Universidade de Aveiro Departamento de Biologia
Ano 2012

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Proteoma de *Aeromonas* em resposta ao stress

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Ana Cristina Esteves, Professora auxiliar convidada do Departamento de Biologia da Universidade de Aveiro e co-orientação científica da Doutora Ana Sofia Duarte, Investigadora em pós-Doutoramento da Universidade de Aveiro.

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palavras-chave

Aeromonas pisciocola AH-3, expressão de proteínas, patogenicidade, *stress*, temperatura, salinidade, pH

resumo

O género *Aeromonas* é frequentemente associado a uma distribuição ampla no meio ambiente, emergindo como importantes patogénicos de seres humanos. No entanto, a complexidade dos mecanismos de patogenicidade permanece desconhecida e nenhum factor de virulência foi até hoje identificado individualmente como responsável pela patogenicidade. Vários factores de virulência bacteriana podem sofrer uma regulação ambiental por meio de diversos factores abióticos, permitindo a bactérias patogénicas coordenarem a expressão de factores de virulência com a existência de condições favoráveis. Análise de respostas ao *stress* é fundamental, especialmente no contexto de bactérias patogénicas. No entanto, investigações sobre proteínas e mecanismos associados à resistência ao *stress* são escassos ou inexistentes para diversas bactérias, principalmente patogénicos incomuns ou oportunistas, como as *Aeromonas*.

Os objectivos deste estudo são a avaliação do proteoma da bactéria, *Aeromonas piscicola,* em resposta ao *stress* e desvendar proteínas com variação na expressõa, envolvidas na resposta ao *stress*. Para atingir estes objectivos procedeu-se à avaliação do proteoma diferencial sob *stress* causado independentemente por temperatura, pH e salinidade.

Através da avaliação do proteoma extracelular foi possível a detecção da presença de 19 proteases nas diferentes condições abióticas e verificar que, 3,5% NaCl e 40 ºC induzem uma evidente redução na expressão de proteases extracelulares. Análise das proteínas secretadas revelou que: a temperatura induz um aumento na expressão de 23 proteínas (4 a 25 ºC, 9 a 37º C e 10 a 40 ºC) para o pH, 8 proteínas (2 a pH 5.0 e 6 a pH 9.0) apresentam um aumento de expressão e para a salinidade, 16 proteínas (7 a 0% e 9 a 3.5% NaCl). Análise das proteínas intracelulares revelou que: a temperatura induz um aumento na expressão de 22 proteínas (5 a 25 ºC, 9 a 37º C e 10 a 40 ºC) para o pH, 8 proteínas (3 a pH 5.0 e 5 a pH 9.0) apresentam um aumento de expressão e para a salinidade, 11 proteínas (4 a 0% NaCl e 7 a 3.5% NaCl). Adicionalmente, verificou-se a expressão de 3 novas proteínas, uma a temperaturas elevadas, outra quando na presença de temperaturas de stress e outra quando exposta ao stress de pH alcalino.

Aeromonas piscicola AH-3 revelou a existência de um sistema de proteção geral pré-existente aquando na presença de condições de stress. Uma conjunto de marcadores de *stress* para cada condição abiótica e marcadores de stress específicos para a modulação de factores abióticos foram detectados. Foi possível concluir que aquando exposta a condições de stress, causadas quer por alterações climáticas ou quando a invasão de um hospedeiro, *Aeromonas piscicola* AH-3 é capaz de expressar os mecanismos necessários para a sobrevivência e crescimento nas condições abióticas testadas.

keywords

Aeromonas piscicola AH-3, protein expression, pathogenicity, stress, temperature, salinity, pH

abstract

Aeromonas are commonly associated with a widely distribution in the environment and as important emerging pathogens for humans. However the complex pathogenesis mechanisms remain poorly understood, and no individual virulence factor has been identified as responsible for the pathogenesis. Several bacterial virulence factors experience an environmental regulation by many abiotic conditions, enabling pathogens to coordinate expression of virulence factors with the existence of favorable conditions. Analysis of stress responses is of fundamental importance, especially in the context of pathogenic bacteria. However, research for proteins and pathways associated with stress tolerance is still lacking for many bacteria and principally for uncommon or opportunistic pathogens, like *Aeromonas.*

The objectives of this study are the evaluation of the proteome response to stress of *Aeromonas piscicola,* and unravel proteins involved in stress response by the evaluation of differential proteome under temperature, pH and salinity stress.

Through the evaluation of the extracellular proteome it was possible to detect the presence of 19 proteases in the different abiotic conditions and verified that, 3.5% NaCl and 40 ºC induces an evident reduction of the expression of extracellular proteases. Analysis of the secreted proteins revealed that: temperature induced the up-regulation of 23 proteins (4 at 25 ºC, 9 at 37º C and 10 at 40 ºC), as for the pH, 8 proteins (2 at pH 5.0 and 6 at pH 9.0) were up-regulated and for salinity 16 proteins (7 at 0% and 9 at 3.5% NaCl) were up-regulated. Analysis of the intracellular proteome revealed that: temperature induced the up-regulation of 22 proteins (5 at 25 ºC, 9 at 37º C and 10 at 40 ºC), as for the pH, 8 proteins (3 at pH 5.0 and 5 at pH 9.0) were up-regulated, and for salinity 11 proteins (4 at 0% and 7 at 3.5% NaCl) were up-regulated. Additionally, 3 newly expressed proteins were observed, one under high temperatures, another when exposed to global temperature stress and one when exposed to alkaline pH stress.

Aeromonas piscicola AH-3 revealed a global pre-existing system of protection, since when in stress conditions, differential expression of proteins occurs in general with the up-regulation of certain proteins. A series of stress markers for each abiotic condition and furthermore some stress markers for specific modulation of abiotic factors were detected. It was possible to conclude that when exposed to stressful conditions, either caused by climate changes or when invading a host, *Aeromonas piscicola* AH-3 is capable of express the necessary mechanisms for survival and growth in the abiotic conditions tested.

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List of abbreviations

AHL – Acyl homoserine lactones

ATR – Acid tolerance response

BCA – Bicinchoninic acid

BSA – Bovine serum albumin

DNA – deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

GCAT – Glycerophospholipid-cholesterol acyltransferase

GSP – Glutamate starch phenol-red agar medium

HCP – Hemolysin corregulated protein

HSPs – Heat shock proteins

sHSP – Small heat shock proteins

LB – Luria-Bertani medium

LPS – Lipopolysaccharides

MALDI – Matrix-assisted laser desorption ionisation

MS – Mass spectrometry

OD – Optical density

PMF – Peptide mass fingerprint

rRNA – Ribosomal ribonucleic acid

SDS – Sodium dodecyl sulfate

SDS-PAGE – SDS-polyacrylamide gel electrophoresis

TEMED – N,N,N',N'-tetrametiletilenodiamine

TCA – Trichloroacteic acid

TOF – Time-of-flight

TRIS – 2-Amino-2-hydroxymethylpropane-1,3-diol

T3SS or TTSS – Type 3 secretion system

T6SS – Type 6 secretion system

VgrG – Valine-glycine repeat protein G

2D-PAGE – Two dimensional polyacrylamide gel electrophores

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Figure 15. SDS-PAGE of intracellular fraction proteins at 25 (A), 30 (B), 37 (C) and 40 ºC (D) of *Aeromonas piscicola* AH-3.

Figure 16. Differential expression of cell proteins by *Aeromonas piscicola* AH-3 grown at different temperatures; analysis was performed by the determination of the optical density percentage for each protein band calculated as a fraction of the total density of every proteins present.

Figure 17. SDS-PAGE of intracellular fraction proteins at pH 5.0 (A), 7.4 (B) and 9.0 (C) of *Aeromonas piscicola* AH-3.

Figure 18. Differential expression of cell proteins by *Aeromonas piscicola* AH-3 grown at different temperatures; analysis was performed by the determination of the optical density percentage for each protein band calculated as a fraction of the total density of every proteins present.

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I. Introduction

1. The Genus *Aeromonas*

The taxonomy of the genus *Aeromonas*, is complex and in unceasing evolution. In 1965, the genus *Aeromonas* was included in the family *Vibrionaceae*. This classification was applied due to the similar phenotypic features, habitats, and diseases caused by both family of bacteria *Aeromonadaceae* and *Vibrionaceae* (1). Later, phylogenetic studies, suggested that a new family should be recognized, the family *Aeromonadaceae* (2). The family *Aeromonadaceae* consists of Gram-negative bacteria rods, facultative anaerobic and oxidase positive (2).

Until the 70s of the XXth century, the classification of the genus *Aeromonas* was based in phenotypic characteristics, mainly upon growth traits and biochemical evaluations. Focusing on these characteristics, *Aeromonas* were divided in two major groups: the mesophilic group and the psychrophilic group. The mesophilic was constituted by species that grew well from 35 ºC to 37 ºC, linked to a diversity of human infections. The second group, the psychrophilic, was constituted with species that grew better at 22 ºC to 28 ºC and primarily caused infections in fish (3). The two groups were phenotypically distinct and could easily be differentiated by the optimal growth temperature, motility, production of indole, and elaboration of a melanin-like pigment on tyrosine agar. The taxonomy of psychrophilic species has remained stable until now but, in the other hand, the number of mesophilic species has increasingly raised (4).

Therefore, the genus *Aeromonas* has become a challenging group due to the changing phylogenetic relationships, evolving taxonomy, and debated role in certain human diseases. There is a large difficulty in identifying the different *Aeromonas* strains due to the phenotypical heterogeneity and growing number of new described species that are continuing to be described, complicating the identification process (1).

1.1. Habitat

The genus *Aeromonas* is widely distributed in the aquatic environment, isolated from rivers, lakes, ponds, seawater, estuaries, drinking waters and all other water associated environments (5). This intimate association between aeromonads and aquatic environment led to the misunderstand that *Aeromonas* would be only associated to water. However, they are ubiquitous in the environment, being able to live in every environmental niche where bacterial ecosystems endure, especially during hot months (6).

Aeromonas are able to grow on a wide range of temperatures, conductivities, pH values, and turbidities. These include aquatic habitats, vertebrates such as fish, birds or pets, processed food, insects and soils (7, 8). *Aeromonas* can be isolated from different types of food, such as meat, fish, seafood, vegetables and processed foods, representing a potential problem in food, due to the ability to grow at low temperatures, wide pH values and in high concentrations of salinity (9).

The majority of isolates were retrieved after enrichment techniques indicating that concentrations of *Aeromonas* were low. Furthermore, the concentration of aeromonads isolated from drinking waters is lower in comparison to the concentration found in food (10).

1.2. Epidemiology

Aeromonas species are predominantly recognized as pathogenic to poikilothermic (cold-blooded) animals, like amphibians, fish and reptiles. Nowadays there are evidences, without any doubt, of an association with infections on birds and mammals (11, 12).

Several species of *Aeromonas* are well known as fish pathogens, causing hemorrhagic septicemia and systemic furunculosis that can lead to major economic losses in aquaculture. *Aeromonas salmonicida* induces typical furunculosis and causes severe septicemia with resultant mortality especially within cold-water fishes (7). Atypical forms of disease can also occur, characterized by dermal ulcerations and external pathology with or without subsequent septicemia (1). Mesophilic species of *Aeromonas*, most notably *Aeromonas hydrophila*, are able to origin similar assortment of diseases in fishes, including hemorrhagic septicemia, red sore disease and ulcerative infections. *Aeromonas* can also cause a variety of serious diseases in other animals, including ulcerative stomatitis in reptiles, “red leg” disease in frogs, septicemia in dogs, and septic arthritis in calves (13).

Although aeromonads have been described for about 100 years, only in the last decades their role as a causative agent of systemic illness in humans has been recognized; however this role is sometimes speculative and subject of debate (14). Infections are mostly common in young children, immunocompetent and immunocompromised persons (15). The exact incidence of *Aeromonas* infections on a global basis is unknown; even less is known about the possible role for newly described species in human disease (1).

Aeromonas usually are absent in the human gut, so transient colonization of the human gastrointestinal tract by aeromonads is most likely a result of the consumption of contaminated foods and drinking waters (8). *Aeromonas* concentration in water increases

with the warmer months of the year. This rise occurs due to the fact that mesophilic aeromonads grow better at elevated water temperatures, leading to increased concentrations of bacteria in aquatic environments (16). The same seasonal tendency was observed for intestinal and extra-intestinal infections (17). It is possible to assume that increased concentrations of aeromonads in aquatic ecosystems during warmer months represent an elevated risk of developing infection and/or colonization (18).

The presence of aeromonads in aquatic environments is a potential public health risk due to the fact that they are capable to persist in water bodies used by humans, like drinking water distribution pipes (19). In developing countries people use untreated waters from rivers and ponds for drinking. An increased concentration of *Aeromonas* species were reported in the floodwater samples following Hurricane Katrina in New Orleans and were the most common cause of skin and soft tissue infections among the survivors of the 2004 Tsunami in Thailand, suggesting that *Aeromonas* can also pose potential public health threats during natural disasters (20, 21). Additionally antibiotic resistance genes and virulence associated traits of bacteria recovered from water for consumption are similar to those present in clinically important *Aeromonas* strains (19).

In addition to these major pathways, ingestion of contaminated water and food, recreational activities such as boating, fishing, and diving can also lead to infection through traumas (22). As urban sprawl continues to invade rural environments, reports describing human illnesses directly related to bites or other penetrating traumas precipitated by vertebrates, such as snakes, and mammals, as bears, suggest that animals can be a reservoir for the exchange of *Aeromonas* species in the environment (23).

1.3. *Aeromonas* associated diseases

Aeromonas species are emerging as important pathogens in humans, causing a variety of systemic infections that can range from relatively mild illnesses to life-threatening conditions: septicemia, triggered by the dissemination of the organism from the intestinal tract, wound infections, infections of tendons, muscles, and bones, infections of the respiratory tract and less frequent, meningitis, peritonitis, eye infections and hemolytic uremic syndrome (24). Based upon incidence, gastrointestinal tract syndromes are the most common, followed by wound and soft tissue infections and all the other are significantly less frequent (1).

Most of the clinical isolates and human infections, about 85%, are identified as *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas veronii* sv. sobria (25).

The most common diseases caused by *Aeromonas* are gastrointestinal syndromes, a phenomenon seen all around the globe both in industrialized and developing countries and affecting all age groups, principally observed in unhealthy persons and patients suffering from pre-condition diseases, including immune disorders. It can vary from a simple diarrhea to a more severe and invasive diarrhea (26). However a discussion over the role of *Aeromonas* as an etiologic agent is still problematic as there are no vast epidemical outbreaks reported and no adequate animal model is available to reproduce the gastrointestinal syndromes (27).

Another invasive disease frequently associated with *Aeromonas* is septicemia (12). This infection is mostly found in imunnocompromised individuals, trauma patients and some cases in healthy persons have been reported (28). Association of septicemia with a major traumatic event, after a penetrating or abrasion injury that occurs in an aquatic environment or in soil, where *Aeromonas* species are present in high numbers, is less frequent (29). In addition to these risk groups, reports have described *Aeromonas* septicemia in apparently healthy adults with no health disorders (30). However most of these individuals were in advanced age, had substantial alcohol consumption, or were exposed to aquatic environments for long periods (1).

Aeromonas species can be encountered in respiratory tract secretions but are not normally considered respiratory pathogens (25). However, there have been reports where respiratory infections are caused by *Aeromonas* either in healthy individuals and individuals with other medical disorders (31). The most frequent respiratory complication associated with the genus *Aeromonas* is pneumonia (32). In healthy individuals respiratory infections are often caused by near-drowning experiences or inhalation of water. However, such cases are extremely rare with *Aeromonas* linked pneumonia and lung abscesses usually being a result of an underlying medical condition such as cardiovascular disease or alcohol abuse (33).

Aeromonas species can also be associated with a variety of skin and soft tissue infections, ranging from mild topical disorders, as pustular lesions, to life-threatening infections, like necrotizing fasciitis with the potential to cause myonecrosis (34). These diseases usually are a consequence of a traumatic injury followed by an exposition to contaminated environments (aquatic related activities in most of the cases), burn injuries or even caused by zoonotic infections through bites of a variety of animals like snakes and bears (25, 35).

A less frequent type of infections caused by *Aeromonas* are intra-abdominal infections such as pancreatitis, and hepatic abscesses as well as peritonitis. In most of these cases, the source of infection is unclear, but commonly found in middle-aged males with one or more underlying diseases (7).

1.4. Pathogenicity

Pathogenicity can be defined as the capacity of microbial agents to cause disease or simply the ability to cause damage to a particular host (36). Features like the inoculum and route of infection, host susceptibility, and virulence characteristics help in the characterization of the pathogenicity for a given strain. One key feature associated with pathogenicity is virulence and can be defined as the ability to instigate disease at a specified end point (37).

An infection caused by a microorganism involves an interaction between the host and the pathogen (36). Bacteria have their own strategies for survival and proliferation bypassing physiologic barriers, to find a particular niche and struggling against the defence mechanisms of the host's immune system (38). Survival of a microorganism upon entering a living organism is associated with virulence, with the number of microorganisms and with an innate capacity for rapid modification of its metabolic activity or adapting to host metabolism (39). Environmental parameters like pH and temperature have an essential role in the selection and promotion of pathogenic species, suggesting that the process of disease production involves a selection of strains with certain characteristics that favor infection (40). Such tolerances may have evolved in the environment and can be considered to be pre-existing adaptations to relatively extreme environmental conditions outside of the natural host (41).

Clinical manifestations observed in recent studies on *Aeromonas* infections suggest the hypothesis that a complex network of mechanisms is involved in the pathogenesis process (25). Therefore pathogenesis pathways of this genus remain poorly understood, and no individual virulence factor can be identified as sole responsible for the pathogenesis. Yet it is know that *Aeromonas* pathogenicity is not simply a random event (42).

The correct way to understand *Aeromonas* pathogenicity is by the comparison with known disease mechanisms in other traditional Gram-negative bacteria, like *Pseudomonas aeruginosa*, and taking rough conclusions about the virulence factors correlated (37).

Consequently, the identification and study of virulence factors has become the norm for the detection of potentially pathogenic strains (43).

Several problems are associated with the knowledge regarding *Aeromonas* pathogenicity. From the different diseases and syndromes that are possible to correlate with *Aeromonas*, only gastroenteritis and wound infections occur predominantly in healthy people (44). However, it is unknown if only the more abundant or all *Aeromonas* recovered from stools are responsible for intestinal symptoms and which features are fundamental for the process of colonization and infection (45). Therefore it is unattainable to differentiate enteropathogenic and non-enteropathogenic strains. Additionally, there is no existence of well-circumscribed outbreaks and an animal model to replicate this syndrome is still lacking. Wound infections pathogenicity has triggered even less interest as a consequence of the less frequent number of episodes (1).

Regarding infections as gastroenteritis, the supposed route of infection occurs through oral ingestion of contaminated foods or/and water (9, 46). *Aeromonas* cells must survive to the adverse gastric acidity and cause infection in the gastrointestinal tract, competing successfully against autochthonous microorganisms (47). When reach the gastrointestinal tract, a series of interrelated processes occur, including directed locomotion, attachment to gastrointestinal epithelium, biofilm formation, colonization, expression of virulence factors and infection (1). Several genes and virulence factors can be involved in the process, but only a few have been studied in great detail, such as *Act*, proteases, flagella and pili (48).

The pathogenicity of *Aeromonas* wound infections may involve similar processes and virulence factors to those described for another Gram-negative wound pathogens, like *Pseudomonas aeruginosa* (1). The process of infection theoretically resides in three steps: i) attachment and colonization of the wound site; ii) expression of proteases and consequent degradation of host proteins and iii) invasion of deeper tissues. Degradation of proteins can serve as an energy source for subsequent multiplication of bacteria (49). When nutrient sources become depleted, a chemotactic gradient develops, with higher protein concentrations in deeper tissues and lower protein concentrations in superficial areas already colonized by aeromonads, triggering rapid migration of *Aeromonas* into subcutaneous regions (50).

In the next chapter, a more detailed description of some of the virulence factors that have been identified for *Aeromonas*, will be presented.

1.5. Virulence in *Aeromonas*

Virulence of *Aeromonas* is dependent on a numerous variety of virulence factors associated with *Aeromonas*, performing an important role on the host-pathogen interactions and enabling bacteria to cause disease. These virulence factors enable colonization, entry, establishment, replication, cause tissue damage, aid in overcoming host defences and eventually causing cell death (38).

Aeromonas species are capable of producing a diversity of virulence factors, similar to virulence factors described in human pathogens, which play important roles during bacterial infections (51). The mainly virulence factors studied are surface polysaccharides, iron-binding systems, extracellular enzymes, secretion systems, adhesins, motility and flagella (7).

1.5.1. Surface polysaccharides

Surface polysaccharides, capsule, lipopolysaccharides and S-layers, can be considered virulence factors in *Aeromonas* (52).

Lipopolysaccharides (LPS) are glycoconjugate surfaces unique to Gram-negative bacteria and promoters of innate immune responses, ranging from local inflammation to disseminated sepsis. Gram-negative bacteria have two membrane layers separated by a periplasmic space, the inner and the outer membrane (53). LPS is an element of the outer leaflet of the outer membrane and is formed by lipid A, core oligosaccharide and O-antigen (42). The O-antigen mediates pathogenicity by protecting bacteria against host's mechanisms of defense like phagocytosis. The primary activity of LPS is its immune stimulatory potency, triggering the complex clinical syndrome of sepsis when the initial host response to an infection becomes deregulated (54).

The capsule is composed by polysaccharides covering the outer membrane of the bacterial cell forming the outer layer of the cell and participates in bacterial interactions with the environment. Capsule is considered a virulence factor for many pathogens since it prevents phagocytosis, favor interactions with other bacteria and host cells, and acts as a physic barrier against hydrophobic toxins (55).

The S-layer is a surface protein layer that forms the outermost cell envelope composed by a single protein or glycoprotein and constitutes the predominant antigen (56). Due to its exposition on the cell surface, S-layers have been associated to pathogenicity, playing a major role in diverse biological functions such as adhesion, protection against phagocytes, antigenic properties, anchoring and bacteriophage receptor (57). In *Aeromonas*

the S-layer is also associated with the protection against proteases and oxidative damage, increasing the capacity of adherence, contributing to the colonization of intestinal mucosa generating a major resistance to opsonophagocytosis, and therefore enabling systemic dissemination after invasion through the gastrointestinal mucosa (58, 59).

1.5.2. Iron-binding systems

In response to infection, the availability of iron is reduced in extracellular and intracellular compartments. The low availability of free iron impairs bacterial growth and pathogenicity. Nonetheless, successful bacterial pathogens have developed several strategies to sequester iron from their hosts or from insoluble polymers on the environment, overcoming the inaccessibility of iron (52).

Although some evidences that high affinity mechanisms for iron acquisition can act as virulence factors, it is possible that some or even all these mechanisms play a central role in the pathogenesis (60). Therefore, acquisition of iron is recognized as a fundamental process for the survival of pathogens within the hosts, and contributes significantly to virulence (61). In *Aeromonas* high affinity mechanisms able to acquire iron can be divided in two: siderophore-dependent and siderophore-independent mechanisms. Siderophores are peptides with low molecular weight, with elevated affinity and specificity towards iron ions. Siderophore-independent mechanisms consist in chelating systems that acquire iron from molecules that contain heme prosthetic group (62).

1.5.3. Extracellular toxins

It has been described that the genus *Aeromonas* are capable of producing a wide range of exotoxins. These toxins described are not produced by all strains: although some strains may possess genes coding for exotoxins, these are only expressed under specific growth conditions. Two main types of enterotoxins have been described in *Aeromonas*: cytotoxic and cytotonic enterotoxins (52).

Cytotoxic enterotoxins induce degeneration of crypts and villi of the small intestine. These toxins can lead to hemolysis, inducing cytotoxicity and enterotoxicity (51). The toxin binds to a glycoprotein on the surface of the target cell and oligomerizes, forming pores in the host's cell membrane leading water to enter the cells, resulting in turgescence and, consequent cell death (63). In addition to cytotoxic hemolytic enterotoxins, some Aeromonas species are also capable of producing two hemolysins without enterotoxic properties: α-hemolysin and β-hemolysin. The α-hemolysin is synthesized in the stationary growth phase

(64). Cytotonic enterotoxins have mechanisms of action similar to cytotoxic enterotoxins but do not lead to degeneration of the epithelium (52).

1.5.4. Extracellular enzymes

Aeromonas are capable of producing a wide variety of extracellular enzymes, some of which are associated with pathogenesis, including proteases, nucleases, amylases, chitinases and lipases (65). Although for some, their role in pathogenicity is still to be determined, they potentially have an important role in adaptation to the host environment (66).

Microbial proteases are known for their contribution to the metabolic versatility of *Aeromonas* spp. allowing them to persist in different habitats and to the promotion of interactions with other organisms. They can be intracellular or extracellular and their production is greatly influenced by abiotic conditions such as temperature, pH and salts (67). Proteases have three main functions in bacterial physiology: i) regulatory function, which involves activation or inactivation of specific proteins by selective proteolysis; ii) general proteolytic function, which consists in a less specific process, resulting in a massive breakdown of cellular proteins. These degrading mechanisms remove denatured proteins and easing adaptive responses by destroying native proteins no longer required by the cell (68); iii) production of nutrients: proteases are released outside the cell (environment) and digest proteins into small peptides, which bacteria can incorporate to survive (66). Proteases are also involved in the pathogenicity and invasion by direct damaging the host tissue or by proteolytic activation of toxins. Proteases can also contribute to the establishment of an infection by overcoming the initial host defenses, disabling the complement system or providing nutrients for cell proliferation (49).

Lipases have been considered virulence factors since interaction can occur with human leukocytes or by affecting several immune systems functions through free fatty acids generated by lipolytic activity (69).

Nucleases are a group with various roles on the bacterial metabolism, some of them being controversial. In fact, the role of nucleases as virulence factors is not well elucidated (66).

1.5.5. Quorum sensing

Some Gram-negative bacteria regulate their virulence factors as a function of population cell density, by other means, through quorum sensing. Quorum sensing is a mechanism that allows bacteria-to-bacteria cell signaling through acyl homoserine lactones

(AHL), which diffuse freely across bacterial membranes (70). When a certain bacterial density is obtained, AHL act as cofactor of transcriptional regulators, allowing coordinated gene expression in an entire bacterial population. This coordinated gene expression concerns survival genes and more importantly, genes coding for virulence factors and biofilm formation (71).

Quorum sensing has been reported in *Aeromonas* spp., and it is a subject of intensive investigation in many bacteria. As an example, protease production can be inhibited by quorum sensing in *Aeromonas salmonicida* strains, as their expression can be associated with high cell densities and may therefore potentially be controlled by Quorum sensing (72).

1.5.6. Biofilm

Aeromonas spp. capable of producing biofilm are more resistant to adverse environmental conditions and therefore important for bacterial survival and simultaneous posing a significant threat to animal and human well-being (73). This features may perform a significant role in the pathogenicity of *Aeromonas*, but are still speculative (74). As an example genes enconding Mg^{2+} and Co^{2+} transport system were suggested to have a role in swarming capability and therefore, in adherence and biofilm formation (74).

1.5.7. Secretion systems

Gram-negative bacteria in order to transport proteins to the cell surface or/and to the extracellular space developed varied secretion systems (75). Type II (T2SS), III (T3SS) and VI (T6SS) secretion systems play a critical role in *Aeromonas* virulence, being activated upon contact with target cells (76-78). They are responsible for delivering proteins directly into the host cells cytosol, the so-called effectors (52). These bacterial proteins injected into the host cells, directly interfere with the host biological processes (79).

The T2SS is used by *Aeromonas* spp. to export proteins like amylases, proteases, as well as virulence factors (77).

The T3SS is a complex system involved in pathogenicity that directly injects proteins from the bacterial cytoplasm trough the inner and outer membrane of the bacterial envelope to the extracellular medium or directly into the eukaryotic cells (78). The T3SS contains three different types of proteins: structural components forming needle-like structures, known as injectisomes; secretion substrates, the effectors and chaperones that assist and protect structural and effector proteins during transport (80). The injectisome is usually induced upon contact with the host cells and allows the transportation of effectors into the eukaryotic

cytosol. The signal that allows effectors recognition and secretion or translocation into the host cells is unknown, although various theories have been suggested. The T3SS expression can also be induced by environmental signals that mimic conditions encountered in the host, such as temperature (81).

A T6SS operon was identified and characterized in *Aeromonas hydrophila*. T6SS specific effectors are toxins that are directly released in host cells and induce cytotoxicity and subsequent apoptosis (82). The T6SS constitute a phage-tail-spike-like injectisome, with the same purpose T3SS of translocations effectors into host cells (76).

1.5.8. Adhesins

The bacterial capacity to adhere the host mucosa is a critical step in the initial stage of the infection process, easing the invasion and colonization of the host (83). Bacterial structures, as pili, flagella and other adhesion factors play an important role in the initial stages of infection as they enable bacteria with motility and adhesion capacities (69). Two classes of adhesins that allow bacteria to bind to specific receptors on the eukaryotic cell surface have been described in *Aeromonas* spp.: filamentous adhesins, associated with filamentous structures and non-filamentous adhesins, associated with proteins of the outer membrane or other structures (84).

One of the filamentous adhesins structures on the bacterial surface known is pili. Attachment to host cells mediated by pili has been described as a potential colonization factor in *A. hydrophila* and *A. veronii* biovar sobria (85). Macromolecules like the LPS and other outer membrane molecules are also considered non-filamentous adhesins (86).

1.6. *Aeromonas piscicola*

The genus *Aeromonas*, as referred before, is a group with a difficult taxonomy due to complex phylogenetic relationships. One of the most evident difficulties is the definition of species from *A. hydrophila* complex, which includes *A. hydrophila*, *A. bestiarum*, *A. salmonicida* and *A. popoffii* (3). The difficulties occurred due to the fact that 16S rRNA gene is highly conserved and sequence analysis of this gene was unable to differentiate members of these species. For example, until 2009 *Aeromonas piscicola* was denominated *Aeromonas hydrophila* AH-3 (87).

Aeromonas piscicola growth occurs between 4 and 37 ºC, being the optimal growth temperature between 25–30 ºC in the presence of 0–3% NaCl (w/v) (87). Some virulence

factors have already been described such as the capability of producing a capsule, lipopolysaccharides, and a functional T3SS, presence of the gene cluster for T6SS and the presence of flagella, fimbriae and pili. The virulence factors described are commonly associated with many Gram-negative pathogenic or opportunistic pathogens (88-91).

2. Stress Response

Stress is a difficult condition to define, because it depends on the definition of what is a non-stressful condition and on what is considered to be the cause of stress. Optimal growth has been regarded as the condition that allows cells to maintain an optimal internal composition and homeostasis. Stress is usually viewed as the lack of homeostasis (92). In one level, the term stress refers to those situations that restrict or prevent growth. In another level a stressful condition is one that differs significantly from the previous growth conditions (93).

Aeromonas spp. are known for their environmental adaptability, being able to grow over a wide range of abiotic conditions such as temperature, pH and salinity (25). This capability of survival is an intriguing biological problem with applications toward understanding bacterial pathogenicity (40). In fact, as stated before, the survival of a microorganism upon entering a living organism is associated with the number of bacterial cells but also, with an innate capacity for rapid modification of metabolic activity in response to host metabolism (39).

In the presence of an adequate concentration of nutrients and optimal growth conditions of temperature, pH, oxygen levels, salinity and other abiotic factors, bacteria will grow at a maximum growth rate. Changes in these conditions may affect the growth rate constituting stress for the bacteria (94). Many of these stress conditions constitute part of the host defence system used to prevent or limit bacterial infections. Depending on the stress levels, bacteria are able to develop adaptive responses, allowing its survival and growth. This capacity of bacteria to sense and adapt to stress conditions is crucial for their survival (95).

Accordingly, several bacterial virulence factors are subjected to an environmental regulation by abiotic conditions, enabling pathogens to coordinate expression of virulence factors with the existence of favourable conditions. Identification of environmental signals responsible for triggering virulence factors has allowed the identification of virulence mechanisms and their characterization (96).

2.1. Thermal stress

Bacteria can grow in a broad range of temperatures. Some are able to grow in temperatures high as 90 ºC, known as thermophilic, while others are able to grow in temperatures below 0 ºC, known as psychrophilic. However the majority of bacteria grow in

less extreme temperatures such as 20 ºC to 40 ºC, known as mesophilic bacteria, that include *Aeromonas* spp. (96).

Mesophilic bacteria are capable of producing a group of proteins called heat shock proteins (HSPs). These proteins play an important role in the pathogenesis of certain hosts and are required for cell growth or survival at elevated temperatures compared to the temperature of their natural habitat, i.e. thermotolerance (97). Therefore heat-shock response is a regulatory system that allows the bacteria to adapt to high temperature changes in the environment (98). Without the ability to produce this unique class of proteins, bacteria would be incapable of producing disease when exposed to increased temperatures, as these proteins are essential for survival of bacteria (99). The production of heat shock proteins in response to different temperatures has been described in environmental *Aeromonas* strains (100).

2.2. Salinity stress

The concentration of solutes in the extracellular medium, like salts, has a critical role in the microbial survival and growth. Regarding the majority of bacteria, low osmolarity is the optimal growth condition. Bacteria maintain a high cytoplasmic concentration of solutes in comparison to the extracellular medium (101). When in hypotonic conditions, such as a decrease in salt concentration, bacteria suffer a rapid intake of water from the extracellular medium to the cytosol resulting in the swelling of the cell and possible lysis. At the same time, cell wall prevents the swelling - turgor pressure. When in hypertonic conditions, water tends to flow to the extracellular medium, and the cell volume is reduced (102). In Gram-negative bacteria regulation of the total osmotic solute pool in the cytoplasm and the relative level of solutes in the periplasm maintain the turgor pressure. The function of bacteria cell wall in response to osmotic stress is to maintain turgor pressure, preserving cell viability (101, 103).

In most halotolerant microorganisms, the osmotic balance is provided by small organic molecules that are either synthesized by the cells or taken up from the medium when available. This strategy is known as compatible-solute strategy. It does not involve the need for specially adapted proteins, and provides a high degree of flexibility for organisms to adapt to significant variations in external osmolarity (104).

Aeromonas spp. are halotolerant, being able to grow in salt concentrations such as the existent in seawater (~3.5% NaCl). However, *Aeromonas* are preferentially found in aquatic environments with low salt concentrations, such as rivers or estuaries. Studies suggest that

salt represses the production of proteases in *Aeromonas* and this fact may influence the survival in conditions with high salinity concentrations like seawater (105).

2.3. pH stress

Bacteria are able to grow in a wide range of pH values. Acidophilic bacteria can survive in environments with pH values as low as 1.0, alkalophilic bacteria grow in environments with pH value as high as 11 and neutralophilic bacteria grow at pH values closer to neutral, between 5.0 and 9.0 (106). Despite this, some neutralophilic bacteria are able to survive and growth under extreme acidic or basic conditions (47, 107).

Pathogenic microorganisms ingested upon consumption of food and water must face an acid pH in the stomach. Therefore an adaption to the low pH is essential to the survival and consequent development of disease. It has been demonstrated that the major mechanisms used by Gram-negative bacteria to control internal pH during growth involves the modulation of the primary proton pumps as well as the K^+/H^+ and Na^+/H^+ antiporters. Other way bacteria respond to a low pH is by the production of enzymes that are involved in the conversion of acidic metabolites to neutral metabolites (103, 106, 107).

In order to *Aeromonas* induce gastrointestinal syndromes, they must be able to adapt to low pH; for accomplishing this, one potential mechanism used is acid tolerance response (ATR). ATR in *A. hydrophila* requires new or increased synthesis of some proteins, followed by the decreased synthesis of other proteins. This suggests that the new proteins (acid shock proteins) may have a significant role in protecting bacteria in low pH conditions (40). ATR proteins may protect cells by several mechanisms: newly synthesized proteins may allow bacteria to maintain internal pH values close to neutral; DNA binding proteins may play a role in adaptation to acid by preventing or repairing DNA damage (108).

3. Microbial proteomics

Through the study of the genome it is not possible to determine the levels of protein expression, their location at sub-cellular level, biological function or which proteins are being produced at a given moment (109). For each growth condition, differences can be observed in the expression, localization or even in the modification of proteins. Therefore, it is not possible to evaluate complete proteomes, but rather the proteome of a microorganism at a certain point in the cell cycle and under certain environmental conditions (110).

The best way to define proteomics is that it is the study of proteins expressed by a genome under a given set of conditions; all the proteins encoded by the organism's genome constitute the proteome of an organism (111). The study of the proteome is essential for understanding functional pathways, expressed phenotypes and to stipulate biological roles for proteins in the context of a complex system (110).

The proteome is very complex, being composed by thousands of proteins. A strategy to struggle against this is the fractionation of the proteome in sub-cellular fractions, such as the extracellular, membrane or intracellular fractions (110). This approach provides additional information about the identified proteins with respect to their biological localization. At the same time, it will facilitate the study of proteins that are present in low abundance (112).

Different sub-cellular fractions have advantages as each fraction provides different information. Intracellular proteins may provide a better understand over the biological mechanisms of the bacteria. Membrane and extracellular proteins are the link between the microorganism and its host and may help to define the mechanisms of pathogenicity (113). The study of membrane proteins can be difficult due to their intrinsically hydrophobic nature and also to the number of trans-membrane proteins leading to difficulties in solubilizing these proteins (114). Additionally, the analysis of secreted proteins can also be difficult due to the presence of contaminating proteins from undefined sources (115).

A method to study the proteome of a certain bacteria is through comparative proteomics. This approach has the objective of comparing the proteome of one microorganism under different conditions, allowing the visualization and identification of proteins that show differential levels of expression (up-regulated, down-regulated or even newly synthetized proteins (116). Comparison can also be performed between pathogenic strains and non-pathogenic strains with the intention of identifying pathogenesis-associated proteins. This strategy provides insights to the physiology of the microorganisms, therefore

allowing to determine which proteins are involved in pathogenicity and also in mechanisms like environmental adaptation (110).

Experimental approaches in proteomics have suffered an increasing development, in the variety of techniques and equipment, allowing the effective separation, identification and quantification of proteins. However for studying proteins several common steps have to be accomplished: protein extraction, quantification, separation and identification (110).

To accomplish protein extraction, several protocols are available, according to the specific fractions of the proteome that is going to be investigated, as well as to the microorganism. After extraction, quantification is essential for comparing samples. For protein separation the most common technique is two dimensional polyacrylamide gel electrophoresis (2D-PAGE) in which proteins are separated according to their isoelectric point and their molecular weight. Other technique used is SDS-PAGE where proteins are separated only according to their molecular weight (117). The major advantages of 2D-PAGE over SDS-PAGE are the high resolution of separation and the identification of modifications in the proteins after translation. In the other hand, SDS-PAGE is less laborious and less expensive (118). After separation of the proteins, the intensity of each protein is detected in the gels and recorded using a suitable software (119). Nowadays, protein identification is achieved mainly by mass spectrometry analysis. The protein is cleaved into peptides by a protease (usually trypsin) and peptide masses are determined by Matrix Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (MALDI-TOF) allowing a protein mass fingerprint (PMF) to be generated; these values are used to search in a database and a match can be used to identify the protein (120).

II. Objectives

Since mesophilic *Aeromonas* are implicated in human diseases, as opportunistic pathogens in most of the cases, it is important to understand the underlying mechanisms responsible for causing disease.

Aeromonas spp. are recognized for their high plasticity in adaptation to different environments (5). To survive to extreme and rapidly changing conditions, bacteria must sense the changes and then respond with appropriate alterations in gene expression and protein activity (95). Abiotic environmental conditions have consequences in the success of host-pathogen interaction, since survival of a microorganism infecting an organism is intrinsically associated with a capacity for rapid modification of adapting to host environmental conditions (39).

Attending to these facts, a question rises: is it possible for the wide plasticity to be related with the increasing potential of *Aeromonas* as a threat public health? Therefore, identification of mechanisms controlling the biological processes that allow bacteria to adjust to and invade the host is an important scientific challenge. Identifying specific mechanisms that contribute to microbial survival under rapidly changing conditions will probably provide insights into stress response systems and therefore possible mechanisms responsible for causing disease (92).

Until now a systematic study on the expression of proteins of *Aeromonas* for different abiotic conditions is still lacking, including for *Aeromonas piscicola* AH-3, known virulent specie of the genus. Therefore there are no concrete insights on the mechanisms that contribute to causing disease and microbial survival regarding this *Aeromonas* sp.

In order to achieve insights on the possible mechanism refereed before, the main goal of this study will be: evaluation of the response proteome to stress of *Aeromonas piscicola* AH-3, and unravel proteins involved in stress response by the detection of differential protein expression.

To achieve this goal the differential proteome under stress will be evaluated. The extracellular fraction and intracellular fraction of the proteome will be evaluated independently and proteins that are up or down-regulated in the different abiotic conditions.

III. Material and Methods

1. Bacterial Strains

The strain used in this work, *Aeromonas piscicola* AH-3, was kindly supplied by Dr. Juan Tomás (University of Barcelona, Spain).

2. Culture Media

All the commercial culture media were prepared according to the manufacturer instructions. Immediately after the preparation of media, these were properly sterilized. The procedure of sterilization consisted on the heating of the medium until it reached a temperature of 121 ºC, with a pressure of 15 psi for 15 minutes.

Table 1. Commercial culture media composition.

	Chemical composition	**Quantity per Litre**
Glutamate Starch Phenol-red Agar Medium (GSP; Merck)*	Sodium-L(+)glutamate	10.0 g
	Soluble Starch	20.0 g
	KH_2PO_4	2.0 g
	$MgSO_4$	0.5 g
	Phenol red	0.36 g
	Agar-agar	12.0 g
Luria-Bertani Medium (LB; Merck)**	Peptone from casein	10.0 g
	Yeast extract	5.0 g
	NaCl	10.0 g

* pH of 7.2 +/- 0.2 at 25 ºC
** pH of 7.0 +/- 0.2 at 25 ºC

When necessary, for the study of abiotic factors, medium preparation followed the following procedure:

- Medium pH adjustment:

After medium preparation, and previously to the sterilization procedure, the medium was heated. After agar dissolution, the pH was adjusted to the pH value pretended with HCl and NaOH.

- Medium salinity adjustment:

Instead of using a commercially prepared culture medium, the culture medium was prepared by adding independently each chemical (according to Table 1), to the appropriated volume of distilled water. The quantity of NaCl used changed according to the percentage of salinity required. Medium was sterilised as described above.

3. Growth conditions

The strain was cultivated on a GSP plate and stored at 4 ºC. Before inoculation for any experiment, a pre-inoculation was performed. From a GSP plate growth, one colony was inoculated in 5 mL of LB medium at 30 ºC overnight with an agitation of 180 rpm. The inoculation was proceeded with a volume of the pre-inocule corresponding to an OD of 1 at 600 nm. Subsequently the volume of pre-inocule was inoculated in 35 mL of LB at the desired temperature, salinity or pH.

4. Growth curves

The effect of temperature, salinity and pH on *Aeromonas piscicola* AH-3 were studied for the following abiotic conditions:

Temperature: 25 ºC, 30 ºC, 37 ºC and 40 ºC;

Salinity: 0%, 1%, 2%, 3.5%, 5% and 7% of NaCl;

pH value: 3.0, 5.0, 6.0, 6.5, 7.0, 7.4, 8.0 and 9.0;

For evaluation of temperature, the incubation temperature changed accordingly to the value desired to evaluate, maintaining the standard medium pH and salinity (as described in Table 1). To evaluate the effect of pH alteration, the pH value of the media changed accordingly to the desired value, maintaining an incubation temperature of 30ºC and the standard medium salinity (Table 1). The evaluation of salinity proceeded by changing NaCl concentration of the medium accordingly to the desired value, maintaining an incubation temperature of 30 ºC and the standard medium pH (Table 1).

Growth curves were performed by measuring the OD values at 600 nm at selected times, after the inoculation, for an amount of determined hours. Growth curves were built using the software GraphPad Prism 5 (GraphPad Software, San Diego California USA).

5. Protein extraction

5.1. Intracellular protein extraction

Intracellular protein extraction was carried out according to Ebanks et. al (81) with alterations, as described below.

1. Grow the strain in 35 mL of LB at 180 rpm; standard conditions were 30 ºC with no adjustment to pH or salinity of LB;
2. When the OD at 600 nm is at the desired value, centrifuge the culture for 10 min at 8000 g, 4 ºC;
3. Wash the pellet 4 times in 10 mL of Washing Buffer (1x PBS 0.1 M, pH 7.4) at 4 ºC;
4. Add 1 mL of denaturing solution[1] (without 2-mercaptoethanol) to the pellet;
5. Sonicate the cells for 2 min (cycles of 2 s with 3 s of interval and a fix cycle of 30 %) in ice;
6. Centrifuge for 40 min at 20000 g at 4 ºC to obtain a pellet with the insoluble components;
7. Collect the supernatant;
8. Store at -80 ºC until analysis.

5.2. Extracellular protein extraction

1. Grow the strain in 35 mL of LB at 180 rpm; standard conditions were 30 ºC with no adjustment to pH or salinity of LB;
2. When the OD at 600 nm is at the desired value, centrifuge the culture for 10 min at 8000 g, 4 ºC;
3. Remove 20 mL of the supernatant;
4. Add 20 mL of TCA/acetone (20 %; 1/1; v/v) with DTT (0.14 %, w/v) to the supernatant and homogenize;
5. Incubate the solution at -20 ºC for 24 h, for protein precipitation;
6. Centrifuge at 10000 g for 20 min, 4 ºC;

[1] See SDS-PAGE protocol, chapter 7 of Material and Methods

7. Discard the supernatant;
8. Wash the pellet with 5 mL of cold acetone;
9. Centrifuge at 10000 g for 10 min, 4 ºC;
10. Carefully remove the acetone;
11. Repeat steps 8-10;
12. Wash the pellet with 5 mL of freeze acetone (80 %; v/v);
13. Centrifuge at 10000 g for 10 min, 4 ºC;
14. Carefully remove the acetone;
15. Re-suspend the pellet with 200 µL of denaturing solution[1] (without 2-mercaptoethanol);
16. Store at -80 ºC.

5.3. Extracellular protein extraction for proteolytic activity detection

1. Grow the strain in 35 mL of LB at 180 rpm; standard conditions were 30 ºC with no adjustment to pH or salinity of LB;
2. When the OD at 600 nm attains the desired value, centrifuge 1 mL of the culture for 10 min at 8000 g, 4 ºC;
3. Remove the supernatant and discard the pellet;
4. Store the supernatant at -80 ºC.

5.4. Protein quantification

Protein quantification was performed using the BCA Protein Assay kit (Thermo Scientific Pierce). The method is based on the biuret reaction: reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline medium. Detection of Cu^{+1} is achieved by a sensitive and selective colorimetric reaction using a reagent containing biocinchoninic acid. This reaction displays a strong absorbance at 562 nm that increases proportionally with protein concentration. Rate of the color development is sufficiently slow to allow large numbers of samples to be assayed at the same time.

A series of dilutions of known protein concentration (standards) are prepared and assayed alongside the samples, the protein concentration of each sample is then determined based on the standard curve created by the standards.

- **Microplate procedure**:

Procedure requires a volume of 10-25 µL of protein sample, however, it offers less capability in lower interfering substances and detect low levels of protein concentration.

1. Prepare the solutions for the standard curve by diluting the contents of a bovine serum albumin (BSA) ampoule (2 mg/ml) in multiple microtubes using the ultrapure H_2O as diluent (Table 2).
2. Prepare the BCA Working Reagent mixing 50 parts of reagent A with 1 part of reagent B.
3. Pipette 50 µL of each BSA standard solution and unknown sample replicate into the correspondent test tubes;
4. Add 200 µL of BCA Working Reagent and agitate the plate approximately for 30 s;
5. Cover the plate and incubate it at 37 ºC for 30 min;
6. Wait to reach room temperature;
7. Read the absorbance at or near 562 nm in the subsequent 10 min;
8. Elaborate the standard curve to determine the concentration of proteins for each sample.

Table 2. Preparation of standard curve for microplate procedure.

Microtube	Volume of Diluent (µL)	BSA Volume	BSA final concentration (µg/mL)
A	0	150 µL of stock	2000
B	62.5	187.5 µL of stock	1500
C	162.5	162.5 µL of stock	1000
D	87.5	87.5 µL of dilution B	750
E	162.5	162.5 µL od dilution C	500
F	162.5	162.5 µL of dilution E	250
G	162.5	162.5 µL of dilution F	125
H	200	50 µL of dilution G	25
I	200	0	0 (Blank)

- **Test tube procedure**:

Slight alterations were introduced to the original protocol, as described below:

1. Prepare the solutions for the standard curve, dilute the contents of a BSA ampoule in multiple microtubes using ultrapure H_2O as diluent (Table 3).
2. Prepare the BCA Working Reagent mixing 50 parts of reagent A with 1 part of reagent B;
3. Pipette 50 µL of each BSA standard solution and unknown sample replicate into the correspondent test tubes;
4. Add 1 mL of BCA Working Reagent to each tube and mix well;
5. Cover the plate and incubate it at 60 ºC for 30 min;
6. Cool down to room temperature;
7. Read the absorbance at or near 562 nm in the subsequent 10min;
8. Prepare the BCA Working Reagent mixing 50 parts of reagent A with 1 part of reagent B;
9. Pipette 50 µL of each BSA standard solution and unknown sample replicate into the correspondent test tubes;
10. Add 1 mL of BCA Working Reagent to each tube and mix well;
11. Cover the plate and incubate it at 60 ºC for 30 min;
12. Cool down to room temperature;
13. Read the absorbance at or near 562 nm in the subsequent 10min;
14. Elaborate the standard curve to determine the concentration of proteins for each sample.

Table 3. Preparation of standard curve for test tube procedure.

Microtube	Volume of Diluent (µL)	BSA Volume	BSA final concentration (µg/mL)
A	350	50 µL of stock	250
B	200	200 µL of dilution A	125
C	225	150 µL of dilution B	50
D	200	200 µL of dilution C	25
E	200	50 µL of dilution D	5
F	200	0	0 (Blank)

6. SDS-PAGE

SDS-PAGE technique is an electrophoresis performed in polyacrylamide gel in the presence of SDS (68). It is commonly used for the determination of the molecular weight of proteins, providing a powerful tool for separating proteins. The gel is a matrix of a polymer of acrylamide, which allows to choose the adequate porosity. The higher the concentration of acrylamide, the smaller the pores of the matrix will be (121).

Previously to electrophoresis, protein conformation (quaternary, tertiary and secondary structures) and charge must be eliminated in order to allow the separation of proteins dependent only on the protein molecular weight. The proteins are mixed with SDS, an anionic detergent that disrupts non-covalent interactions in native proteins, instigating the denaturation of the proteins. In the absence of SDS, proteins with the same molecular mass can have different migrations due to the different charges and tridimensional structures. Complete denaturation of proteins is achieved by chemical (urea and 2-mercaptoethanol) and thermal denaturation (122).

Depending on the size, each protein will migrate differently through the gel: small proteins will migrate rapidly while big proteins will have difficulty to migrate through the gel, migrating slowly. Therefore, it is possible to achieve a separation based on the protein molecular size. When the electrophoresis is completed, the proteins in the gel can be visualized by staining them, revealing the band patterns (123).

The system used was the Mini-Protean® 3 Cell, from Bio-Rad, linked to an Electric Potential Difference generator, PowerPac 300 from Bio-Rad.

6.2. Protocol

Electrophoretic separation of proteins in a polyacrylamide gel on the presence of SDS was performed following the method described by Laemmli (1970).

- **Sample denaturation:**

1. Add 2-mercaptoethanol to the protein sample (2 %; v/v); vortex to insure complete protein solubilisation;
2. Heat the sample at 100 ºC for 3 to 5 min;
3. Cool down the sample at room temperature;
4. Load the samples in the gel or store at -80 ºC.

- **Electrophoresis:**

1. Assemble the gel supports;
2. Verify if the supports are well assembled pouring ultrapure H_2O;
3. Prepare the separation gel (10 %)[2]:

Ultrapure H_2O	1.45 mL
Tris 1.5 M pH 8.8	1.68 mL
Acrylamide: bisacrylamide 40 %	1.88 mL
SDS 10%	100 µL
Ammonium persulfate 10 %	50 µL
TEMED	2.5 µL

4. Homogenize gently and pour 4.5 mL the gel quickly to each support;
5. Cover the gel with isopropanol 50% (v/v);
6. Wait for the gel to polymerize (approximately 30min);
7. Remove the isopropanol and wash with ultrapure H_2O;
8. Prepare the concentration gel (4%)[2]:

Ultrapure H_2O	1.63 mL
Tris 0.626 mM pH 6.8	0.63 mL
Acrylamide: bisacrylamide 40%	244 µL
SDS 10%	50 µL
Ammonium persulfate 10%	25 µL
TEMED	1.25 µL

9. Homogenize gently and pour the concentration gel over the separation gel and place the comb carefully to avoid the formation of air bubbles;
10. Wait for the gel to polymerize (approximately 30min);
11. Place the gel in the electrophoretic chamber;
12. Fill the electrophoretic chamber with 700-1000 mL of running buffer;
13. Remove the comb, load the samples (always with the same concentration) and add 2.5 µL of molecular marker (Precision plus protein standards, Bio-Rad) in the wells using a Hamilton syringe;
14. In case of necessary add very carefully more running buffer;
15. Close the electrophoretic chamber and start the running at 120 V during 165 min, approximately at a temperature of 4 ºC;
16. After finished the run, incubate the gel for 1 hour in a staining solution, Coomassie

[2] Add the reagents for each gel in the exact order. Immediately before loading the gel to the support, add ammonium persulfate and TEMED; always mix the solution carefully.

Brilliant Blue R-250;

17. Place the gel in ultrapure H_2O to remove the remaining staining solution;

18. Distain the gel by using a distaining solution, until bands become visible in a colorless background;

19. Place the gel in ultrapure H_2O to remove the remaining distaining solution;

20. Digitalize the gel with a densitometer (GS-800 densitometer, Biorad).

6.3. Reagents

Table 4. Preparation of running buffer.

Running buffer	Tris:Bicine (1:1) 100 mM
	SDS 10 % (w/v)

Table 5. Preparation of staining solution.

Staining solution	Coomassie Brilliant Blue R-250 0.4 % (w/v)
	Ethanol 50 % (v/v)
	Acetic acid 10 % (v/v)

Table 6. Preparation of distaining solution.

Distaining solution	Ethanol 25 % (v/v)
	Acetic acid 5 % (v/v)

Table 7. Preparation of denaturing buffer.

Denaturing buffer	2-Mercaptoethanol 2 % (v/v)
	SDS 2 % (w/v)
	Urea 8 M
	Tris:Bicine (1:1) 100 mM
	Blue bromophenol (traces)

7. Zymography

Zymography is a technique for the detection and characterization of proteases in samples (68). The standard method is based on a two-stage technique involving protein separation by a native-SDS-PAGE co-polymerized with a protein substrate (predominantly gelatin or casein), followed by detection of proteolytic activity (124). Like in SDS-PAGE, it has been stated that zymography allows separation based on the proteases molecular size: small proteases will migrate rapidly while big proteases will have difficulty to migrate through the gel, migrating slowly (125). Nonetheless, since proteins are not fully denatured, molecular weight determined by zymograpohy should be considered a rough estimation (68).

After the electrophoresis, removal of SDS from the gel, using a non-ionic detergent, allows the proteases to be renatured. One should take into account that some proteases may not renature and therefore cannot be detected by this technique (125). Later by hatching the gel with a specific buffer at a controlled temperature, proteases are able to degrade the copolymerized substrate. These digested areas (bands) originate a translucent zone in contrast with a blue background after the staining of the gel.

The main variable when performing a zymography is the time of incubations. Increasing incubation time represents an increase of the method sensitivity. However, if incubations are prolonged, protease bands will diffuse and merge, eliminating the possibility of detecting all proteases in the sample.

The system used was the Mini-Protean® 3 Cell, from Bio-Rad, linked to an Electric Potential Difference generator, PowerPac 300 from Bio-Rad.

7.2. Protocol

- **Electrophoresis:**

1. Assemble the gel supports;
2. Verify if the supports are well assembled placing ultrapure H_2O;
3. Prepare the separation gel (10%)*:

Ultrapure H_2O	1.45 mL
Tris 1.5 M pH 8.8	1.68 mL
Gelatine 1 %	250 µL
Acrylamide: Bisacrylamide 40%	1.88 mL
SDS 10 %	100 µL
Ammonium Persulfate 10 %	50 µL
TEMED	2.5 µL

4. Homogenize gently and pour 4.5 mL of the gel quickly to each support (after removing the ultrapure H_2O);
5. Cover with isopropanol 50 % (v/v);
6. Wait for the gel to polymerize (approximately 30 min);
7. Remove the isopropanol and wash with ultrapure H_2O;
8. Prepare the concentration gel (4 %)[2]:

Ultrapure H_2O	1.63 mL
Tris 0.626 mM pH 6.8	0.63 mL
Acrylamide: Bisacrylamide 40 %	244 µL
SDS 10 %	50 µL
Ammonium Persulfate 10 %	25 µL
TEMED	1.25 µL

9. Homogenize gently and pour the concentration gel over the separation gel and place the comb carefully to avoid the formation of air bubbles;
10. Wait for the gel to polymerize (approximately 30 min);
11. Place the gel in the electrophoretic chamber;
12. Fill the electrophoretic chamber with approximately 700 mL of running buffer;
13. Remove the comb, load the sample (always with the same concentration) and add 2.5 µL of molecular marker (Precision plus protein standards, Bio-Rad) in the wells using a Hamilton syringe;
14. Close the electrophoretic chamber and start the run at 120 V for 2 h 45 min,

approximately at a temperature of 4 ºC;

15. Afterwards, submerge the gel in Triton X-100 (0.25%, v/v) at 37ºC for 15min, in order to remove all the SDS at 50rpm;
16. Repeat step 15;
17. Incubate the gel in reaction buffer for 2 h 30 min at 37 ºC;
18. Incubate the gel for 1 h in staining solution, Coomassie Brilliant Blue R-250;
19. Remove the staining solution and wash the gel with ultrapure H_2O to remove the remaining staining solution;
20. Distain the gel with distaining solution;
21. When, digested areas become visible in a blue background, replace the distainig solution by ultrapure H_2O;
22. Digitalize the gel with a densitometer.

7.3. Reagents

Table 8. Preparation of gelatin.

Gelatin	Gelatin 1% (w/v)

Table 9. Preparation of sample buffer.

Sample buffer	Tris 1.5 M pH 8.8
	SDS 10 % (w/v)
	Glycerol 20 % (v/v)

Table 10. Preparation of renaturation buffer.

Renaturation buffer	Triton X-100 2.5 % (v/v)

Table 11. Preparation of running buffer.

Running buffer	Tris:Bicine (1:1) 100 mM
	SDS 10 % (w/v)

Table 12. Preparation of denaturing buffer.

Denaturing buffer	2-Mercaptoethanol 2 % (v/v)
	SDS 2 % (w/v)
	Urea 8 M
	Tris:Bicine (1:1) 100 mM
	Blue bromophenol (traces)

Table 13. Preparation of staining buffer.

Staining solution	Coomassie Brilliant Blue R-250 0.4 % (w/v)
	Ethanol 50 % (v/v)
	Acetic acid 10 % (v/v)

Table 14. Preparation of distaining buffer.

Distaining solution	Ethanol 25 % (v/v)
	Acetic acid 5 % (v/v)

Table 15. Preparation of reaction buffer.

Reaction buffer	Tris 50 mM pH 8.8
	NaCl 5 mM
	$CaCl_2$ 10 mM
	$ZnCl_2$ 1 µM

8. Gel analysis

Gel images were acquired on a Bio-Rad GS-800 Calibrated Densitometer. After scanning of the gels, software Quantity One (Bio-Rad) was used to determine the molecular weight and optical density of each protein.

The molecular weight of the proteins was determined by comparison with the Precision Plus Protein Standard from Bio-Rad, a molecular weight calibration kit.

9. Protein Identification

Proteins separated by SDS-PAGE or by zymography were excised from the gels and subsquently reduced and alkylated before analysis by mass spectrometry, according to Bart Samyn et al. (126). The protocol is described below:

1. Distain the bands overnight with 30 % methanol (v/v) solution;
2. Wash the gel pieces with 150 µL of 200 mM NH_4HCO_3, 50 % acetonitrile for 30 min at 30 ºC in a water bath;
3. Rinse the bands with 30 µL ultrapure H_2O for 10 min;
4. Wash the gel pieces with 150 µL 200 mM NH_4HCO_3, 50 % acetonitrile for 30 min at 30 ºC;
5. Dry the gel pieces in a speedvac at room temperature for 15 min;
6. Reduction of proteins: add 15 µL of 10mM DTT in 7 M GuHCl, 0.3 M Tris (pH 9.0) during 45 min at 55 ºC;
7. Alkylation of proteins: add 5 µL of 55 mM iodoacetamide, 200 mM NH_4HCO_3 (pH 7.0) and incubate the mixture in the dark for 45 min at room temperature (20–25 ºC);
8. Remove excess of salts: wash the gel twice with 150 µL of 200 mM NH_4HCO_3, 50% acetonitrile in ultrapure H_2O during 30 min at 30 ºC;

9. Dry the gel pieces in a speedvac at room temperature for 15 min;
10. Store the bands at -20 ºC.

After this treatment, gel bands were analyzed by MALDI-TOF/TOF, a mass spectrometry approach.

Proteins are digested using trypsin and the resultant peptides are then subjected to analysis by MALDI-TOF/TOF. In this mass spectrometry technique the sample is embedded in a matrix, a crystalline structure of small organic compounds, and placed on a conductive support. Then the support is irradiated with a nanosecond laser beam. The laser energy causes structural decomposition of the irradiated crystal and generates a plume from which ions are extracted by an electric field, resulting in the disintegration of the crystal. Following acceleration through the electric field, the ions drift through a field-free path and finally reach the detector. Ion masses are typically calculated by measuring their time of flight (TOF), which is longer for larger molecules than for smaller ones (120, 126).

The tryptic peptides provide a characteristic peptide mass fingerprint (PMF) unique to each protein, allowing identification of the analyzed protein. Although this approach is useful to identify proteins in simple mixtures, peptide sequence information obtained MS/MS is required to identify individual proteins in more complex samples. Complex algorithms have been developed to aid in this process, starting from peptide MS/MS data whereby peptides are identified by correlating the uninterpreted MS/MS spectra with predicted product ion spectra derived from peptides of the same mass contained in the databases. For proteins not contained within sequence databases, it is necessary to determine partial or complete amino acid sequences using either manual or automated *de novo* peptide sequence analysis methods (120, 126).

IV. Results and Discussion

1. Growth characterization

Recognition of environmental signals responsible for modulation of the expression of proteins is essential for a better understanding and detailed characterization of the mechanisms involved in adaptation and pathogenesis (96). Therefore in order to analyse the response proteome to stress, first is necessary to define which abiotic conditions should be evaluated.

The abiotic factors selected for evaluation were temperature, pH and salinity. These abiotic factors were chose regarding their importance in virulence since they may inhibit the growth of some pathogenic microorganisms (127). Additionally, since the main habitat of *Aeromonas* spp. is water, the changes that this environment experience, due to the predicted climate changes, will have an important impact on bacteria. Principal changes on the water bodies will consist in the acidification of the ocean, already corroborated (128), and the rise of temperature and salinity (129).

To perform this study, *A. piscicola* AH-3 was grown in different abiotic conditions and growth curves were determined. As the objective of this study is the evaluation of *A. piscicola* AH-3 stress response, the strain should be able to grow in the selected conditions. All assays were performed in triplicate.

1.1. Temperature

With the previously knowledge, that AH-3 has a temperature growth range between 4 and 37 ºC and an optimal growth between 25 and 30 ºC (87), the following temperatures were selected to evaluation: 25 ºC, 30 ºC, 37 ºC and 40 ºC.

Temperature 37 ºC was selected since it corresponds to the temperature of the human body and 40 ºC represents the temperature of the human body in a febrile state, possibly in response to an infection. Therefore, for AH-3, upon entering a human host, to be able to survive in and colonize the host, it must successfully adapt to these temperatures.

The results obtained showed that growth occurred at all temperatures evaluated (Fig. 1). The temperature 30 ºC revealed to be the optimal growth condition, as expected accordingly to a previous study on AH-3 (87).

At the temperature of 25 ºC growth was considerably slower than that determined at 30, 37 or even 40 ºC. In fact, at 25 ºC the growth in the exponential phase was slower than in the other conditions tested and the OD_{600nm} was always inferior to the OD_{600nm} obtained at

temperatures of 30 and 37 ºC. Such differences in the growth rate between 25 and 30 ºC temperatures were not expected since the optimal growth should occur between these ranges.

According to Beaz-Hidalgo, R. et al. (87) the maximum temperature at which AH-3 is able to grow is 37 ºC. However, the results obtained in this work showed that growth occurred at 40 ºC and that growth at 37 ºC was similar to the growth at the optimal growth condition (30 ºC). Despite that the results observed were in discordance with the previous study referred, these results are expectable as Schubert RH. et al. (130) and Statner B. et al. (131) showed that low temperature-adapted *Aeromonas* spp. when exposed to elevated temperatures, grew faster when in comparison to species that were already adapted to these temperatures. Therefore this ability would be advantageous for aquatic strains when encountering a homoeothermic host, like humans for example.

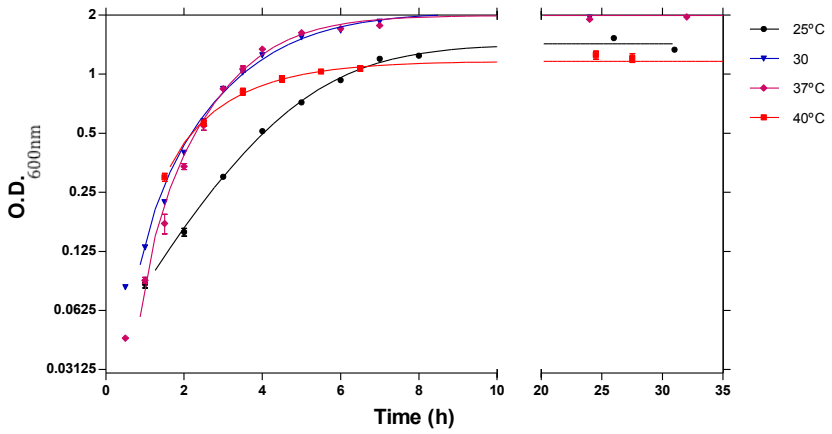

Figure 1. Effect of temperature in the growth of *Aeromonas piscicola* AH-3, presented with media and standard deviation. The growth curves were obtained using the software Graphpad 5.0.

Regarding the results obtained for the temperature of 40 ºC, the exponential phase was similar to that observed at 37 and 30 ºC but the stationary phase was reached earlier with an OD_{600nm} inferior to the one attained at 30 and 37 ºC. Differences between growth curves at 30 and 37 ºC are barely noticed: only in the beginning of the exponential phase a better growth occurs at 30 ºC, being overlapped at the final stage of exponential phase. At the stationary phase the growth rates are equal and OD_{600nm} is approximately the same. Therefore it is possible to affirm that 37 ºC does not represent a major stress condition for

AH-3 while 40 ºC represents a stressful condition in comparison to the other evaluated temperatures.

The results obtained suggest that, to the contrary to what is described in the literature, temperatures of 25 ºC and of 40 ºC can represent stressful environmental conditions for AH-3. Therefore, proteomic analysis was conducted at 25 ºC, 40 ºC and also at 30 and 37 ºC for comparison purposes.

1.2. pH

No data related to the effect of medium pH on AH-3 growth has been described until now. However, it is known that *Aeromonas* are able to grow in a range of pH values from 5.o to 9.0. Some species, such as *A. hydrophila,* are even able to survive at lower pH values. As referred previously, adaptation to low pH is essential to *Aeromonas* as they are a well-known causing agent of gastrointestinal diseases (40). Since the information available regarding the influence of the extracellular pH value on growth of AH-3 is scarce, a survey was conducted, in a range of pH 3.0-9.0.

Regarding pH 3.0, this value corresponds to approximately the pH value of the stomach, while the pH found in the intestinal track is between values of 5 to 7.4 and pH 7.4 represent approximately the pH of the human body tissues (132, 133). Also alkaline pH, up to pH 9, can be found in open wounds and chronic wounds (134). Therefore AH-3, when in an human host, must be able to survive and adapt to these wide pH values (40). The results obtained can be observed in Figure 2.

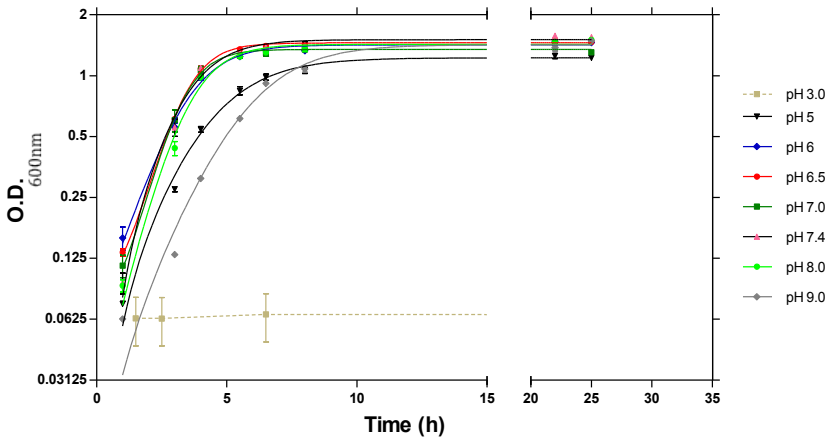

Figure 2. Effect of pH in the growth of *Aeromonas piscicola* AH-3, presented with media and standard deviation. The growth curves were obtained using the software Graphpad 5.0.

Data showed that growth occurred in all pH values without major differences, except for pH 3.0 in which growth was not detected. It is difficult to define the optimal growth pH due to the fact that between the pH range 6 to 8 the growth curves have nearly the same behaviour (same OD values). As pH 7.4 represents an important pH value, from the biological point of view, it will be defined as optimal growth condition. Nonetheless, our data shows that *A. piscicola* AH-3 is able to grow without significant differences in the range of 6.0-8.0, while at pH values of 5.0 and 9.0, AH-3 grew at a slower rates. At pH 5.0 the stationary phase is reached at a lower cell concentration.

Data shows that *Aeromonas piscicola* is able to survive and grow in similar conditions to those existent the intestinal tract. However to reach the intestinal tract *Aeromonas* must survive to the stomach pH. Although it was not possible to detect cell growth at pH 3.0 by spectrophotometry (Fig. 2), the occurrence of aggregates of cells was noticed. These aggregates are most probably a response to stress due to the extremely acidic conditions of the medium, enabling the survival of the bacteria at pH 3.0 and therefore surviving to the stomach pH. In other bacteria these aggregates are associated with an important adaptive strategy to colonize adverse environments (135) and can be also associated with chronic infection (136).

To verify the possibility that aggregates represent a mechanism of AH-3 to survive in adverse conditions and later leave this state and proliferate, the aggregates were dispersed through sonication and cultivated in GSP medium. After incubation it was possible to verify that growth occurred. Therefore it is possible to assume that these aggregates can ensure cell viability through stressful conditions.

The results observed suggest that pH 3.0, 5.0 and 9.0 are stressful environmental conditions in *A. piscicola*, to which it has mechanisms to overcome. Therefore, we chose 3 conditions (pH 5.0; pH 9.0 and the control, pH 7.4) for subsequent proteomic analysis.

1.3. Salinity

AH-3 is known to have a growth range in salinity between 0 and 3% of NaCl (w/v) (87). The percentage of NaCl between 0% and 7% were selected to evaluate the growth. Condition of 1% NaCl represents approximately the percentage of NaCl present in the human body and 3.5% approximately the NaCl concentration present in seawater (105). The results obtained can be observed in Figure 3.

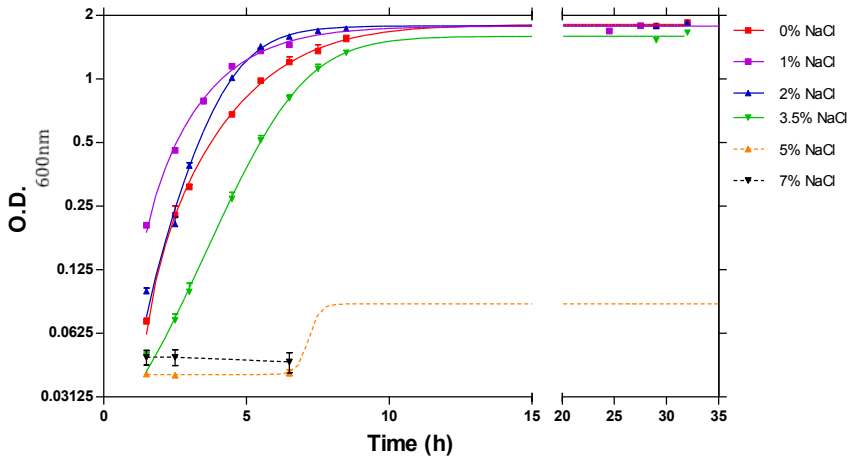

Figure 3. Effect of salinity in the growth of *Aeromonas piscicola* AH-3, presented with media and standard deviation. The growth curves were obtained using the software Graphpad 5.0.

The results obtained show that AH-3 grows at NaCl concentrations of 0%-3.5%, but not at 7%. Condition of 1% NaCl corresponds to the optimal growth, followed by condition of 2% NaCl and 0% NaCl. At 3.5% NaCl, growth occurred slower than at 0, 1 or 2% NaCl, representing a possible stressful condition. These results are according to others studies where *Aeromonas* are predominantly found in freshwater, but can also be found in the epipelagic layer of the sea, most often in estuaries (105).

However, in the description of *A. piscicola* by Beaz-Hidalgo, R. et al., it is referred that growth only occurs between 0 and 3% NaCl, which is not in agreement with our results, as in condition 5% NaCl the growth occurred in the form of aggregates. It was possible to detect viable aggregates similar to those detected at pH 3.0; at 7% NaCl no aggregates were detected. These data suggest that AH-3 is able to survive at concentrations of NaCl higher than those initially described.

Having into account these results, 0%, 1% and 3.5% NaCl were selected for proteomic analysis.

The results obtained for the characterization of AH-3 growth in the different abiotic conditions unravel new insights in the growth range parameters. It was possible to determine that previous study on the characterization of the bacteria were not accurate: maximum growth temperature described was of 37 ºC and the results obtained show that at least growth occurs at 40 ºC; maximum concentration of NaCl described was of 3% but it was verified that normal growth occurs at least until 3.5% and growth in the form of aggregates

occurred at 5%. This characteristic, the capability of producing aggregates was also not described in literature, however it as been already associated to some Gram-negative bacteria. AH-3 is also capable of growing either in acidic pH (at pH 5.0 and at pH 3.0 in the form of aggregates) and alkaline pH (at least pH 9.0).

In resume, AH-3 is able to grow in a variety of conditions, including possible stress conditions that can be found either in the environment or when upon contact with human hosts. When encountering an extreme stress condition has the capability of growth and maintain viability by the formation of aggregates.

2. Proteome characterization

In order to evaluate the response of AH-3 proteome to stress, the proteome was sub-fractioned in extracellular and cellular proteome. The extracellular proteome was characterized by evaluating the proteolytic proteome (by zymography) as well as the total extracellular protein profile (by SDS-PAGE). The cellular proteome was characterized by evaluation of the total cellular protein content (by SDS-PAGE). The methods used for the characterization of the proteome, SDS-PAGE and zymography, were described in the chapter III - "Material and Methods". All the procedures were performed in triplicate.

2.1. Extracellular proteome

Although a number of genes, encoding groups of secreted proteins have been cloned and sequenced, little is known on why *Aeromonas* possesses them in such abundance and variety. While many of proteases-encoding genes so far studied in *Aeromonas* have homologs in other bacteria, some are entirely novel. Such novel extracellular proteins should provide interesting subjects for basic research as well as have potential valuable applications in medicine, agriculture and in industry (66).

2.1.1. Extracellular proteases

Extracellular proteases are involved in the capability of *Aeromonas* to persist in different environments by easing adaptive responses through the destruction of native proteins no longer required by the cell and production of nutrients by the digestion of proteins into small peptides. Extracellular proteases are also involved in the promotion of interactions with other organisms, contributing to pathogenicity, invasion and establishment

of an infection (66), and also in maintaining mutualistic associations, as described for *Aeromonas veronii*, one of the leech symbionts (137).

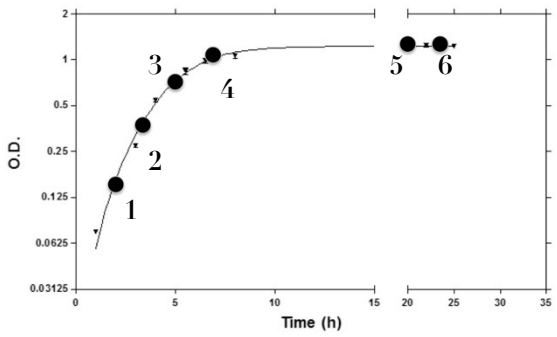

Figure 4. Specific points on the growth curve selected for extracellular proteases evaluation.

In order to carry out the characterization of extracellular proteases we have selected specific time-points during the exponential and the stationary phases. Stress conditions were compared with non-stressful conditions (optimal growth conditions): aliquots of the extracellular medium were withdrawn at the same position on the growth curves, four points during the exponential phase and two points during the stationary phase (Fig. 4).

2.1.1.1. Temperature

The results of the evaluation of extracellular proteases expressed by AH-3 grown at 25, 30, 37 and 40 ºC can be observed in Figure 5.

For each zymography a control sample was used (an aliquot of the 3rd point of growth at 30 ºC), enabling the normalization of the gels. Each gel was digitalized and analyses proceed as described in Material and Methods section. 16 proteases bands were able to be detected with different molecular weights, in the different conditions tested. Results can be observed in table 16.

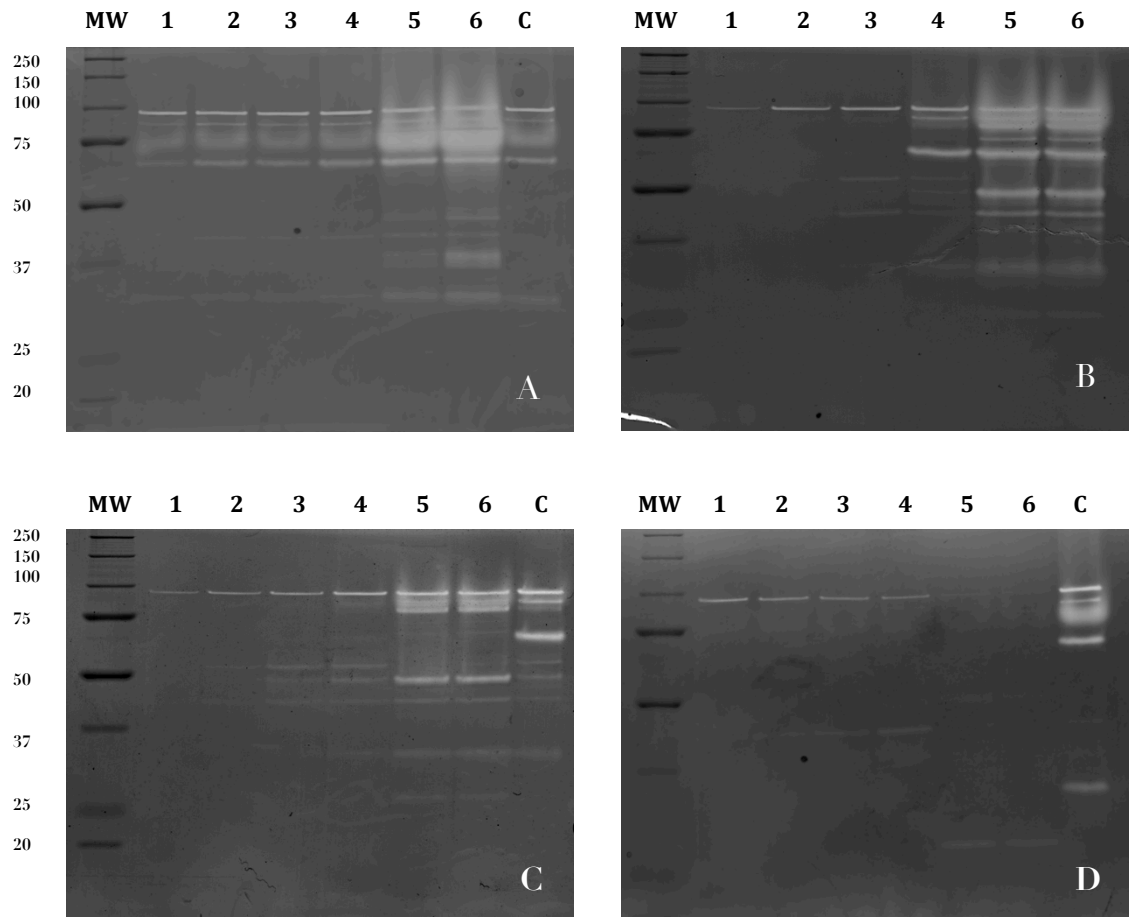

Figure 5. Zymography of extracellular proteases at 25 (A), 30 (B), 37 (C) and 40 ºC (D) of *Aeromonas piscicola* AH-3.

Temperature of 25 ºC is the condition in which more proteases were expressed. Additionally, two proteases bands were only detected at this temperature, at the stationary phase. Temperature of 40 ºC is the condition with a lower number of proteases expressed: only 4 proteases bands could be detected. Besides the proteases detected, it is possible to visualize that intensity of the proteases bands is higher in lower temperatures, having the maximum intensity at 25 ºC and with almost no intensity at 40 ºC, meaning that the concentration of these proteases is lower and/or that their activity is less significant than the concentration/activity of the proteases expressed by AH-3 at lower temperatures. Despite the growth at 25 ºC is lower comparing to the other conditions this did not implied a decrease of the protease expression.

Table 16. Extracellular proteases bands of *Aeromonas piscicola* AH-3 detected for the different temperatures: (-) absence of the protease band; (e) presence in exponential phase; (E) presence in stationary phase.

Molecular weight (kDa)	25 ºC	30 ºC	37 ºC	40 ºC
98	e/E	e/E	e/E	e/E
85	e/E	e/E	E	-
80	e/E	e/E	E	-
72	e/E	e/E	E	-
65	e/E	e/E	E	-
62	E	-	-	-
59	E	E	-	-
54	-	e	e/E	-
49	E	e/E	e/E	E
45	E	E	-	-
43	e/E	e/E	e/E	e
39	E	E	-	-
37	E	-	-	-
33	E	e/E	-	-
32	e/E	e/E	e/E	-
25	-	E	E	E

When comparing the different conditions for differential proteases expression no obvious stress markers for temperature stress could be assigned. Yet it is possible to confirm that temperature plays an important role on the expression of extracellular proteases by AH-3, as with the rise of temperature it was noticeable a downshift on the protease expression and with the decrease of temperature an increase of protease expression occurs.

These results are in concordance with other studies performed for some *Aeromonas* species. Environmental isolates from *A. hydrophila* produced significantly less extracellular proteases when grown at 37 ºC compared to lower temperatures (138). Therefore, these results suggest that growth rate and extracellular protease expression are not directly related, as the slower growth condition registered the highest protease expression (even in comparison with the optimal growth condition) and conditions with similar growth rates to the optimal condition registered a lower protease expression.

The results obtained for the expression of proteases under different temperatures values are also in concordance with previous studies, where environmental changes modulate the expression of proteases (139). The temperature of the human body is

approximately 37 ºC. For this reason, strains that produce virulence factors at this temperature are likely to be more significant as human pathogens, as is the fact that AH-3 is capable of producing extracellular proteases at 37 ºC (7).

2.1.1.2. pH

The results of the evaluation of extracellular proteases expressed by AH-3 at pH values 5.0, 7.4 and 9.0 can be observed in the Figure 6.

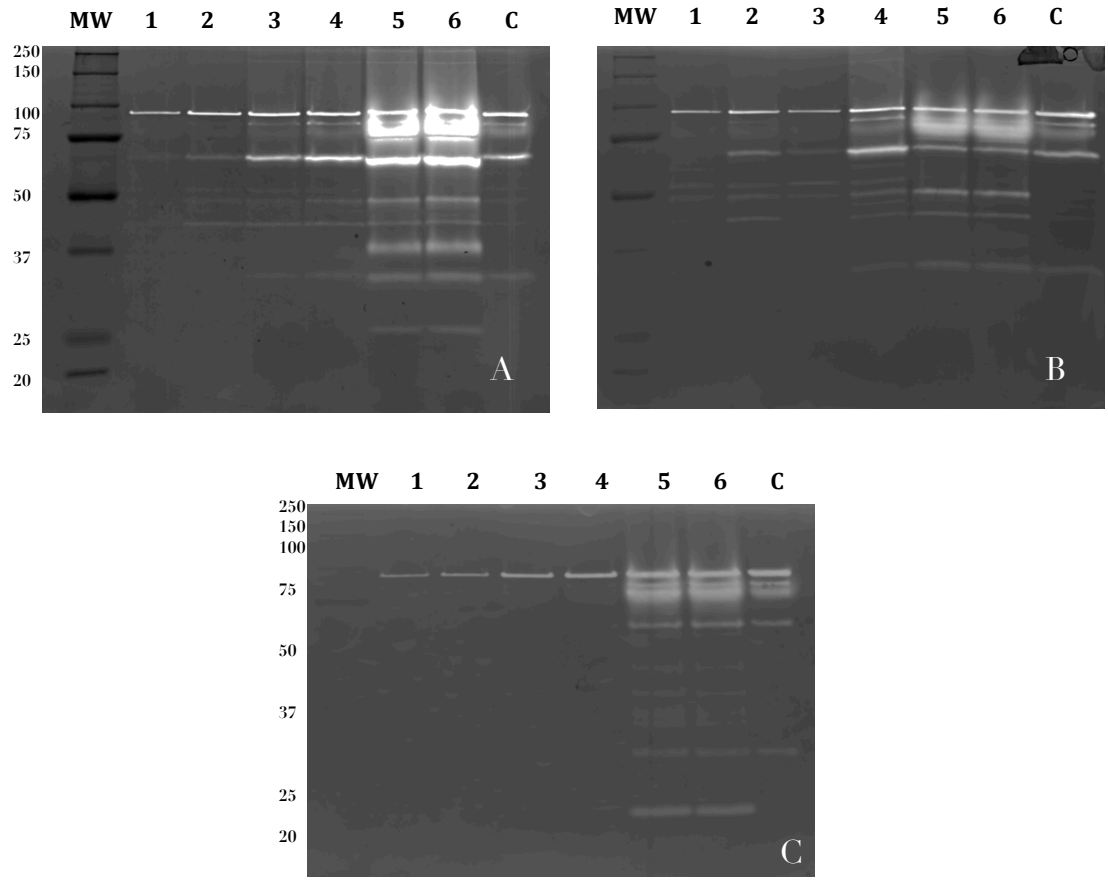

Figure 6. Zymography of extracellular proteases at pH 5.0 (A), 7.4 (B) and 9.0 (C) of *Aeromonas piscicola* AH-3.

For each zymography, a control sample was used (an aliquot of the 3rd point of growth at 30 ºC), enabling the normalization of the gels. Each gel was digitalized and analyses proceed as described in Material and Methods. Eighteen protease bands were detected with different molecular weights, in the different conditions tested. Results can be observed in table 17.

Table 17. Extracellular proteases bands of *Aeromonas piscicola* AH-3 detected for the different pH values: (-) absence of the protease band; (e) presence in exponential phase; (e*) presence in the end of exponential phase; (E) presence in stationary phase.

Molecular weight (kDa)	pH 5.0	pH 7.4	pH 9.0
98	e/E	e/E	e/E
85	e/E	e/E	e*/E
80	e/E	e/E	e*/E
72	E	E	-
65	e/E	e/E	e*/E
62	E	e*	E
59	-	e	-
54	-	e/E	-
51	e	-	E
49	e/E	e/E	E
47	-	e*/E	E
45	e*/E	e*	E
43	e/E	e/E	-
39	-	e*/E	-
37	E	e*/E	E
33	e/E	e/E	-
32	-	-	E
25	E	-	E

Regarding the results for the optimal growth condition, pH 7.4, only 3 proteases bands detected in the other conditions are not expressed in this condition. Comparing the conditions of pH 5.0 and pH 7.4, no major differences can be observed, only with a slight raise on the protease expression at pH 5.0. For the condition of pH 9.0 it is possible to visualize a down-regulation on the expression of proteases: in the exponential phase only 4 active proteases bands could be detected and their intensity in comparison to other conditions is very low. In the stationary phase, the expression of proteases is higher, more closely to the protein activity detected on the other conditions.

When comparing the different conditions for differential proteases expression no obvious stress markers for pH stress, can be assigned. However, considering the previous results it is possible to verify that medium pH has influence in the expression of proteases. pH 9.0 promoted the major effects in terms of protease expression, leading to down-regulation of extracellular proteases. Like the results observed for temperature there is no correlation between the growth and protease expression, as pH 5 expressed slight more proteases bands in comparison to optimal growth condition of pH 7.4.

The results obtained for the expression of proteases under different pH values are in concordance with previous studies, where pH have an important role in the expression of proteases (139).

Considering that extracellular proteases are involved in the capability of *Aeromonas* to adapt to low pH of the gastrointestinal track, as referred previously, and the extracellular protease expression at low pH, it is likely to assume the potential ability of *A. piscicola* as a causing agent of gastrointestinal disease.

2.1.1.3. Salinity

The results of the evaluation of extracellular proteases expressed by *A. piscicola* AH-3 grown at salinity values, 0, 1 and 3.5% of NaCl can be observed in the Figure 7.

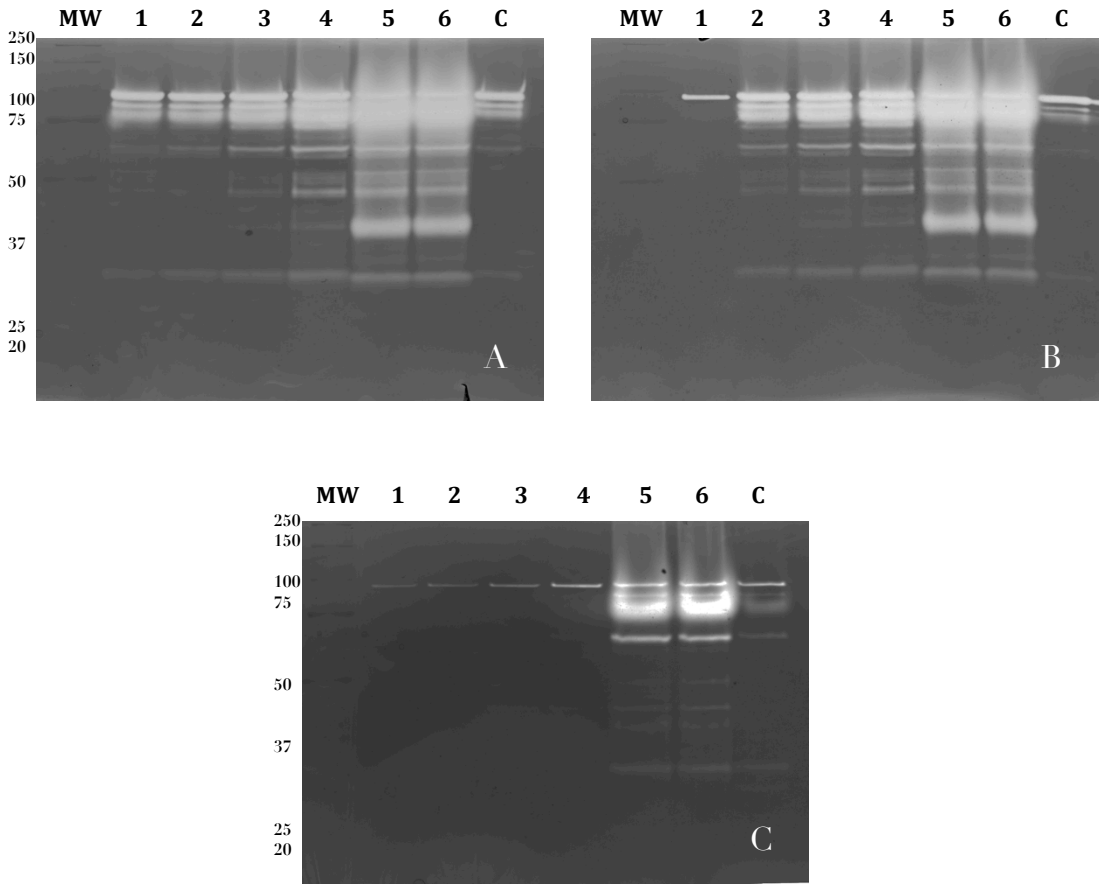

Figure 7. Zymography of extracellular proteases at 0%(A), 1%(B) and 3.5% NaCl(C) of *Aeromonas piscicola* AH-3.

For each zymography, a control sample was used (an aliquot of the 3rd point of growth at 30 ºC), enabling the normalization of the gels. Each gel was digitalized and analyses proceed as described in Material and Methods. Nineteen proteases bands were detected with different molecular weights, in the different conditions tested. Results can be observed in table 18.

Conditions of 0 and 1% NaCl are very similar in the expression of extracellular proteases, presenting a high intensity of the proteases expressed, in both exponential and stationary phases. On the other hand, data obtained for condition 3.5 % NaCl show differences on the expression of proteases, with more significance on the exponential phase, where few proteases are expressed. Furthermore, the intensity of the proteases expressed is lower, in the exponential and stationary phases. Nevertheless, when comparing the different conditions for differential proteases expression, no obvious stress markers for salinity stress could be assigned.

Table 18. Extracellular proteases bands of *Aeromonas piscicola* AH-3 expressed in the presence of different percentages of salinity: (-) absence of the protease band; (e) presence in exponential phase; (e*) presence in the end of exponential phase; (E) presence in stationary phase.

Molecular weight (kDa)	0% NaCl	1% NaCl	3.5% NaCl
98	e/E	e/E	e/E
85	e/E	e/E	e*/E
80	e/E	e/E	e*/E
72	e/E	e/E	E
65	e/E	e/E	e*/E
62	e/E	e/E	E
59	e/E	e/E	E
54	-	e/E	-
51	e/E	e/E	-
49	e*/E	e/E	E
47	e/E	e/E	E
45	e*/E	e/E	E
43	e*/E	e/E	e*/E
39	e/E	e/E	-
37	-	e/E	e/E
33	e*/E	e	-
32	e/E	e/E	-
30	-	E	-
25	-	-	E

These results are in concordance with other studies preformed out with other *Aeromonas* species, where the results imply that high salt concentrations repress the production of proteases by *Aeromonas*. The depressed protease activity of *Aeromonas* in seawater may influence the growth and/or survival of *Aeromonas* in seawater. The growth in the presence of high concentrations of NaCl is slow compared with that in low concentrations of NaCl, consequently, the number of *Aeromonas* in seawater is low compared with that in river environments (105). Having into account the results obtained it is possible to assume that salinity has an important role in the expression of extracellular proteases in AH-3, and that in the presence of high concentrations of NaCl the expression of extracellular proteases is repressed.

The depressed protease activity of AH-3 in high concentrations of salinity may influence the behaviour of AH-3 in seawater. However Khan et al. suggested that the decreased protease activity influenced also the growth of *Aeromonas* in seawater. This fact is not in concordance with the results obtained, as the growth at 3.5% (sea water salinity) was very similar to the growth in the optimal growth condition (Fig. 3) and it was verified a big depression on proteases expression (Fig. 7).

If a direct and solo correlation between proteases expression, survivability and growth occurred, it was expectable to see a profile of protease expression in the exponential growth phase very similar between conditions of 1% and 3.5%, since the growth is similar in this phase but that is not verified. However, it is possible that other abiotic and biotic factors present in the environment together with high salinity represent a more stressful condition for bacteria and therefore affects negatively the growth of *Aeromonas* spp. and its survival. Yet, survivability of AH-3 was not affected with an increase of salinity concentrations as bacteria as the capability to growth and survive in the form of aggregates at extreme salinity concentrations, at least up to 5% NaCl.

A study concerning the differential expression of extracellular proteases by *Aeromonas*, under different abiotic conditions, during the different growth phases, had never been performed until now. However, diverse extracellular proteases have been identified and characterized on *Aeromonas* spp., as well in other Gram-negative bacteria. Two types of proteases have been described in *A. hydrophila* and other species as the major proteolytic activities of motile *Aeromonas*: a metalloprotease thermo-stable of 65-68 kDa and a serine-protease thermo-sensitive of 38 kDa (140, 141). A serine protease was also identified by Esteve (142), and other 3 distinct protease bands with 31, 44 and 60 kDa representing a

metalloprotease thermo-stable. Beside these proteases, a zinc-metalloprotease of 19 kDa was detected (143) and also a serine protease thermo-stable of 22 kDa in a strain of *A. hydrophila* (144).

Analysing the molecular weight of the extracellular proteases bands detected for AH-3 in the different abiotic conditions there is an evident correlation with the previously described proteases. The molecular weight of the proteases detected is not exactly the same, but this is a technical problem intrinsic to the methodology approach of zymography. It was possible to detect a protease band with ~65 kDa, that may correspond to the metalloprotease thermo-stable of 65-68 kDa identified in other studies (140). Proteases band with ~37 kDa can correspond to the serine protease identified with 38 kDa in other studies (141). Protease bands with 32, 43 and 59 kDa were also observed similar to the molecular weights 31, 44 and 60 kDa observed for a metalloprotease thermo-stable (146). The protease band detected with 25 kDa can be associated with the serine protease thermo-stable of 22 kDa identified in other studies (144).

From all the abiotic conditions evaluated, only 1 protease band was expressed in all these conditions during the exponential growth phase, the protease with ~98 kDa. Therefore, it is possible to assume that this protease has an important role in the growth and adaptation to different environments. However, in the condition of 40 ºC, this protease tends to disappear along the exponential growth with almost no expression on the stationary phase, followed by the expression of other proteases. It is possible that this protease is being degraded along the growth and resulting from that is the appearance other proteases bands in late stage of exponential phase and in the stationary phase. Currently, there is no protease identified with this molecular weight.

Nevertheless, in a non-published study in development (personal communication), a gene encoding a putative new metallo-serine type collagenase, was identified in *Aeromonas hydrophila* ATCC 7966 genome with an apparent molecular weight of 100 kDa. Expression of extracellular collagenases by bacteria can be related with virulence and nutrition. Therefore, it is possible to assume that the protease band detected of 98 kDa likely corresponds to this collagenase. Further studies should be conducted to confirm this hypothesis

Another factor that has a great influence over the expression of extracellular proteases is cellular density and quorum sensing (72). This could justify the fact that a higher expression of proteases in stressful conditions occurs in the stationary phase, when cell density is higher, enabling cells to express higher concentrations of proteases.

Table 19. Association of described proteases in the literature with proteases band detected for *Aeromonas piscicola* AH-3 zymography in different conditions.

Described proteases	Proteases band detected
Metalloprotease thermo-stable ~65-68 kDa (Leung KY, Stevenson RM. 1988)	~65 kDa
Serine protease ~38 kDa (Nieto TP, Ellis AE. 1986)	~37 kDa
Metalloprotease thermo-stable ~31, 44 and 60 kDa (Esteve C, Alcaide E, Blasco MD. 2012)	~32, 43 and 59 kDa
Serine protease thermo-stable ~22 kDa (Rodriguez LA, Ellis AE, Nieto TP. 1992)	~25 kDa
Metallo-serine type collagenase ~100 kDa	~98 kDa

2.1.2. Extracellular proteins

Extracellular proteins are recognized for contribute significantly to the wide distribution and great adaptability of *Aeromonas spp.* to environmental changes, through direct contact with host tissues and may therefor mediate important pathogen–host interactions. As a result, extracellular proteomes have been used to identify and characterize secreted virulence factors for various *Aeromonas spp.* (147).

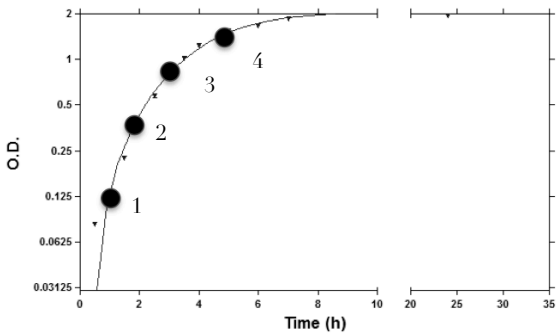

Figure 8. Specific points on the growth curve selected for protein evaluation.

In order to proceed to the characterization of extracellular proteins, exponential phase was evaluated by SDS-PAGE. To compare stress conditions with the optimal growth condition, extraction of extracellular proteins proceeded at four points during the exponential phase at the same point on the growth curves (Fig. 8), therefore allowing an evaluation of the proteins secreted to the extracellular medium along the exponential phase of the growth curve.

2.1.2.1. Temperature

The results of the evaluation of extracellular proteins expressed by AH-3 grown at temperature values, 25, 30, 37 and 40 ºC can be observed in the Figure 9.

Each gel was digitalized and analyses proceed as described in Material and Methods. Using the software Quantity One (Bio-Rad) the analysis of the gels was proceeded with the determination of the molecular weight for each protein band detected.

A total of 44 proteins bands were detected ranging approximately from a maximum molecular weight of 180 kDa to a minimum molecular weight of 13 kDa. Afterwards, the same software was used to evaluate the optical density of each protein band, and different conditions were compared. Results can be observed in Figure 10.

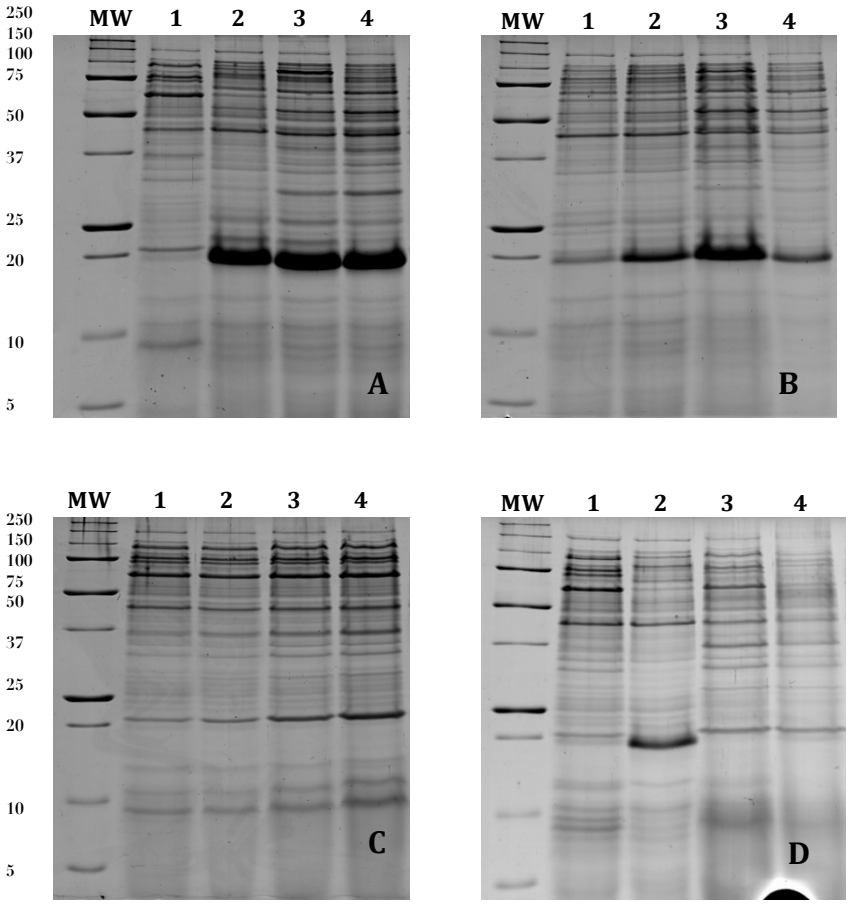

Figure 9. SDS-PAGE analysis of extracellular proteins at 25 (A), 30 (B), 37 (C) and 40 ºC (D) secreted by *Aeromonas piscicola* AH-3.

By evaluation of Figure 10, it was possible to observe that newly synthetized proteins did not occur in the stressful conditions (temperatures of 37 ºC and 40 ºC) and that the major differences occurred in the up or down-regulation of protein expression. It is also possible to notice an evolution of the differences in density, particularly for the temperature of 25 ºC, by the gradual increase of expression for some proteins during the exponential growth. In opposite, temperature of 40 ºC registered globally a decrease on the expression of some proteins. This decrease of protein expression at 40 ºC is in concordance with the results obtained for extracellular proteases expression, where a gradual down-shift on the expression of extracellular proteases was also detected at 40 ºC. It is possible to affirm that at high temperatures, high as 40 ºC, there is a drastic down-regulation on the secretion of proteins. Therefore, high temperatures have a great impact on the expression of extracellular proteins.

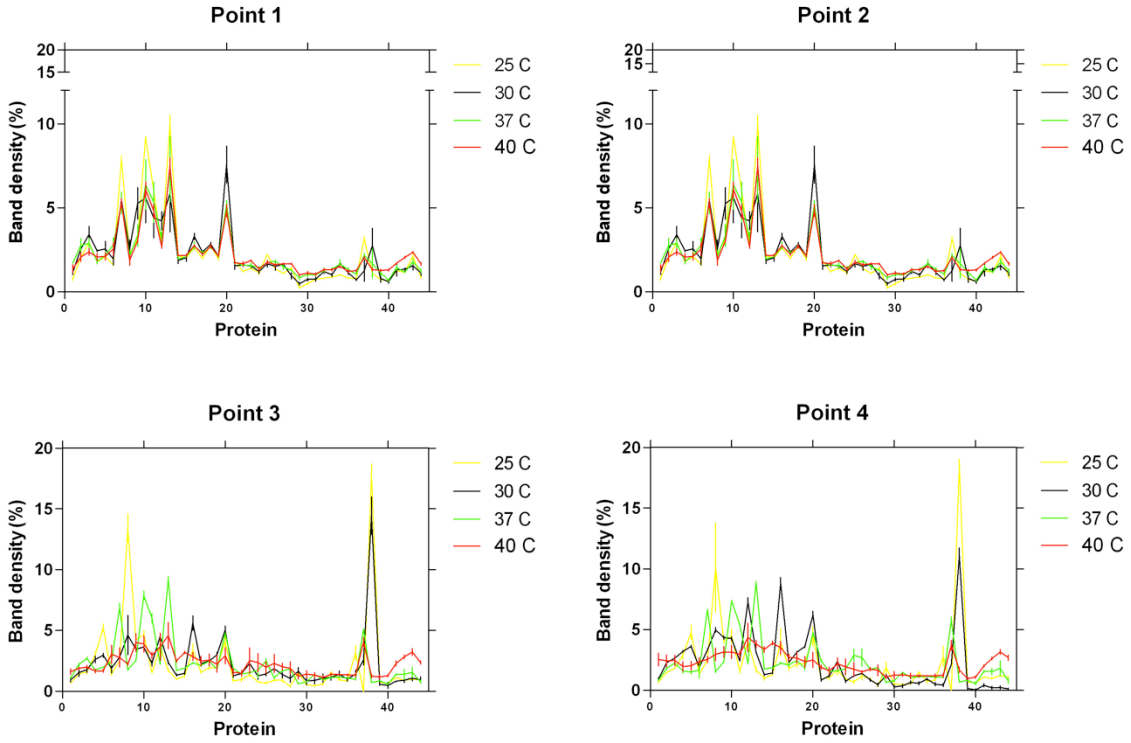

Figure 10. Differential expression of extracellular proteins of *Aeromonas piscicola* AH-3 grown at 25, 30, 37 and 40 ºC through the exponential growth phase; analysis was performed by the determination of the optical density percentage for each protein band calculated as a fraction of the total optical density of every proteins present.

Using a two-way ANOVA statistical test, differential expressions of proteins were statistically analysed. In the Annexe 1.1.1, results for the expression of each protein along the exponential growth can be visualized for a detailed interpretation of the results. The results showed the following statistical significance when compared with other conditions:

Regarding the condition of 25 ºC, 5 proteins (protein 12 with a $p < 0.001$, protein 16 with a $p < 0.001$, protein 19 with a $p < 0.01$, protein 20 with a $p < 0.01$ and protein 37 with a $p < 0.001$) were down-regulated and 4 proteins (protein 5 with a $p < 0.001$, protein 8 with a $p < 0.001$, protein 36 with a $p < 0.001$ and protein 38 with a $p < 0.001$) were up-regulated. For the condition of 37 ºC, 6 proteins (protein 9 with a $p < 0.01$, protein 12 with a $p < 0.001$, protein 16 with a $p < 0.001$, protein 19 with a $p < 0.01$, protein 20 with a $p < 0.01$ and protein 38 with a $p < 0.001$) were down-regulated and 9 proteins (protein 7 with a $p < 0.001$, protein 10 with a $p < 0.001$, protein 11 with a $p < 0.001$, protein 13 with a $p < 0.001$, protein 21 with a $p < 0.001$, protein 27 with a $p < 0.001$, protein 37 with a $p < 0.001$, protein 41 with a $p < 0.001$ and protein 43 with a $p < 0.001$)were up-regulated. In the condition of 40 ºC, 6 proteins (protein 12 with a $p < 0.001$, protein 16 with a $p < 0.001$, protein 19 with a $p < 0.001$, protein

20 with a $p < 0.001$, protein 37 with a $p < 0.001$ and protein 38 with a $p < 0.001$) were down-regulated and 10 proteins (protein 14 with a $p < 0.001$, protein 15 with a $p < 0.001$, protein 21 with a $p < 0.001$, protein 27 with a $p < 0.001$, protein 37 with a $p < 0.001$, protein 39 with a $p < 0.001$, protein 40 with a $p < 0.001$, protein 41 with a $p < 0.001$, protein 42 with a $p < 0.001$ and protein 43 with a $p < 0.001$)were up-regulated.

Evaluating the differential expression of proteins it was possible to identify 10 protein bands as stress markers. From these 10 protein bands, 5 protein bands (proteins 21, 27, 37, 41 and 43) are over-expressed in conditions of 37 and 40 ºC, therefore can be considered as stress markers for high temperatures. And 5 protein bands (proteins 12, 16, 19 and 38) have differential expression for all stress conditions; therefore can be considered as stress markers for temperature.

The results observed are in concordance with another study performed for other *Aeromonas* species where the extracellular proteome was evaluated under the influence of different temperatures and the expression of extracellular proteins was reduced at 37 ºC in comparison to 25 ºC. Through differential analysis of the proteome, different proteins have been identified as being down-regulated under growth of 37 ºC: serine protease, chitin-binding protein, S-layer, flagella proteins, metalloprotease, and other unknown proteins A hemolysin was also identified as being up-regulated in the extracellular proteome, together with other set of unknown proteins (147). Observing the results obtained in this study, some of the proteins down-regulated at 37 and 40 ºC are possibly those also described as down-regulated at 37 ºC by Yu et al. (38) as being down-regulated at 37 ºC. More specifically, protein 38 is probably one of these proteins, since it is up-regulated at 25 ºC but down-regulated at 37 and 40 ºC.

The decreased expression of a serine protease and metalloprotease at 37 ºC is consistent with the results observed in the analysis of the extracellular proteases at this temperature, where a decreased was observed on the expression of protease bands. Also a decreased expression of flagellins can be associated as a mechanism to evade host immune system through Toll-like receptor 5 (128), and therefore AH-3 may have the capability to evade the host immune system by this mechanism.

Additionally, in the current study a high number of proteins detected down-regulated a bigger number of proteins up-regulated was detected (128). As referred before in a previous study of differential protein expression, only a hemolysin was detected as being up-regulated at 37 ºC. Therefore, the 9 proteins up-regulated of AH-3 under growth of 37 ºC may represent novel virulence factors. Regarding the fact at high temperatures, TTSS is induced (81), is also expected that some of the proteins up-regulated at both temperatures of 37 ºC

and 40 ºC are associated with the expression of TTSS. Proteins that are up-regulated at 40 ºC but not at 37 ºC are therefore not related with the expression of TTSS, and can be proteins associated with stress response to extreme temperatures or even represent novel virulence factors.

2.1.2.2. pH

The results of the evaluation of extracellular proteins expressed by *Aeromonas piscicola* AH-3 grown at pH values, 5.0, 7.4 and 9.0 can be observed in Figure 11.

Gels were digitalized and analyses proceed as described in Material and Methods. Through the usage of software Quantity One (Bio-Rad) the analysis of the gels was performed by the detection of the molecular weight for each protein band detected.

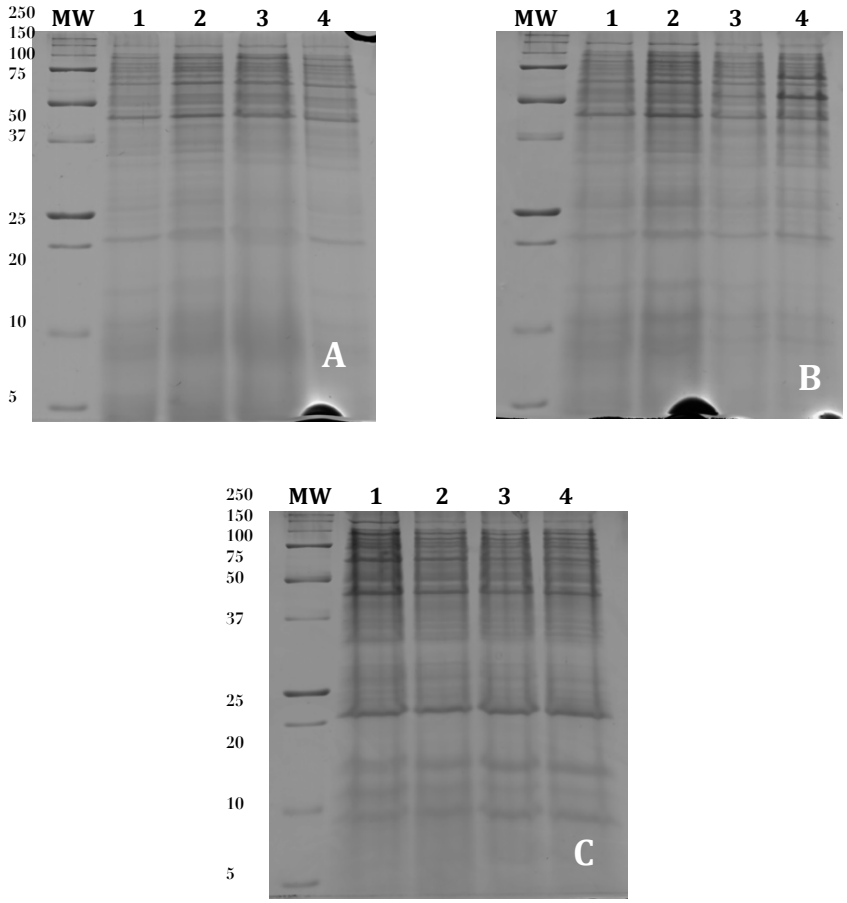

Figure 11. SDS-PAGE of extracellular proteins at pH 5.0 (A), 7.4 (B) and 9.0 (C) of *Aeromonas piscicola* AH-3.

A total of 36 proteins bands were detected ranging approximately from a maximum molecular weight of 180 kDa to a minimum molecular weight of 13 kDa. The same software

was used to proceed to the evaluation of the optical density of each band. Afterwards, different conditions were compared for identification of differential expression of proteins and results can be observed in Figure 12.

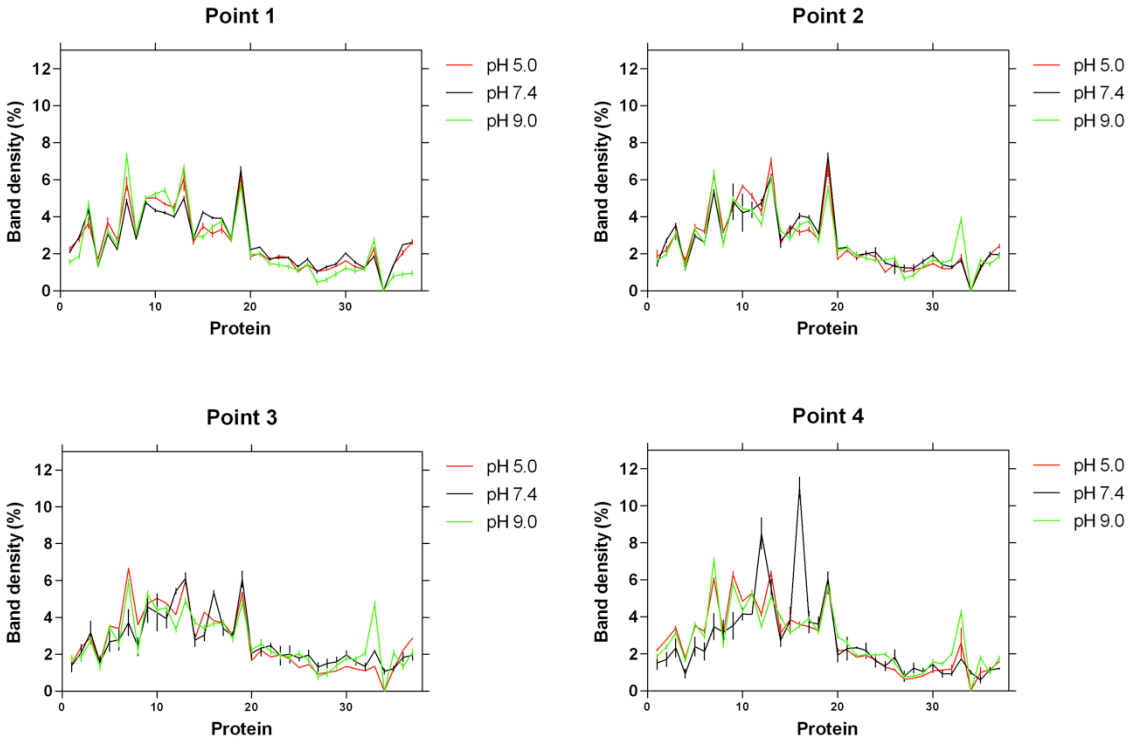

Figure 12. Differential expression of proteins secreted by *Aeromonas piscicola* AH-3 grown at different pH; analysis was performed by the determination of the optical density percentage for each protein band calculated as a fraction of the total optical density of every proteins present.

Using a two-way ANOVA statistical test, differential expressions of proteins were statistically analysed. In the Annexe 1.1.2., graphics of each protein along the exponential growth with statistical analysis can be visualized for a detailed interpretation of the results. The results showed the following statistical significance when compared with other conditions: In condition of pH 5.0, 3 proteins (protein 12 with a $p < 0.001$, protein 16 with a $p < 0.001$ and protein 34 with a $p < 0.001$) were down-regulated and 2 proteins (protein 7 with a $p < 0.05$ and protein 11 with a $p < 0.01$) were up-regulated. In the condition of pH 9.0, 5 proteins (protein 12 with a $p < 0.001$, protein 16 with a $p < 0.001$, protein 19 with a $p < 0.01$, protein 27 with a $p < 0.05$ and protein 34 with a $p < 0.001$) were down-regulated and 6 proteins (protein 7 with a $p < 0.001$, protein 11 with a $p < 0.01$, protein 14 with a $p < 0.01$,

protein 32 with a $p < 0.001$, protein 33 with a $p < 0.001$ and protein 35 with a $p < 0.001$) were up-regulated.

It is possible to visualize that differences between conditions occurred in the up or down-regulation of protein expression, without the occurrence of newly synthetized proteins (Figure 12). The differences presented are very restricted along the exponential phase, with no obvious differences in the protein differential expression. However it is possible to visualize an increase of protein expression on the 4th point for the condition pH 9.0.

Evaluating the differential expression of proteins, 5 proteins (proteins 7, 11, 12, 16 and 34) have differential expression both at pH 5.0 and pH 9.0. Therefore it is possible to propose these specific proteins as stress markers for pH stress. However, no specific markers for acid pH or alkaline pH stress could be assigned as only 1 condition for each pH state was evaluated. Evaluation of additional specific pH conditions (acid and alkaline conditions) would provide further specific stress markers for alkaline and acid pH.

With the results observed it is possible to affirm that alkaline pH has a bigger impact on the extracellular protein expression than acidic pH, inducing an increase on up and down-regulated expression of proteins. Therefore, adaptation for alkaline pH seems to require a higher adaptation relatively to acidic pH adaptation.

According to Francis et al. (31) TTSS can be induced under low pH. Therefore if TTSS system was induced in AH-3 under growth of pH 5.0, proteins that are up-regulated at pH 5.0 (protein 7 and 11) should be related to this secretion system. Since *Aeromonas spp.* are commonly associated with being the causative agent of gastrointestinal disease (32), it is possible that TTSS represents a mechanism expressed at low pH, assisting bacteria in the process of infection. However, proteins 7 and 11 are also up-regulated at pH 9.0, therefore it is possible to assume that TTSS may be also induced in AH-3 at pH 9.0. Supporting this hypothesis is the fact of *Aeromonas spp.* are commonly found has a agent causative of infections in tissues wounds (19). The pH present in this type of wounds has been described as alkaline pH with values ranging from pH 8.5 to pH 9.0 (33). Therefore, TTSS could represent a possible mechanism expressed in this type of wounds, assisting *Aeromonas spp.* in the process of infection.

2.1.2.3. Salinity

The results of the evaluation of extracellular proteins expressed by AH-3 grown at 0, 1 and 3.5% NaCl can be observed in the Figure 13.

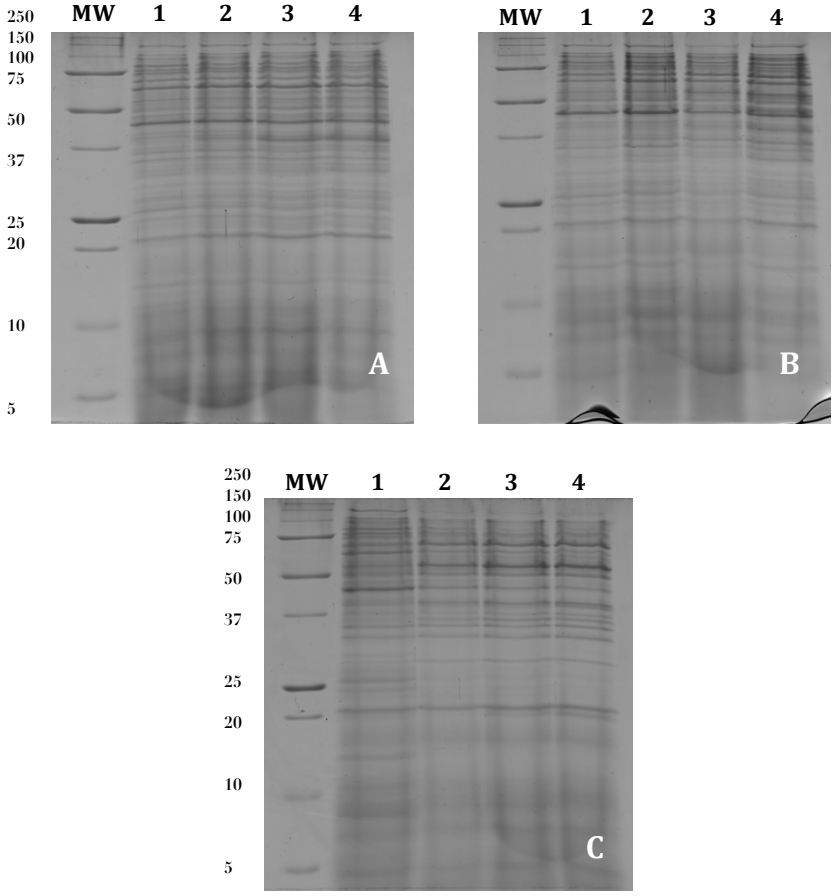

Figure 13. SDS-PAGE of extracellular proteins at 0%(A), 1%(B) and 3.5% NaCl(C) of *Aeromonas piscicola* AH-3.

Each gel was digitalized and analyses proceed as described in Material and Methods. The analysis of the gels proceeded with the determination of the molecular weight of each protein band detected, using the software Quantity One (Bio-Rad). A total of 53 proteins bands were detected ranging approximately from a maximum molecular weight of 180 kDa to a minimum molecular weight of 13 kDa. The software Quantity One was also used in the evaluation of the optical density for each band, and different conditions were compared for identification of the differential expression of proteins. Results can be observed in Figure 14.

Figure 14 shows that the differences between conditions occurred in the up or down-regulation of protein expression. Both the conditions of 0% and 3.5% NaCl present very similar values of protein expression compared to the optimal growth condition of 1% NaCl, however more differential expression occurs in 3.5% NaCl, specially during the development of the exponential growth phase. With these results it is possible to affirm that high salinity concentrations modulate the expression of extracellular proteins.

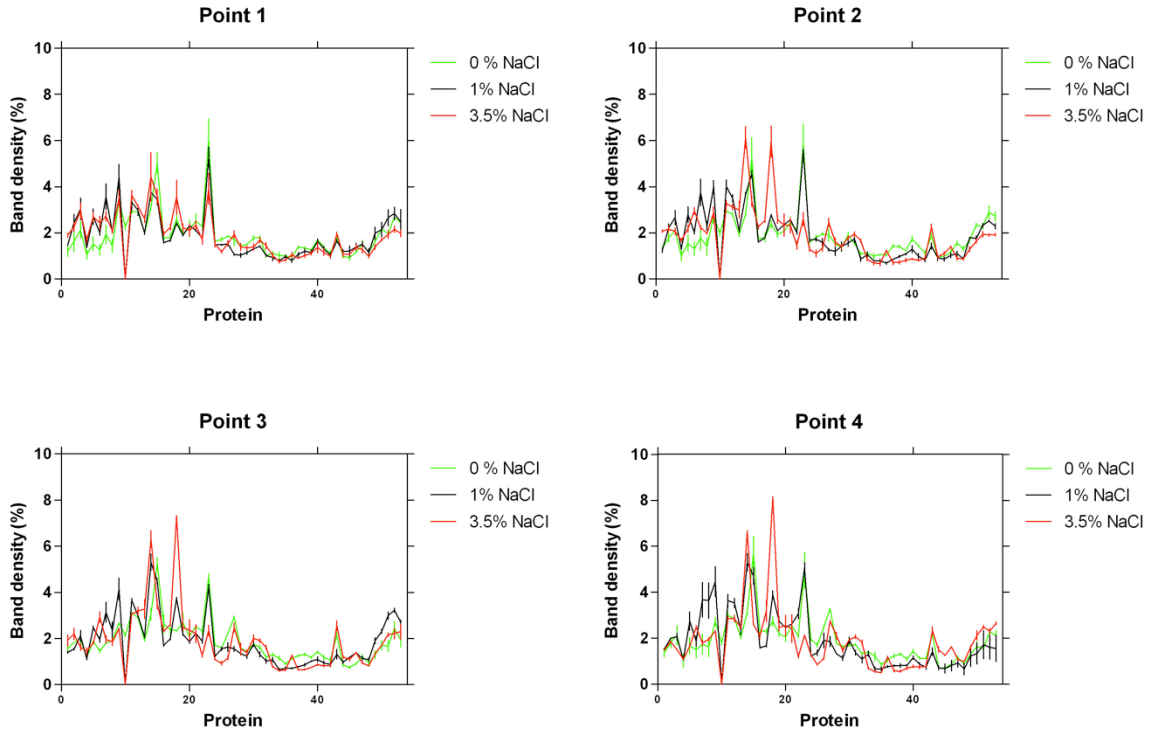

Figure 14. Differential expression of proteins secreted by *Aeromonas piscicola* AH-3 grown at different salinities; analysis was performed by the determination of the optical density percentage for each protein band calculated as a fraction of the total density of every proteins present.

Using a two-way ANOVA statistical test, differential expressions of proteins were statistically analysed. In the Annexe 1.1.3., the expression of each protein detected along the exponential growth of AH-3, can be visualized for a detailed interpretation of the results.

The results showed that: at 0% NaCl, 3 proteins (protein 6 with a $p < 0.05$, protein 9 with a $p < 0.01$ and protein 14 with a $p < 0.01$) were down-regulated and 7 proteins (protein 10 with a $p < 0.001$, protein 24 with a $p < 0.001$, protein 27 with a $p < 0.001$, protein 36 with a $p < 0.01$, protein 37 with a $p < 0.001$, protein 38 with a $p < 0.001$ and protein 43 with a $p < 0.01$) were up-regulated. At 3.5% NaCl, 5 proteins (protein 7 with a $p < 0.05$, protein 9 with a $p < 0.001$, protein 22 with a $p < 0.001$, protein 23 with a $p < 0.01$ and protein 25 with a $p < 0.001$) were down-regulated and 9 proteins (protein 6 with a $p < 0.05$, protein 13 with a $p < 0.001$, protein 18 with a $p < 0.001$, protein 27 with a $p < 0.001$, protein 32 with a $p < 0.001$, protein 36 with a $p < 0.001$ and protein 43 with a $p < 0.001$) were up-regulated.

Evaluating the differential expression between the different abiotic conditions it was possible to identify different stress markers. Protein 6 and 14 are possible stress markers of high salinity since their expression was up-regulated in 3.5% NaCl but at 0% NaCl were down-regulated. Proteins 6, 7, 9, 27, 36, 43 are possible stress markers to general osmotic unbalance.

Markers of high salinity stress are possibly related to specific mechanisms to respond to a high increase of NaCl concentration in the medium, while stress markers of general osmotic unbalance are not specific mechanisms since they are altered in the same way either in 0% and 3.5% NaCl conditions. These general stress markers are possibly molecules that are synthesized by the cells to balance the osmolarity. This strategy is known as compatible-solute strategy and does not involve the need for specially adapted proteins, providing a high degree of flexibility to organisms to adapt to significant variations in external osmolarity (34). However, other strategies to adapt to these stressful conditions are being used since specific proteins were up-regulated in both conditions of stress, especially at 3.5% NaCl.

Differential changes in the protein expression, that were more prominent in the condition of 3.5% NaCl, can be linked to the fact that *Aeromonas spp.* are preferentially found in aquatic environments with low NaCl concentrations (14). Therefore, the adaptions to these environments pose a stress situation for bacterial growth, that is corroborated by the expression of more up-regulated proteins at 3.5% NaCl than at 0% NaCl.

2.2. Intracellular proteome

Through the evaluation of the cellular proteome, a wider understand on the mechanisms of action in response to stress factors can be provided and therefore it will be possible to gain more insights on the mechanisms responsible for fitness and adaptation (35).

In order to proceed to the characterization of the cellular protein fraction, exponential phase was evaluated by SDS-PAGE. To compare stress conditions with the optimal growth condition, four points along the exponential phase of the growth curve were selected and protein extraction was performed (Figure 10).

2.2.1. Temperature

The results of the evaluation of intracellular proteins expressed by *A. piscicola* AH-3 grown at 25, 30, 37 and 40 ºC can be observed in the Figure 15.

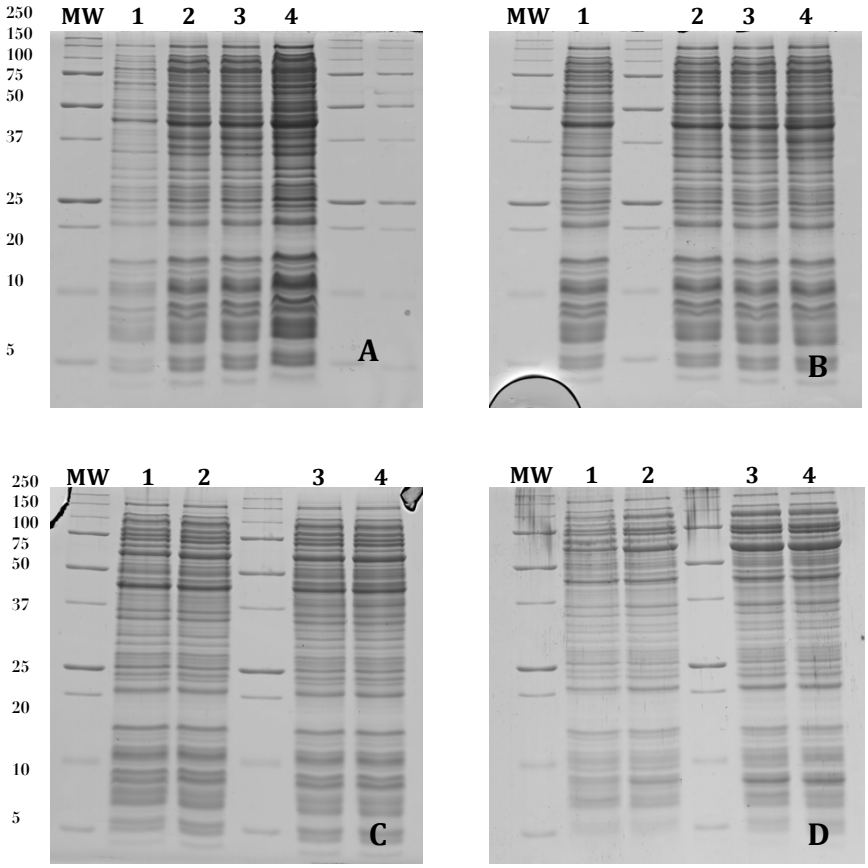

Figure 15. SDS-PAGE of intracellular fraction proteins at 25 (A), 30 (B), 37 (C) and 40 ºC (D) of *Aeromonas piscicola* AH-3.

Each gel was digitalized and analyses proceed as described in Material and Methods. Using the software Quantity One (Bio-Rad) the analysis of the gels proceeded with the determination of the molecular weight of each protein detected.

A total of 77 proteins bands were detected ranging approximately from a maximum molecular weight of 180 kDa to a minimum molecular weight of 9 kDa. Afterwards, the same software was used to evaluate the optical density of each band, and different conditions were compared for identification in differential expression of proteins. Results are shown in Figure 16. By evaluating Figure 16, it is possible to state that only 2 newly synthetized proteins were observed in stressful conditions (protein 39 expressed only at 37 and 40 ºC and protein 30 expressed at 25, 37 and 40 ºC) and that the major differences that occurred corresponds to up or down-regulation of protein expression. Using a two-way ANOVA statistical test, differential expressions of proteins were analysed. In the Annexe 1.2.2., data concerning to the expression of each protein is shown.

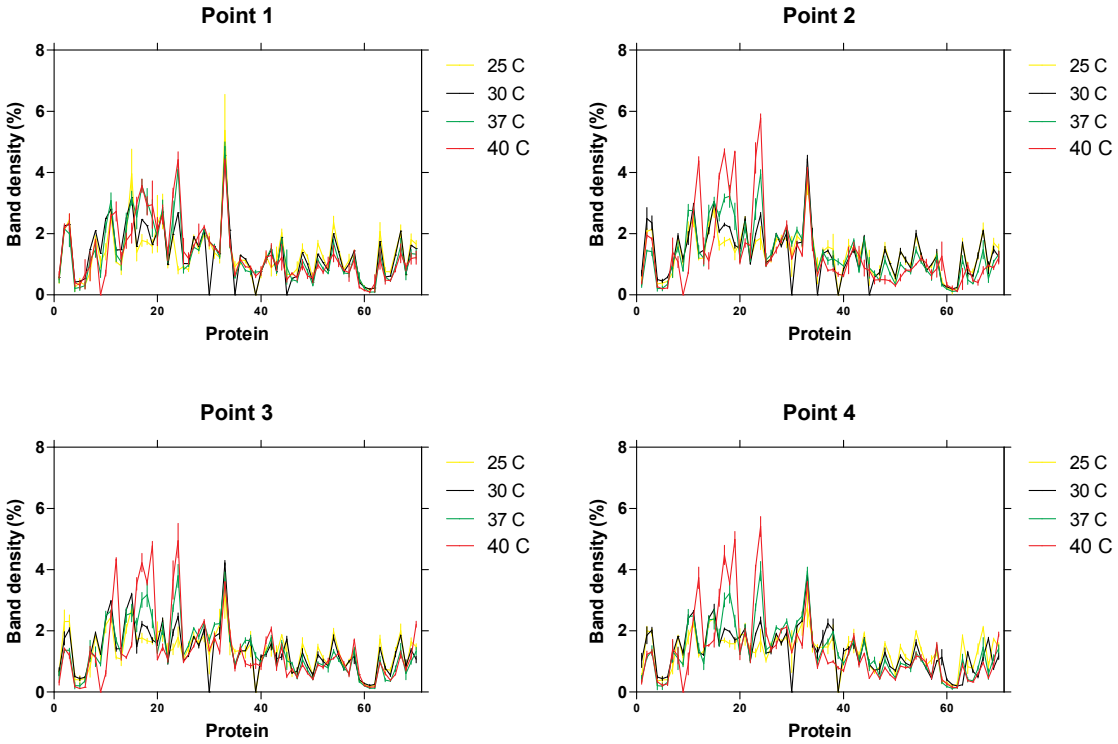

Figure 16. Differential expression of cell proteins by *Aeromonas piscicola* AH-3 grown at different temperatures; analysis was performed by the determination of the optical density percentage for each protein band calculated as a fraction of the total density of every proteins present.

The results showed the following statistical significance when compared with other conditions: at 25 ºC, 6 proteins were down-regulated (protein 10 with a $p < 0.01$, protein 17 with a $p < 0.05$, protein 24 with a $p < 0.001$, protein 37 with a $p < 0.001$, protein 63 with a $p < 0.001$ and protein 71 with a $p < 0.001$) and 5 proteins (protein 35 with a $p < 0.001$, protein 44 with a $p < 0.01$, protein 66 with a $p < 0.001$, protein 67 with a $p < 0.01$ and protein 69 with a $p < 0.001$) were up-regulated. At 37 ºC, 8 proteins were down-regulated (protein 2 with a $p < 0.001$, protein 3 with a $p < 0.01$, protein 8 with a $p < 0.05$, protein 37 with a $p < 0.01$, protein 40 with a $p < 0.01$, protein 54 with a $p < 0.01$, protein 63 with a $p < 0.01$ and protein 66 with a $p < 0.001$) and 9 proteins were up-regulated (protein 10 with a $p < 0.05$, protein 16 with a $p < 0.01$, protein 17 with a $p < 0.001$, protein 18 with a $p < 0.001$, protein 24 with a $p < 0.01$, protein 35 with a $p < 0.001$, protein 27 with a $p < 0.01$, protein 46 with a $p < 0.01$ and protein 71 with a $p< 0.001$). At 40 ºC, 15 proteins were down-regulated (protein 3 with a $p < 0.01$, protein 8 with a $p < 0.01$, protein 10 with a $p < 0.001$, protein 15 with a $p < 0.001$, protein 14 with a $p < 0.001$, protein 38 with a $p < 0.05$, protein 27 with a $p < 0.001$, protein 37 with a $p <$

0.001, protein 40 with a $p < 0.001$, protein 44 with a $p < 0.001$, protein 54 with a $p < 0.001$, protein 63 with a $p < 0.001$, protein 66 with a $p < 0.001$, protein 67 with a $p < 0.001$ and protein 69 with a $p < 0.01$) and 10 proteins were up-regulated (protein 12 with a $p < 0.001$, protein 16 with a $p < 0.001$, protein 17 with a $p < 0.001$, protein 18 with a $p < 0.001$, protein 19 with a $p < 0.001$, protein 23 with a $p < 0.001$, protein 24 with a $p < 0.001$, protein 35 with a $p < 0.01$, protein 70 with a $p < 0.001$ and protein 71 with a $p < 0.001$).

Furthermore 7 of these proteins were differentially expressed only at 37 ºC and 40 ºC (protein 3, 8, 16, 18, 39, 54 and 71), suggesting that these proteins may have a significant role in protecting the cells from stress caused by high temperatures, representing therefore stress markers for high temperatures. Also, 8 proteins (proteins 10, 17, 24, 35, 30, 37, 63 and 66) presented differential expression at all temperatures beside the optimal growth condition, and therefore represent general stress markers for changes in temperature.

Furthermore, it is possible to state that temperature plays an important role on the expression of intracellular proteins by AH-3: with a decrease of the temperature it was noticeable a general decrease on the protein expression and with the rise of temperature an increase of proteins expression occurs. These results are in contrast with the results obtained for the extracellular proteins where the expression of proteins decreased with the rise of the temperature. In opposite, temperature of 25 ºC registered global decrease on the expression of some proteins.

The results observed are in concordance with other studies conducted with *Aeromonas spp.* An exposure of cells to elevated temperatures decreases the synthesis of normally expressed proteins and simultaneously induces an overproduction of a specific group of proteins, the HSPs (36), that are typical of the heat shock responses described for other bacteria. Therefore with the results obtained by the analysis of the proteome response to stress induced by temperature, it is possible to assume that up-regulated proteins detected might be HSPs.

Furthermore, protein 39 is only expressed at 37 ºC and 40 ºC, therefore is a specific response to high temperatures. This specific response can be associated with HSPs. However even at 30 ºC AH-3 express HSPs, for example it has been reported in *A. salmonicida* a HspA homologue up-regulated at 28 ºC (37) and therefore only a overproduction of the HSPs occur at 37 ºC and 40 ºC with few newly HSPs expressed at this conditions.

HSPs are classified according to their molecular weight and are divided into six families: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (sHSPs, ranging in size from 15 kDa to 30 kDa) (37). Twelve proteins (protein 12 with ~ 94 kDa, protein 16 with ~ 77 kDa, protein 17 with ~ 73 kDa, protein 18 with ~72, protein 19 with ~ 69 kDa, protein 23

with ~ 61 kDa, protein 24 with ~ 59 kDa, protein 35 with ~ 41 kDa, protein 39 with ~ 37 kDa, protein 46 with ~ 31 kDa, protein 70 with ~ 13 kDa and protein 71 kDa with ~ 12 kDa) were up-regulated only at 37 ºC and/or 40 ºC. The molecular range of these proteins are between 94 and 12 kDa and therefore if some of these proteins are HSPs they are representative of 5 families, HSP90, HSP70, HSP60, HSP40 and HSPs. These results are in concordance with previous studies where diverse HSPs of these families were identified in different *Aeromonas* spp. (38).

HSPs are recognized as molecular chaperones, responsible for protective mechanisms towards intracellular molecules essential to biological processes, playing an important role in protein-protein interactions such as folding and promoting proper protein conformation and prevention of unwanted protein aggregation (37). Therefore, HSPs protective mechanisms towards the cell are consistent with the increase of intracellular proteins when AH-3 is grown under high temperatures. As the exponential phase develops, it is possible that the constant thermal stress induces bacteria to express more HSPs needed for protection, enabling growth and survival, while repressing the expression of extracellular proteins not fundamental for growth and survival.

2.2.2. pH

Evaluation of the cellular fraction proteins expressed by AH-3 grown at pH values, 5.0, 7.4 and 9.0 can be observed in the Figure 17. Gels were digitalized and analysed as described in Material and Methods. The analysis of the gels proceeded with the determination of the molecular weight for each protein band detected (Quantity One, Bio-Rad).

A total of 70 protein bands could be detected approximately with a maximum molecular weight of 180 kDa to a minimum molecular weight of 9 kDa. The same software was used for determination of the optical density of each band. Subsequently, different conditions were compared for identification of differential expression of proteins. Results can be observed in Figure 18.

Acidification (pH 5.0) and basification (pH 9.0) induced up and down-regulation of several proteins and only one newly synthetized protein was detected (Figure 18). Nonetheless, the differences found are very restricted, with no obvious changes of differentially expressed proteins. Therefore, both the conditions of pH 5.0 and pH 9.0 present very similar values of protein expression comparing to the optimal growth condition, pH 7.4.

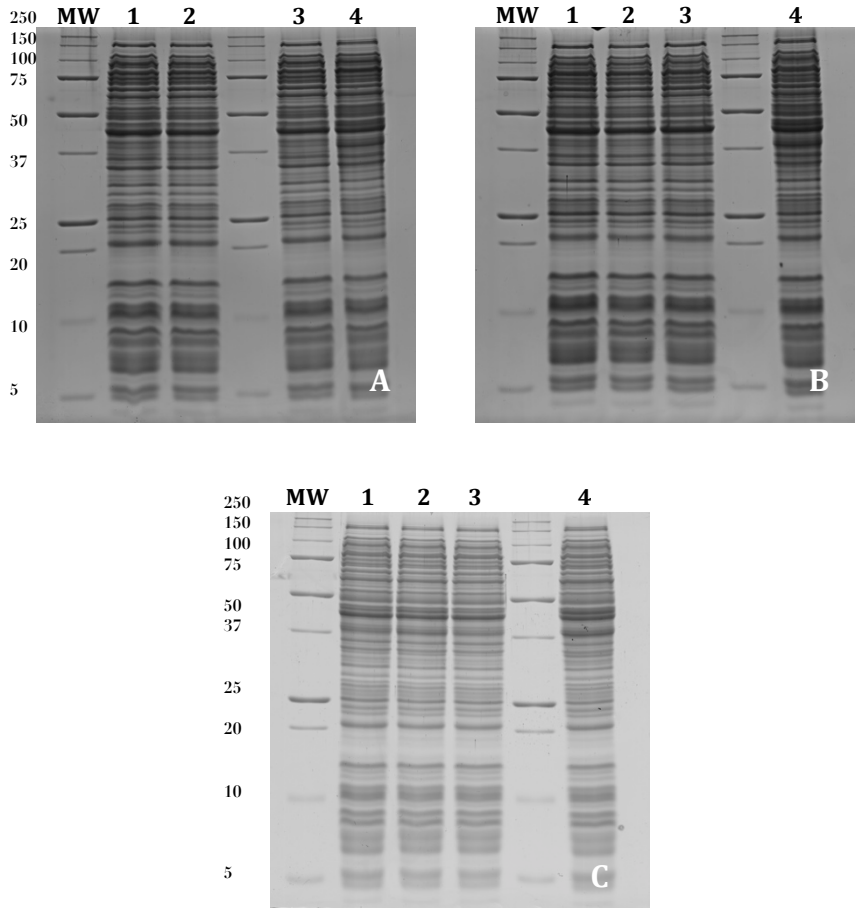

Figure 17. SDS-PAGE of intracellular fraction proteins at pH 5.0 (A), 7.4 (B) and 9.0 (C) of *Aeromonas piscicola* AH-3.

Using a two-way ANOVA statistical test, differential expressions of proteins were analysed. In the Annexe 1.2.2., the optical density of each protein along the exponential growth, can be visualized for a detailed interpretation of the results.

The results showed the following statistical significance when compared to the control condition: at pH 5.0, 1 protein was down-regulated (protein 12 with a $p < 0.001$) and 3 proteins were up-regulated (protein 26 with a $p < 0.001$, protein 30 with a $p < 0.01$ and protein 52 with a $p < 0.05$). At pH 9.0, 3 proteins were down-regulated (protein 56 with a $p < 0.01$, protein 60 with a $p < 0.01$ and protein 62 with a $p < 0.001$) and 5 proteins were up-regulated (protein 26 with a $p < 0.001$, protein 29 with a $p < 0.001$, protein 30 with a $p < 0.001$, protein 52 with a $p < 0.05$ and protein 55 with a $p < 0.001$).

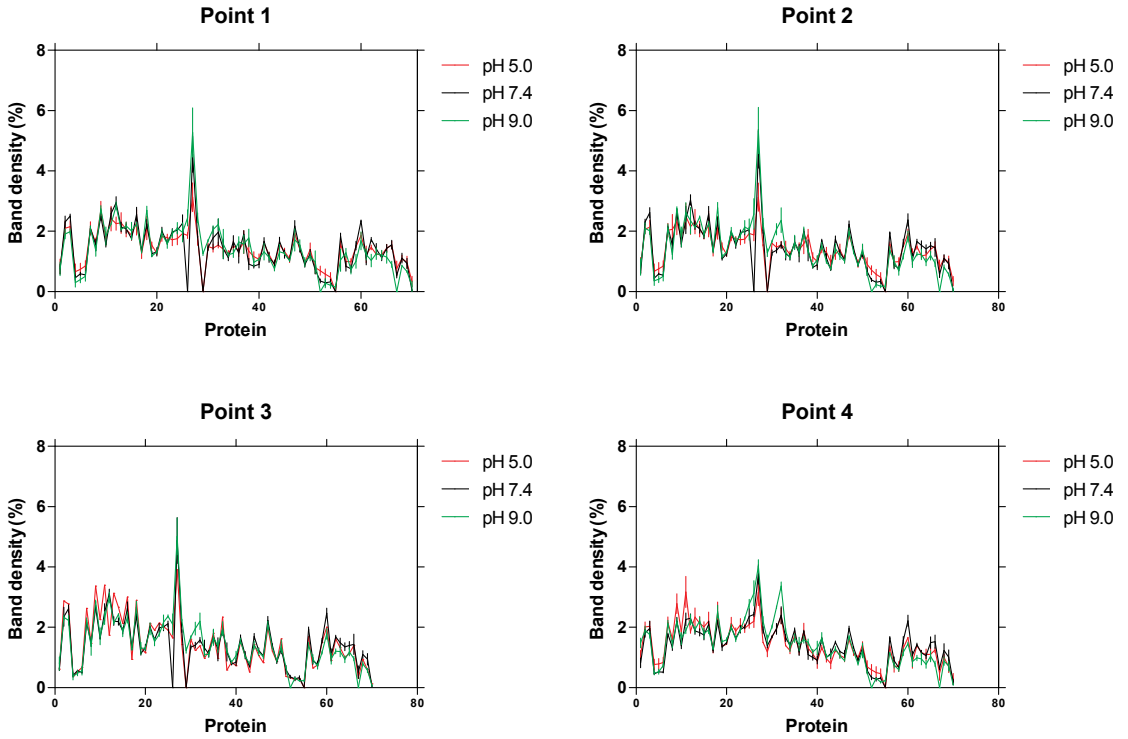

Figure 18. Differential expression of cell proteins by *Aeromonas piscicola* AH-3 grown at different temperatures; analysis was performed by the determination of the optical density percentage for each protein band calculated as a fraction of the total density of every proteins present.

Furthermore, 3 of these proteins were up-regulated both in pH 5 and pH 9, suggesting that these proteins may have a significant role in protecting the cells from stress caused by pH, maintaining the haemostasis of the cells. These proteins are possible stress markers for general pH stress, either acid or alkaline. Having in consideration that these stress marker proteins detected at pH 5.0 were also up-regulated at pH 9.0, it can be hypothesized that the mechanism of stress response is the same. From the different mechanisms described in the literature to respond to changes in the medium pH, the most plausible to be involved is the boost of pH homeostasis for AH-3, enabling the cells to maintain internal pH close to neutral even at extremely low or high external pH (39). These proteins can be involved with mechanisms used by Gram-negative bacteria in response to external pH changes: primary proton pumps, K^+/H^+ and Na^+/H^+ antiporters and production of enzymes that are involved in the conversion of acidic/alkaline metabolites to neutral metabolites (40). Additionally, protein 55 was newly synthetized in condition of pH 9.0, suggesting that this protein has a significant and specific role in adaptive response to alkaline environment. Therefore it is

plausible to assume this protein as a specific stress marker for alkaline pH, and perform an important role on protecting the cell.

The detection of possible ATR proteins, in response to exposure at pH 5.0 was expected; however, it was possible to detect only one protein up-regulated at pH 5.0 with no expression at pH 9.0 and therefore it is possible to assume that this specific protein is an ATR protein. These proteins are induced by acid stress and are able to protect cells by one or more possible mechanisms. Newly synthesized proteins boost the cell to maintain pH homeostasis. Chaperonin proteins could also be involved in protection of proteins from acid denaturation or damage, for example, heat shock proteins GroEL and DnaIi have been identified as being induced in *S. typhimztriztm* under acidic conditions (41). DNA binding proteins may perform a function in adaptation to acid by preventing or repairing DNA damaged (42). Detection of only one ATR protein is possibly related to the fact that their detection requires a pre-exposure to acid pH, normally pH 5.0 and then transition to pH 3.0 (8). Therefore since the evaluation performed did not involved this gradual transition to more acidic pH, and proteomic evaluation was performed at pH 5.0 and not at pH 3.0, absence of ATR proteins would be expected.

Differential protein expression at acidic and basic pH was rather small, suggesting that AH-3 did not require a high adaptation to pH stress (acid or alkaline) and protection of AH-3 to acid stress and alkaline stress does not require protein synthesis and probably relies essentially on the physiologically controlled pH homeostasis mechanism, including Na^+ /H^+ and K^+ /H^+ antiporters. Therefore, it is plausible to assume that pH has a minor impact on the expression of cell-associated proteins in AH-3, implying that survival and growth in these stress conditions is achieved without great impact on these biological processes.

2.2.3. Salinity

The results of the evaluation of cell-associated proteins expressed by *A. piscicola* AH-3 grown at 0, 1 and 3.5% NaCl can be observed in the Figure 19.

Each gel was digitalized and analyses proceed as described in Material and Methods. The analysis of the gels proceeded with the determination of the molecular weight for each protein band detected (Quantity-One, Bio-Rad). A total of 65 protein bands were detected ranging approximately from a maximum molecular weight of 180 kDa to a minimum molecular weight of 9 kDa.

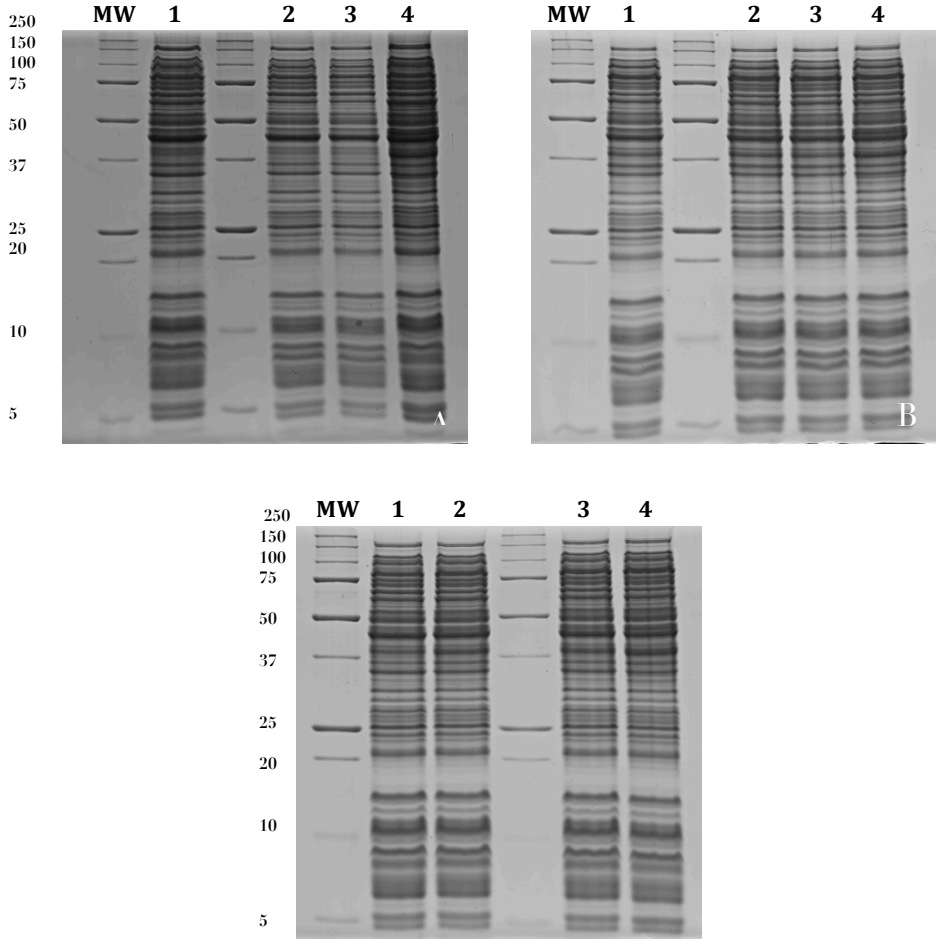

Figure 19. SDS-PAGE analysis of intracellular proteins at 0%(A), 1%(B) and 3.5% NaCl(C) of *Aeromonas piscicola* AH-3.

The same software was used to determine the optical density of each band, and different conditions were compared for identification of the differential expression of proteins. Results can be observed in Figure 20.

Figure 20 shows the differences on the optical density (%) of each protein detected between conditions. Both the conditions of 0% and 3.5% NaCl presents very similar values of protein expression comparing to the optimal growth condition of 1% NaCl.

Using a two-way ANOVA statistical test, differential expressions of proteins were analysed. In the Annexe 1.2.3., data for the optical density of each protein along the exponential growth, can be visualized for a detailed interpretation of the results. The results showed the following statistical significance when compared with other conditions: at 0% NaCl, 2 proteins were down-regulated (protein 9 with a $p < 0.05$ and protein 12 with a $p < 0.01$) and 4 proteins were up-regulated (protein 1 with a $p < 0.001$, protein 3 with a $p <$

0.001, protein 40 with a $p < 0.001$ and protein 62 with a $p < 0.01$). At 3.5% NaCl, 2 proteins were down-regulated (protein 9 with a and protein 12) and 7 proteins were up-regulated (protein 21 with a $p < 0.001$, protein 40 with a $p < 0.001$, protein 41 with a $p < 0.001$, protein 43 with a $p < 0.01$, protein 45 with a $p < 0.001$, protein 50 with a $p < 0.01$ and protein 62 with a $p < 0.001$).

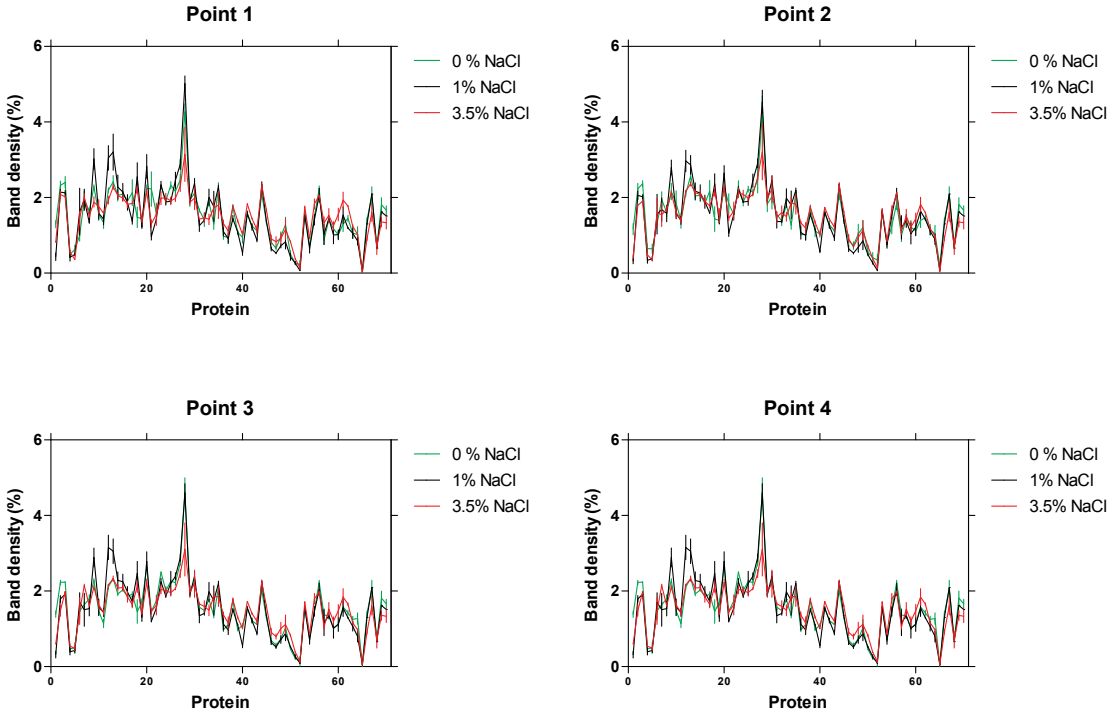

Figure 20. Differential expression of cell proteins by *Aeromonas piscicola* AH-3 grown at different salinities; analysis was performed by the determination of the optical density percentage for each protein band calculated as a fraction of the total density of every proteins present.

When evaluating the differential expression of proteins it is possible to state that 5 proteins (proteins 1, 9, 12, 40 and 62) present differential expression towards optimal growth condition. Therefore, these proteins can be assigned as stress markers for salinity stress. However, no visualization of proteins up-regulated at 3.5% NaCl and down-regulated at 0% NaCl (or opposite) could be detected, and therefore mechanisms responsible for osmotic regulation apparently are the same when AH-3 growth occurs in the presence of low and high concentrations of NaCl.

Nevertheless, a limited number of proteins were up-regulated only at one specific condition. At 0% NaCl one protein was found only up-regulated (protein 3) in this condition; at 3.5% NaCl 5 proteins were found only up-regulated (proteins 21, 41, 43, 45 and 50) in this condition. Consequently these proteins may represent specific responses to low and high concentrations of NaCl, respectively. However regarding these possible specific responses, more conditions of high and low concentrations should be evaluated for further discussion and conclusions.

In some Gram-negative bacteria, osmotic balance is provided by organic molecules that are either synthesized by the bacteria or are retrieved from the medium when available, and do not involve the need for expression of specific proteins (43). This mechanism of adaptation to osmotic unbalance justifies the low proteome alterations detected, as no new synthetized proteins are required if bacteria is able to retrieve from the medium molecules necessary or is already able to produce these molecules and only an increase of the production is necessary.

Differential changes in the protein expression were more prominent in the condition of 3.5% NaCl than at 0% NaCl, therefore high salinity concentrations pose a greater stress than low concentrations. This can be linked to the fact that *Aeromonas spp.* are preferentially found in aquatic environments with low salt concentrations and therefore, the adaption to these environments pose a stress situation for bacterial growth (14). However, in general NaCl (salinity) stress conditions do not pose a major stressful environment to AH-3, as these bacteria can easily adapt to this conditions at intracellular level.

Table 20. Possible related functions and/or associations of proteins detected with differential expression at intracellular and extracellular level.

	Extracelullar proteins	**Intracellular proteins**
↑ 37 e 40 ºC	T3SS	HSPs
↓ 37 e 40 ºC	Serine protease Chitin-binding protein S-layer Flagella proteins Metalloprotease	-
↑ pH 5.0 e 9.0	T3SS	Primary proton pumps Antiporters K^+/H^+ e Na^+/H^+
↑ pH 5.0	-	ATR
↑ 0% e 3.5% NaCl	Osmotic balance	Osmotic balance

3. Protein Identification

Protein identification was realized according to the protocol described in Material and Methods and the analysis by mass spectrometry conducted in the Laboratory for Protein Biochemistry & Biomolecular Engineering, Department of Biochemistry and Microbiology, Ghent University.

Regarding the extracellular protease activity, since no evident stress markers could be observed by the comparison of the different condition gels or either by comparing

different abiotic conditions, all the proteases detected, in total 19 proteases bands, were submitted for mass spectrometry analysis. This analysis is still in progress.

For the evaluation of the cellular fraction of the proteome all the proteins displaying statistical significance in differential protein expression (up and down-regulated) were selected for protein identification by mass spectrometry. This analysis is still in progress.

V. Conclusions

The objectives proposed for the study were achieved with also a few hints for the hypothesis of wide plasticity spectrum to environmental conditions of *Aeromonas piscicola* AH-3, could be withdrawn.

The growth ranges of AH-3 reported in the literature were stricter than these detected in the study: AH-3 grew at higher temperature and salinity values than what was reported earlier.

It was possible to evaluate the effect of different abiotic conditions, temperature, salinity and pH, and their consequent impact in the extracellular proteome (proteases and proteins) and intracellular proteome. High temperatures revealed to be the most stressful abiotic factor to AH-3.

Consequently, proteins up and down-regulated by the stress conditions in comparison with the optimal growth condition were detected. From these proteins it was possible to define a series of stress markers for each abiotic condition and furthermore some stress markers for specific modulation of abiotic factors were detected.

No conclusions relating protease expression and their role in the capability of adaptation to stress conditions could be drawn. However, it was possible to verify that high concentrations of salt (≥ 3.5% NaCl) and high temperatures (> 40 ºC), induces the reduction of the expression of extracellular proteases by AH-3. Yet it was also possible to conclude that growth rate does not correlates with protease expression.

In extreme conditions of stress, the formation of aggregates was observed, that have been associated with chronic infections as well as to an adaptive strategy to colonize adverse environments (135, 136). Therefore is possible to state that AH-3 can survive in the presence of non-favorable environments with the formation of aggregates, and serve at the same time as a strategy for ensuring growth of AH-3 population.

Regarding the results for the analysis of the intracellular proteome, temperature induced the highest differential expression of proteins, corresponding to the abiotic factor with major impact in the proteome. Temperature induced the up-regulation of 22 proteins that are probably involved in response (adaptation) mechanisms to stress. As for the pH, 8 proteins were up-regulated, and for salinity 11 proteins were up-regulated. Additionally, 3 newly synthetized proteins were observed, one when AH-3 was exposed to high temperatures, another when exposed to global temperature stress and 1 when exposed to alkaline pH stress.

Regarding the results for the analysis of the extracellular proteome, temperature induced the highest differential expression of proteins, corresponding to the abiotic factor with the major impact in the proteome response to stress. Temperature induced the up-

regulation of 23 proteins that can probably be involved in response (adaptation) mechanisms to stress. As for the pH, 8 proteins were up-regulated and for salinity 16 proteins up-regulated. No newly synthetized proteins were observed at extracellular level.

The identification by mass spectrometry of the proteins detected would lead to more specific conclusions on the mechanisms that contribute to microbial survival under rapidly changing conditions. The identification of down-regulated proteins are also of interest since they can provide a large picture of the biological processes that are occurring in the bacteria when under stress.

It is evident that *Aeromonas piscicola* AH-3 have a global pre-existing system of protection, since in general when in stress conditions AH-3 differential expression of proteins occurs with the up-regulation of certain proteins and not with newly synthetized proteins. This fact is probably related with the high capability of plasticity towards environmental conditions presented by *Aeromonas*. Therefore it is possible to conclude that, AH-3 when exposed to stressful conditions either caused by climate changes or when invading a host, is capable of express the necessary mechanisms for survival and growth in the abiotic conditions encountered.

VI. Future perspectives

In the line of the results obtained it would be of great interest the improvement on the knowledge of mechanisms associated with the stress response of *Aeromonas piscicola* AH-3. To accomplish this, it will be essential to identify the proteins that were up- and down-regulated in the different abiotic conditions evaluated, as well as identify the extracellular proteases. Furthermore, evaluation of the membrane proteome under the abiotic conditions used for evaluation of the other fractions of the proteome, may help in the identification and definition of adaptation mechanisms. In this way, it will be possible to obtain data concerning the physiology of fitness regulation and possibly understand features favouring the emergence of these microorganisms as human pathogens. Additionally other abiotic conditions, such as antibiotics, atmosphere restriction and nutrient starvation could be added for proteomic evaluation.

Study of other extracellular enzymes, than proteases, like lipases, could be of great interest since their involvement in some *Aeromonas* spp. virulence has been described.

Other future work may involve the study of the observed aggregates of the bacterial cells in extreme stress conditions through a proteomic approach, in order to a better understand of the specific mechanisms in response to stress conditions.

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VIII. Annexes

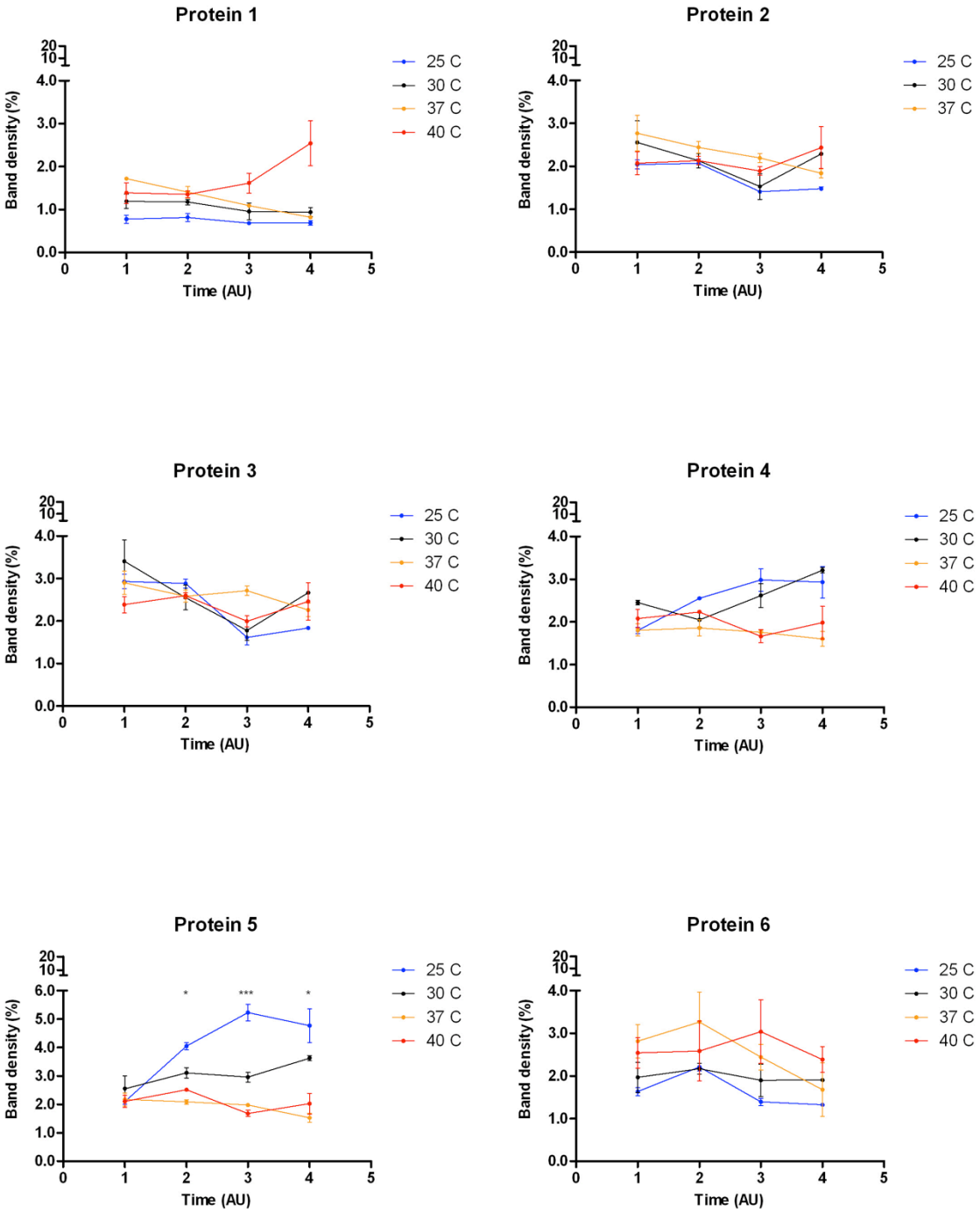
1. Differential expression of protein bands

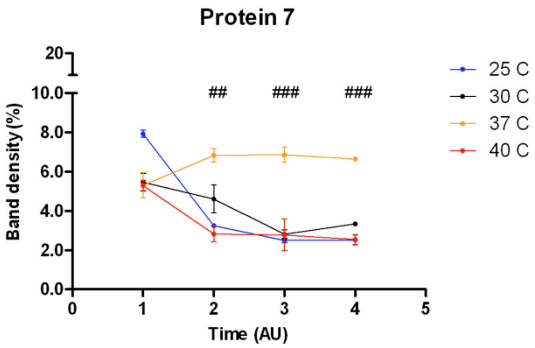
Using a Two-way ANOVA statistical test, differential expressions of proteins were statistically analysed. Evaluation of differential expression of protein bands graphics can be visualized in the annexed graphics. Statistical analysis of the differential expression is described with the following leged:

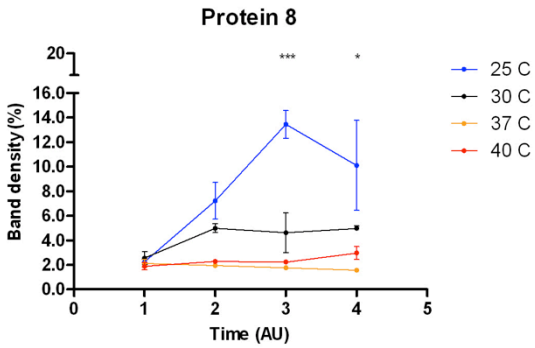
***** - $p < 0.05$ for conditions of 25 ºC, 0% NaCl and pH 5.0;
****** - $p < 0.01$ for conditions of 25 ºC, 0% NaCl and pH 5.0;
******* - $p < 0.001$ for conditions of 25 ºC, 0% NaCl and pH 5.0;
# - $p < 0.05$ for conditions of 37 ºC, 3.5% NaCl and pH 9.0;
- $p < 0.01$ for conditions of 37 ºC, 3.5% NaCl and pH 9.0;
- $p < 0.001$ for conditions of 37 ºC, 3.5% NaCl and pH 9.0;
+ - $p < 0.05$ for conditions of 40 ºC;
++ - $p < 0.01$ for conditions of 40 ºC;
+++ - $p < 0.001$ for conditions of 40 ºC;

1.1. Extracellular proteins

1.1.1. Temperature




Protein 7
Band density (%)
Time (AU)
##
###
###
25 C
30 C
37 C
40 C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Band density (%)
Time (AU)
***
*
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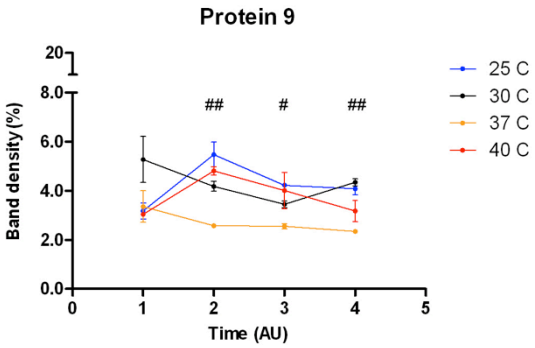
30 C
37 C
40 C


Protein 9
Band density (%)
Time (AU)
##
#
##
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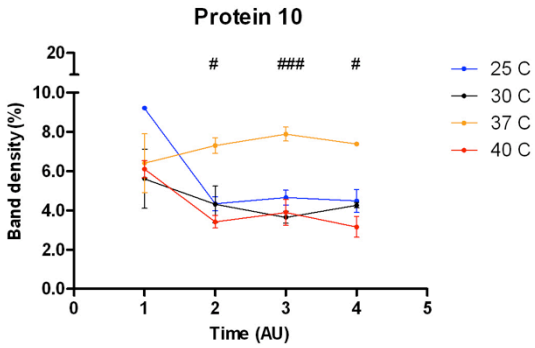
30 C
37 C
40 C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Band density (%)
Time (AU)
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#
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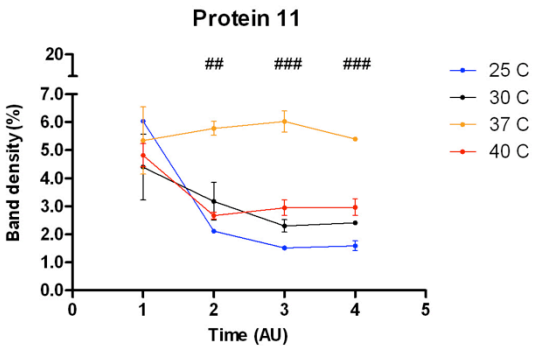
30 C
37 C
40 C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Band density (%)
Time (AU)
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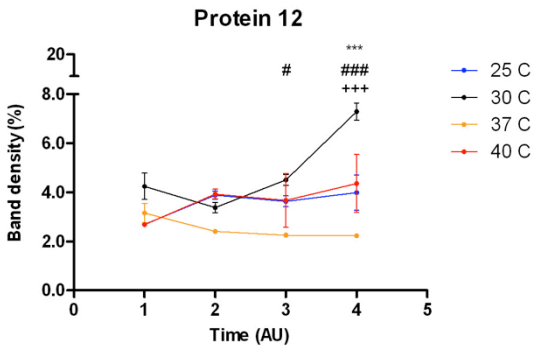
37 C
40 C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Band density (%)
Time (AU)
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+++
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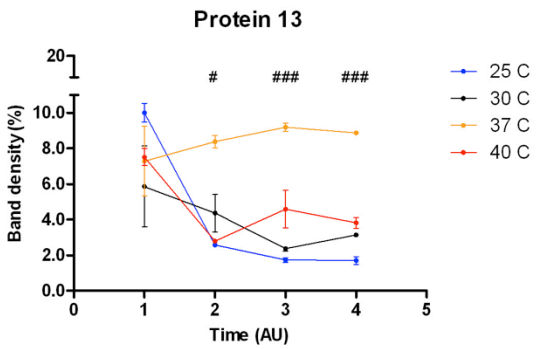
30 C
37 C
40 C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Band density (%)
Time (AU)
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30 C
37 C
40 C
#
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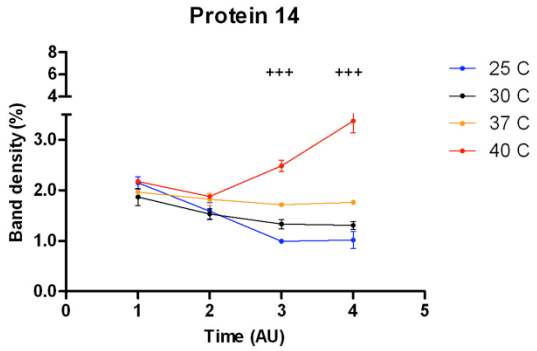
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Band density (%)
Time (AU)
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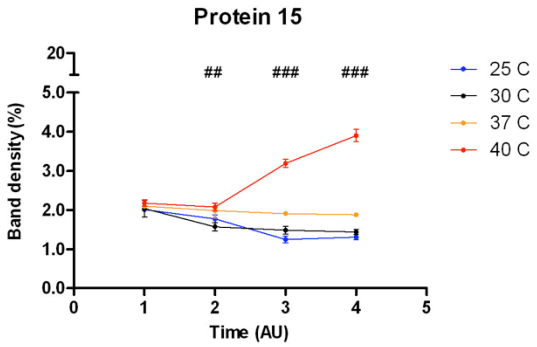
30 C
37 C
40 C
+++
+++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Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
##
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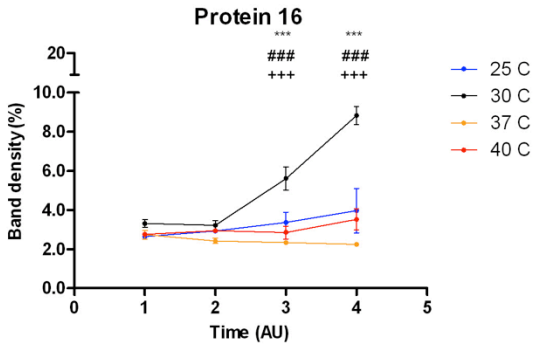
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Band density (%)
Time (AU)
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37 C
40 C
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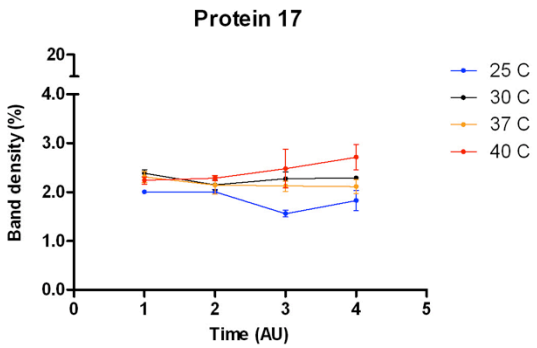
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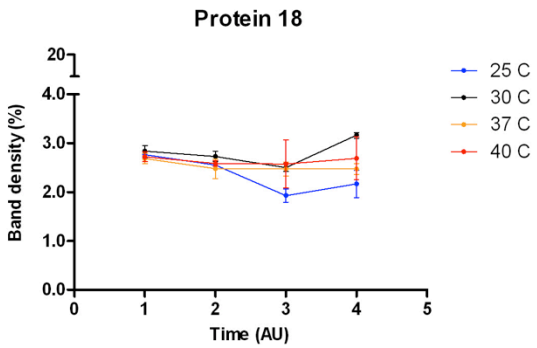
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Band density (%)
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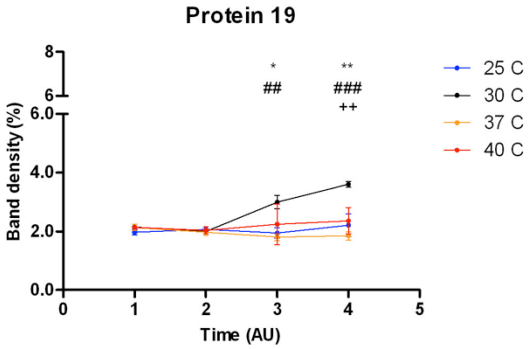
40 C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Band density (%)
Time (AU)
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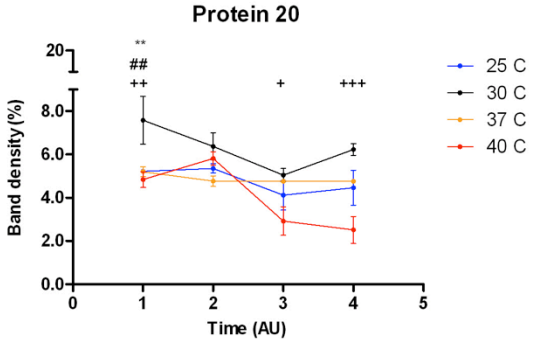
40 C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Band density (%)
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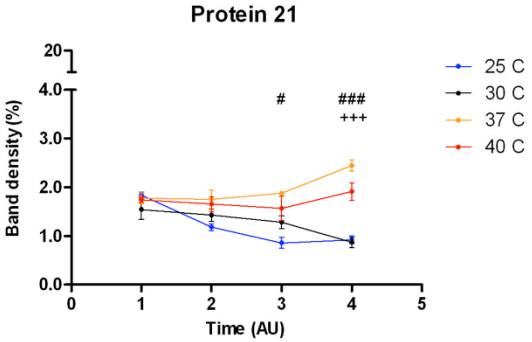
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Band density (%)
Time (AU)
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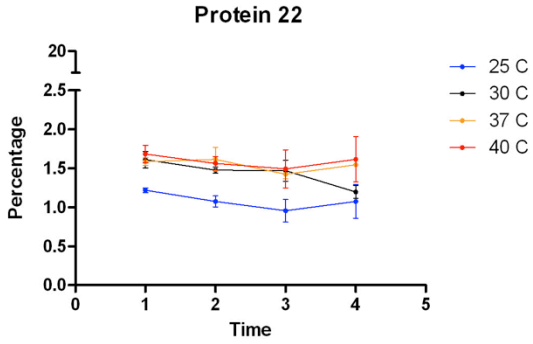
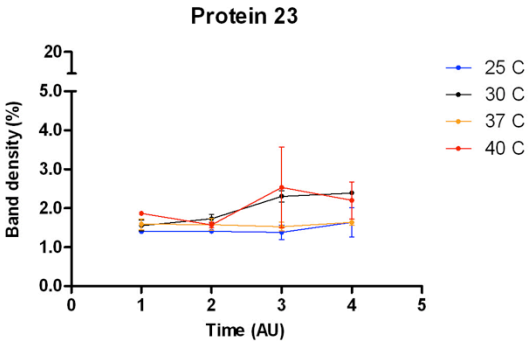
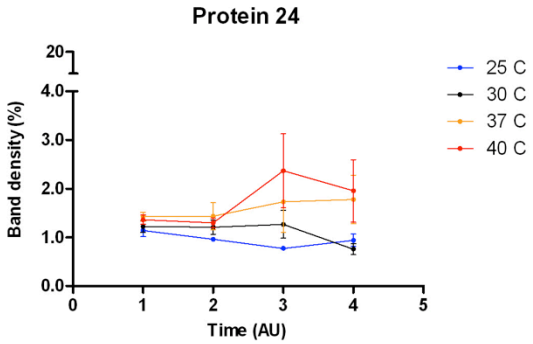
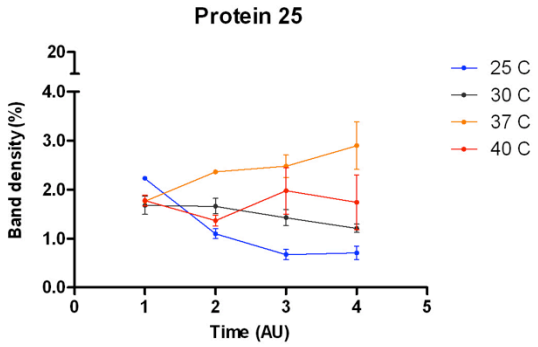
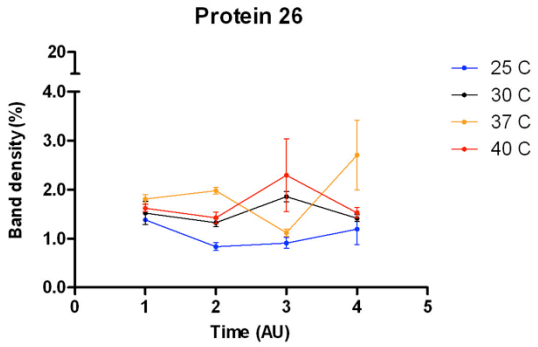
30 C
37 C
40 C

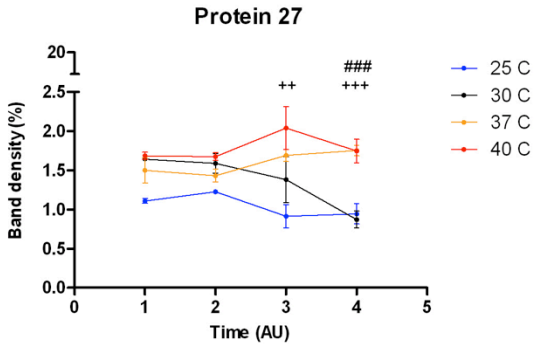

Protein 22
Percentage
Time
25 C
30 C
37 C
40 C


Protein 23
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 24
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 25
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 26
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 27
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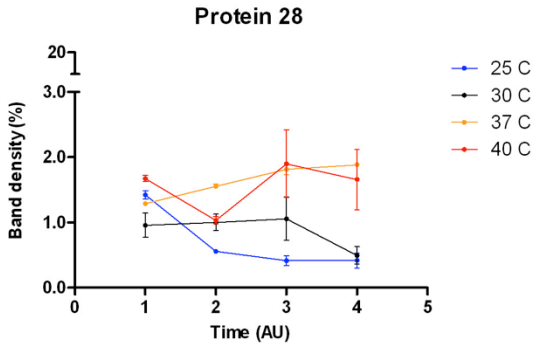
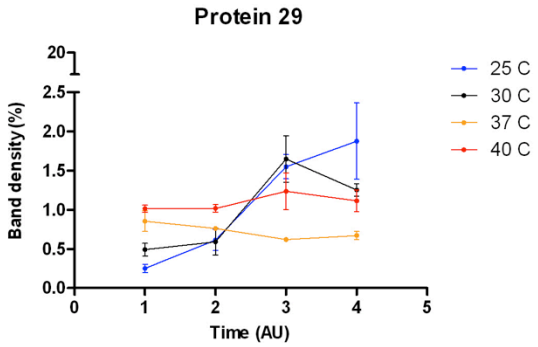
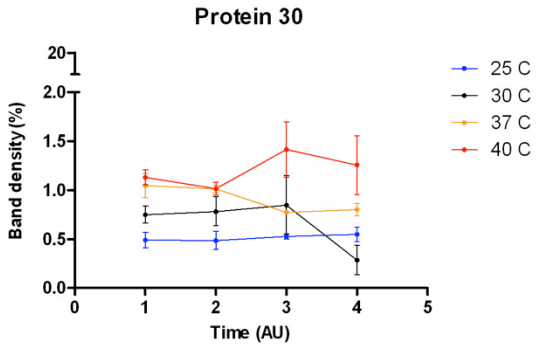
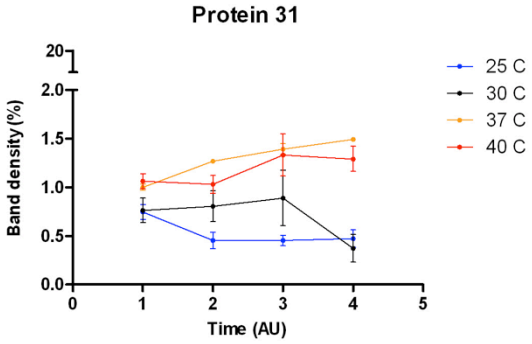
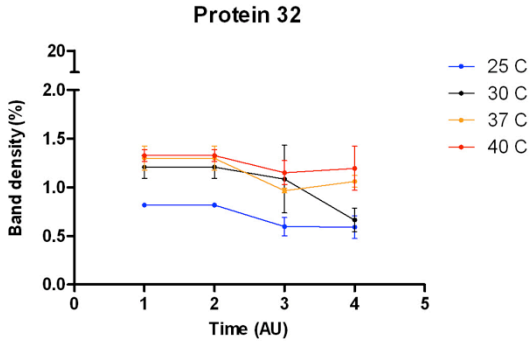
+++
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

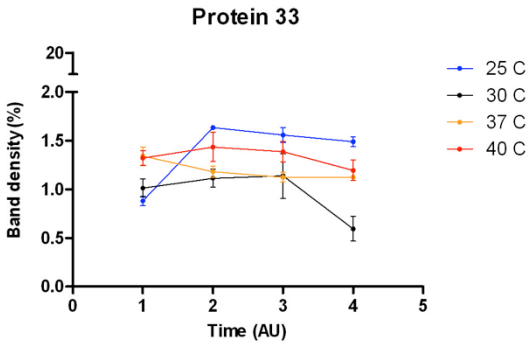

Protein 28
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

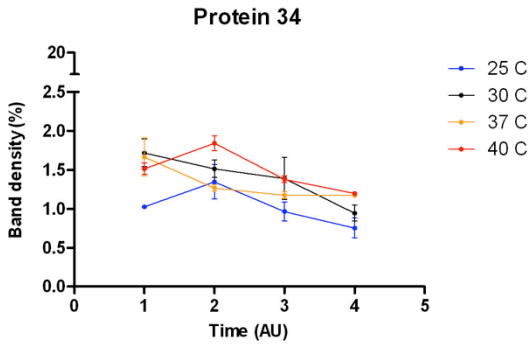

Protein 29
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

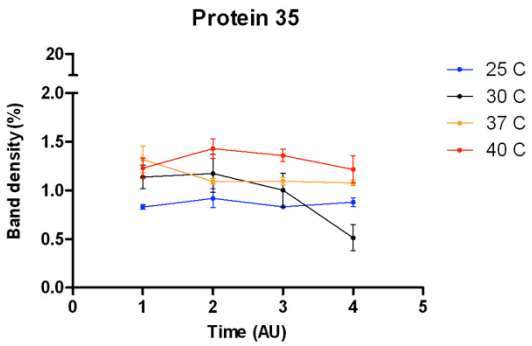

Protein 30
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

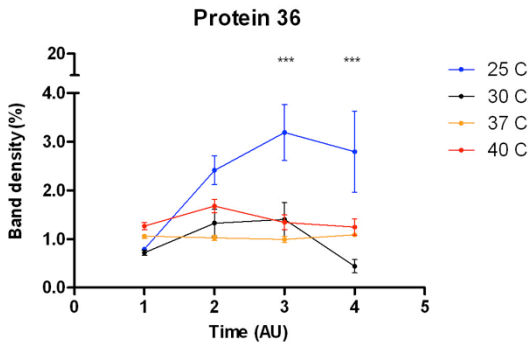

Protein 31
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 32
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 33
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 34
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 35
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 36
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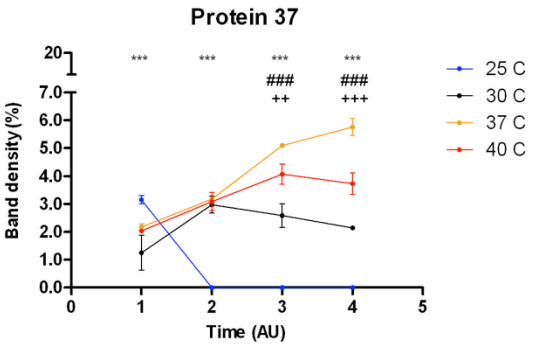
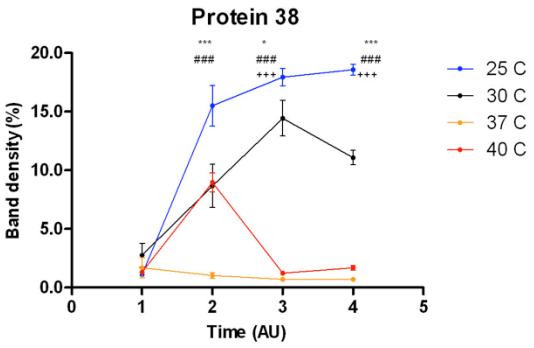
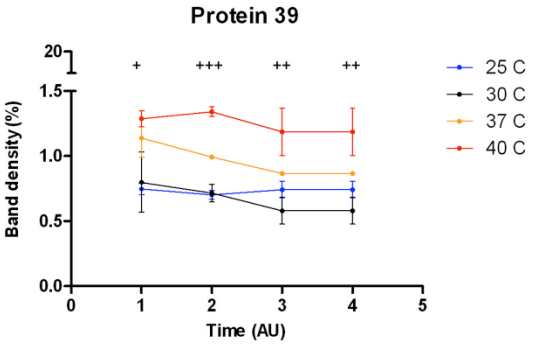
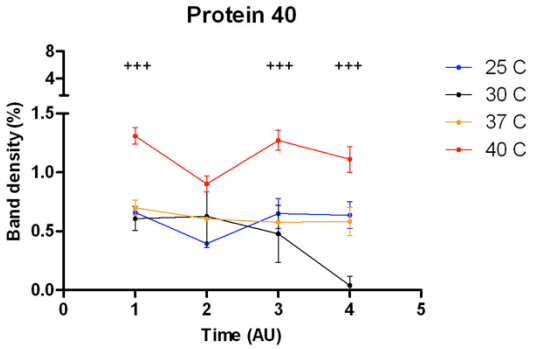
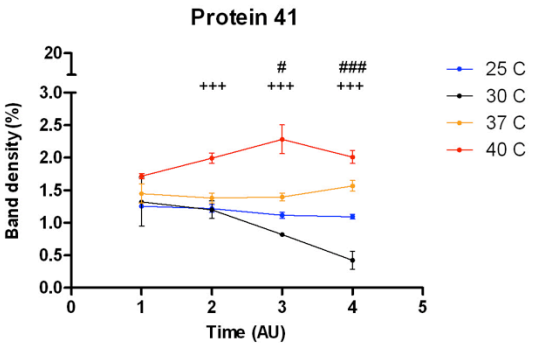
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

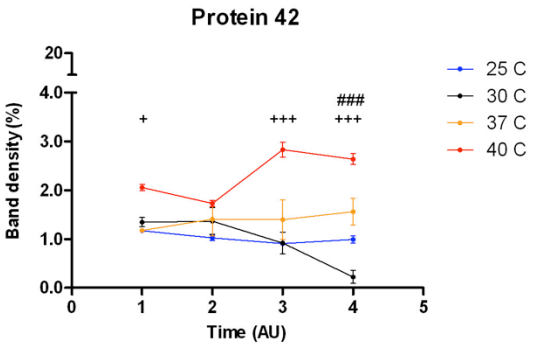

Protein 37
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 38
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

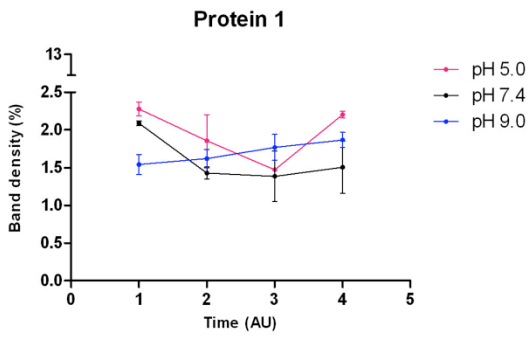

Protein 39
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

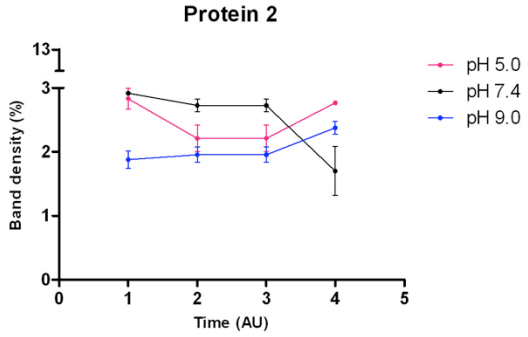

Protein 40
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

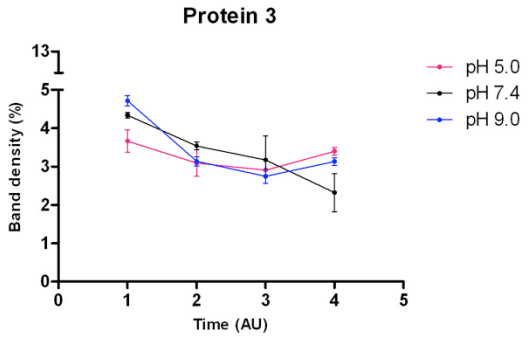

Protein 41
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

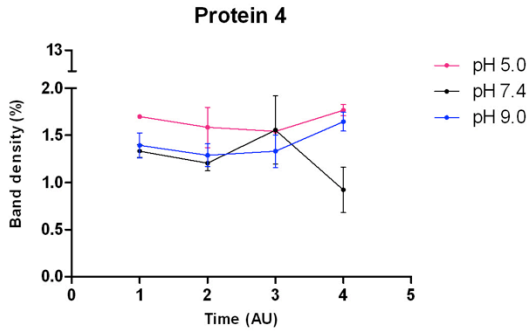

Protein 42
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

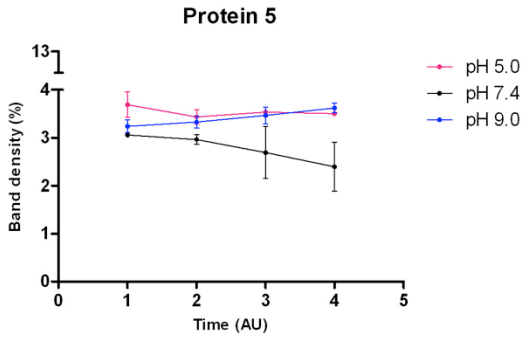
1.1.2. pH

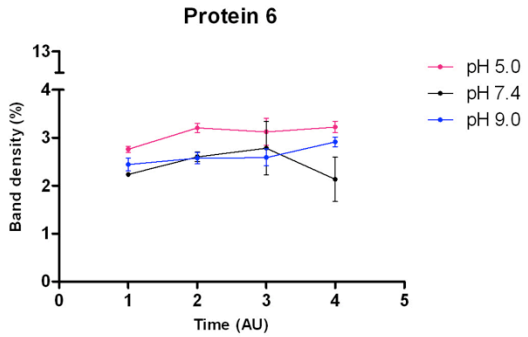

Protein 1
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

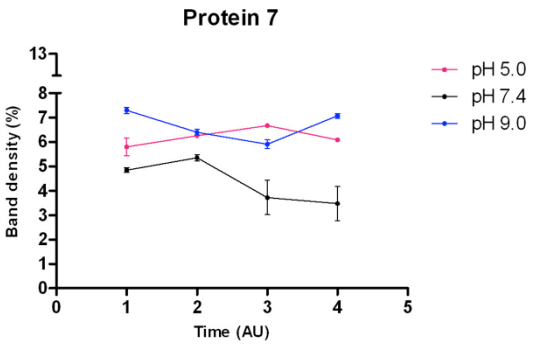

Protein 2
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

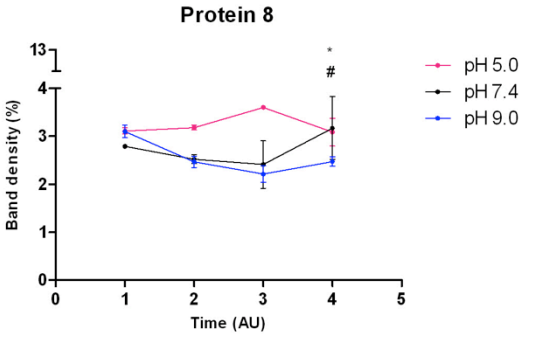

Protein 3
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 4
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 5
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 6
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 7
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 8
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
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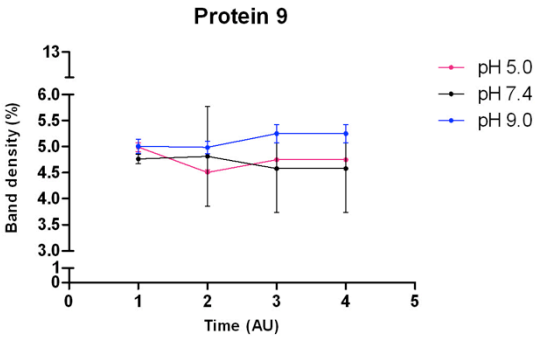
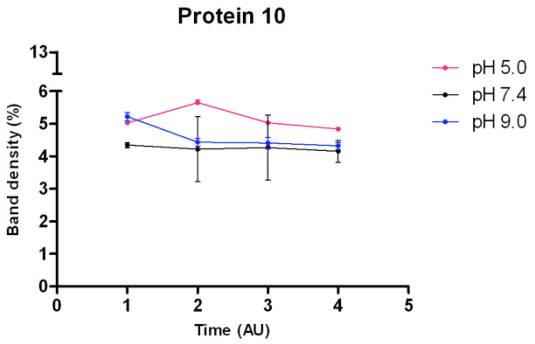
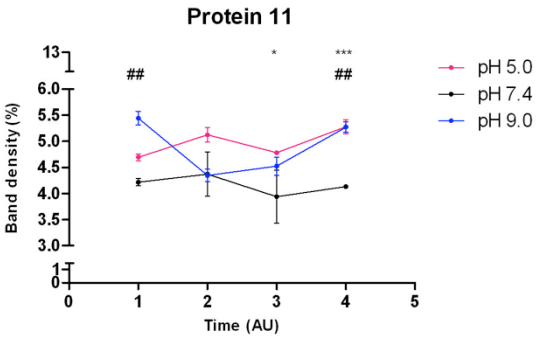

Protein 9
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 10
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 11
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
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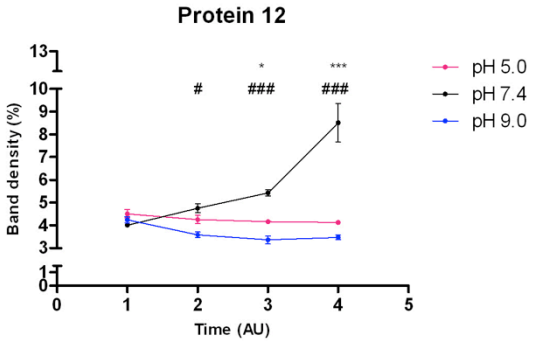
Protein 12
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
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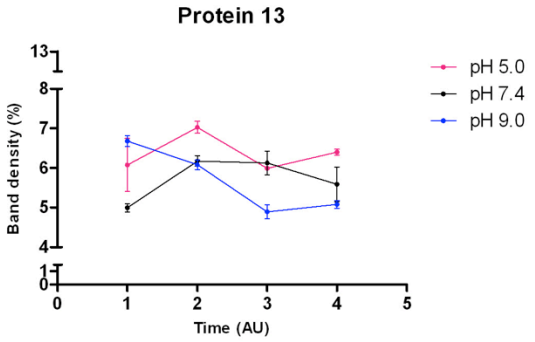
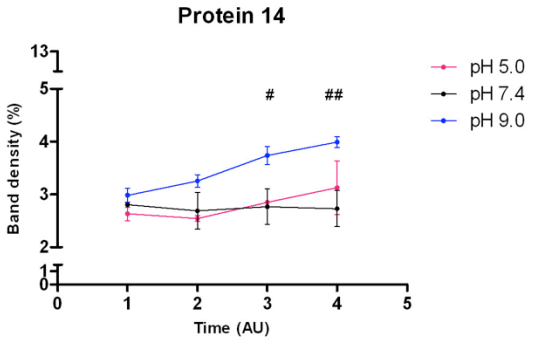
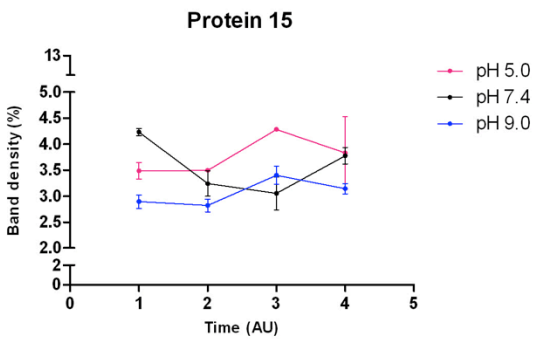
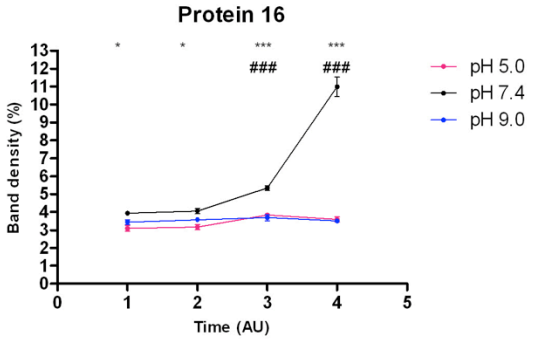

Protein 13
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 14
Band density (%)
Time (AU)
#
##
pH 5.0
pH 7.4
pH 9.0


Protein 15
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 16
Band density (%)
Time (AU)
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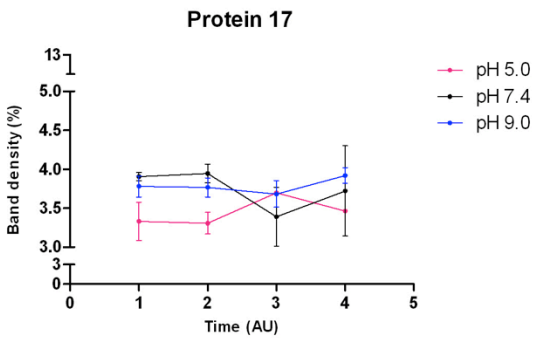
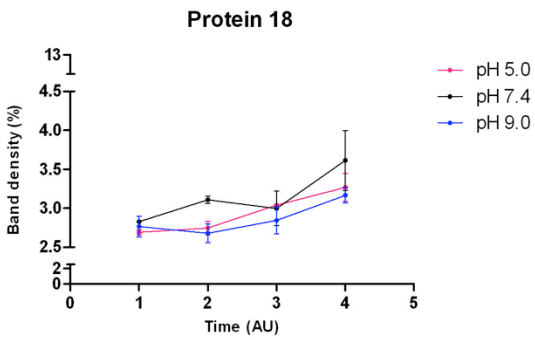
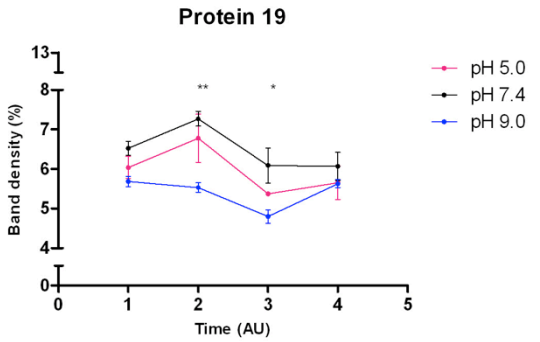
pH 5.0
pH 7.4
pH 9.0


Protein 17
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 18
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 19
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
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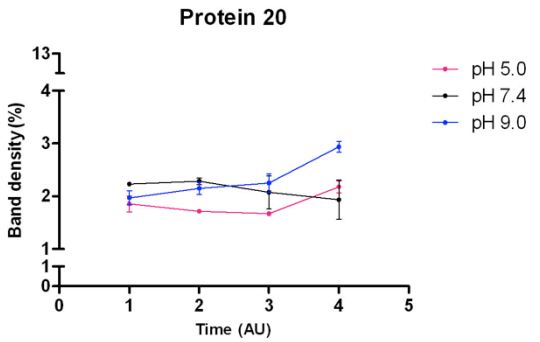
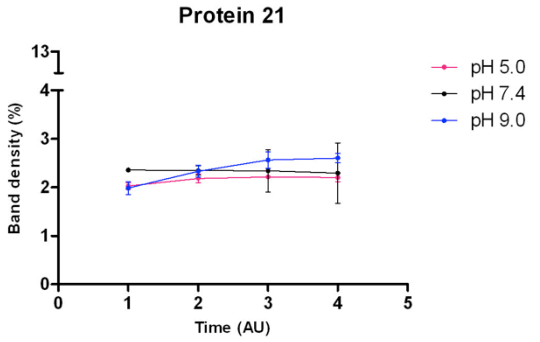
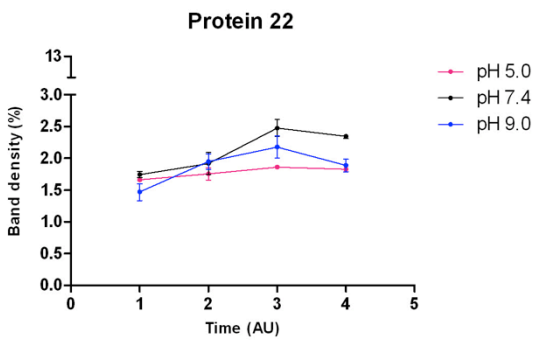
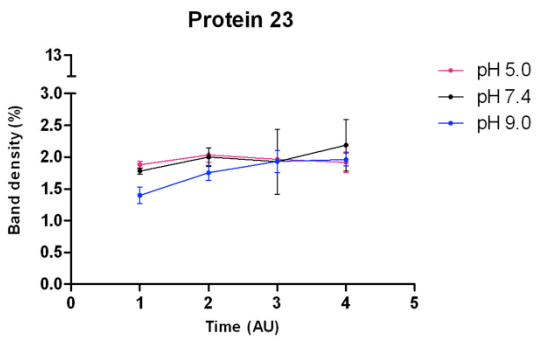
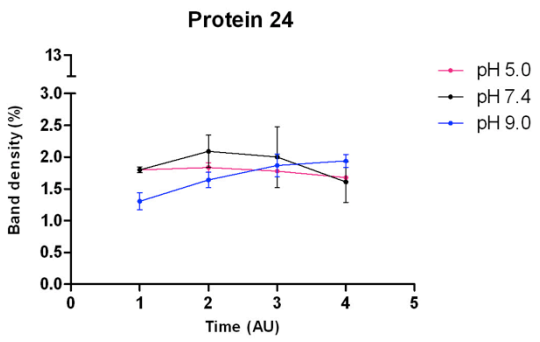
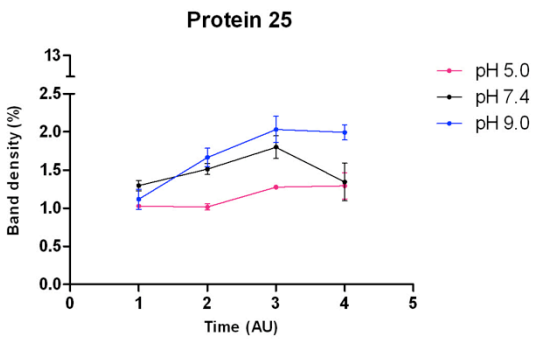

Protein 20
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

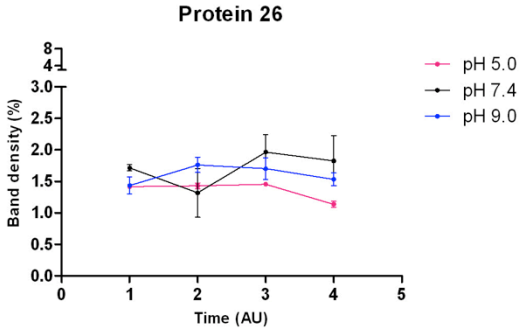

Protein 21
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

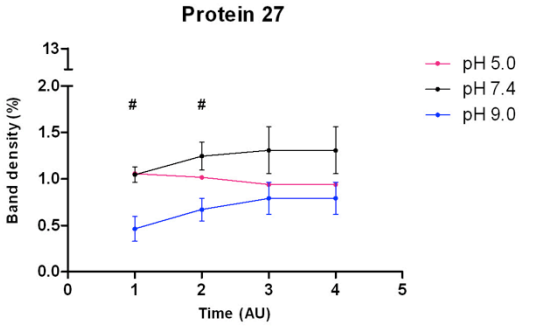

Protein 22
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

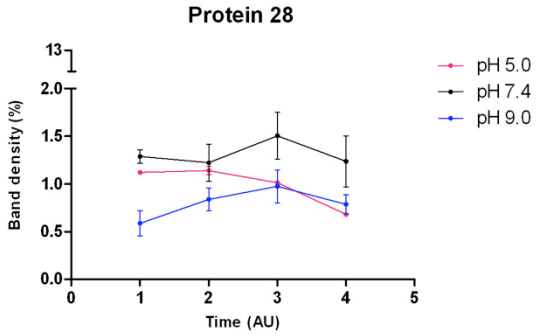

Protein 23
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

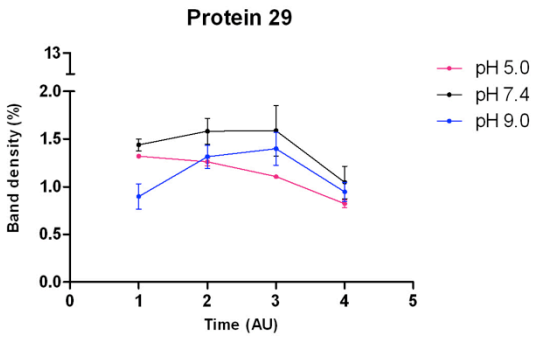

Protein 24
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

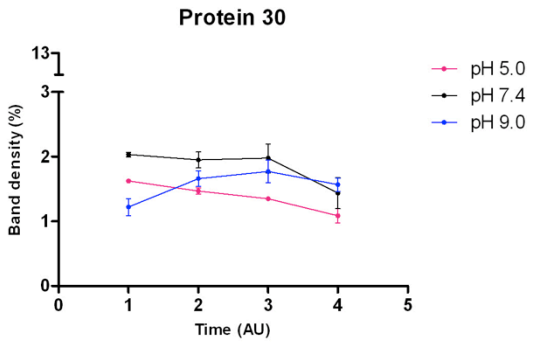

Protein 25
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

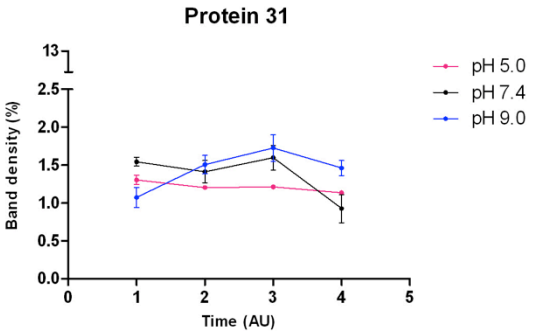

Protein 26
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

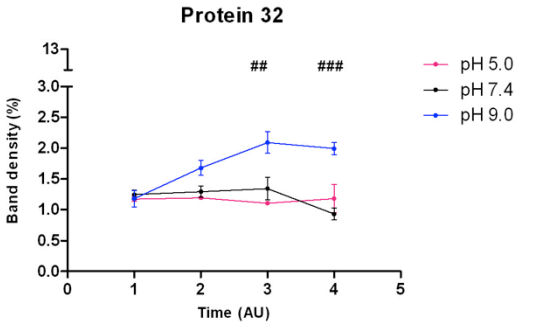

Protein 27
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
#
#

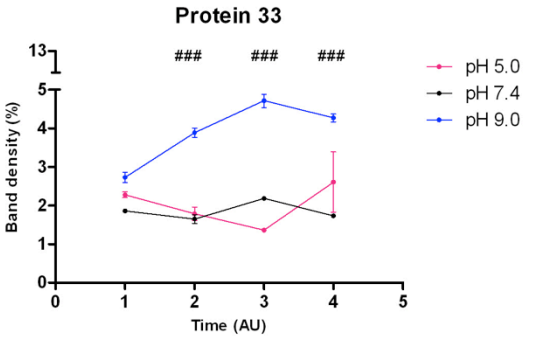

Protein 28
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

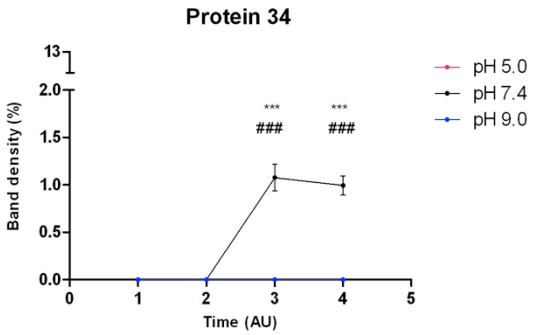

Protein 29
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

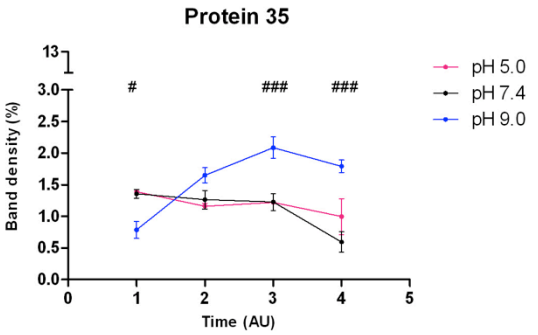

Protein 30
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

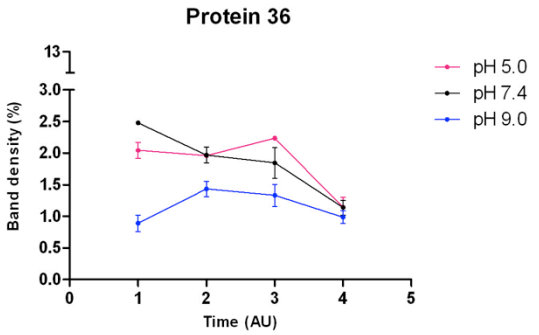

Protein 31
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

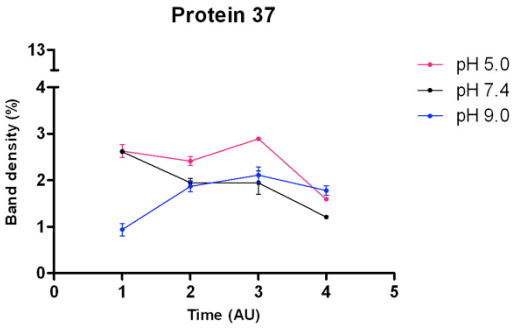

Protein 32
##
###
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 33
###
###
###
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

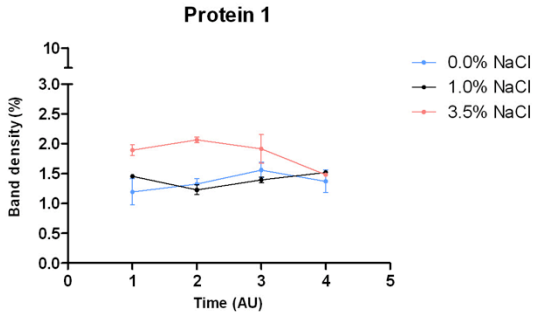

Protein 34
***
***
###
###
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

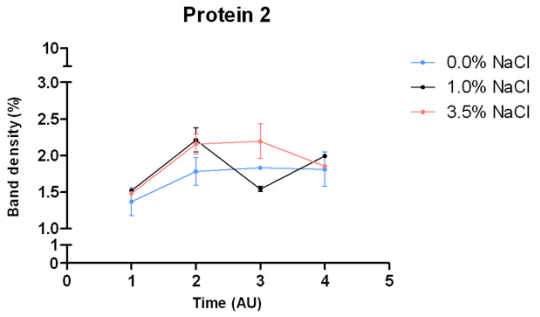

Protein 35
#
###
###
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

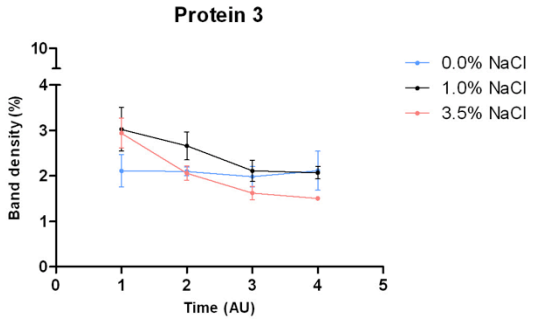

Protein 36
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

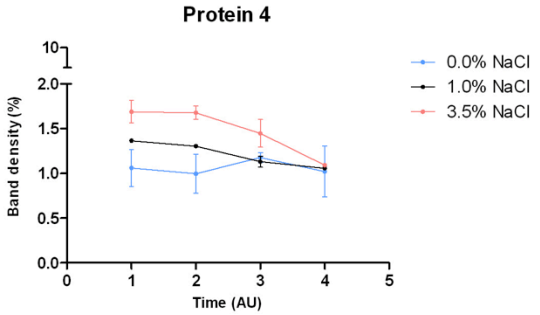

Protein 37
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

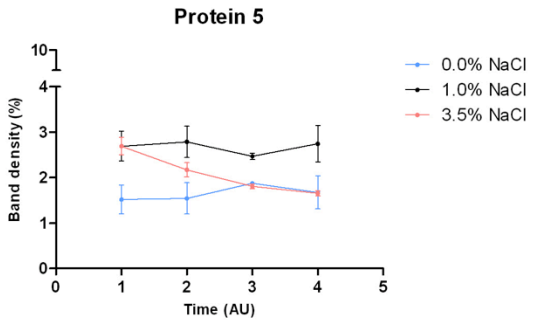
1.1.3. Salinity

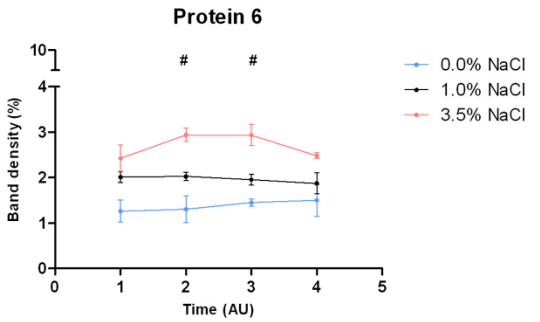


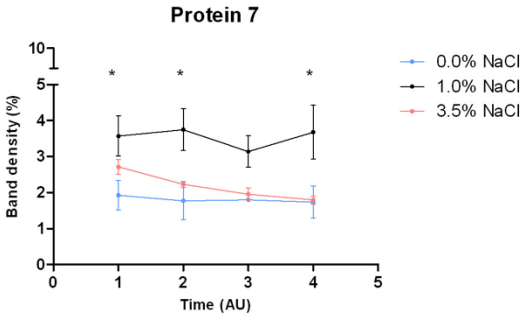


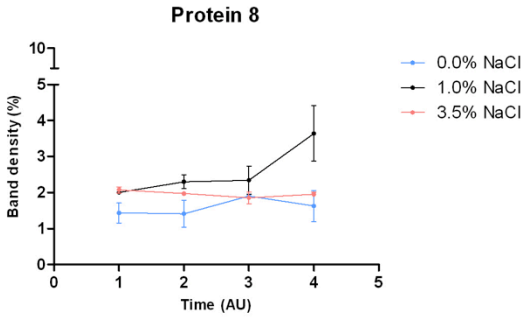


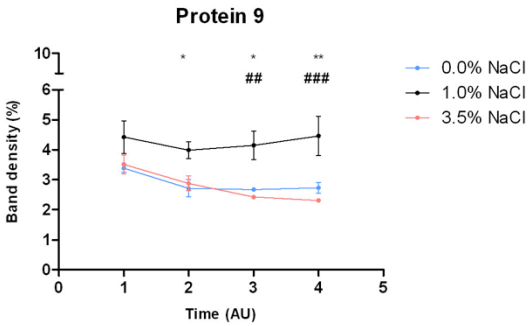


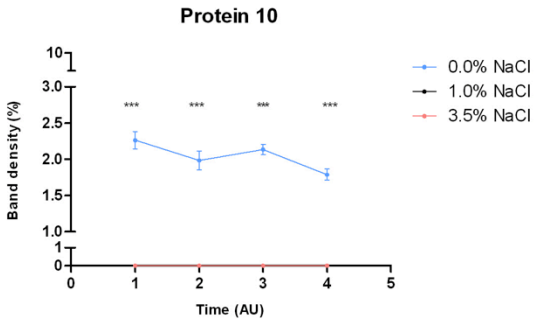


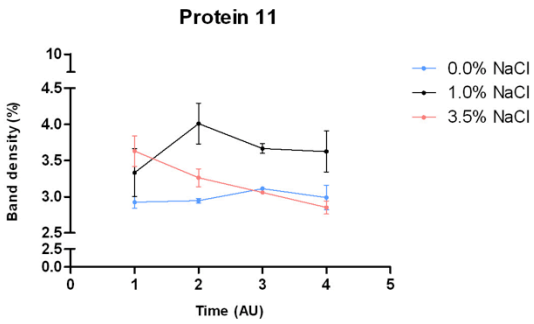


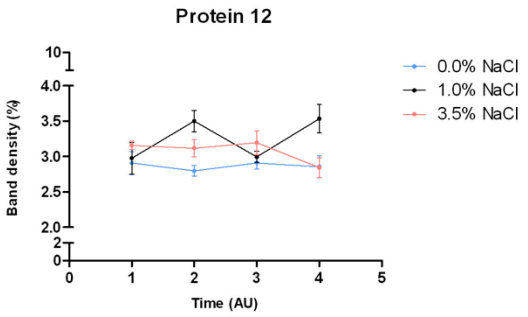

Protein 7
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

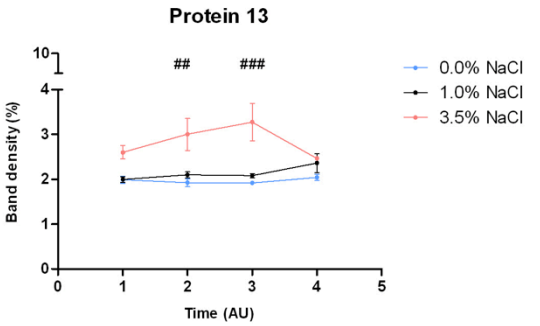

Protein 8
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

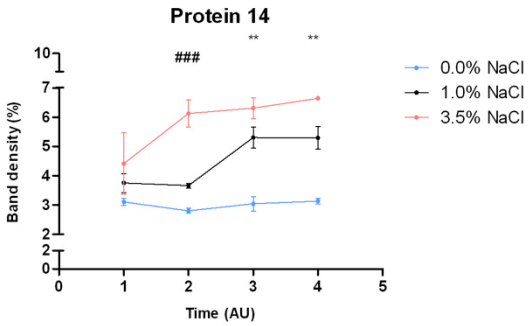

Protein 9
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

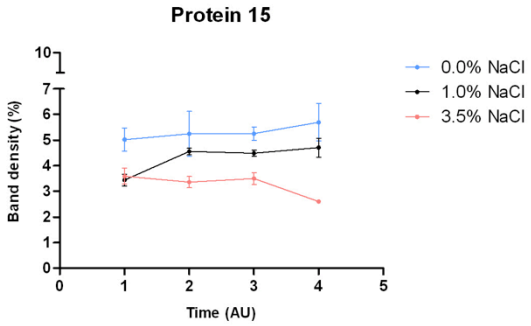

Protein 10
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

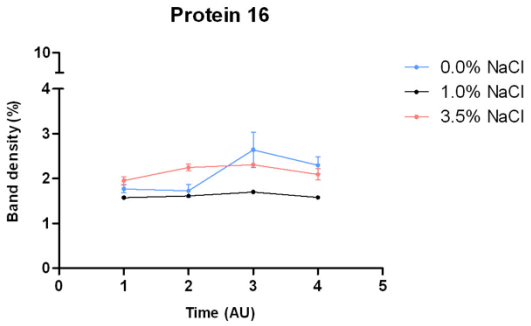

Protein 11
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

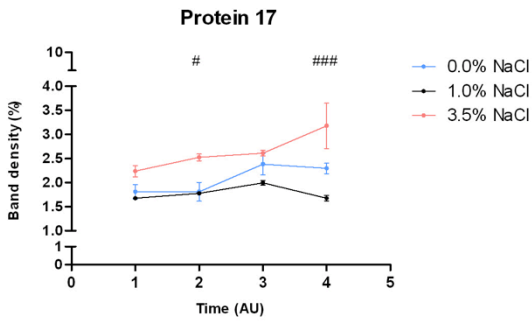

Protein 12
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

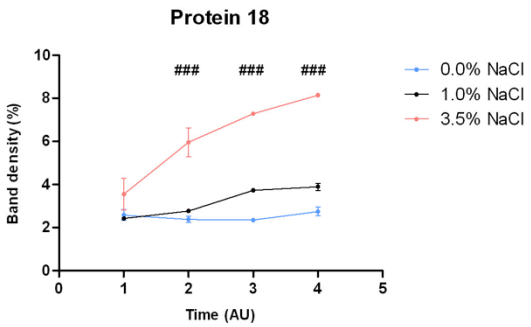

Protein 13
##
###
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

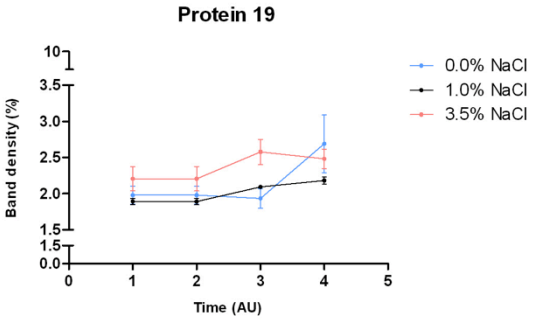

Protein 14
###
**
**
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

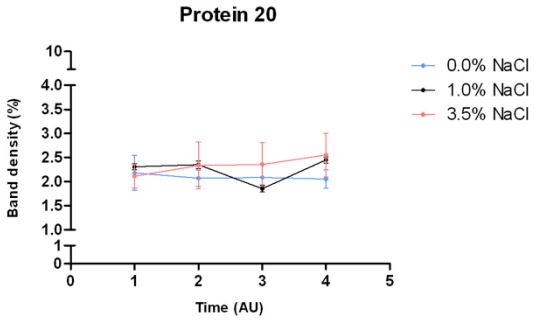

Protein 15
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

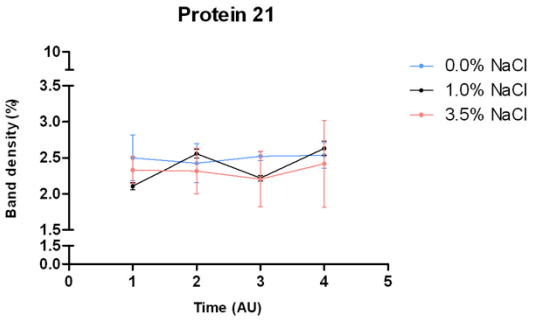

Protein 16
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

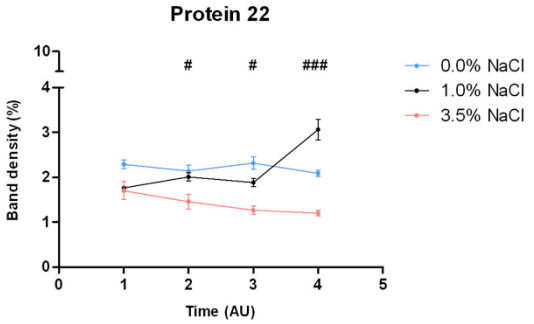

Protein 17
#
###
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

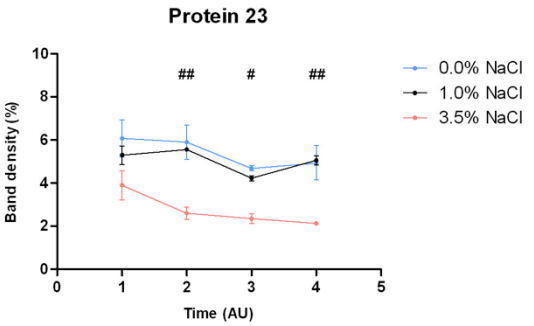

Protein 18
###
###
###
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

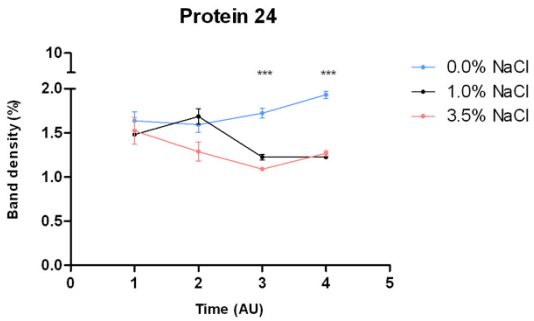

Protein 19
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

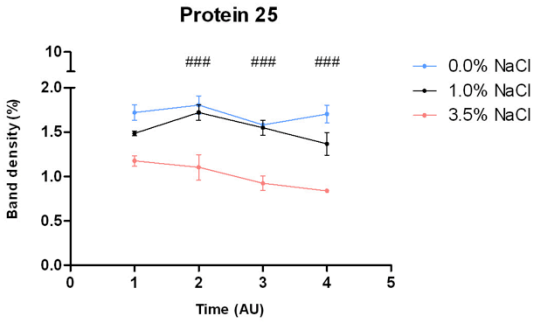

Protein 20
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

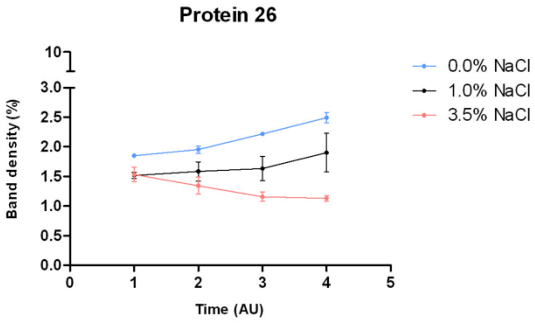

Protein 21
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

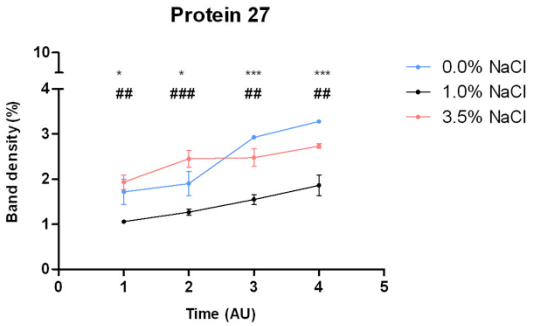

Protein 22
#
#
###
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

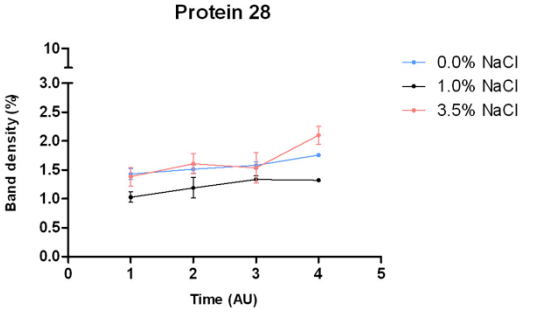

Protein 23
##
#
##
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

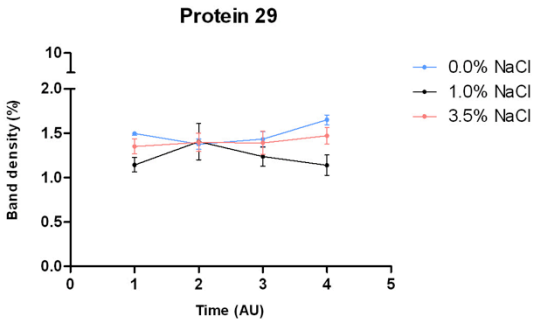

Protein 24
***
***
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

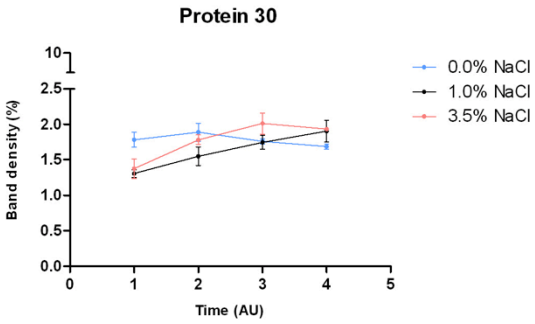

Protein 25
###
###
###
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

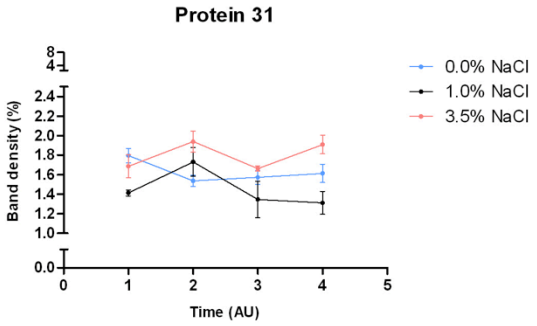

Protein 26
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

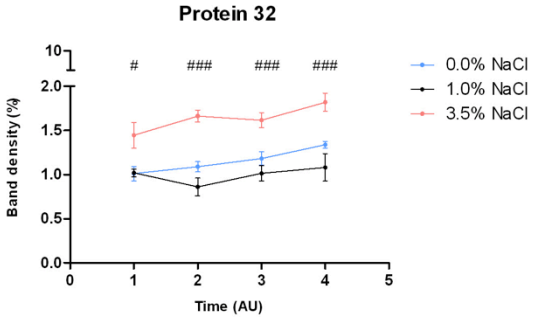

Protein 27
*
*
***
***
##
###
##
##
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

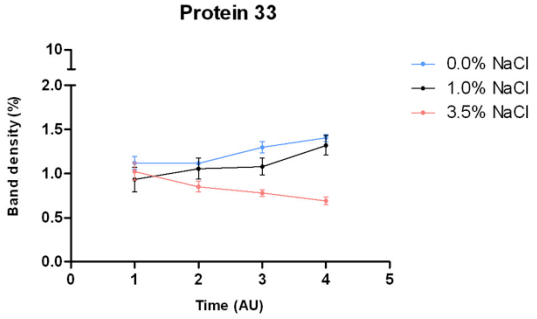

Protein 28
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

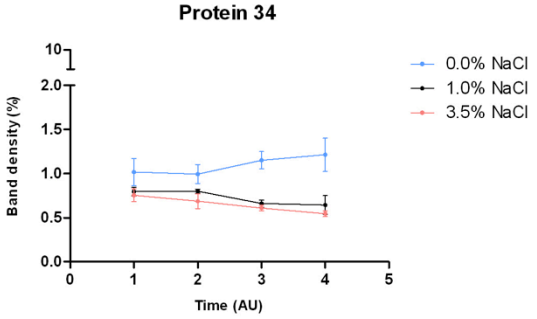

Protein 29
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

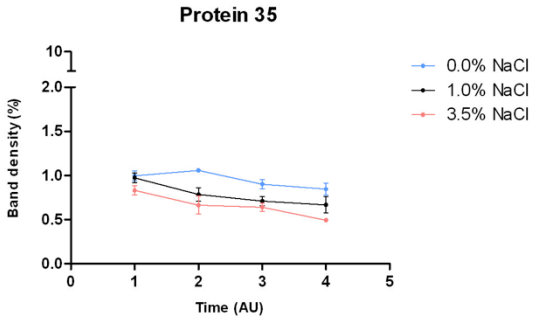

Protein 30
0.0% NaCl
1.0% NaCl
3.5% NaCl
Band density (%)
Time (AU)

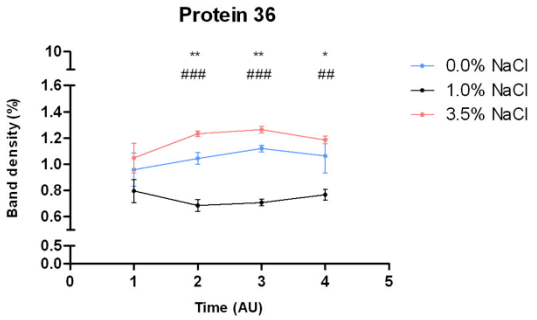

Protein 31
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

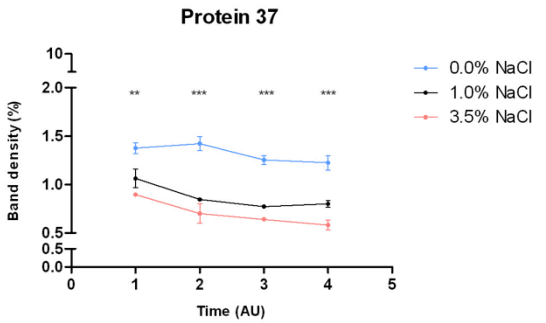

Protein 32
#
###
###
###
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

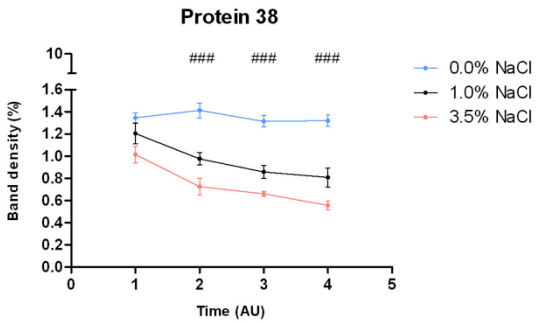

Protein 33
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

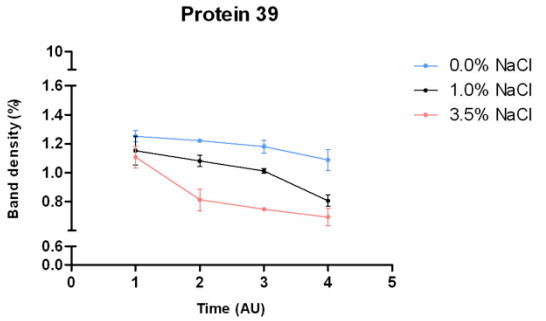

Protein 34
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

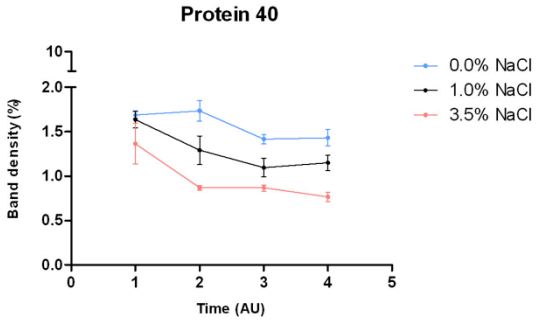

Protein 35
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

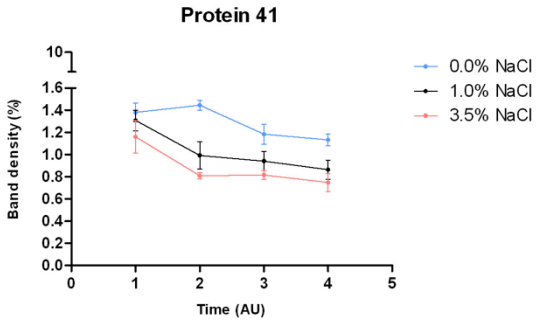

Protein 36
**
**
*
###
###
##
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

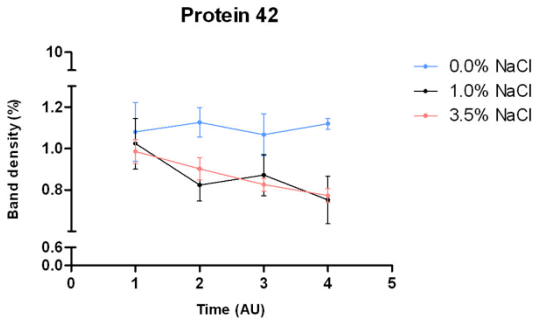

Protein 37
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl
**
***
***
***

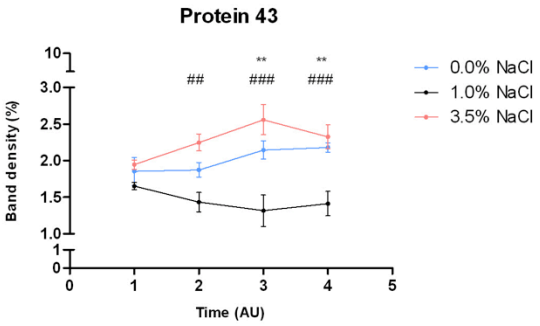

Protein 38
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl
###
###
###

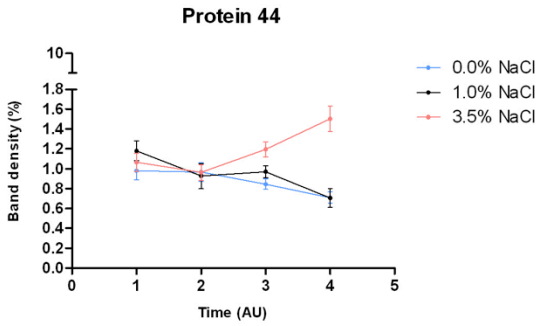

Protein 39
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

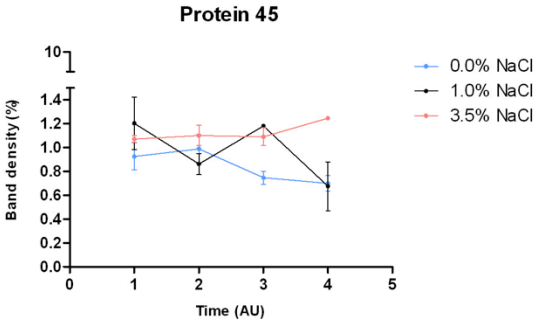

Protein 40
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

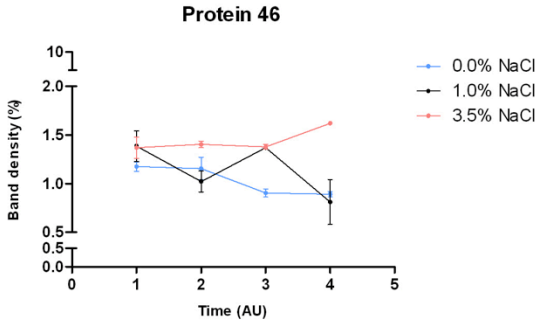

Protein 41
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

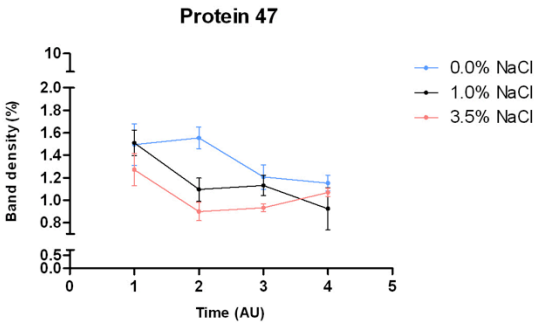

Protein 42
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

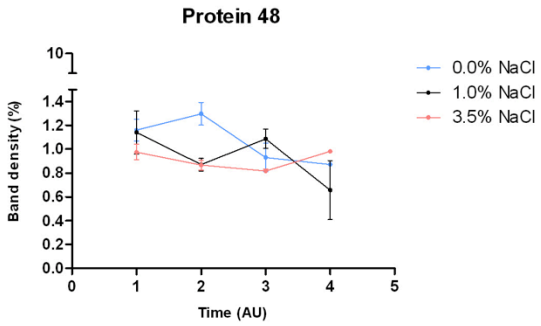

Protein 43
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl
##
**
###
**
###

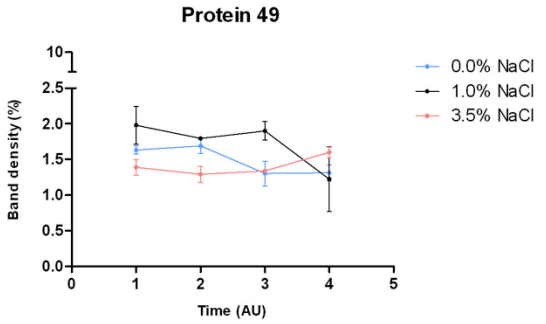

Protein 44
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

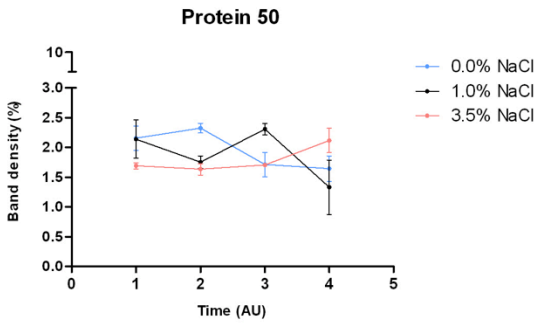

Protein 45
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

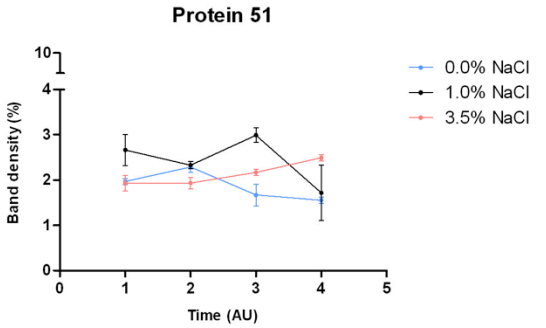

Protein 46
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

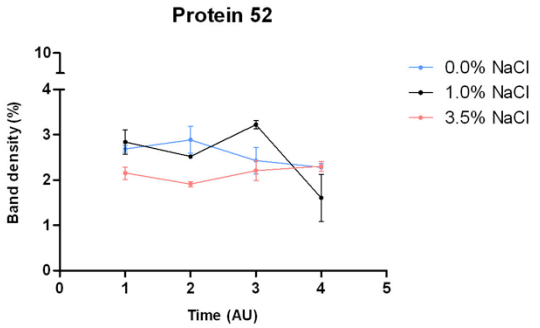

Protein 47
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

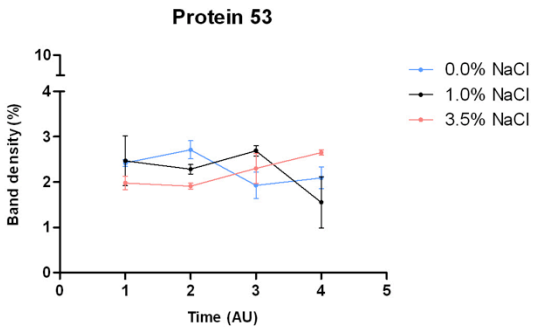

Protein 48
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 49
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 50
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

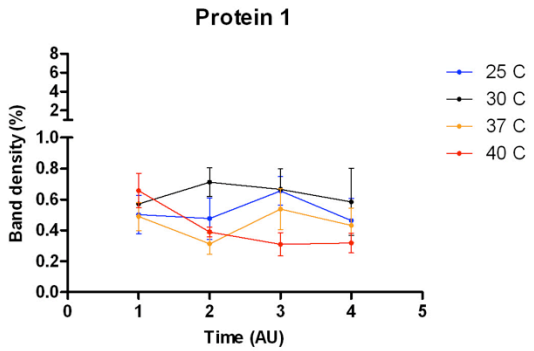

Protein 51
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

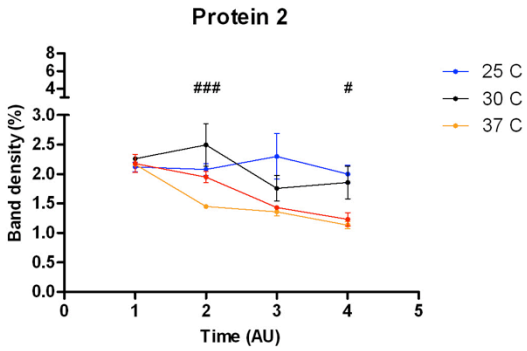

Protein 52
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

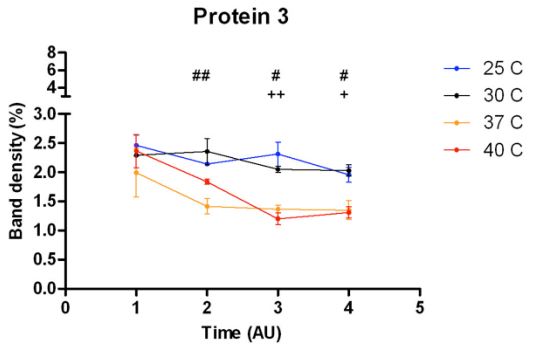

Protein 53
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

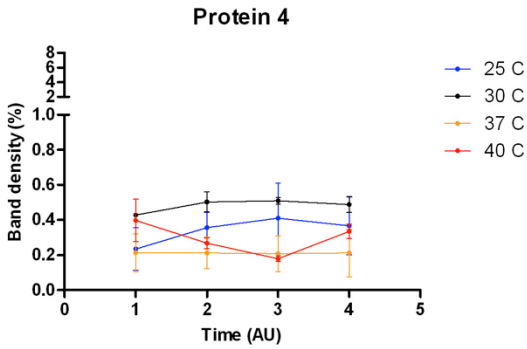
1.2. Intracellular proteins

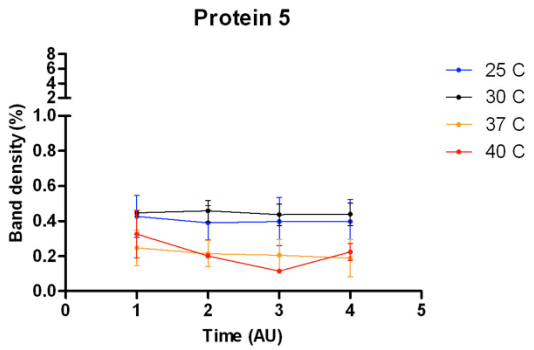
1.2.1. Temperature

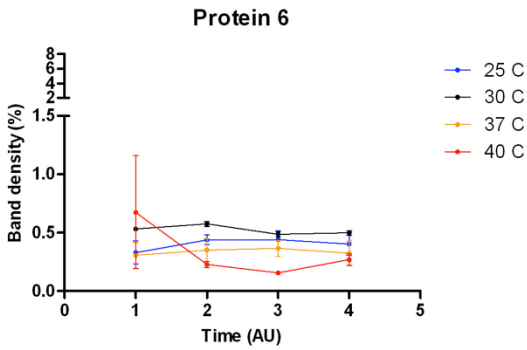


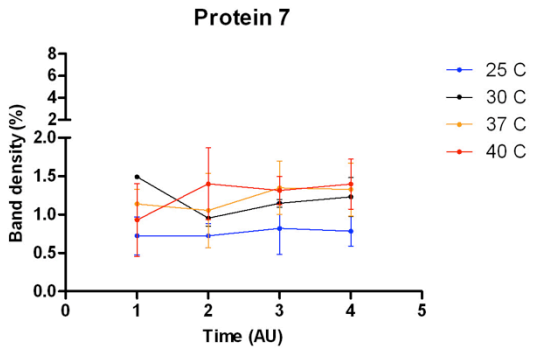


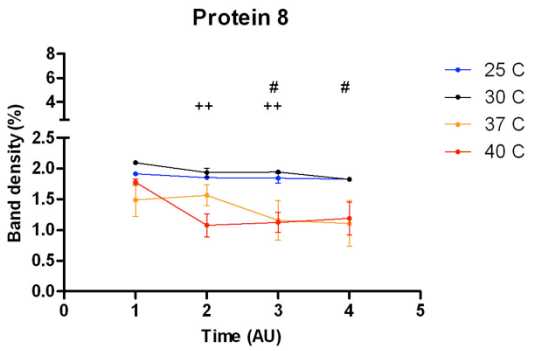


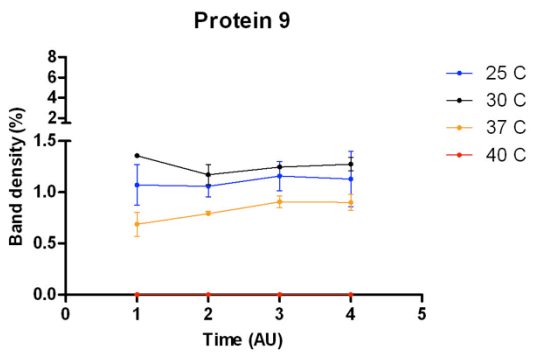


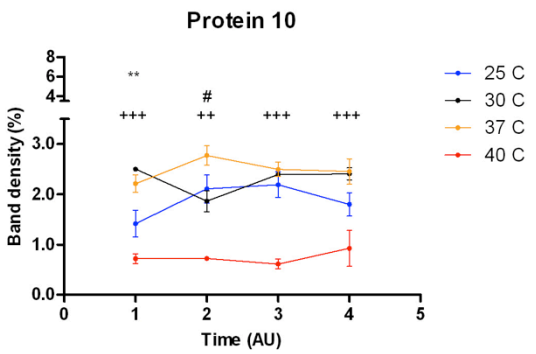


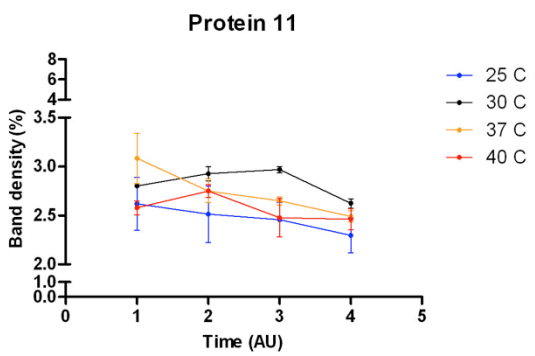


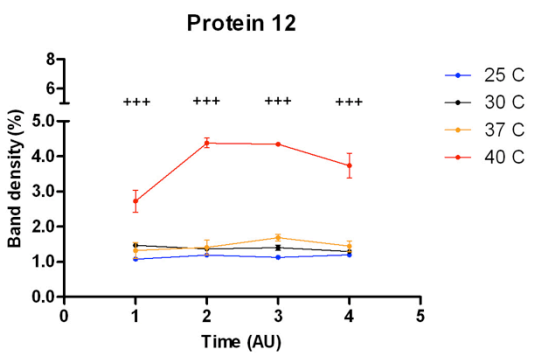

Protein 7
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

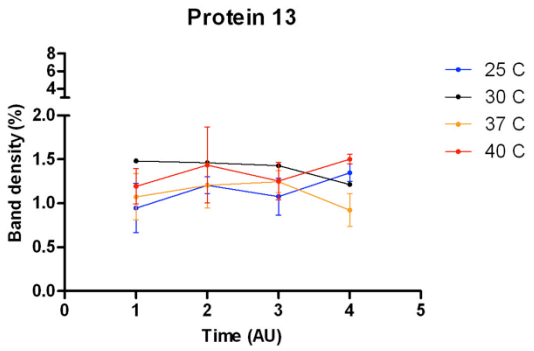

Protein 8
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

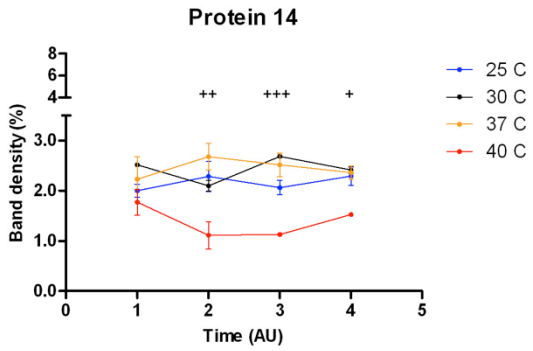

Protein 9
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

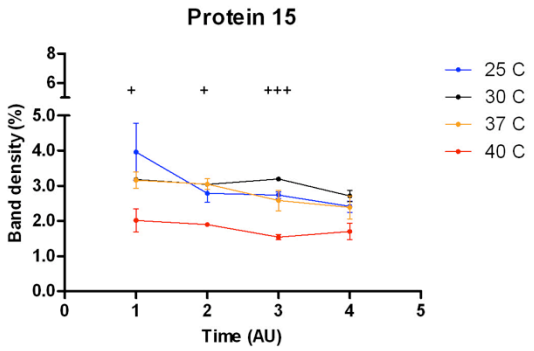

Protein 10
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

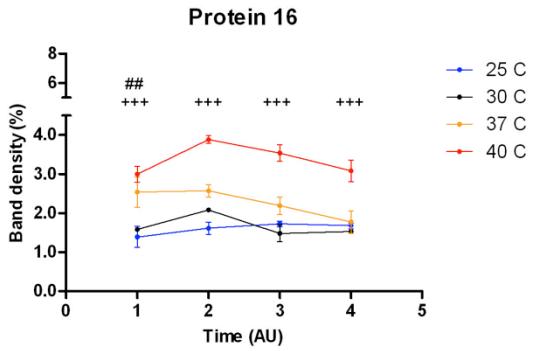

Protein 11
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

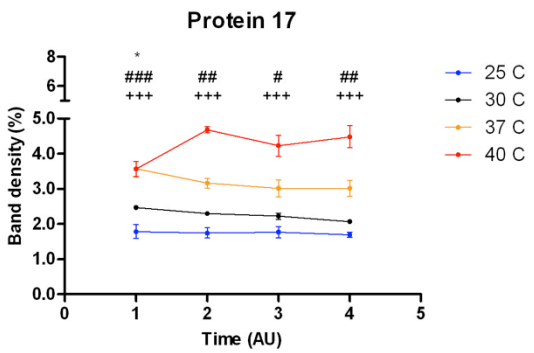

Protein 12
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

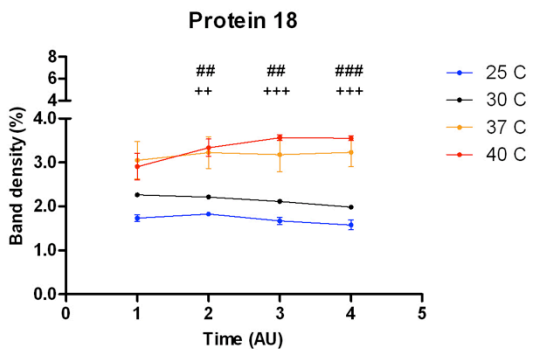

Protein 13
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

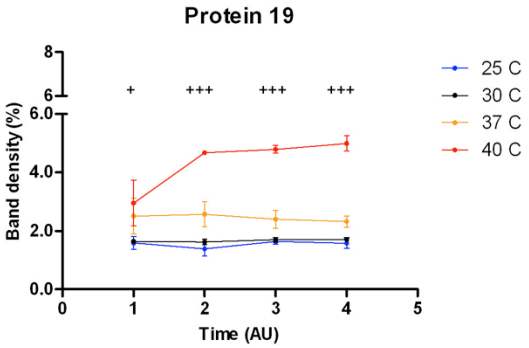

Protein 14
++
+++
+
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

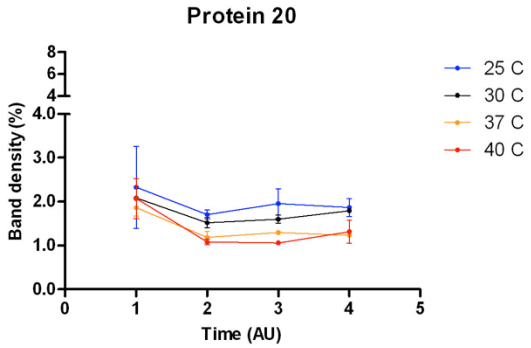

Protein 15
+
+
+++
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

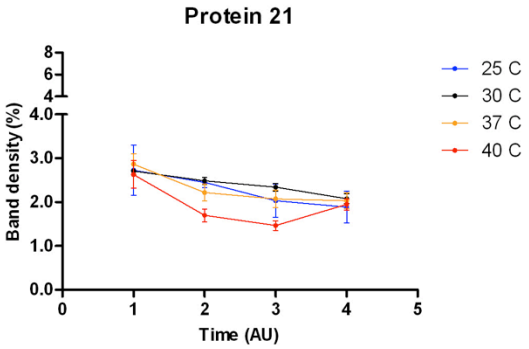

Protein 16
##
+++
+++
+++
+++
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

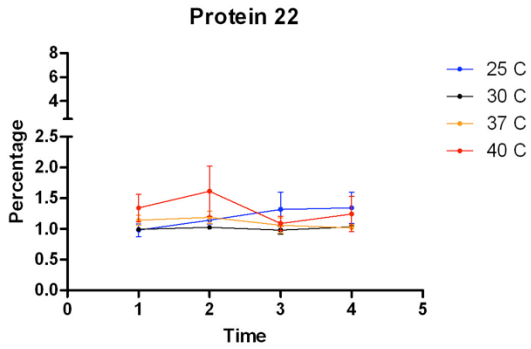

Protein 17
*
###
+++
##
+++
#
+++
##
+++
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

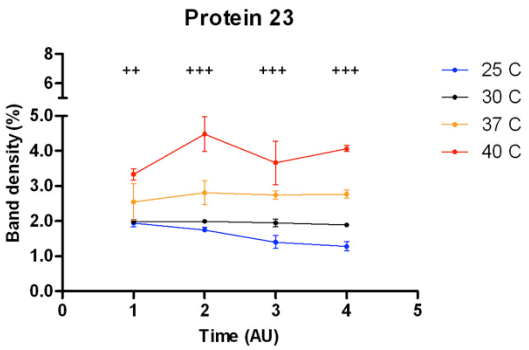

Protein 18
##
++
##
+++
###
+++
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

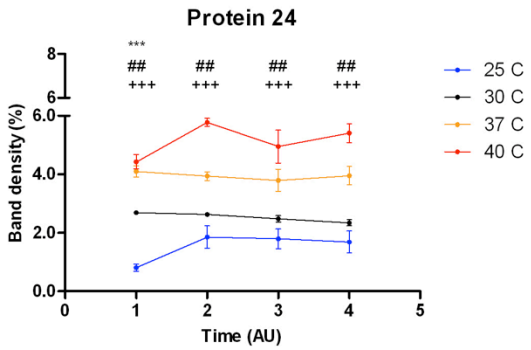

Protein 19
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
+
+++
+++
+++

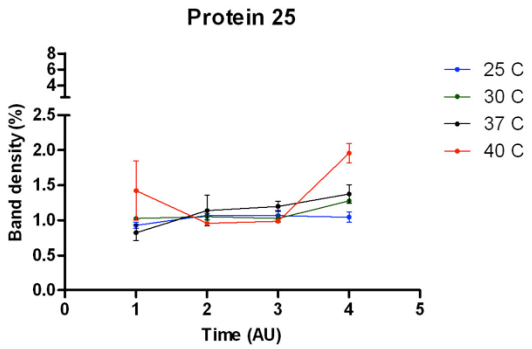

Protein 20
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

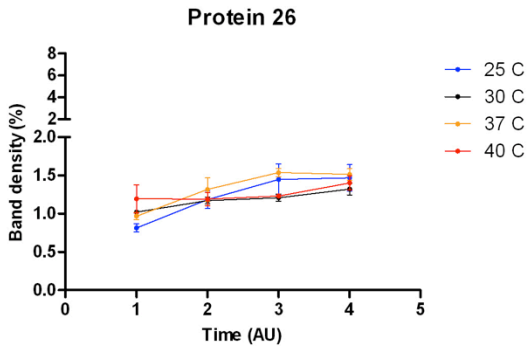

Protein 21
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

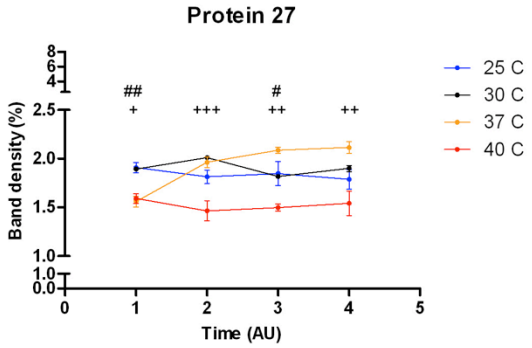

Protein 22
Percentage
Time
25 C
30 C
37 C
40 C

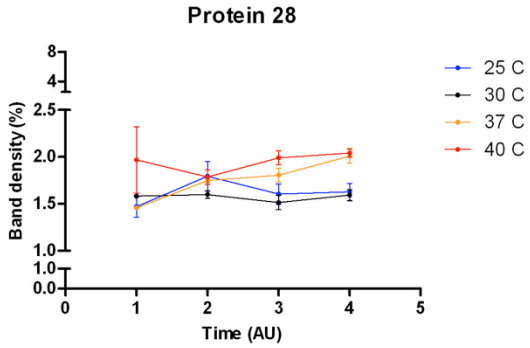

Protein 23
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
++
+++
+++
+++

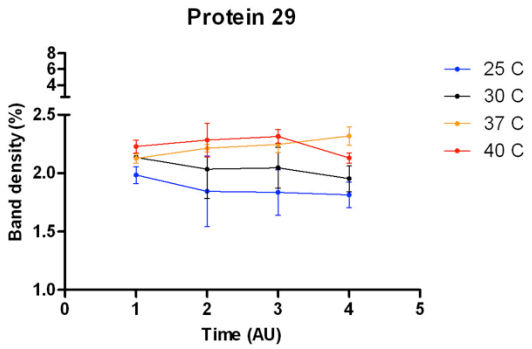

Protein 24
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
***
##
+++

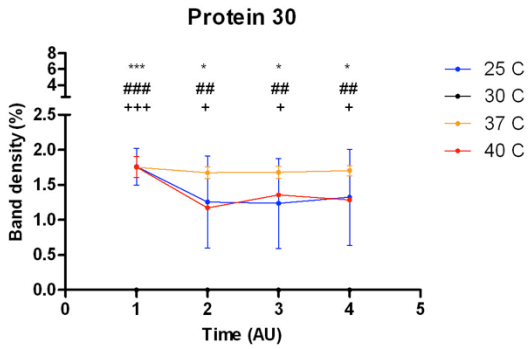

Protein 25
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

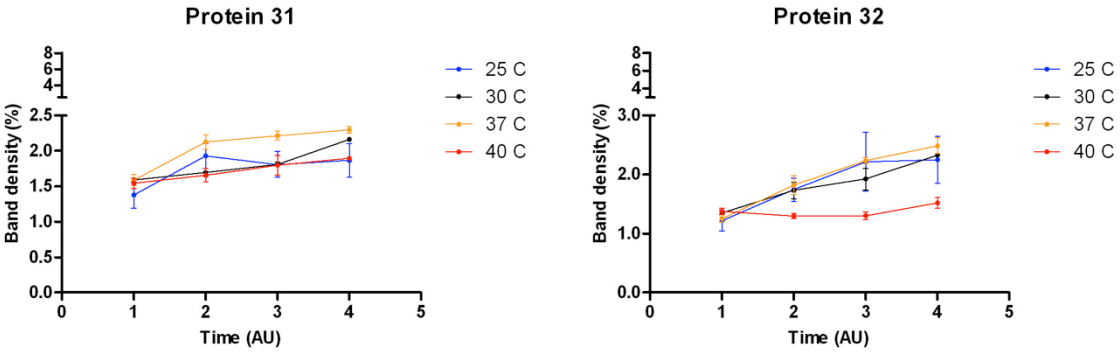

Protein 26
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 27
##
+
+++
#
++
++
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

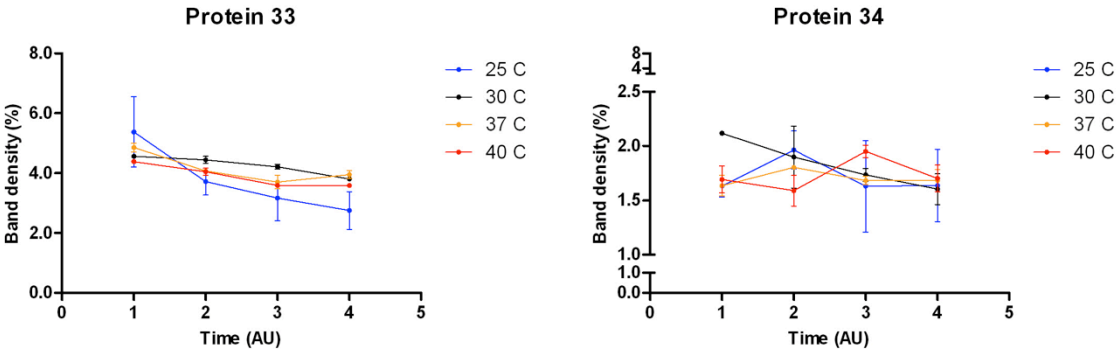

Protein 28
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 29
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

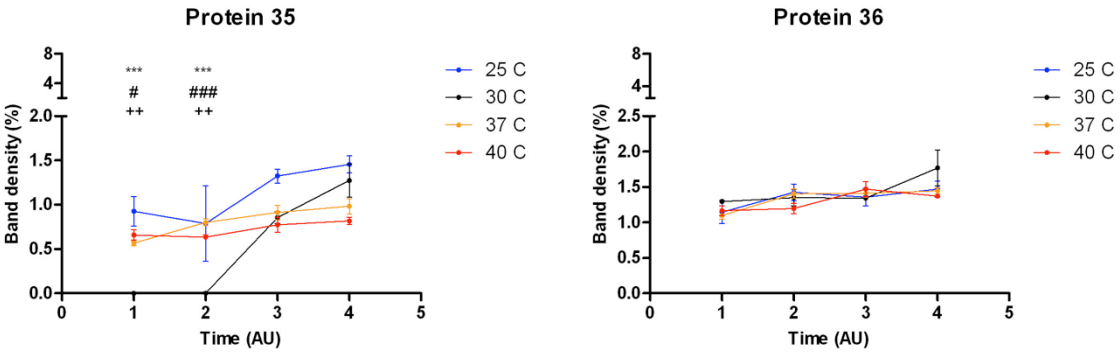

Protein 30
***
###
+++
*
##
+
*
##
+
*
##
+
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 31
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

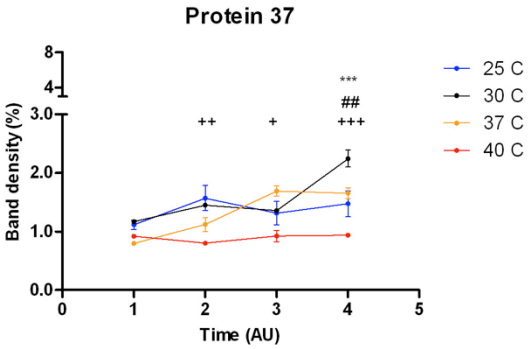
Protein 32
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

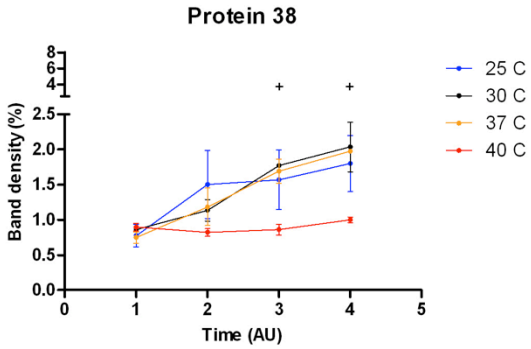

Protein 33
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

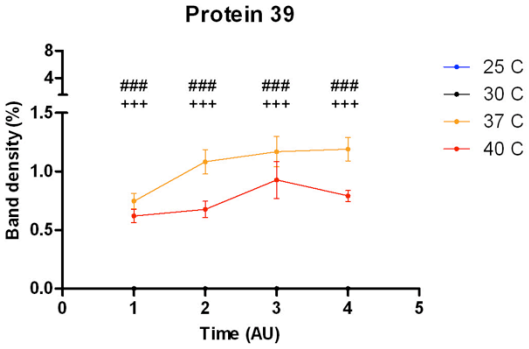
Protein 34
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

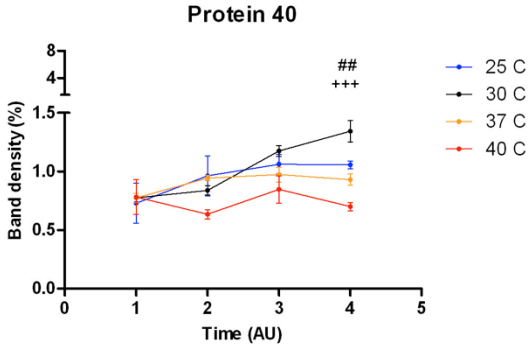

Protein 35
***
***
#
###
++
++
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

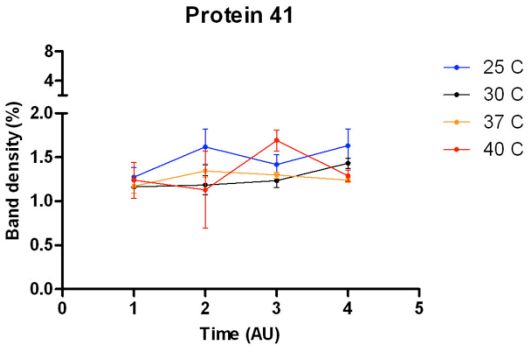
Protein 36
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

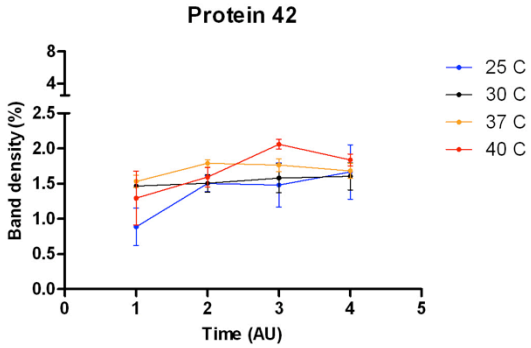

Protein 37
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
++
+
***
##
+++

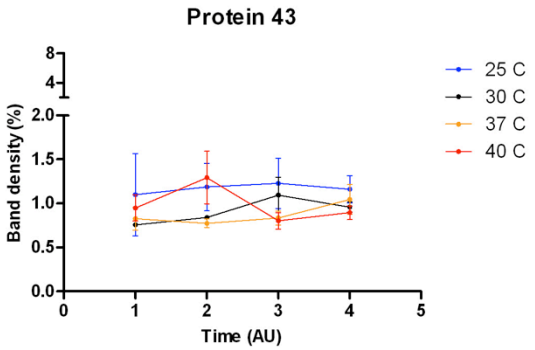

Protein 38
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
+
+

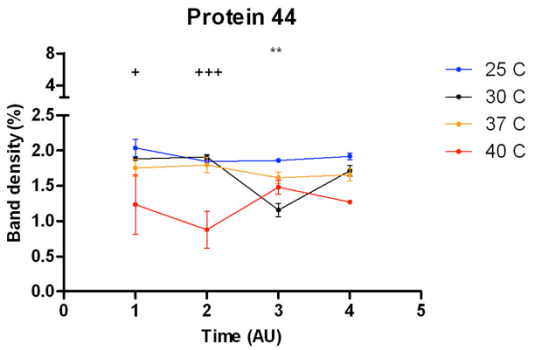

Protein 39
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
###
+++

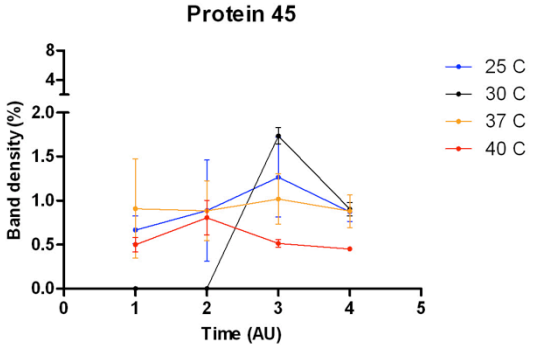

Protein 40
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
##
+++

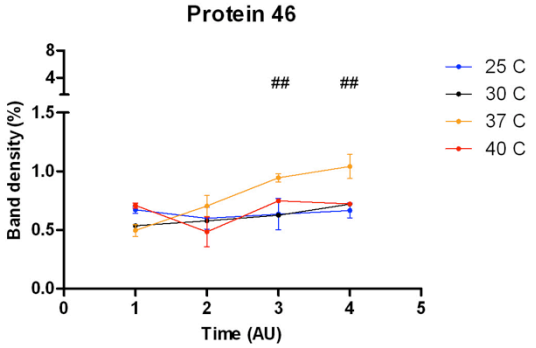

Protein 41
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

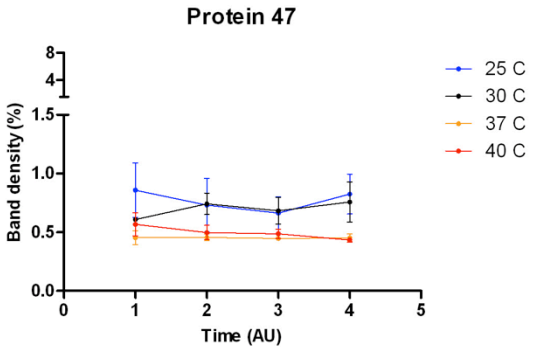

Protein 42
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

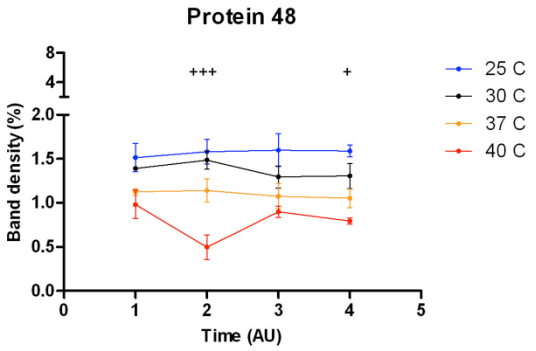

Protein 43
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

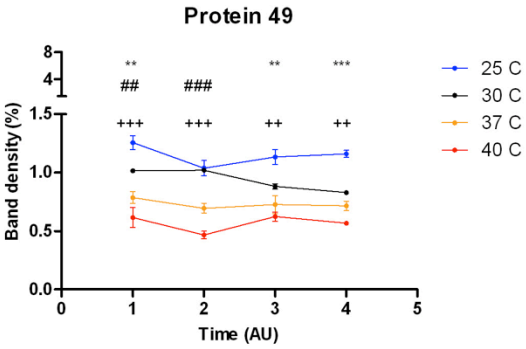

Protein 44
+
+++
**
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

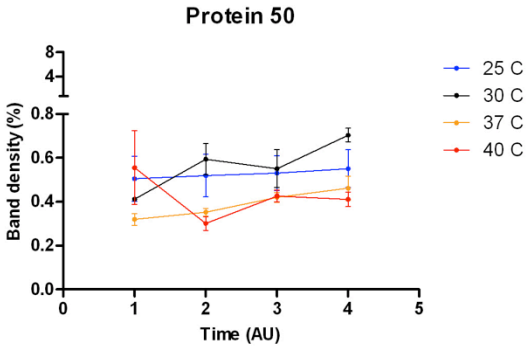

Protein 45
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

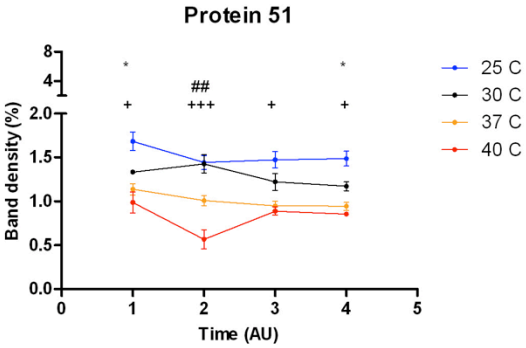

Protein 46
##
##
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

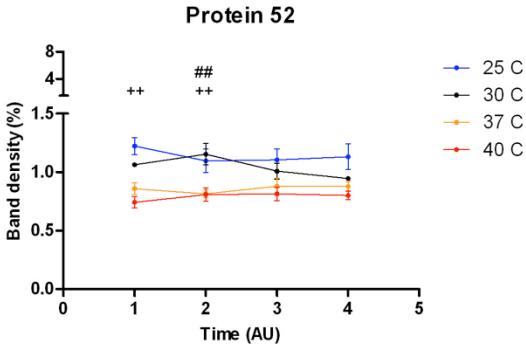

Protein 47
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

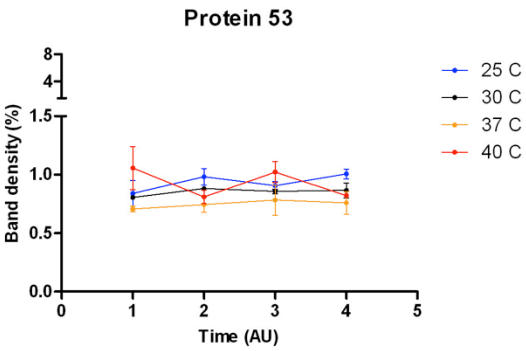

Protein 48
+++
+
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

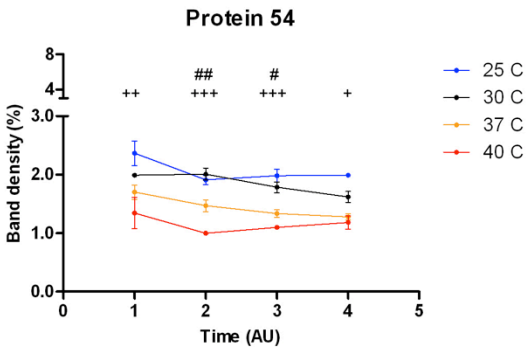

Protein 49
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

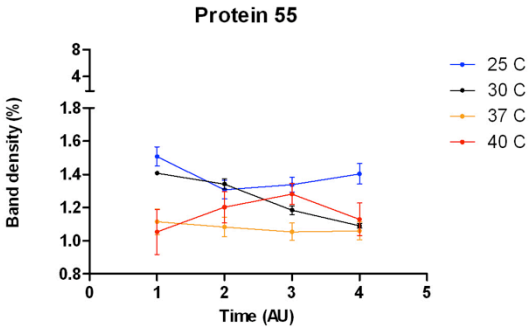

Protein 50
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

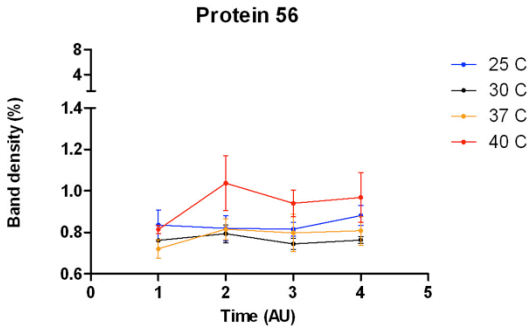

Protein 51
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

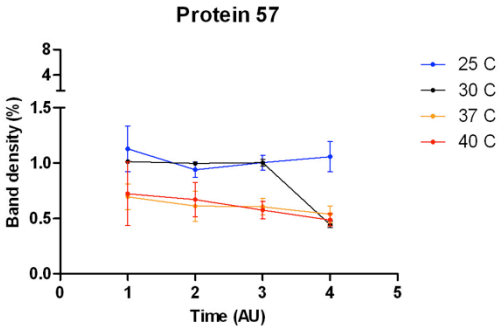

Protein 52
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

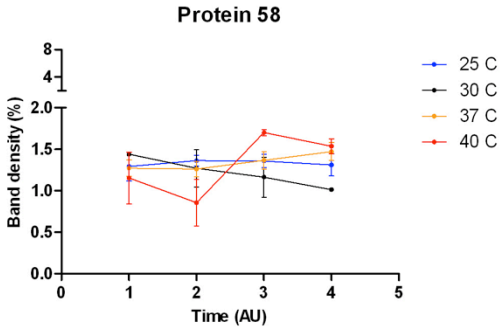

Protein 53
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

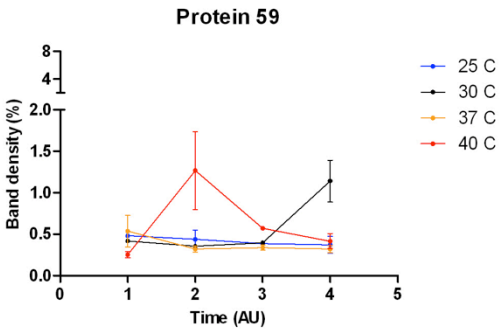

Protein 54
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

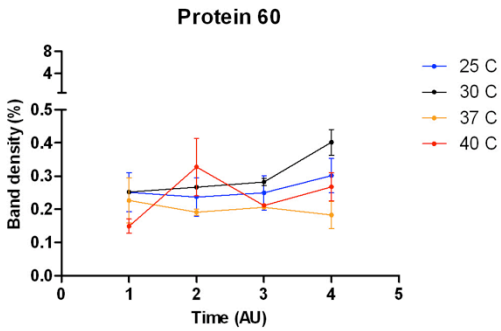

Protein 55
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

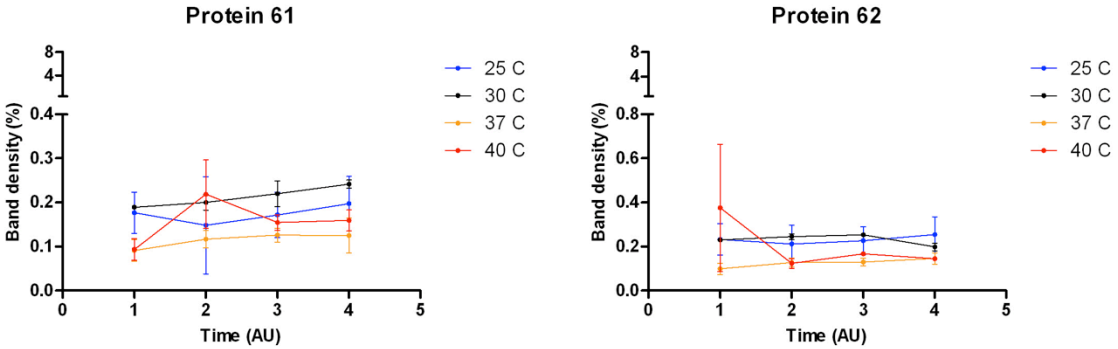

Protein 56
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 57
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

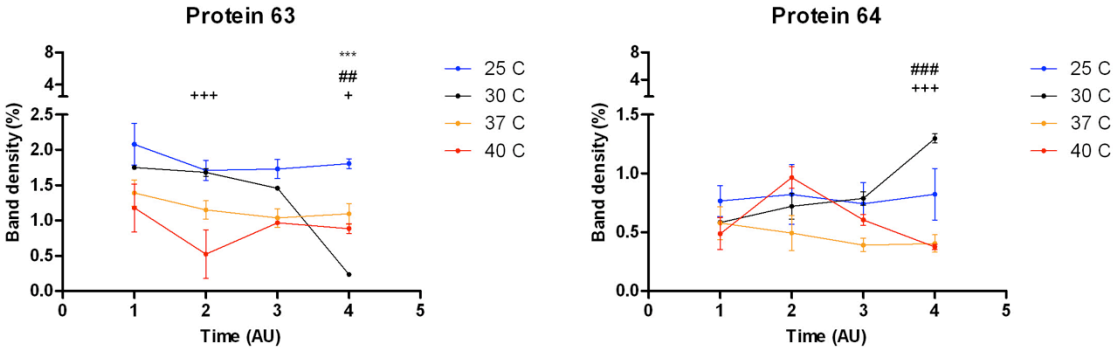

Protein 58
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 59
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

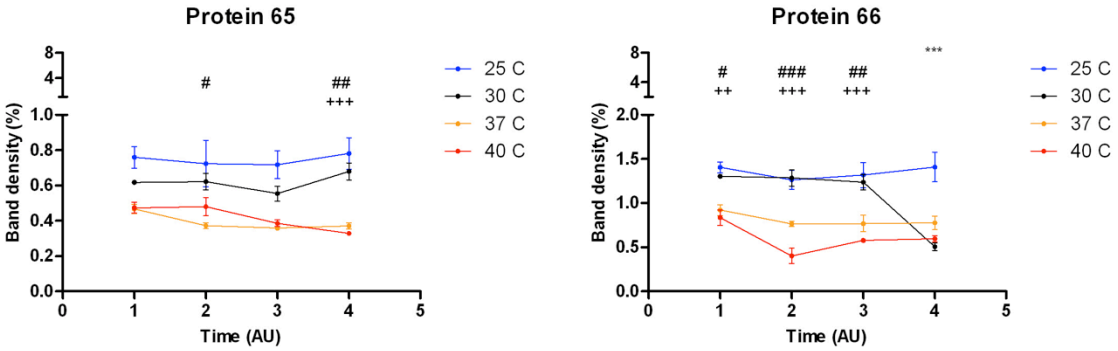

Protein 60
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 61
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

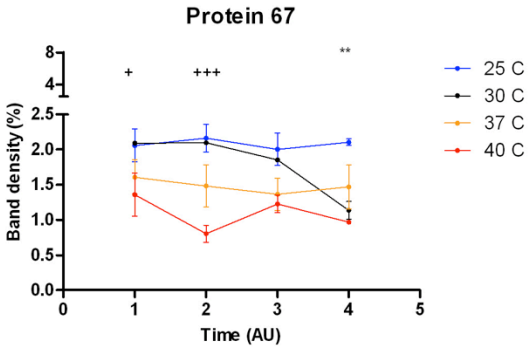
Protein 62
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

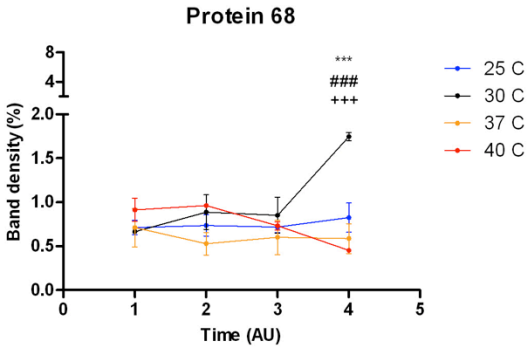

Protein 63
+++
***
##
+
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

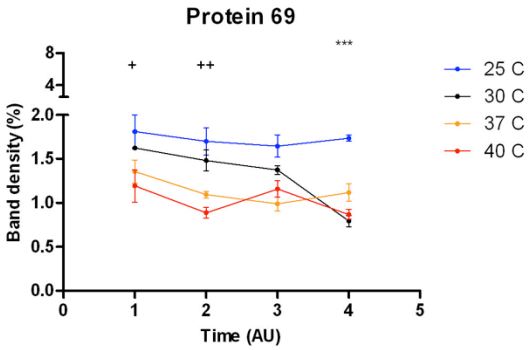
Protein 64
###
+++
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

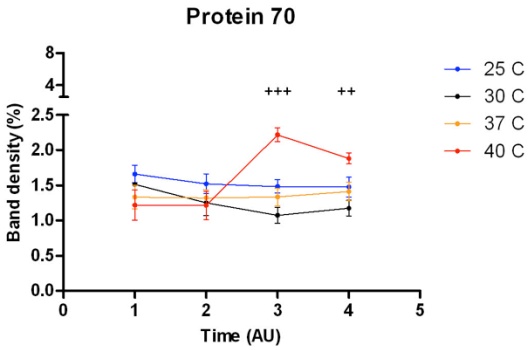

Protein 65
#
##
+++
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

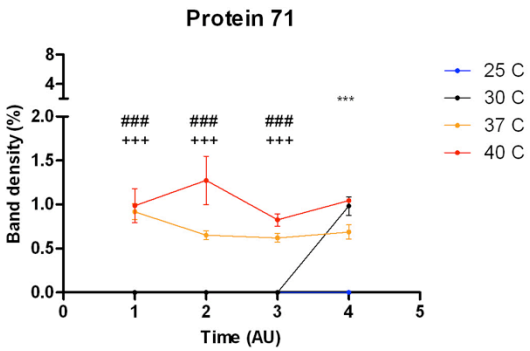
Protein 66
#
++
###
+++
##
+++
***
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

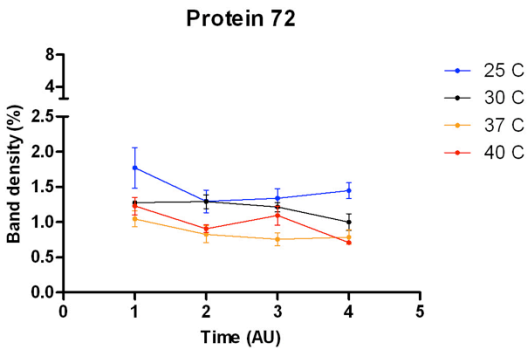

Protein 67
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
+
+++
**

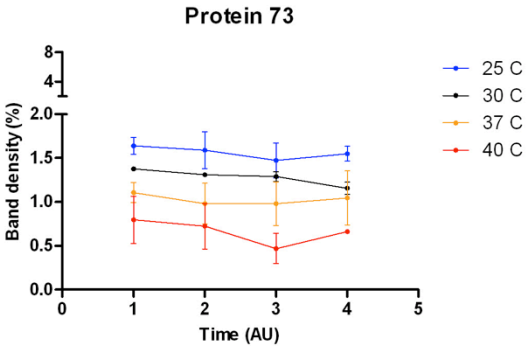

Protein 68
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
***
###
+++

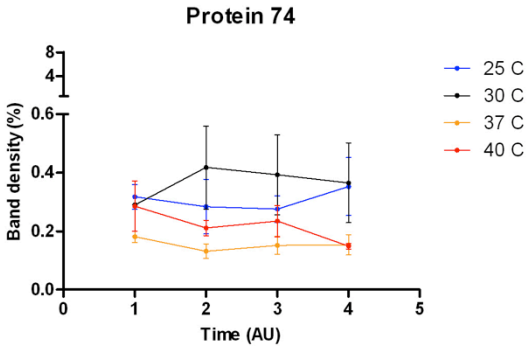

Protein 69
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
+
++
***

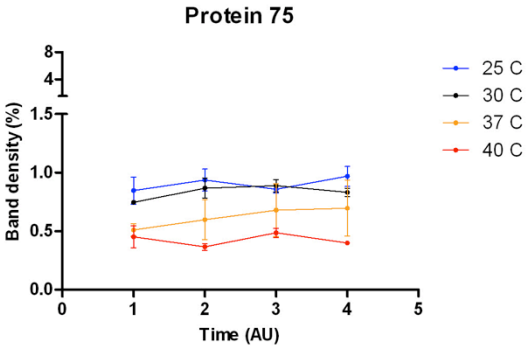

Protein 70
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
+++
++

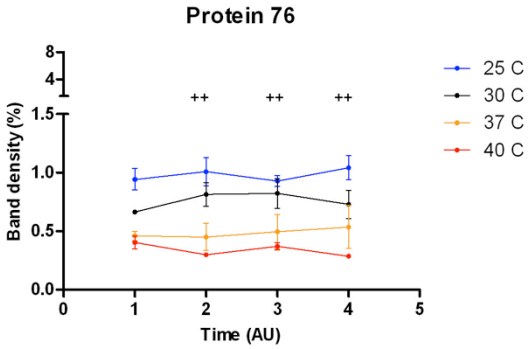

Protein 71
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C
###
+++
###
+++
###
+++
***

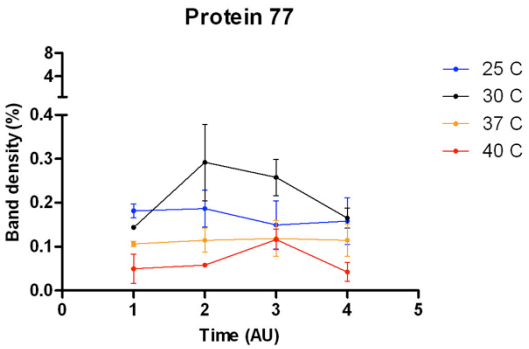

Protein 72
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C


Protein 73
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

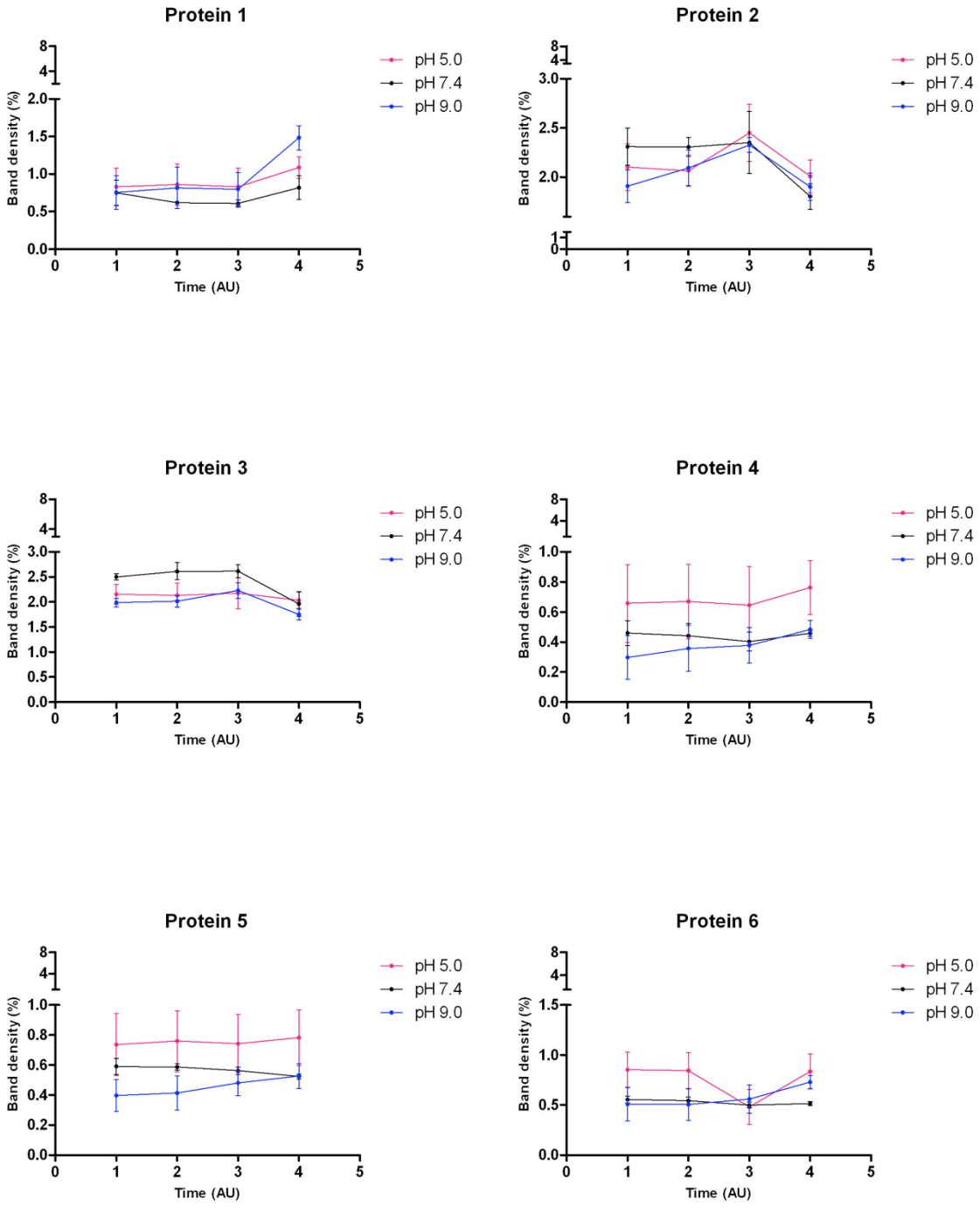

Protein 74
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

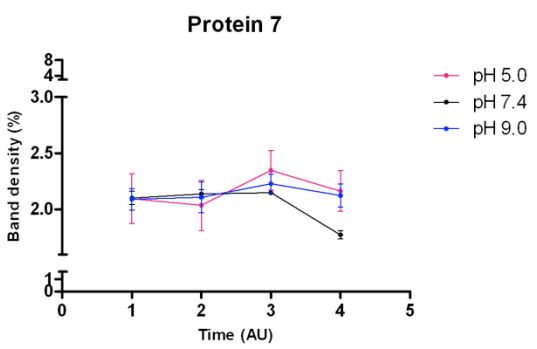

Protein 75
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

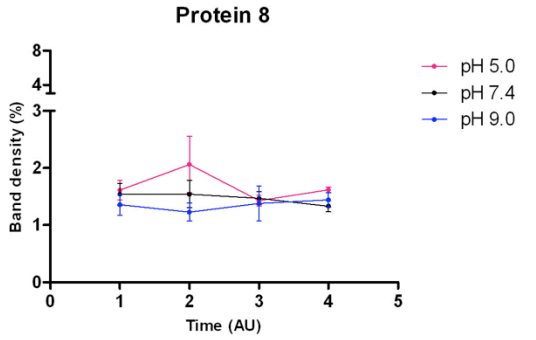

Protein 76
++
++
++
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

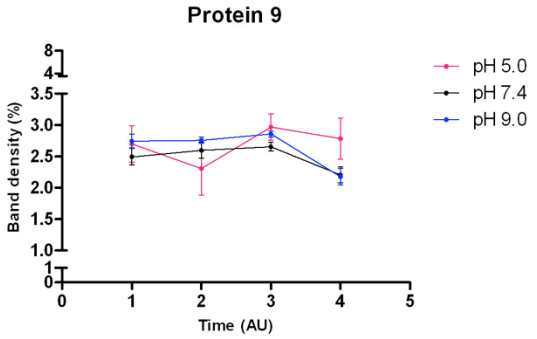

Protein 77
Band density (%)
Time (AU)
25 C
30 C
37 C
40 C

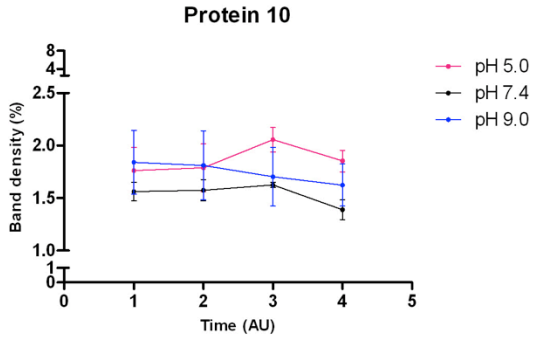
1.2.2. pH

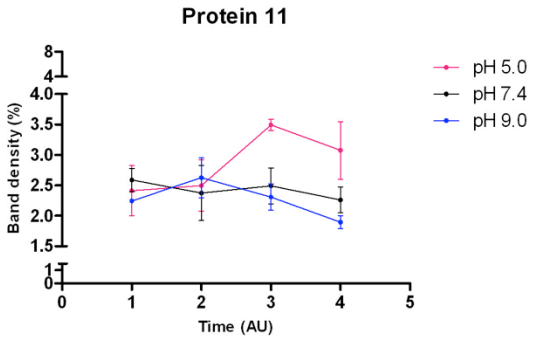

Protein 1
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
Protein 2
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
Protein 3
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
Protein 4
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
Protein 5
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
Protein 6
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

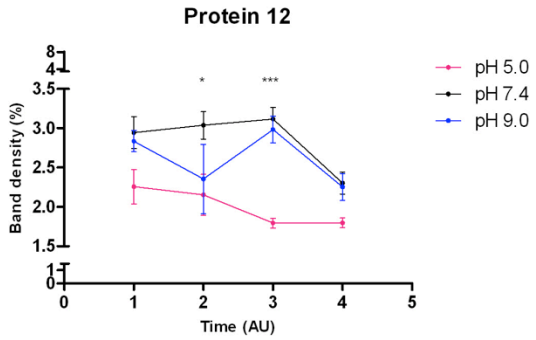

Protein 7
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

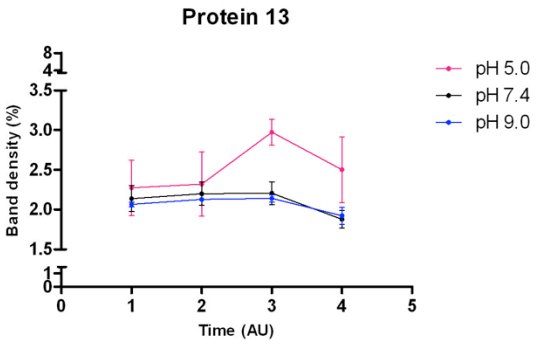

Protein 8
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

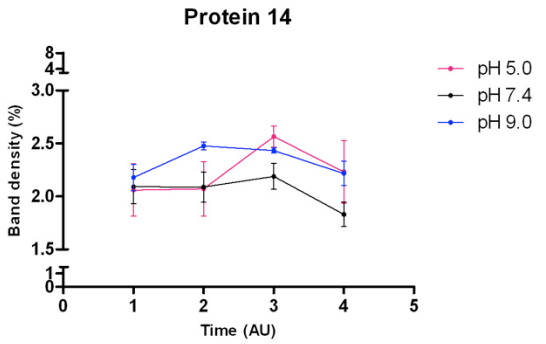

Protein 9
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

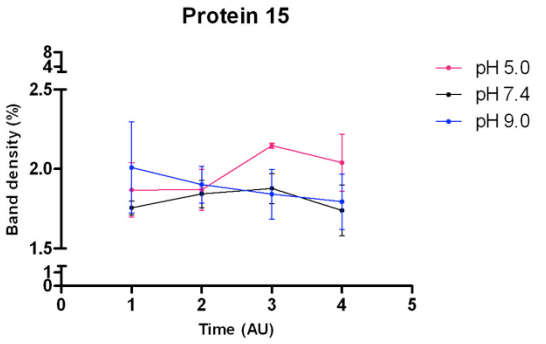

Protein 10
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

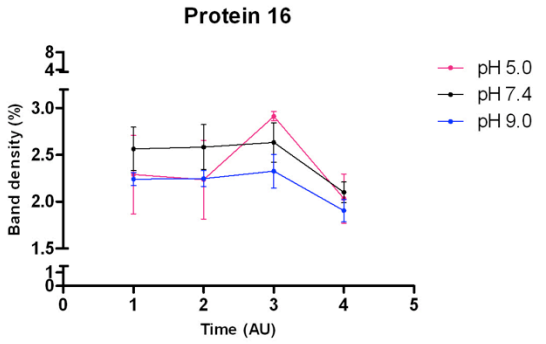

Protein 11
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

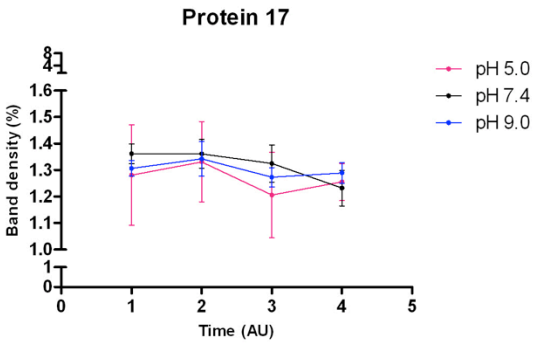

Protein 12
*
***
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

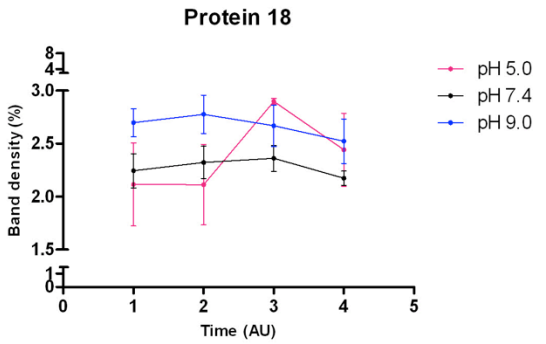

Protein 13
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

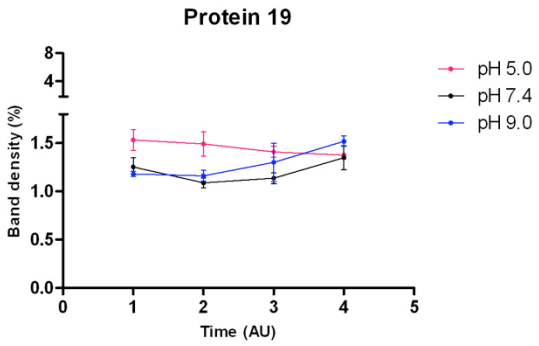

Protein 14
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

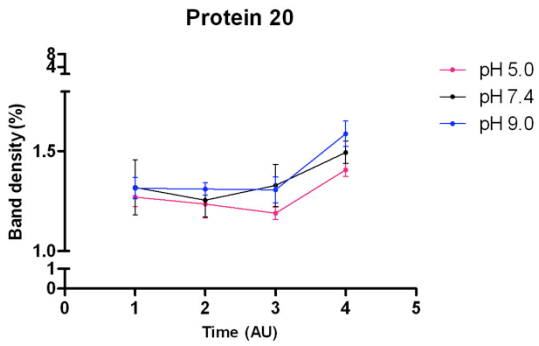

Protein 15
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

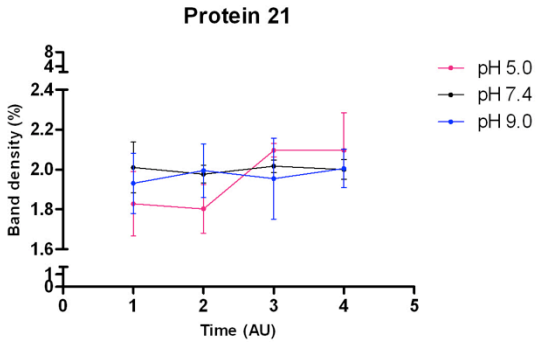

Protein 16
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

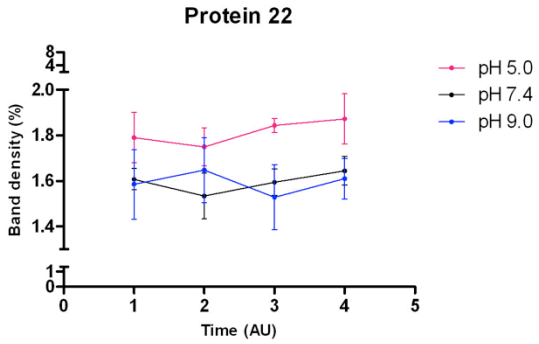

Protein 17
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

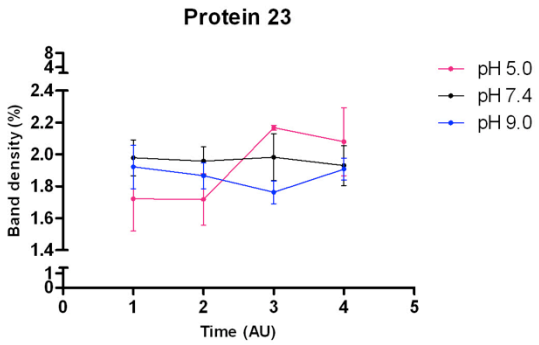

Protein 18
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

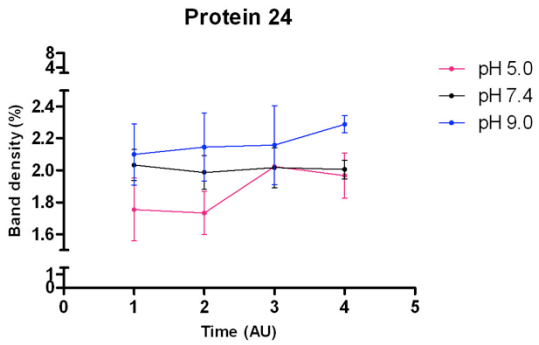

Protein 19
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

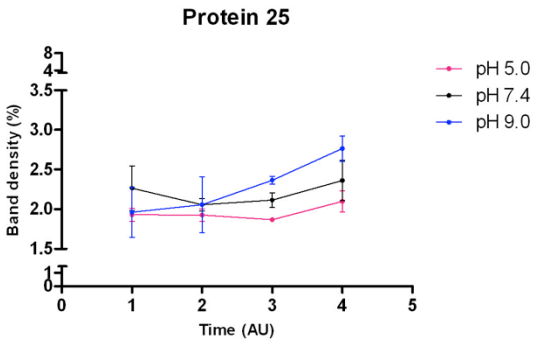

Protein 20
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

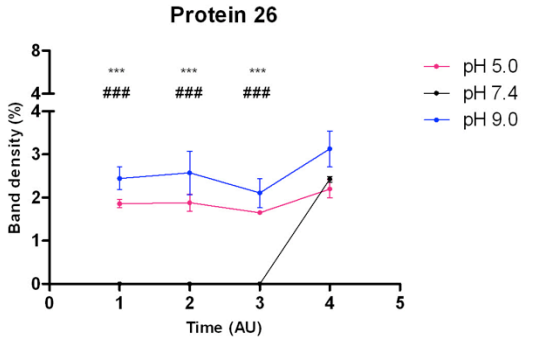

Protein 21
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 22
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 23
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 24
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 25
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 26
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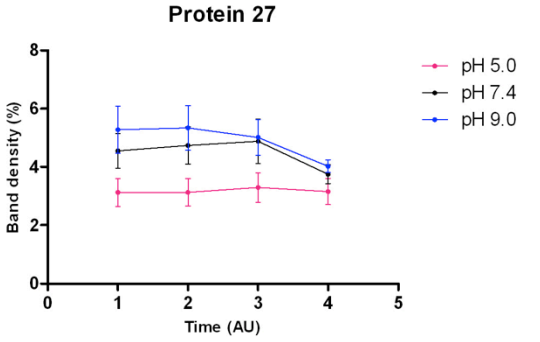
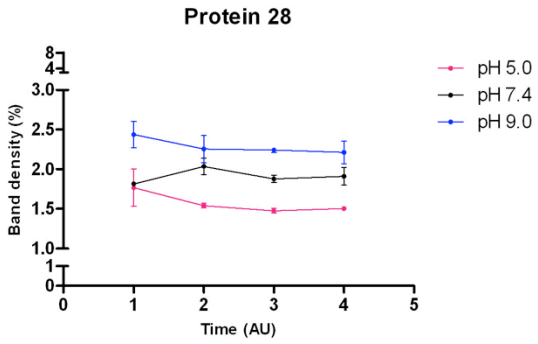
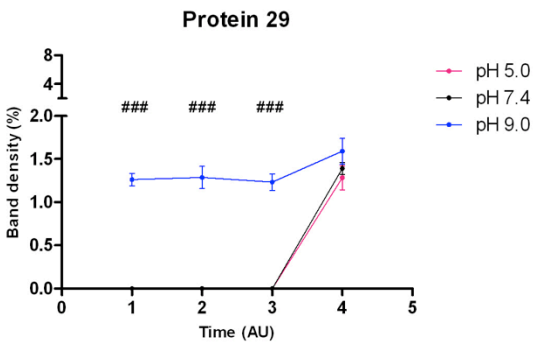
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 27
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 28
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 29
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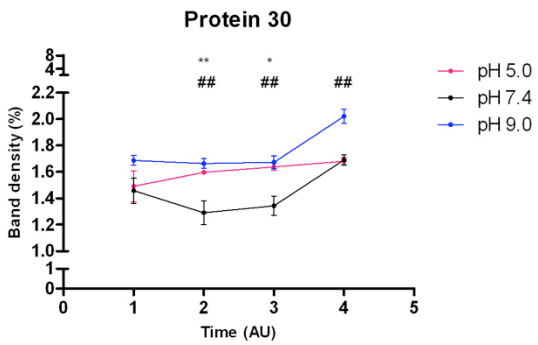
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 30
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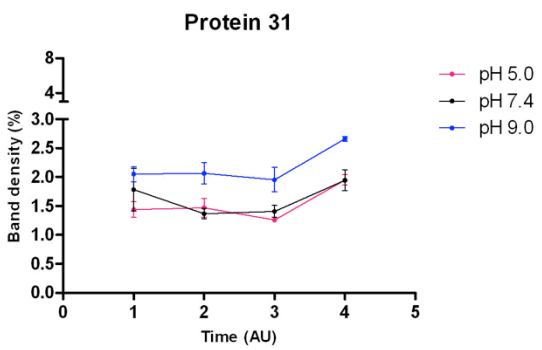
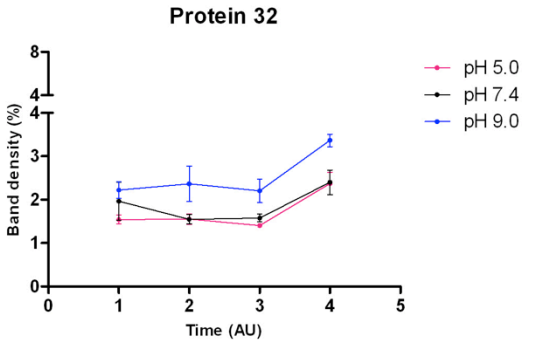
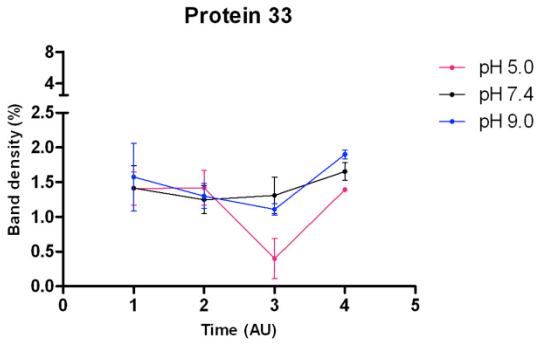
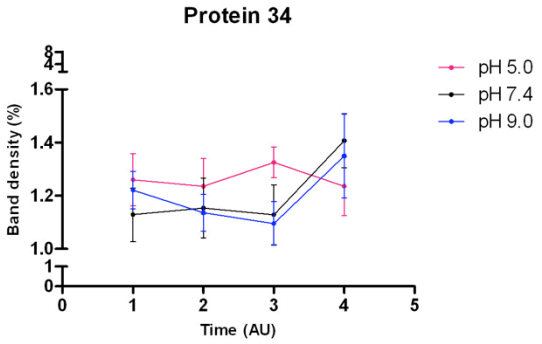
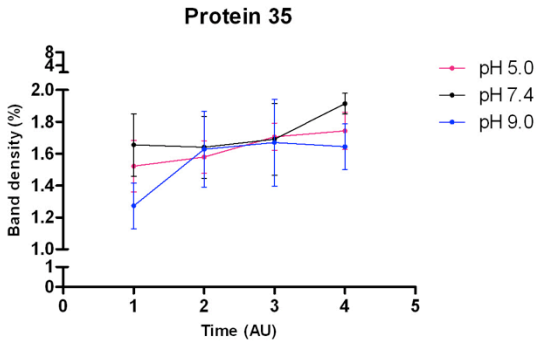
*
##
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

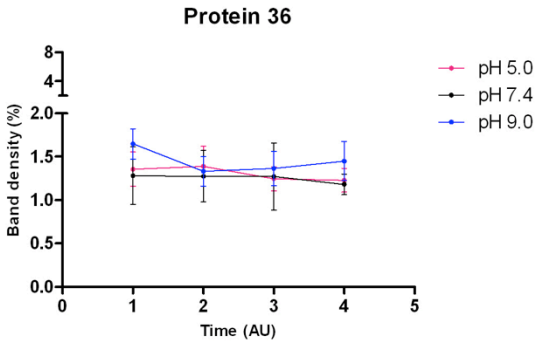

Protein 31
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

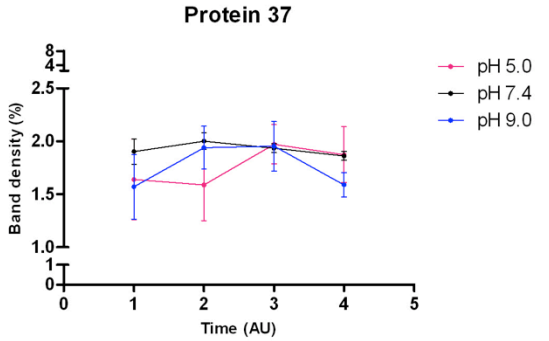

Protein 32
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

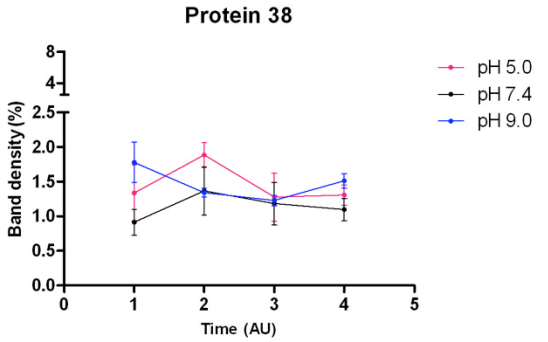

Protein 33
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

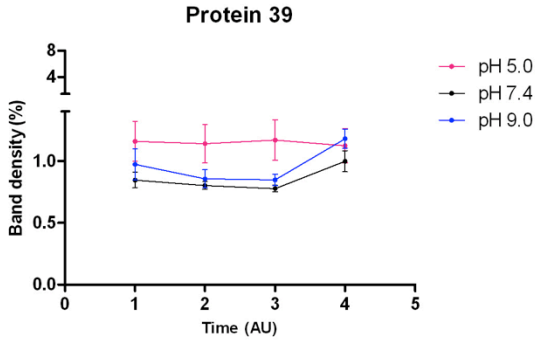

Protein 34
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

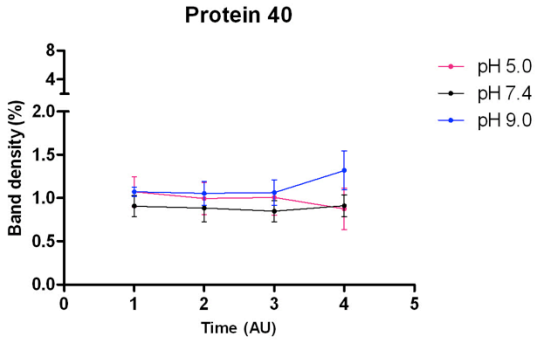

Protein 35
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

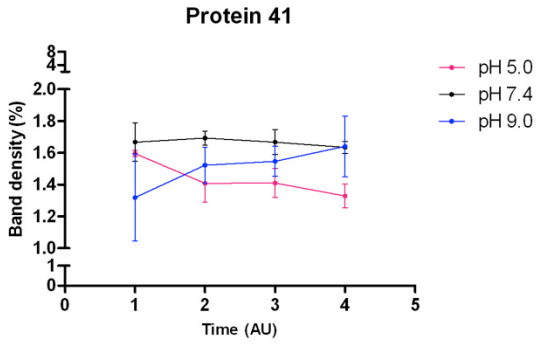

Protein 36
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 37
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 38
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 39
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 40
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0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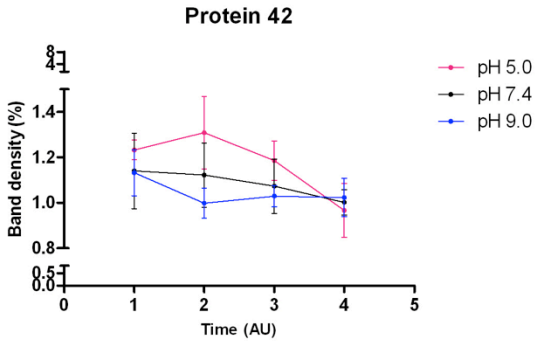
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0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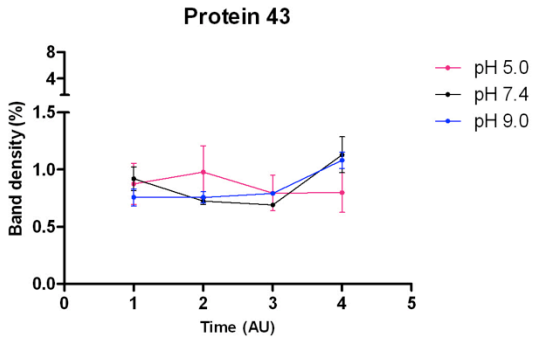
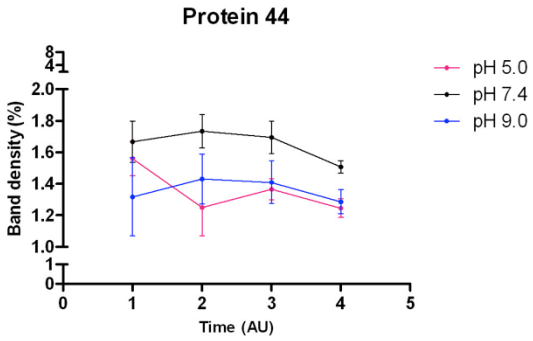
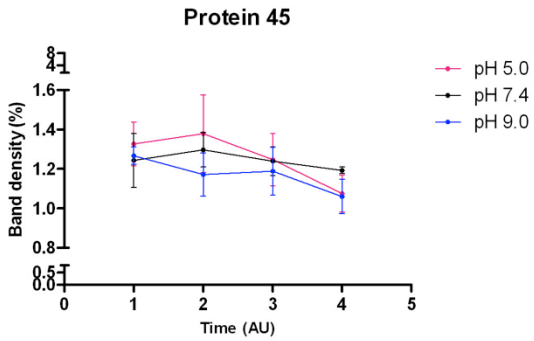
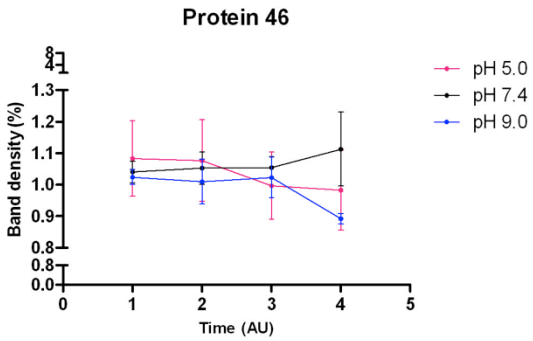
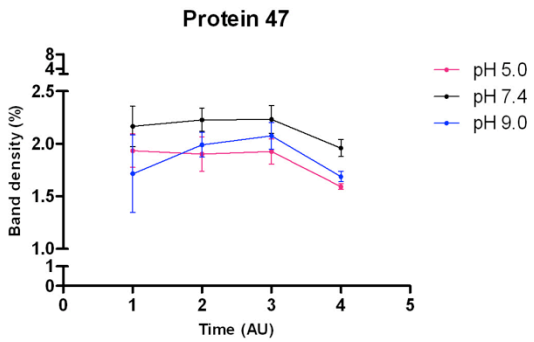
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

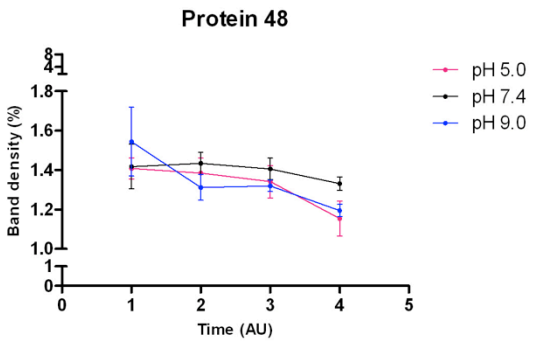

Protein 43
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

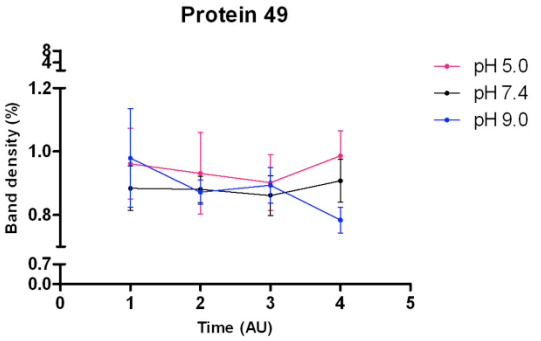

Protein 44
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

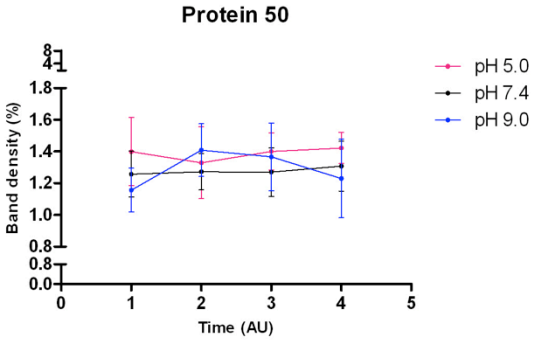

Protein 45
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

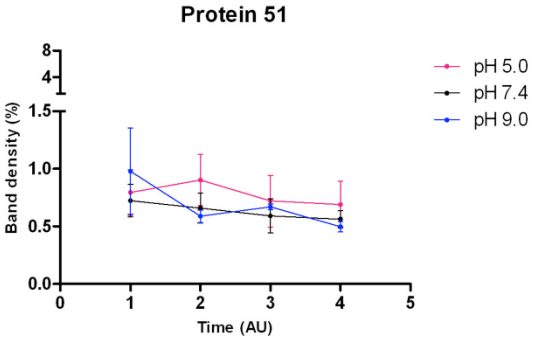

Protein 46
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

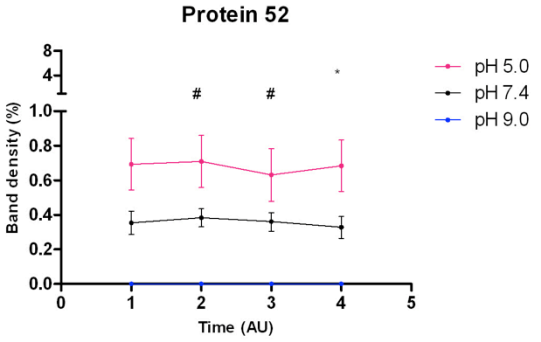

Protein 47
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

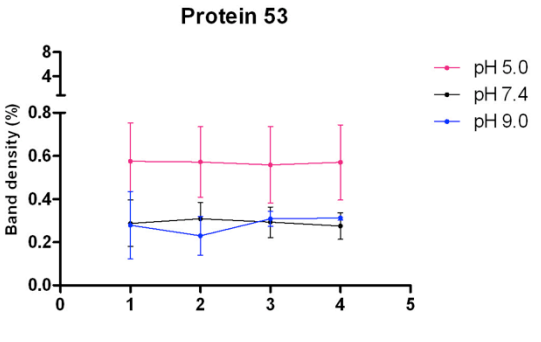

Protein 48
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

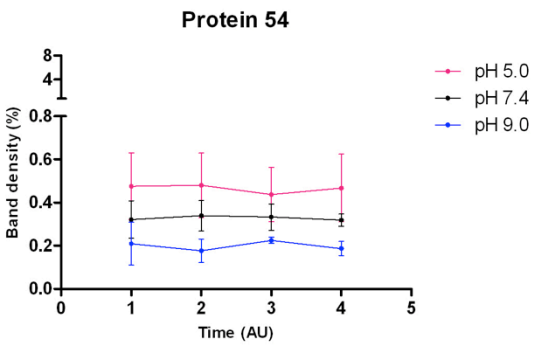

Protein 49
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

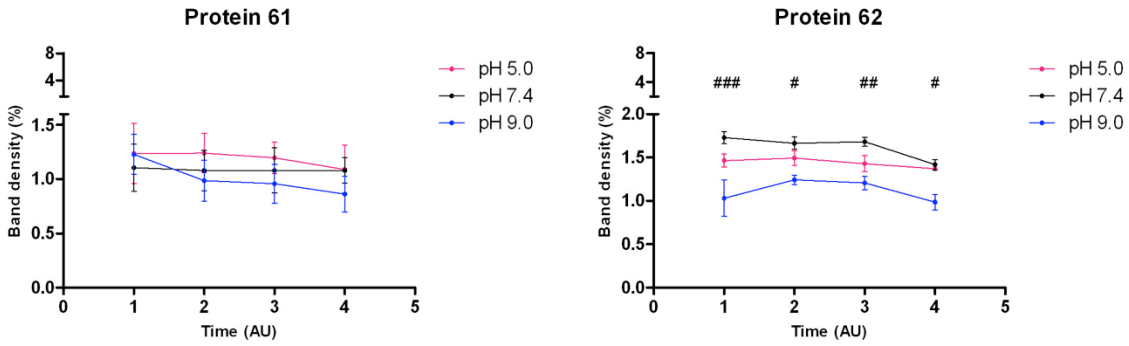

Protein 50
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 51
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 52
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 53
Band density (%)
pH 5.0
pH 7.4
pH 9.0


Protein 54
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 61
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

Protein 62
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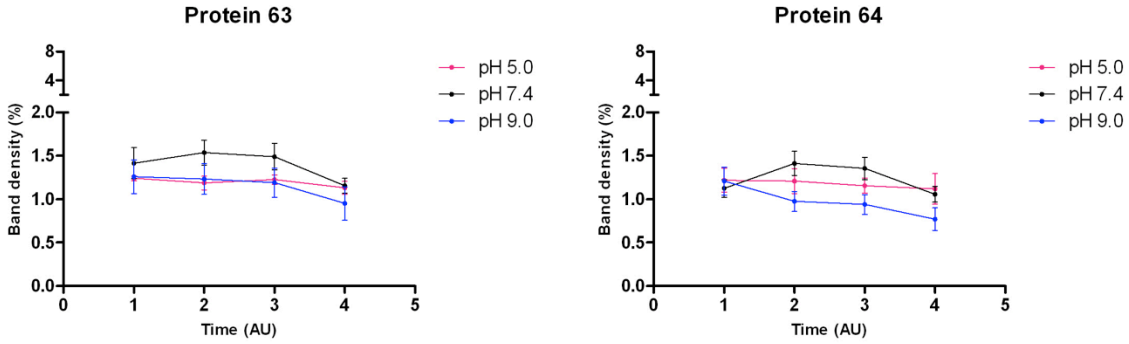
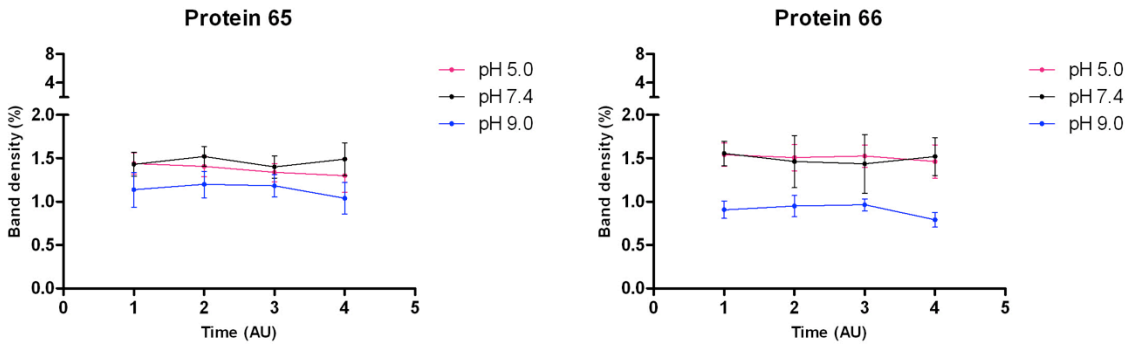
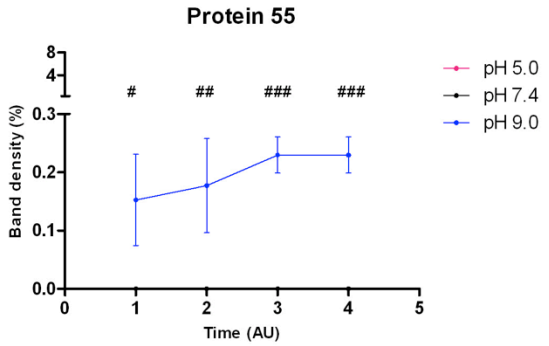
#
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 63
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

Protein 64
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 65
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

Protein 66
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 55
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
#
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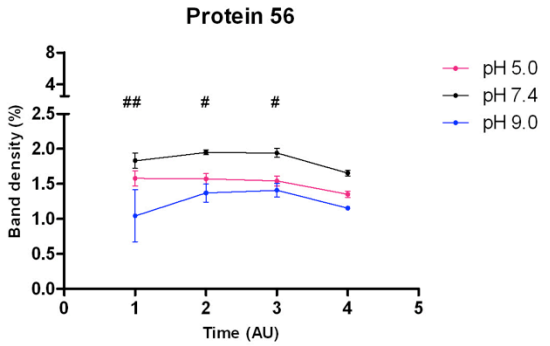
Protein 56
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
##
#
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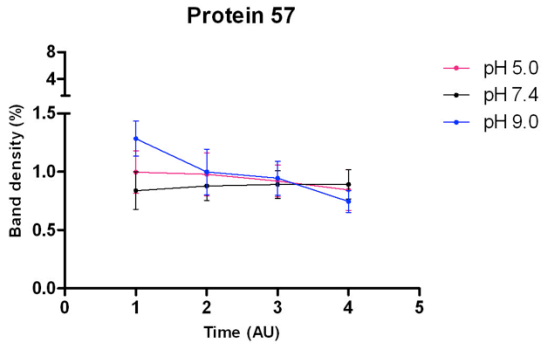
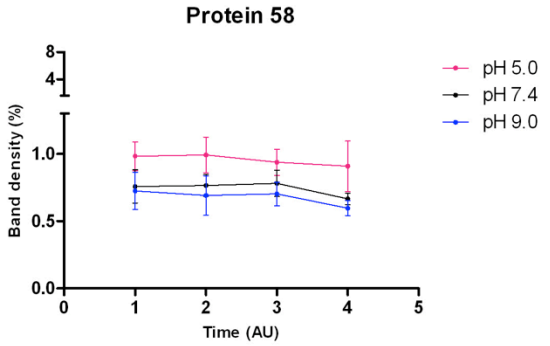
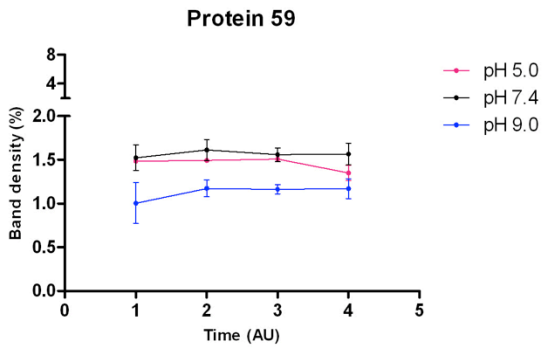
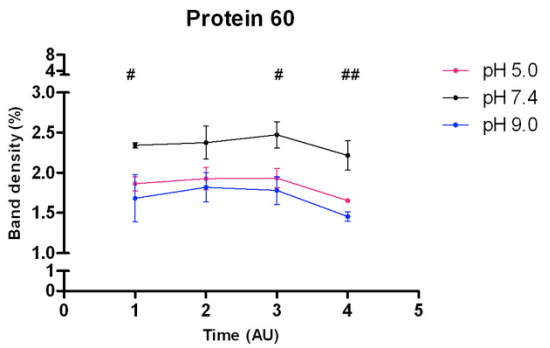

Protein 57
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 58
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 59
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0


Protein 60
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0
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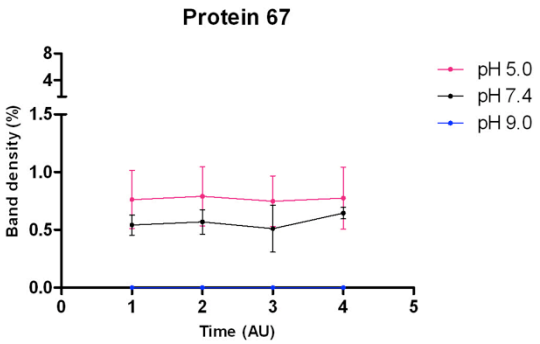
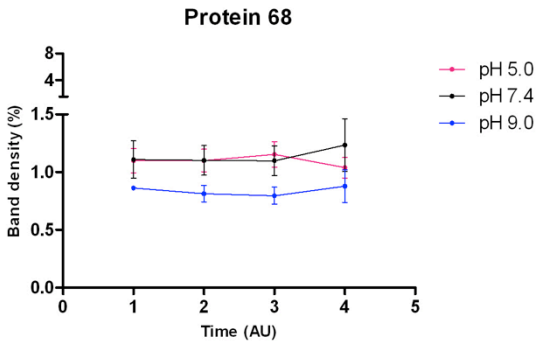
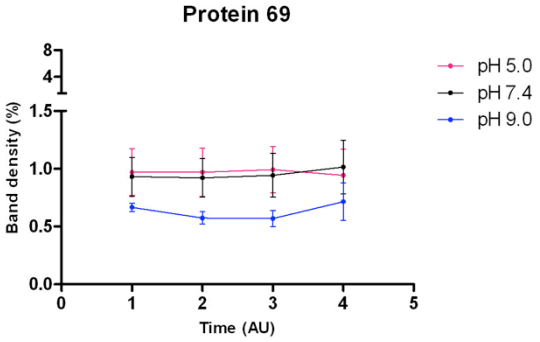
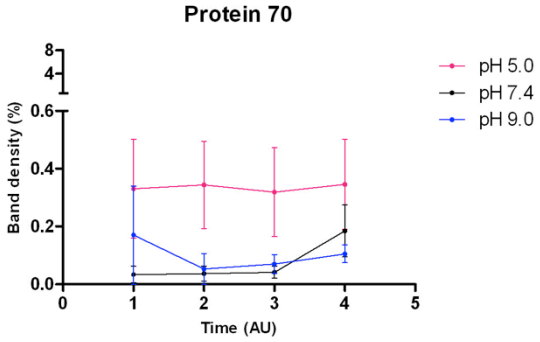
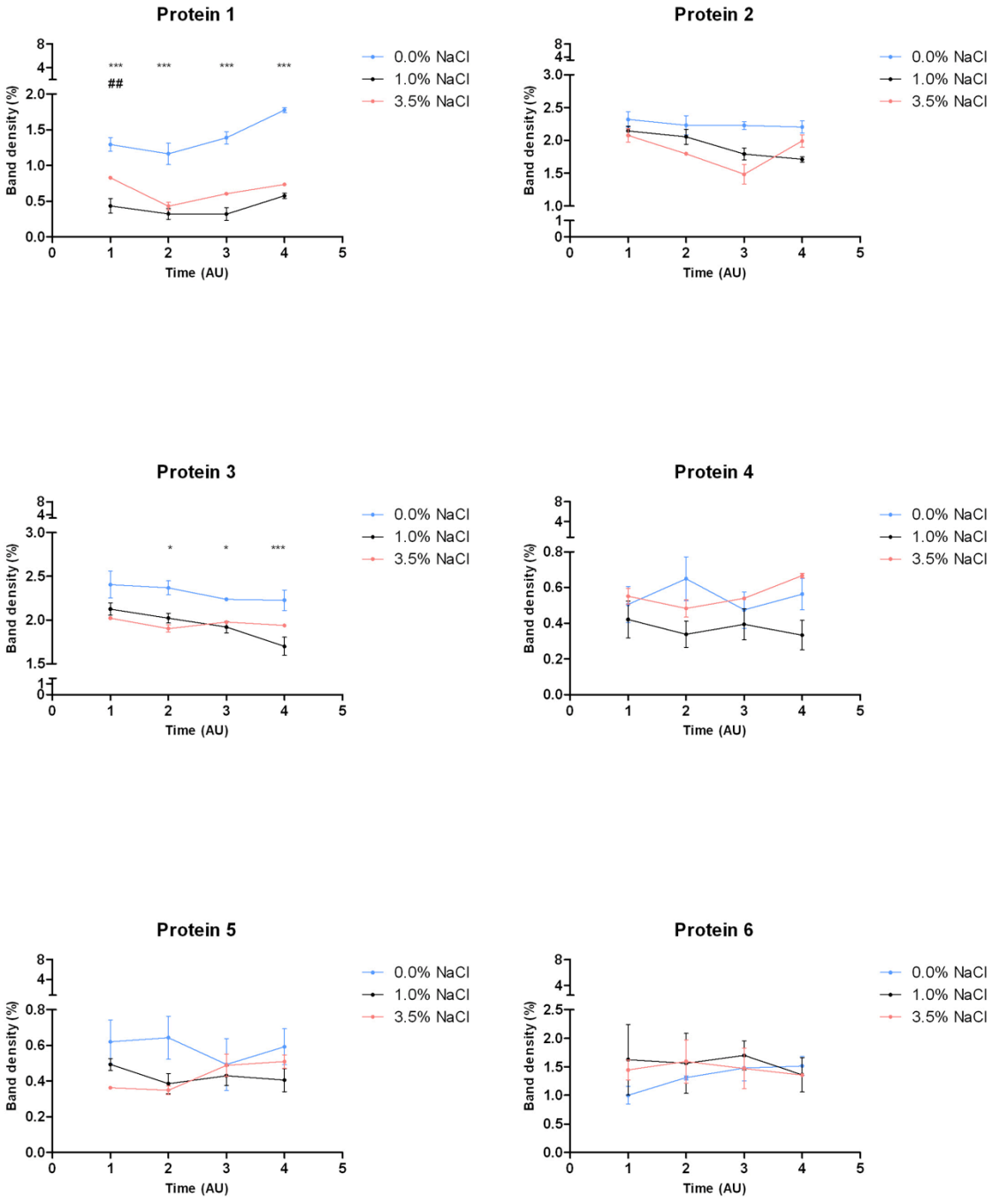

Protein 67
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

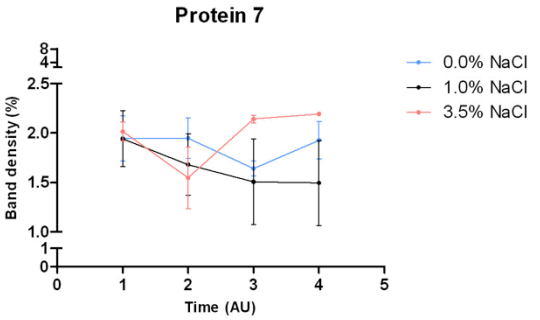

Protein 68
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

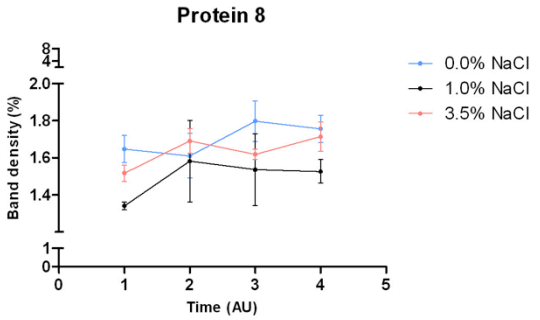

Protein 69
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

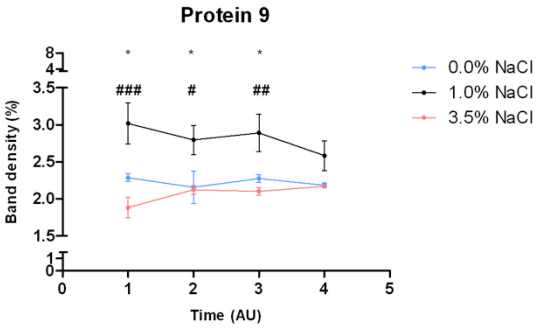

Protein 70
Band density (%)
Time (AU)
pH 5.0
pH 7.4
pH 9.0

1.2.3. Salinity




Protein 7
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 8
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 9
Band density (%)
Time (AU)
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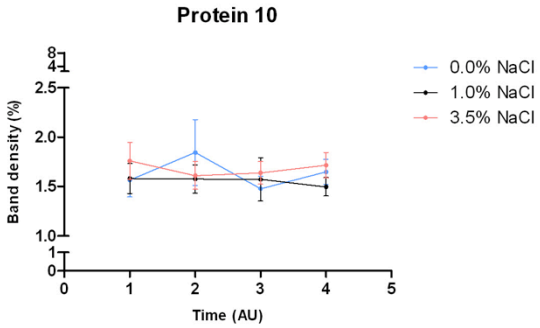
1.0% NaCl
3.5% NaCl


Protein 10
Band density (%)
Time (AU)
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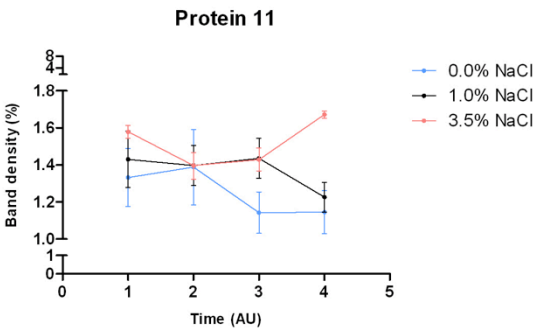
1.0% NaCl
3.5% NaCl


Protein 11
Band density (%)
Time (AU)
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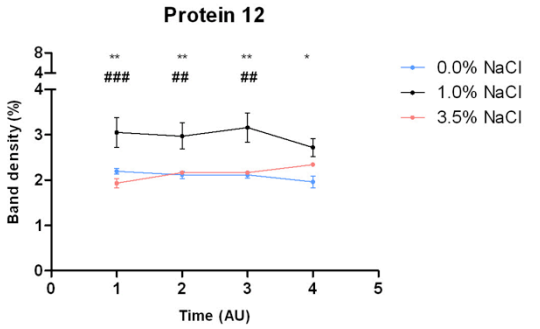
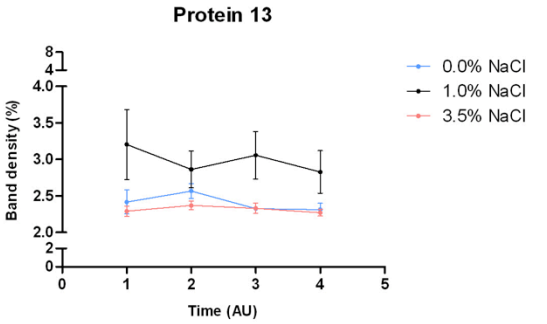
1.0% NaCl
3.5% NaCl


Protein 12
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 13
Band density (%)
Time (AU)
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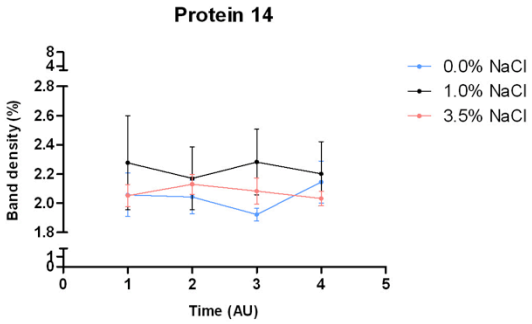
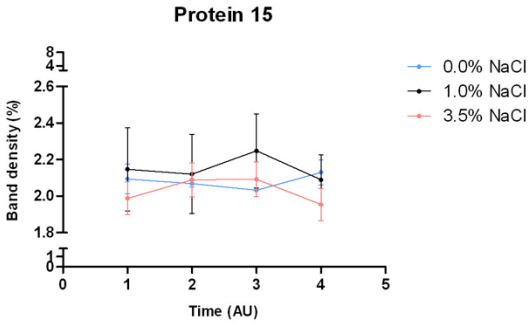
1.0% NaCl
3.5% NaCl


Protein 14
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 15
Band density (%)
Time (AU)
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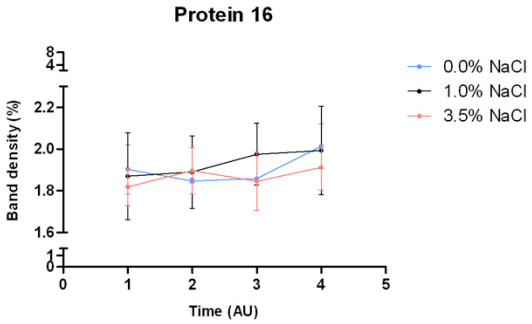
1.0% NaCl
3.5% NaCl


Protein 16
Band density (%)
Time (AU)
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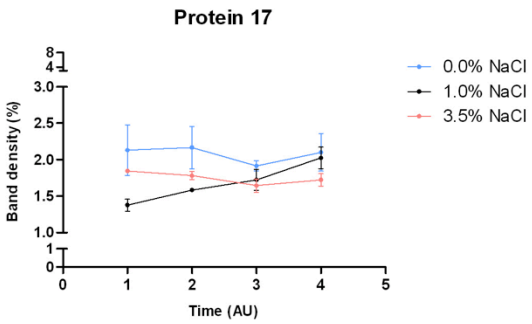
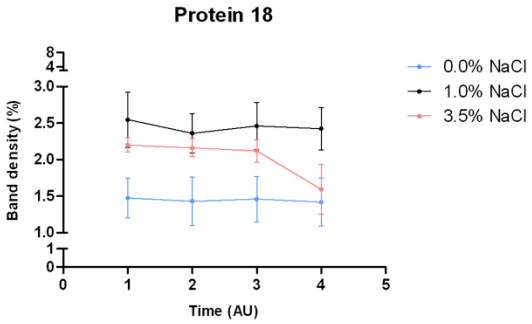
1.0% NaCl
3.5% NaCl


Protein 17
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 18
Band density (%)
Time (AU)
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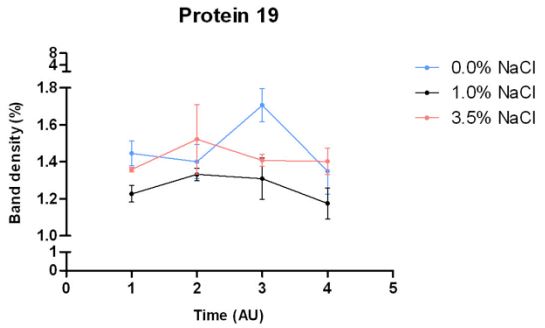
1.0% NaCl
3.5% NaCl


Protein 19
Band density (%)
Time (AU)
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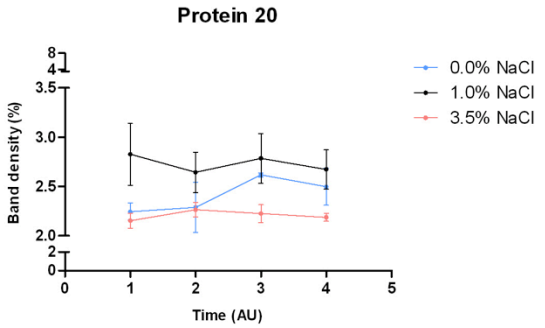
1.0% NaCl
3.5% NaCl


Protein 20
Band density (%)
Time (AU)
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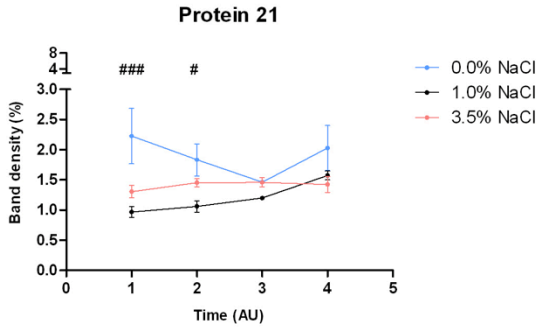
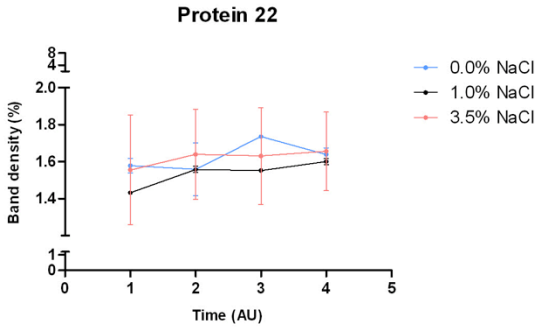
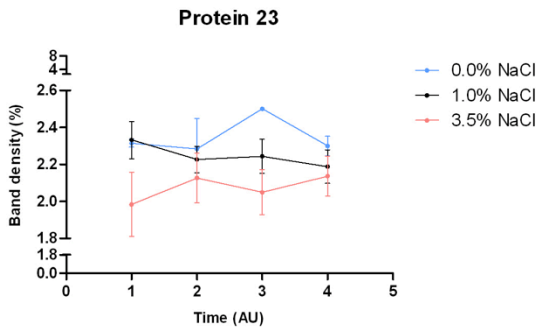
1.0% NaCl
3.5% NaCl


Protein 21
###
#
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 22
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 23
Band density (%)
Time (AU)
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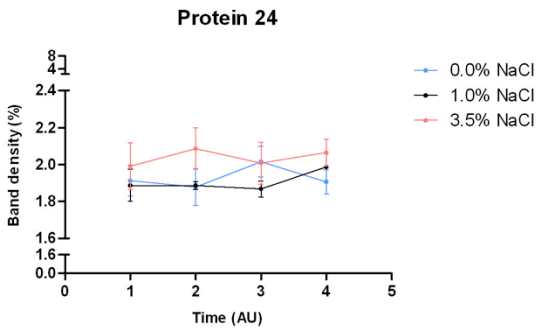
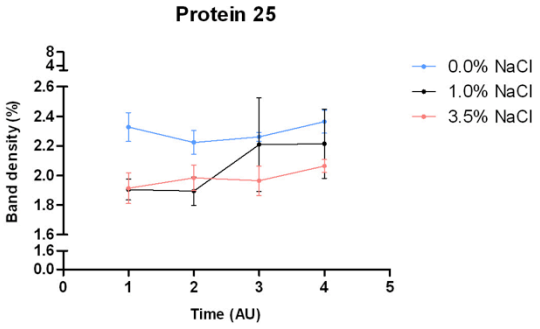
1.0% NaCl
3.5% NaCl


Protein 24
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 25
Band density (%)
Time (AU)
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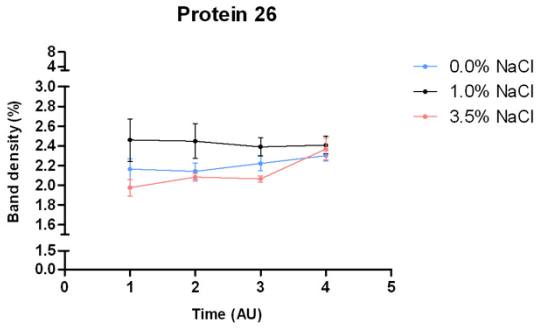
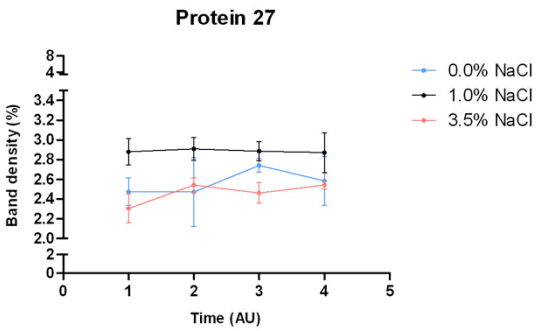
1.0% NaCl
3.5% NaCl


Protein 26
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 27
Band density (%)
Time (AU)
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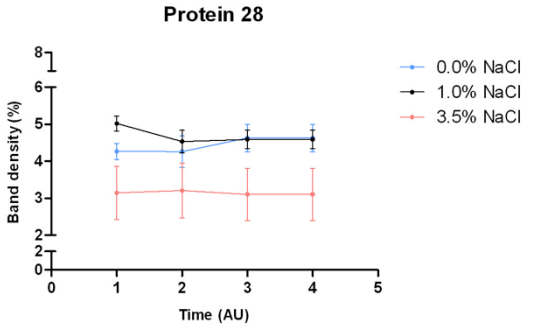
1.0% NaCl
3.5% NaCl


Protein 28
Band density (%)
Time (AU)
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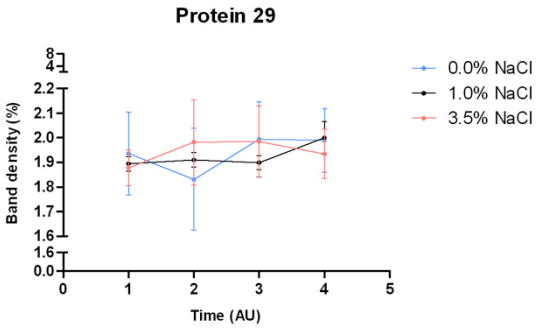
1.0% NaCl
3.5% NaCl


Protein 29
Band density (%)
Time (AU)
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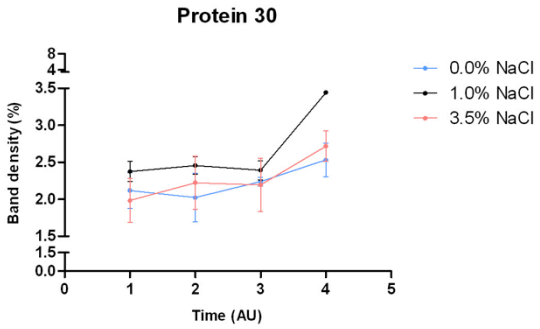
1.0% NaCl
3.5% NaCl


Protein 30
Band density (%)
Time (AU)
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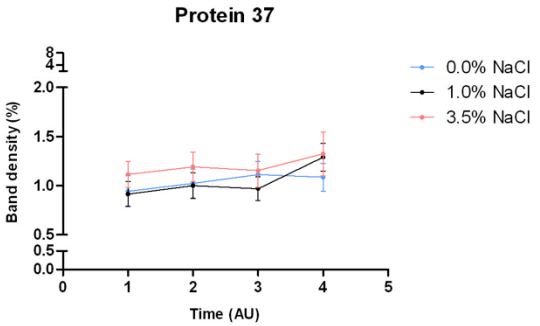
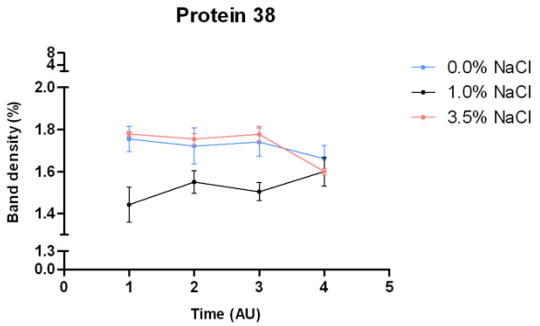
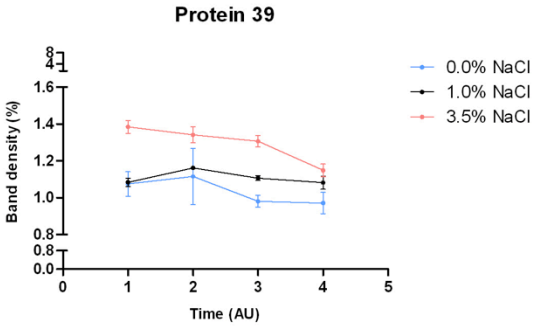
1.0% NaCl
3.5% NaCl


Protein 37
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 38
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 39
Band density (%)
Time (AU)
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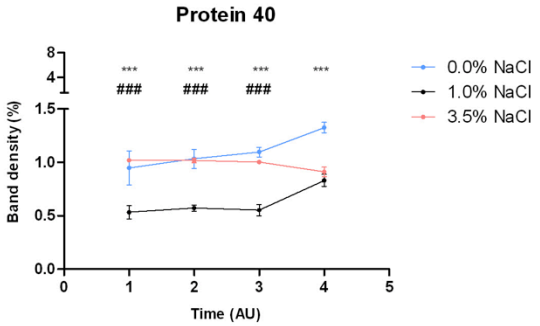
1.0% NaCl
3.5% NaCl


Protein 40
***
###
Band density (%)
Time (AU)
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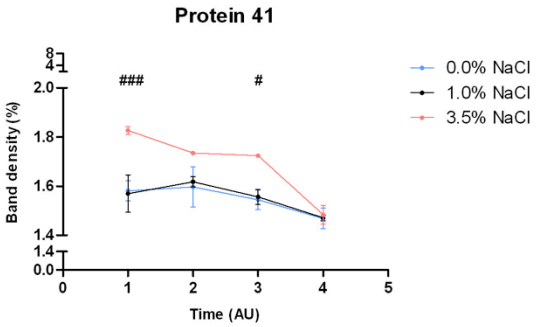
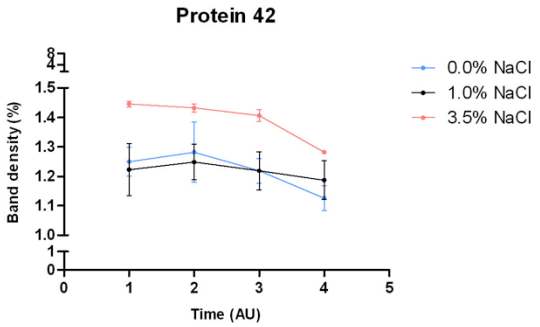
1.0% NaCl
3.5% NaCl


Protein 41
###
#
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 42
Band density (%)
Time (AU)
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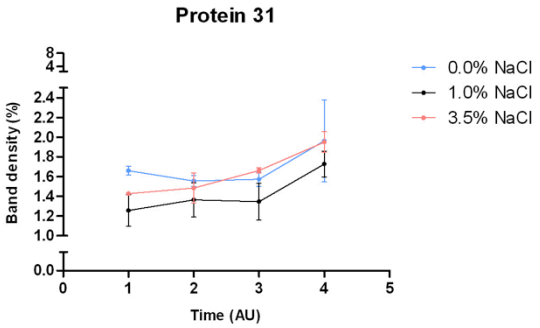
1.0% NaCl
3.5% NaCl


Protein 31
Band density (%)
Time (AU)
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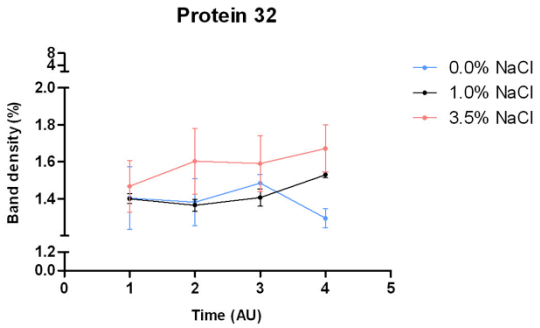
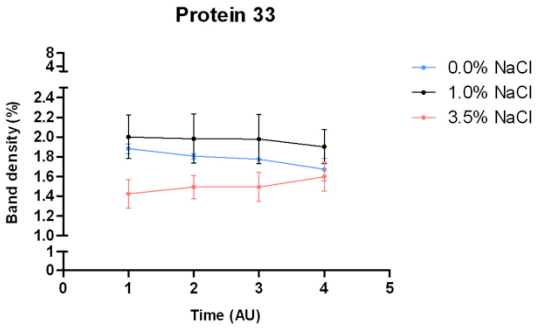
1.0% NaCl
3.5% NaCl


Protein 32
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 33
Band density (%)
Time (AU)
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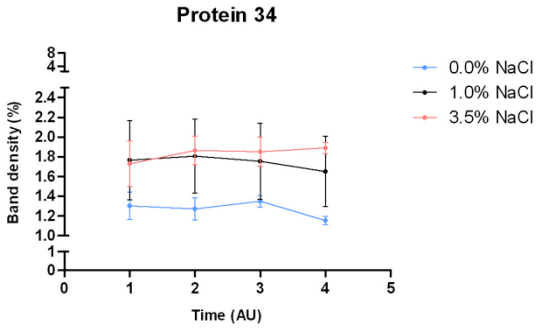
1.0% NaCl
3.5% NaCl


Protein 34
Band density (%)
Time (AU)
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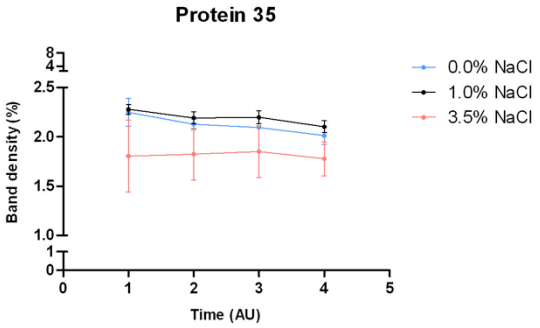
1.0% NaCl
3.5% NaCl


Protein 35
Band density (%)
Time (AU)
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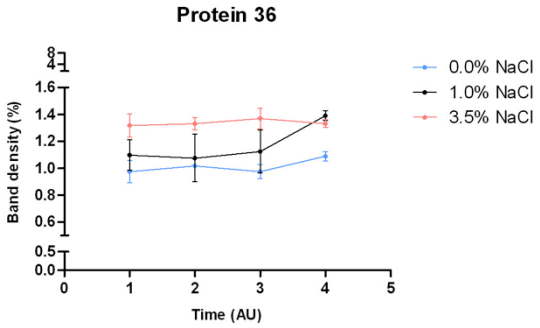
1.0% NaCl
3.5% NaCl


Protein 36
Band density (%)
Time (AU)
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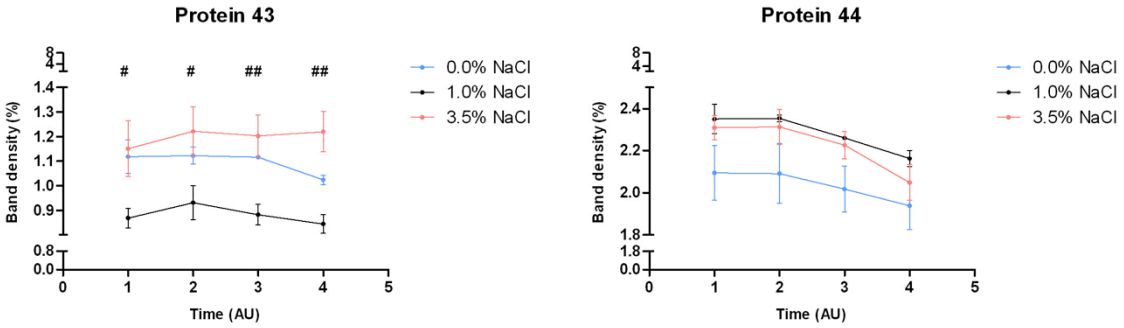
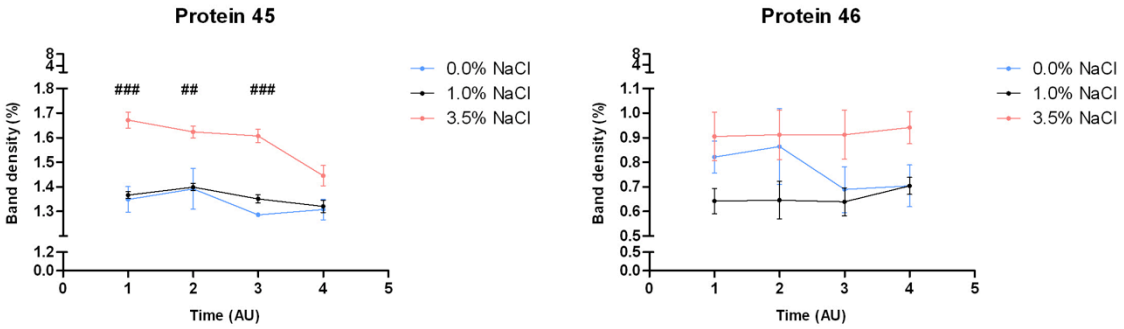
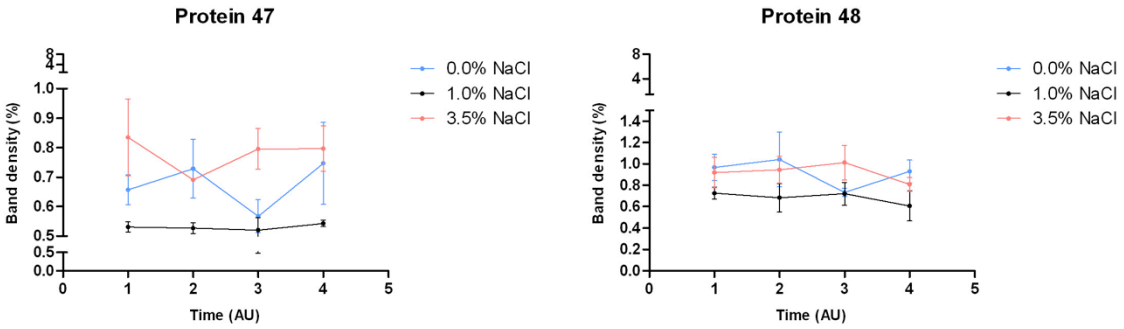
1.0% NaCl
3.5% NaCl


Protein 43
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl
##

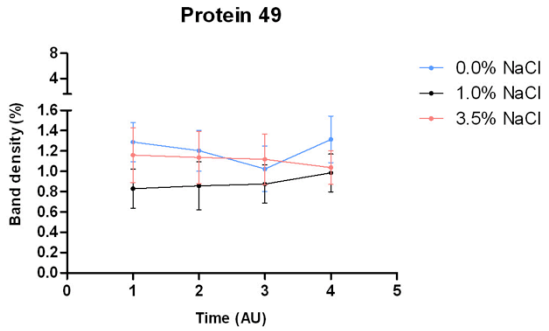
Protein 44
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

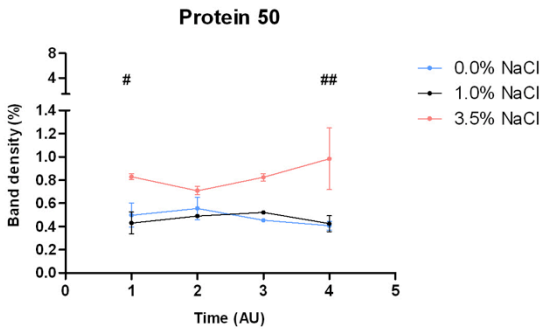

Protein 45
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl
###

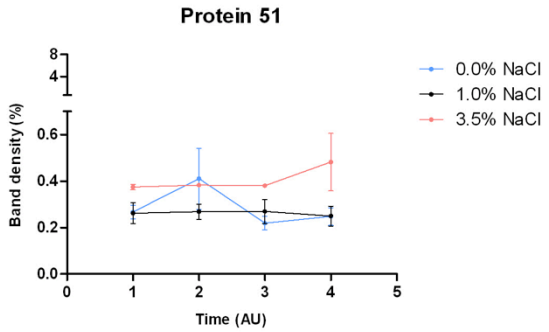
Protein 46
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

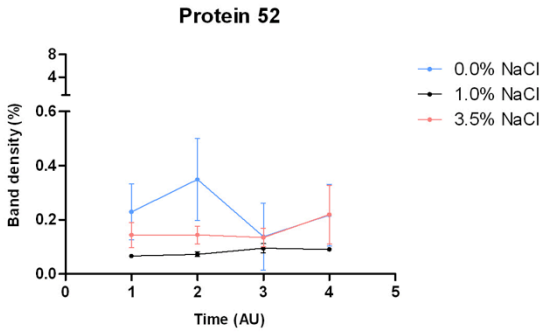

Protein 47
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

Protein 48
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 49
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 50
#
##
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 51
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 52
Band density (%)
Time (AU)
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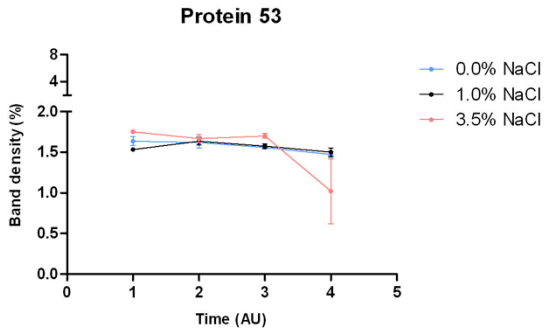
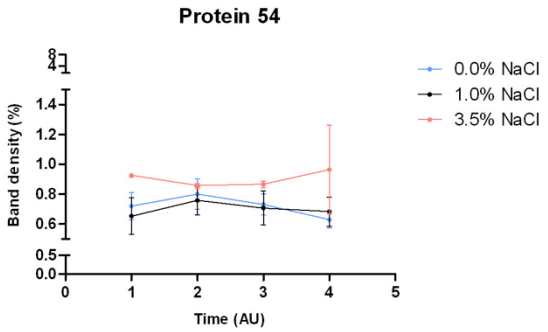
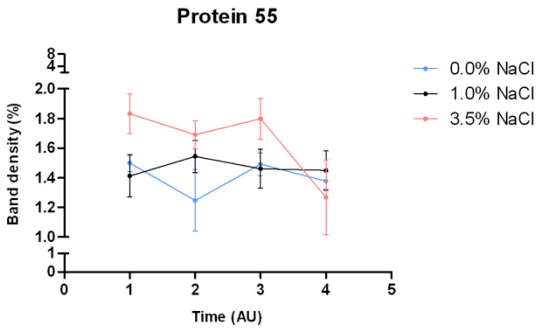
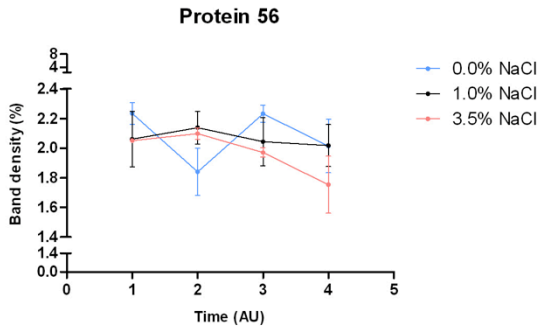
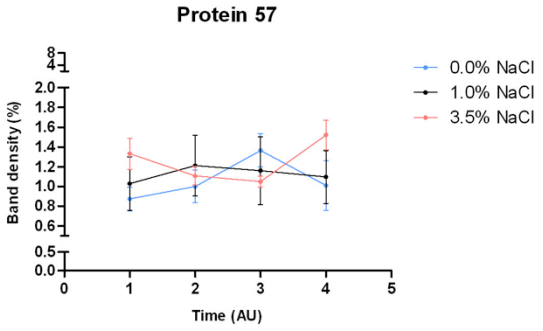
1.0% NaCl
3.5% NaCl

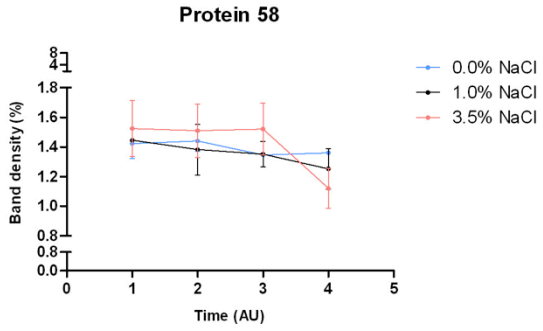

Protein 53
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

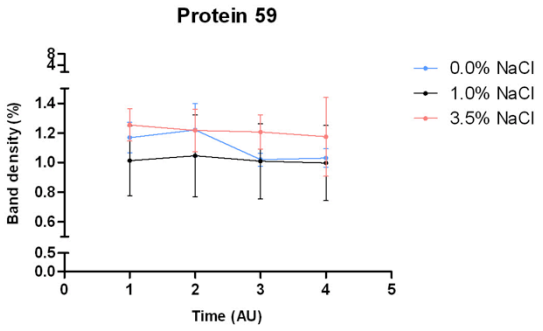

Protein 54
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

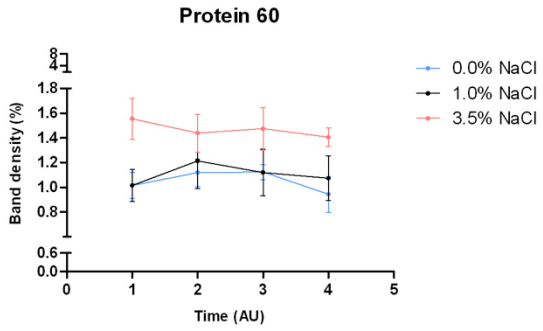

Protein 55
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

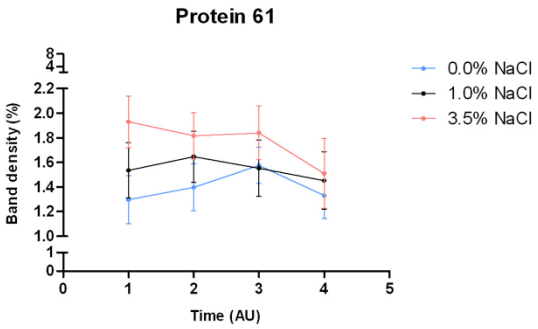

Protein 56
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

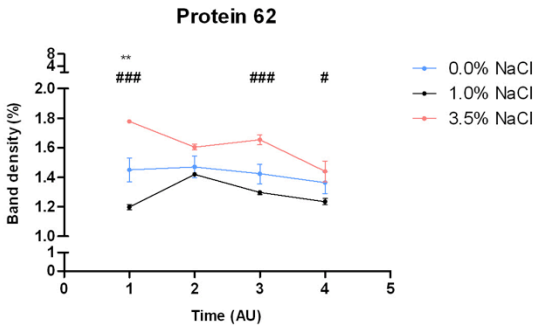

Protein 57
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

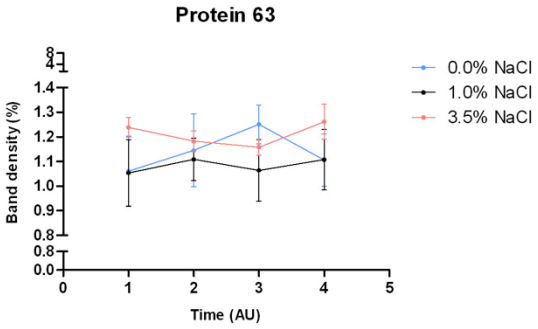

Protein 58
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

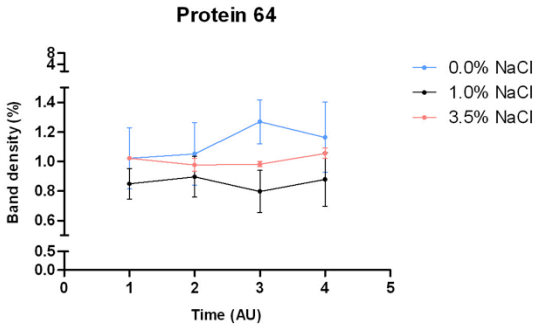

Protein 59
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

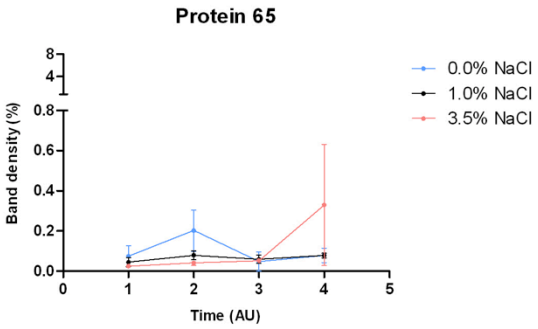

Protein 60
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 61
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 62
**
###
###
#
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 63
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 64
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl


Protein 65
Band density (%)
Time (AU)
0.0% NaCl
1.0% NaCl
3.5% NaCl

2. Molecular weight of proteins detected by SDS-PAGE

Table 21. Molecular weight of extracellular proteins detected in Temperature conditions.

Protein	~ Molecular weight (kDa)	Protein	~ Molecular weight (kDa)
1	180.995	42	15.598
2	154.083	43	15.008
3	142.975	44	13.237
4	118.367		
5	106.688		
6	97.341		
7	93.346		
8	90.457		
9	84.061		
10	76.497		
11	72.906		
12	68.763		
13	62.018		
14	58.898		
15	57.299		
16	54.603		
17	52.214		
18	49.597		
19	46.961		
20	44.571		
21	43.422		
22	41.212		
23	40.054		
24	37.745		
25	36.603		
26	36.304		
27	34.340		
28	32.323		
29	31.747		
30	29.784		
31	28.826		
32	27.989		
33	27.000		
34	26.388		
35	25.289		
36	24.088		
37	21.488		
38	20.498		
39	17.529		
40	17.066		
41	16.159		

Table 22. Molecular weight of extracellular proteins detected in pH conditions.

Protein	~ Molecular weight (kDa)
1	188.264
2	161.392
3	145.824
4	118.165
5	104.982
6	99.219
7	94.552
8	90.104
9	84.837
10	78.921
11	73.750
12	69.763
13	63.829
14	60.379
15	59.271
16	53.037
17	50.356
18	47.078
19	45.118
20	44.224
21	41.648
22	38.349
23	36.595
24	35.291
25	33.922
26	32.876
27	32.021
28	30.375
29	28.767
30	26.754
31	25.379
32	23.700
33	21.485
34	17.101
35	16.044
36	14.303

Table 23. Molecular weight of extracellular proteins detected in Salinity conditions.

Protein	~ Molecular weight (kDa)	Protein	~ Molecular weight (kDa)
1	180.029	42	23.284
2	134.682	43	21.527
3	125.699	44	20.597
4	110.956	45	19.316
5	99.488	46	18.291
6	94.993	47	17.482
7	93.540	48	16.845
8	91.637	49	16.119
9	83.974	50	15.767
10	81.844	51	14.874
11	78.954	52	14.307
12	74.503	53	12.668
13	73.275		
14	69.020		
15	62.887		
16	59.631		
17	58.648		
18	55.059		
19	53.259		
20	51.007		
21	50.000		
22	47.336		
23	44.703		
24	44.041		
25	40.974		
26	39.375		
27	38.695		
28	37.932		
29	36.570		
30	35.786		
31	34.381		
32	33.087		
33	32.269		
34	32.001		
35	29.836		
36	28.761		
37	28.050		
38	27.771		
39	26.949		
40	26.371		
41	25.251		

Table 24. Molecular weight of intracellular proteins detected in Temperature conditions.

Protein	~ Molecular weight (kDa)	Protein	~ Molecular weight (kDa)
1	180.863	42	35.871
2	177.240	43	35.298
3	164.432	44	34.365
4	150.278	45	33.816
5	138.011	46	32.804
6	130.099	47	32.051
7	113.676	48	31.091
8	108.982	49	29.468
9	101.015	50	28.484
10	95.734	51	27.582
11	94.323	52	26.805
12	91.109	53	26.472
13	89.765	54	25.496
14	85.006	55	23.958
15	82.927	56	22.960
16	77.372	57	21.675
17	75.854	58	20.877
18	71.877	59	20.158
19	70.921	60	19.501
20	68.359	61	19.012
21	67.225	62	17.864
22	64.363	63	17.283
23	61.830	64	16.828
24	59.996	65	16.448
25	59.199	66	15.712
26	56.679	67	15.279
27	53.905	68	14.809
28	51.610	69	13.835
29	49.934	70	13.282
30	49.189	71	12.345
31	47.020	72	12.096
32	46.203	73	11.416
33	44.055	74	10.811
34	43.289	75	10.049
35	41.797	76	9.713
36	40.661	77	8.845
37	39.655		
38	38.868		
39	38.192		
40	37.247		
41	36.322		

Table 25. Molecular weight of intracellular proteins detected in pH conditions.

Protein	~ Molecular weight (kDa)	Protein	~ Molecular weight (kDa)
1	180.863	42	35.871
2	177.240	43	35.298
3	164.432	44	34.365
4	150.278	45	33.816
5	138.011	46	32.804
6	130.099	47	32.051
7	113.676	48	31.091
8	108.982	49	29.468
9	101.015	50	28.484
10	95.734	51	27.582
11	94.323	52	26.805
12	91.109	53	26.472
13	89.765	54	25.496
14	85.006	55	23.958
15	82.927	56	22.960
16	77.372	57	21.675
17	75.854	58	20.877
18	71.877	59	20.158
19	70.921	60	19.501
20	68.359	61	19.012
21	67.225	62	17.864
22	64.363	63	17.283
23	61.830	64	16.828
24	59.996	65	16.448
25	59.199	66	15.712
26	56.679	67	15.279
27	53.905	68	14.809
28	51.610	69	13.835
29	49.934	70	13.282
30	49.189	71	12.345
31	47.020	72	12.096
32	46.203	73	11.416
33	44.055	74	10.811
34	43.289	75	10.049
35	41.797	76	9.713
36	40.661	77	8.845
37	39.655		
38	38.868		
39	38.192		
40	37.247		
41	36.322		

Table 26. Molecular weight of intracellular proteins detected in Salinity conditions.

Protein	~ Molecular weight (kDa)	Protein	~ Molecular weight (kDa)
1	180.863	42	35.871
2	177.240	43	35.298
3	164.432	44	34.365
4	150.278	45	33.816
5	138.011	46	32.804
6	130.099	47	32.051
7	113.676	48	31.091
8	108.982	49	29.468
9	101.015	50	28.484
10	95.734	51	27.582
11	94.323	52	26.805
12	91.109	53	26.472
13	89.765	54	25.496
14	85.006	55	23.958
15	82.927	56	22.960
16	77.372	57	21.675
17	75.854	58	20.877
18	71.877	59	20.158
19	70.921	60	19.501
20	68.359	61	19.012
21	67.225	62	17.864
22	64.363	63	17.283
23	61.830	64	16.828
24	59.996	65	16.448
25	59.199	66	15.712
26	56.679	67	15.279
27	53.905	68	14.809
28	51.610	69	13.835
29	49.934	70	13.282
30	49.189	71	12.345
31	47.020	72	12.096
32	46.203	73	11.416
33	44.055	74	10.811
34	43.289	75	10.049
35	41.797	76	9.713
36	40.661	77	8.845
37	39.655		
38	38.868		
39	38.192		
40	37.247		
41	36.322		