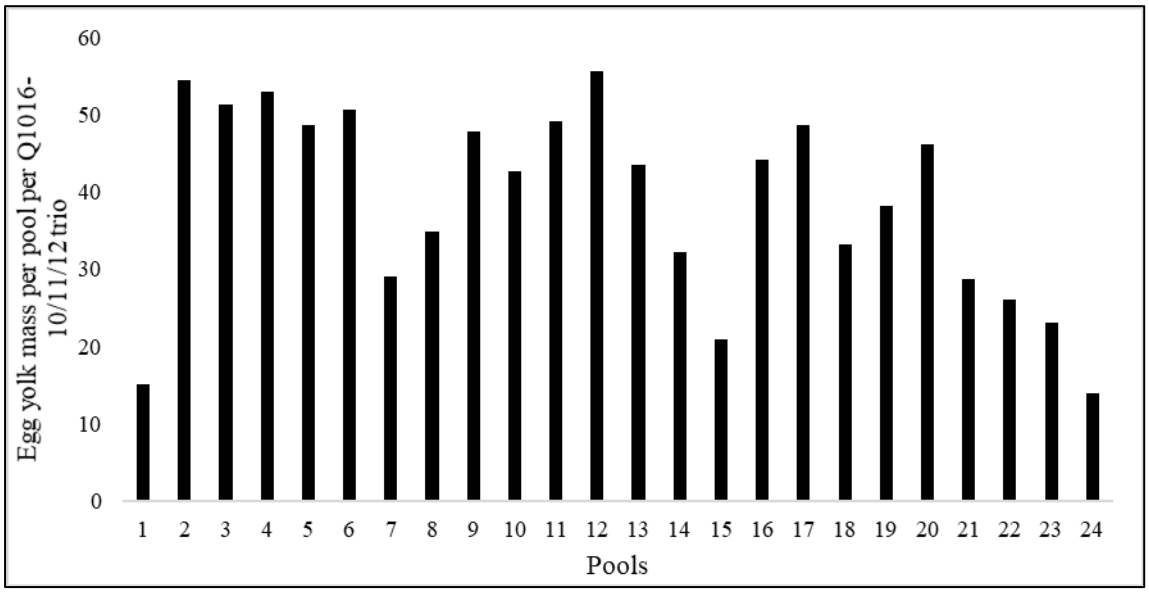

Figure A. 13. Egg yolk pool mass from the Q1016-10/11/12 trio (PL protocol), during the immunization protocol.

6.9. Clustal Omega protein sequence alignment results and Percent Identity matrix

The results obtained from the Clustal Omega alignment using the aminoacid sequence of TolC *E. coli*, TolC *V. anguillarum* and VceC from *V. cholerae* were then analysed through the ESPript 3.0. (Easy Sequencing in PostScrip) software. This program enhances graphically the sequences similarities between the three analysed sequences.

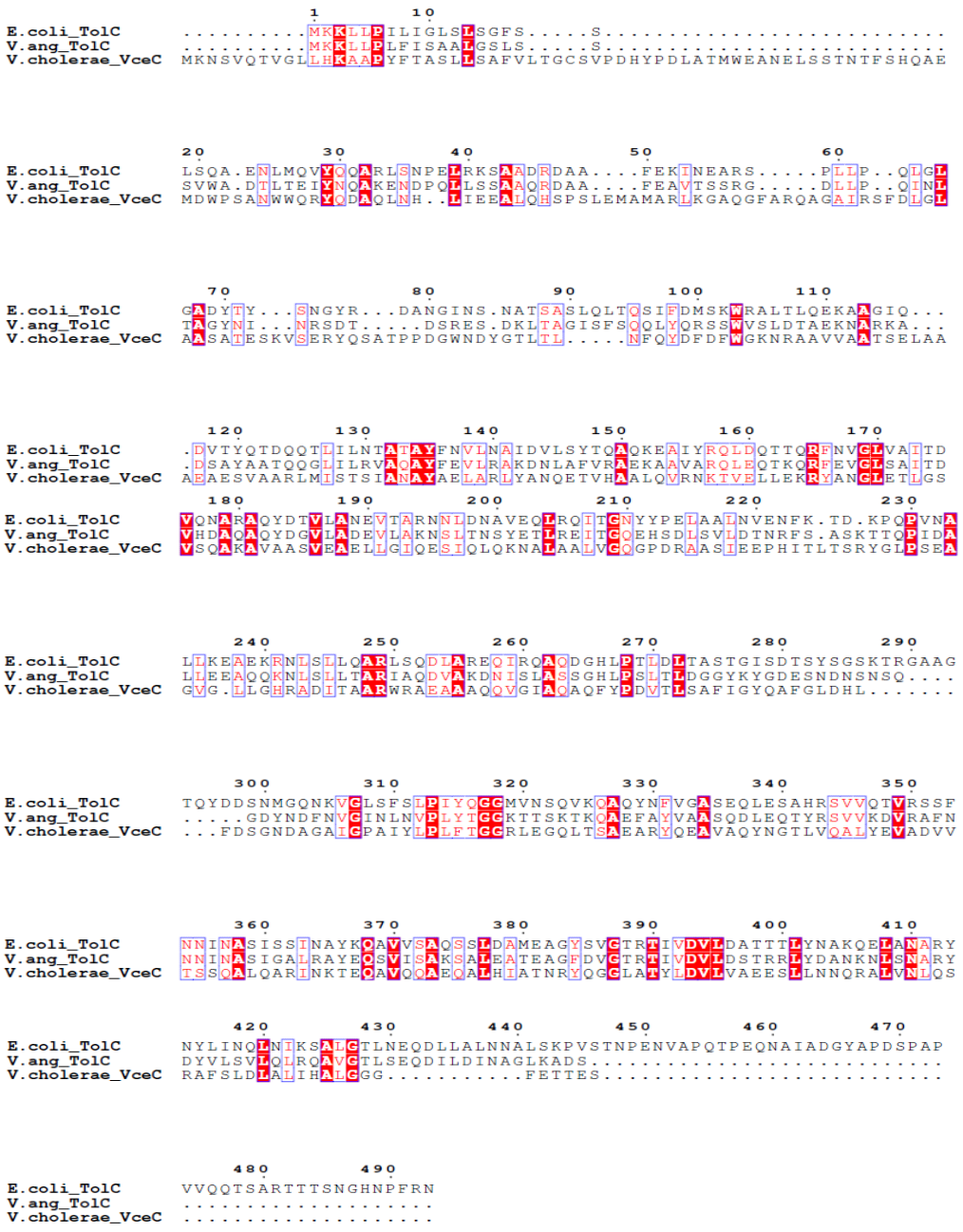

Figure A. 14. Full protein sequence alignment of TolC-like outer-membrane proteins.

Sequence alignment of *E. coli* TolC, *V. anguillarum* TolC and *V. cholerae* VceC outer-membrane proteins. Alignment was obtained in Clustal Omega (57) and ESPript 3.0 (58) was used for graphical representation.

Through the previous software it was also possible to calculate the percent identity between each of the analysed sequences. The result is presented in the Figure A.15.., where VIBCH, refers to the VceC sequence from *V. cholerae*, VIBAN, TolC from *V. anguillarum* and TolC_ECOLI, TolC from *E. coli*.

```
Percent Identity  Matrix - created by Clustal2.1


  1: tr|Q9KS51|Q9KS51_VIBCH          100.00   20.82   21.82
  2: tr|A0A191W654|A0A191W654_VIBAN   20.82  100.00   46.31
  3: sp|P02930|TOLC_ECOLI             21.82   46.31  100.00
```

Figure A. 15. Percent Identity matrix between the VceC, TolC *V. anguillarum* and TolC *E. coli* proteins.