Catarina Ribeiro Correia

Degree in Cellular and Molecular Biology

Evaluation of pathogenic potential of *Aeromonas spp.* strains using *in vitro* methodologies

Dissertation to obtain a Master Degree in
Molecular Genetics and Biomedicine

Supervisor: Ana Luísa Ferreira Simplício, Ph.D IBET/ITQB-UNL
Co-Supervisor: Maria Teresa Crespo, Ph.D IBET/ITQB-UNL

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Presidente: Prof. Doutora Paula Maria Theriaga Mendes Bernardo Gonçalves
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ABSTRACT

Aims: To contribute to the evaluation of the pathogenic potential of *Aeromonas*, through the test of 24 *Aeromonas spp.* strains of Portuguese origin, for the adherence, invasion and cytotoxicity abilities in mammal cells.

Rationale: Studies on other enteropathogens indicate that a pathogen must be able to attach to host target cells to cause gastrointestinal disease [Finlay and Falkow, 1997; Scoglio *et al.*, 2001], via either toxin production or host cell invasion, or both [Knutton, *et al.* 1987].

Results: 19 (79%) and 12 (50%) strains were found to have the ability to adhere and invade differentiated cells respectively while 22 (92%) and 13 (54%) strains had ability to adhere and invade undifferentiated Caco-2 cells. These results indicate that most *Aeromonas spp.* strains interact optimally with cultured human intestinal cells at cellular sites expressed in the brush border early in the differentiation process of Caco-2 cells. In 13 (54%) strains it was observed an aggregative adhesion pattern as observed in other enteropathogens, including all clinical strains. 6 (25%) isolates express both adherence and extracellular cytotoxicity, but preheating caused a decrease in the citotoxicity of the supernatants of 5 of these strains suggesting that the remainder clinical strain (A255) has the ability to produce extracellular heat-stable toxins. 17 (71%) isolates express cell contact dependent cytotoxicity, but only 13 of these strains were able to invade Caco-2 cells, indicating the presence of others mechanisms of cell lysis not yet determined.

Conclusions: *Aeromonas spp.* strains isolated from water, food and food processing surfaces showed adhesive, invasive and cytotoxic patterns similar or larger than clinical strains, suggesting that environmental *Aeromonas spp.* stains have the potential to cause human illness and that food and water sources may act as dissemination vehicles of this human pathogen with implication in the public health in Portugal.

KEYWORDS: *Aeromonas,* Cytotoxicity, Adherence, Invasion, Caco-2 Cells.

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RESUMO

Objetivos: Contribuir para a avaliação do potencial patogénico das *Aeromonas*, através da análise de 24 estirpes de *Aeromonas spp.*, com origem em Portugal, da capacidade de aderência, invasão e citotoxicidade em células mamíferas.

Base: Estudos sobre outros enteropatógenos indicam que um agente patogénico deve ser capaz de aderir a célula alvo para causar doenças gastrointestinais [Finlay and Falkow, 1997; Scoglio *et al.*, 2001], quer através da produção de toxinas ou quer pela invasão da célula hospedeira, ou por ambas [Knutton, *et al.* 1987].

Resultados: 19 (79%) e 12 (50%) estirpes apresentaram capacidade de aderir e invadir células diferenciadas, respetivamente, enquanto 22 (92%) e 13 (54%) estirpes apresentaram capacidade de aderir e invadir células indiferenciadas Caco-2. Estes resultados indicam que a maioria das estirpes de *Aeromonas spp.* interagem otimamente com células intestinais humanas cultivadas em locais celulares expressas nas microvilosidades no início do processo de diferenciação das células Caco-2. Em 13 (54%) estirpes observou-se um padrão de adesão agregativa igual ao observado em outros enteropatogénicos, incluindo todas as estirpes clinicas. 6 (25%) estirpes expressam tanto aderência como citotoxicidade extracelular, mas o pré-aquecimento causou uma diminuição na citotoxicicidade dos sobrenadantes de 5 dessas estipes, o que sugere que a restante estirpe clinica (A255) têm a capacidade de produzir toxinas termorresistentes. 17 (71%) estirpes expressam citotoxicidade dependente de contacto celular, mas apenas 13 dessas estirpes foram capazes de invadir células Caco-2, indicando a presença de outros mecanismos de lise celular ainda não determinados.

Conclusão: As estirpes de *Aeromonas spp.* isoladas a partir de água, alimentos e superfícies de processamento de alimentos apresentaram padrões de adesão, invasão e citotóxicos semelhantes ou maiores que as das estirpes com origens clínicas, sugerindo que as estirpes *Aeromonas spp.* com origens ambientais têm o potencial de causar doenças humanas e que alimentos e água podem podem atuar como veículo de difusão destes patogénicos humanos com implicação na saúde pública em Portugal.

PALAVRAS-CHAVES: *Aeromonas,* Citotoxicidade, Aderência, Invasão, Células Caco-2.

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Chapter 1   Introduction

1.1. Taxonomy of genus *Aeromonas*

The genus *Aeromonas* belongs to a single family of bacteria, *Aeromonodaceae*, of the order *Aeromonadales*. They consist of straight, coccobacillary to bacillary gram-negative bacteria with rounded ends measuring 0.3-1.0 x 1.0-3.5 μm [Martin-Carnahan and Joseph, 2005]. They occur singly, in pairs, and rarely as short chains. *Aeromonas spp.* are facultative anaerobic, catalase positive, oxidase positive, chemoorganotrophic bacteria that exhibit both oxidative and fermentative metabolism of carbohydrates. *Aeromonas spp.* are waterborne and grow optimally within 22-35ºC, but some species can growth within 0-45ºC [Mateos *et al.*, 1993], they tolerate a pH range from 4.5 to 9 and have a optimally growth at sodium chloride concentrations between of 0-4% [Isonhood and Drake, 2002].

Aeromonas spp. can be divided in two major groups, according to different phenotypical characteristics. The strains that grow at 35ºC to 37ºC and are motile by polar flagella, belong to mesophilic group and are commonly responsible for numerous human infections, being subdivided into *Aeromonas hydrophila, Aeromonas caviae* and *Aeromonas sobria.* The psychrophilic group comprises non-motile strains that grow better between 22ºC and 28ºC are responsible for causing fish infection, and are designated by *Aeromonas salmonicida* [Janda and Abbott, 1998].

Currently there are thirty one recognized *Aeromonas* species at list of prokaryotic names with standing in nomenclature: genus *Aeromonas* [Euzéby, 2013]*.*

1.2. Occurrence

Aeromonas species are autochthonous (natural inhabitants) to the aqueous environment and due to their high adaptation capacity, are widely distributed in nature occurring in many different habitats, namely freshwater, marine and estuarine waters, sewages and ground waters [Havelaar *et al.*, 1992; Holmes *et al.*, 1995; Nielsen *et al.*, 2001; Rahman *et al.*, 2007 in USEPA, 2006] where they may be pathogenic for poikilotherms (cold-blooded animals) [Janda and Abbott, 1998].

Aeromonas spp. have been isolated in several countries from chlorinated drinking water supplies [Hazen *et al.*, 1978; Burke *et al.*, 1984; Van der Kooij, 1988; Fernandez *et al.*, 2000; Figueras *et al.*, 2005 in USEPA, 2006] and in concentrations typically below 10 CFU/mL [Havelaar *et al.*, 1990; Van der Kooij, 1991 in USEPA, 2006]. An Australian study correlated the increased incidence of gastroenteritis in households where the water supply system had significant *Aeromonas* biofilm buildup [Kirk *et al.,* 1997]. Multiple strains are frequently found [Kuhn *et al.*, 1997; Sen and Rodgers, 2004 in USEPA, 2006] and they occur in biofilms where they mainly may be protected from disinfection [Holmes and Nicolls, 1995; Van der Kooij *et al.*, 1995 in USEPA, 2006]. In the Netherlands a drinking water standard of 2 CFU/mL at 25ºC has been established [Havelaar *et al.*, 1990 in USEPA, 2006], a bottled water standard is in effect in Canada [Warburton *et al.*, 1998; Warburton, 2000 in

USEPA, 2006] and in the U.S. *Aeromonas hydrophila* is listed by EPA (Protection Agency's) on the Candidate Contaminant List (CCL) since 1998 [USEPA, 1998].

Isonhood and Drake (2002) reviewed *Aeromonas spp.* in foods. While *Aeromonas spp.* have been isolated from fish, shellfish, meats (chicken, beef, lamb, pork, etc.), vegetables, dairy products (cheese, raw milk, etc.) and ready to eat foods, few foodborne outbreaks have been reported and most resulted from ingestion of fish or shellfish. However a growing body of epidemiological evidence supports the possibility of *Aeromonas* causing foodborne gastroenteritis. While plethora of putative virulence factors has been postulated and demonstrated in food isolates, the exact role and mechanism of *Aeromonas* in causing diarrheal illness has not been elucidated. Evidence suggests that a high infective dose is necessary to produce gastrointestinal disease in a susceptible host, however, the fact that *Aeromonas* may survive and grow at refrigerator temperatures raises the concern that a reservoir of bacteria that may be created and able to achieve an infective dose when foods are mishandled.

As mentioned before, *Aeromonas* form biofilms on surfaces (including within water distributing systems) and this may pose a threat of contamination in food processing. Bal'a *et al.* (1998) found that heat and chlorine were effective against biofilms on stainless steel surfaces, but older biofilms were more resistant recently established biofilm to heat. An eight-day old biofilm was destroyed by heating to 60ºC and by exposure to 75 mg/L chlorine for 1 minute.

1.3. Health effect in humans

Figueras (2005) reviewed human infections caused by *Aeromonas spp*. They have multiple virulence factors that are typically associated with gastrointestinal diseases in other pathogenic bacteria, but while the production of extra-intestinal disease in humans is undisputable, the role of *Aeromonas spp.* as gastroenteritis agents is controversial, and has been based on extrapolation from anecdotal case reports, case-control studies, and a handful of outbreaks epidemiologically associated with food or water ingestion. The direct relationship between the presence of most of these virulence factors and gastrointestinal disease has not been proven. No epidemiological studies have indisputably linked *Aeromonas* with outbreaks of diarrheal disease. The association is strongest in children under the age of 2 years, adults over 50 and the immunocompromised, but a high number of asymptomatic persons carry *Aeromonas* in their gastrointestinal tract.

Fecal isolation rates of *Aeromonas* in asymptomatic persons, in developed countries, range between 0% to 4.0% [Millership *et al.*, 1983; Agger *et al.*, 1985; Svenungsson *et al.*, 2000 in USEPA, 2006]; while in symptomatic persons it ranges between 0.8 to 7.4% [Agger *et al.*, 1985; Moyer, 1987; Albert *et al.*, 2000 in USEPA, 2006]. In lower developed regions like in Southeast Asia, asymptomatic carriage rates are as high as 27.5%, while in patients with diarrhea rates have been reported as high as 34% [Pazzaglia *et al.*, 1990 in USEPA, 2006].

Aeromonas hydrophila, Aeromonas veronii biovar Sobria *and Aeromonas caviae* are the most frequently associated with gastrointestinal disease. Gastrointestinal infections are usually self-limiting, but antibiotic therapy may be required with prolonged infection.

Aeromonas. hydrophila, Aeromonas caviae, Aeromonas veronii biovar Sobria*, Aeromonas veronii* biovar Veronii*, Aeromonas jandaei, Aeromonas trota* and *Aeromonas schubertii* are also known to be pathogenic to humans. They cause a variety of extra-intestinal infections such as wound infection, memingitis, osteomyelitis, septic arthritis, endocarditis, peritonitis, eye and urinary tract infections. Those extra-intestinal infections are usually serious and potentially life-threatening and for a successful recovery is necessary an aggressive antibiotic therapy after an antimicrobial susceptibility test, because multi-drug resistance is common among *Aeromonas.*

1.4. Health effect in animals

Gosling (1996) reviewed animal infections caused by *Aeromonas spp.* Diseases caused by *Aeromonas* represent a significant source of loss to the aquaculture industry. The disease mechanisms of *Aeromonas* that cause animal disease are essentially the same ones that produce disease in humans.

1.5. Virulence properties

Janda and Abbott (2010) reviewed the virulence factors produced by *Aeromonas spp.* and the pathogenicity of *Aeromonas* is complex and multifactorial and incompletely understood despite decades of intense investigation. Although an established animal model that faithfully reproduces the syndrome associated with *Aeromonas* does not exist, testing with isogenic mutants in animal cells show that many virulence factors produced by *Aeromonas* are associated with its pathogenesis. Factors contributing to virulence are present in two forms, cell-associated structures and extracellular products.

1.5.1. Cell-associated virulence factors

Structural factors have been discovered in *Aeromonas ssp.* that promote their attachment (pili, flagella) and colonization (adhesins, outer membrane proteins (OMPs)). Other factors protect *Aeromonas* from host response (S-layer, lipopolysaccharide (LPS), capsule) and are responsible for delivering virulence factors directly into the host cell (Type II and Type III secretion systems) [USEPA, 2006].

A vital step for the enteropathogens to initiate infection is through adherence to host cells, allowing localization and subsequent colonization of the appropriate target tissues [Finlay and Falkow, 1997; Scoglio *et al.*, 2001]. It is essential to cause gastrointestinal disease, *via* either toxin production or host cell invasion, or both [Knutton *et al.,* 1987].

Flagella have been referred to have a crucial role in adhesion, biofilm formation, and colonization of several other pathogenic bacteria, such as *Pseudomonas aeruginosa* [Stanley, 1983 in USEPA, 2006], *Salmonella enterica* [Ciacci-Woolwine *et al.*, 1998 in USEPA, 2006], *Escherichia coli* [Pratt and Kotler, 1998 in USEPA, 2006], *Helicobacter pylori* [Eaton *et al.*, 1996 in USEPA, 2006] and *Vibrio cholerae* [Gardel and Mekalanos, 1996 in USEPA, 2006].

Only a limited group of bacteria is capable of expressing two types of flagellar systems such as *Aeromonas spp.* [Kirov *et al.,* 2004; Merino *et al.,* 2006; Sen and Lye, 2007], being well studied in

Aeromona hydrophila and they do not seem to share structural genes or regulatory [McCarter, 2004 in Merino *et al.,* 2006]. Polar flagella and lateral flagella were described by Rabaan *et al.* (2001) and Kirov *et al.* (2002). Kirov (2003) reviewed the expression of lateral flagella and their multi-functional role in pathogenesis.

The swimming motility has been linked to a single polar unsheathed flagellum in all mesophilic *Aeromonas*, which is expressed constitutively and highly regulated by a number of environmental factors [Merino *et al.,* 2006]. They respond to sensory stimuli (chemotaxis), allowing locomotion toward new substrates and, therefore, confer an adaptive advantage in the colonization of different environments [Merino *et al.,* 2006]. The swarming motility has been linked to many unsheathed peritrichous lateral flagella in 50 to 60% of *Aeromonas* [Kirov *et al.,* 2004], which are expressed when grown in matrices that do not allow motility by a single polar flagellum, like viscous environments or over surfaces and solid media [Kirov et al., 2003; Naharro *et al.,* 2011].

In this bacterial genus both types of flagella function as adhesins enabling adherence of human cell lines by most *Aeromonas* isolates, observed in experimental tests with Caco-2, HEp-2 e Henle 407 cells [Kirov *et al.,* 2004; Sen and Lye, 2007]. Strains that loose polar flagella are virtually nonadherent to cell lines, while strains lacking lateral flagella have a reduced capacity for cell binding. Flagellar mutants were shown to have decreased binding capacity by more than 80% [Kirov *et al.,* 2004]. In addition, the flagella have been described as colonization factors and biofilm formation in different surfaces [Kirov *et al.,* 2004].

Several studies have shown a relationship between the adhesive patterns and the increased potential for virulence of bacteria [Parsot, 2005; Mitache *et al.,* 2009; Mora *et al.,* 2009]. Because they are well studied, adhesive patterns obtained with *Escherichia coli* serves as a model for studies of infection of other bacteria in eukaryotic cells. Diffuse adhesion (DA), can be observed in *Escherichia coli* as well as localized (LA) and aggregative (AA) with "stacked-brick" appearance. It is believed that the latter is related to the higher bacterial virulence, in contrast to other patterns which are seen in less virulent bacteria [Challapalli *et al.,* 1988; Singh *et al*, 1992; Parras *et al.,* 1993].

Several studies suggest that attachment to host cells is mediated by pili, extracellular filamentous appendages, and were described as potential colonization factors in *Aeromonas hydrophila* and *Aeromonas veronii* biovar Sobria [Hokama and Iwanaga, 1991 in USEPA, 2006]. Kirov (1993) reported that pili were important adhesive factors for mucosal surface attachment and described filamentous and nonfilamentous adhesins. Two morphotypes of pili have been observed in *Aeromonas spp.*, short rigid pili (S/R type) similar to those of *Escherichia coli* Type I and Pap pili [Ho *et al.,* 1992 in USEPA, 2006] and long wavy flexible pili (L/W type) belong to a class of Type IV bundle-forming pili (Bfp) [Kirov and Sanderson, 1996 in USEPA, 2006]. Removal of pili or neutralization of attachment sites by homologous antibody treatment limits or defeats adherence properties in cell culture systems in *Aeromonas* [Iwanaga and Hokama, 1992 in USEPA, 2006].

Several studies also suggest that non-pilar adhesins play a major role in adhesion of *Aeromonas*. Rocha de Souza *et al.* (2003) studied interaction of adherence and invasion properties of *Aeromonas caviae* using Caco-2 cells and observed that the 43 kDa OMP facilitated cell binding. Transmission

electron microscopy (TEM) did not demonstrate fimbrial structures on cell surfaces of highly-adherent *Aeromonas caviae* strains. These data suggest that OMPs mediate adherence in *Aeromonas caviae* instead of pili. Some OMP have hemagglutination activity, while other OMPs are thought to have pore-forming capability.

One of the possible mechanisms involved in the *Aeromonas* pathogenesis is associated with the production of type III (T3SS) or injectisome secretion system, which was reviewed by Coburn (2007). A broad clinical spectrum of diseases have been referred as being caused by T3SS containing pathogens, for example, infections with enteropathogenic *Escherichia coli, Shigella, Salmonella* and *Yersinia* species which result in serious intestinal diseases.

There is high structural similarity between the bacterial flagellum and the T3SS, many structural proteins are clearly homologous. This system is composed of several rings, the basal area is anchored in the inner membrane forming a canal that crosses the outer membrane and projected outwards, this way allows adherence to cell membranes and injection of bacterial toxins (effector proteins) directly in the cytosol of host cells [Alberts *et al.,* 2002]. Some of these effector proteins have multiple biological functions, such as changing the cytoskeleton to facilitate the invasion or activation of intracellular signaling cascades within the host cells, eventually causing lysis of host epithelial cells and contributing to the degradation of tissues host [Alberts *et al.,* 2002; Sha *et al.,* 2005; Krzymińska *et al.,* 2012].

Several studies have shown invasive ability of *Aeromonas* to equal that of *Campylobacter* [Nishikawa et al., 1994; Shaw et al., 1995 in USEPA, 2006], while intracellular bacteria have been demonstrated by electron microscopy, no gene or product has been identified specifically with invasion [USEPA, 2006].

1.5.2. Extracellular virulence factors

Extracellular factors have been discovered in *Aeromonas ssp.* that promote their ability to obtain nutrients that allow the proliferation and dissemination of the microorganism in the host, such as enterotoxins, proteases, phospholipases and hemolysins associated [Khajanchi *et al.* 2010].

DNases can act as food enzymes used to obtain phosphorus and nitrogen [Pemberton *et al.,* 1997] and provide a barrier to the entry of foreign DNA in the host cell, playing an important role in microbial defense mechanisms [Kamble and Deshmukh, 2012], but can also act as virulence factors due to the ability to degrade extracellular fibers released by neutrophils, preventing phagocytosis [Brinkmann *et al.,* 2004].

Lipases can act as food enzymes used to obtain compounds as carbon source, they are classified as hydrolases that can act on ester bonds, promoting triacylglycerol hydrolysis and causing the release of fatty acids and glycerol [Hedstrom and Nisson, 1975 in Chuang *et al.,* 1997], but can also act as virulence factors due to the concentration increased of free fatty acids interferes in various immune system functions [Buttke and Cuchens, 1984, Eftimiadi *et al.,* 1987 in Chuang *et al.,* 1997]. Some strains appear to express more than one gene whose product has lipolytic activity, but the products of

gcat, pla, and *apl-1* genes encode phospholipases often associated with intestinal lesions, while the *lipA* and *lipH3* genes encode lipases without phospholipid action [Chuang *et al.,* 1997].

Elastases have elastolytic and caseinolytic activities. In the *Aeromonas* genus, the caseinolytic activity is mainly a result of the AhyA serine protease, *ahyA* gene product which a weak elastolytic activity, and it has been associated with the acceleration of the maturity of the AhyB elastase, extracellular metalloprotease encoded by the *ahyB* gene, that the elastolytic activity has been associated [Cascón *et al.,* 2000a, 2000b]. The role of AhyB elastase in *Aeromonas spp.* is not yet completely elucidated, but revealed a 52% aminoacid identity when compared to the sequence of the LasB elastase identified in *Pseudomonas aeruginosa*, which can degrade many components of the immune system, including chemokines and cytokines [Horvat *et al.,* 1989; Kevin *et al.,* 2003 in Kuang *et al.,* 2011] and antimicrobial peptides [Schad *et al.,* 1987 in Kuang *et al.,* 2011].

Enterotoxins produced by *Aeromonas spp.* include into two categories, cytotoxic and cytotonic [Krzymińska *et al.*, 2003; Von Gravaenitz, 2007]. Chopra and Houston (1999) reviewed enterotoxins of *Aeromonas spp.* associated with production of gastrointestinal disease.

The cytotoxic enterotoxins may cause extensive damage to the epithelia, include heat-labile and stable compounds, with hemolytic and cytotoxic activities, like pore-forming toxin aerolysin and different α- and β-hemolysins [Galindo *et al.*, 2006; Von Gravaenitz, 2007], while progress is being made in understanding aerolysin activity, the actual mechanism is complex and incompletely understood. The cytotoxin Act, which is structural and functional closely related to the cytotoxin Aerolisina [Martin-Carnahan e Joseph, 2005], is consensually considered the most important virulence factor associated with the genus *Aeromonas.* It's secreted by the type II secretion system (T2SS), expressing hemolytic and cytotoxic activity, which involves the formation of pores in the target cell membrane and consequently occurs the influx of water, resulting in cell lysis [Khajanchi *et al.,* 2010]. The cytotoxin HlyA is a non-channel forming β-hemolisina similar to the *Vibrio cholerae* hemolysin, widespread in the genus *Aeromonas* and is virtually ubiquitous in *Aeromonas hydrophila* [Naharro *et al.,* 2011].

The cytotonic enterotoxins, on the other hand, cause increase in the level of cAMP in intestinal epithelial cells, like cholera toxin [Galindo *et al.* 2006], resulting in fluid secretion from intestinal cells, not causing the degeneration of crypts and villi of the small intestine [Sha *et al,* 2002]. The cytotonic Ast and Alt cause fluid accumulation in ligated ileal loops in animal models and probably have an undescribed role in causing diarrhea in humans [Sha *et al.,* 2002]. Laohachai *et al.* (2003) reviewed the role of bacterial toxins that induce changes in membrane transport leading to diarrheal disease.

The cytotoxic properties have been considered a major virulence factor presented by *Aeromonas*, making essential to assess the cytotoxicity of different strains in order to evaluate their pathogenic potential [Ghatak *et al.*, 2006].

1.6. Caco-2 cell line as a model of intestinal barrier

Several infection *in vitro* studies in animal cell lines support the ability of these bacteria to cause cellular damage, in addition to its ability to adhere and to invade cells [Schiavano *et al.,* 1998; Martins *et al.*, 2002; Balaji *et al,* 2004].

Caco-2 cells, which are derived from a human colon adenocarcinoma, have gained great attention in recent years as an *in vitro* model of the intestinal epithelium. In culture Caco-2 cells spontaneously differentiate and organize as a polarized monolayer with tight junctions and microvilli, mimicking the *in vivo* enterocyte, expressing relatively high levels of digestive brush border enzymes and display other morphological, structural and functional properties similar to intestinal enterocytes [Pinto *et al.*, 1983 in Delie and Rubas, 1997]. Due to these characteristics, the Caco-2 cell culture is now widely used as a tissue model for studying adhesion and invasion of probiotic bacteria or entericpathogens [Delie and Rubas, 1997]. For example, Panigrahi *et al.* (1990) showed that the adhesion of non-01 *Vibrio cholerae* to Caco-2 cells correlated with human intestinal colonization and disease.

Traditional Caco-2 cell culture requires a 21-day period to attain a differentiated monolayer. During the growing phase Caco-2 cells remain undifferentiated and immediately after they reach the status of confluence, cells start the differentiation program that will be finished 18-21 days later [Delie and Rubas, 1997]. According to the morphological and functional grade of differentiation they can be divided into three subgroups:

1) cells homogeneously undifferentiated (sub confluent population);
2) cells heterogeneously polarized and differentiated (intermediate phase);
3) cells homogeneously polarized and differentiated (>15 days after seeding).

A number of enzymes typical of the mature enterocyte are located on the microvilli forming the brush border such as: sucrase-isomaltase, lactase, aminopeptidase N, dipeptilpeptidane IV and alkaline phosphatase, and the level of expression is comparable to the *in vivo* level, increasing with the different stages of differentiation as in the human small intestine. They also express the polarized membrane receptors for growth factors and a number of transport activities located either on apical or basolateral membrane [Delie and Rubas, 1997].

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Chapter 2   Objectives

In the last decade *Aeromonas* have been referred as an emergent pathogen of gastrointestinal disease in humans and normally associated to ingestion of contaminated water or food [Figueras, 2005]. However the presence of virulence genes does not correlate with expression of gene products or with manifestations of disease in animals or humans [Bondi *et al.*, 2000] and therefore the precise combination of virulence factors that invariably confer virulence on a particular strain has not been determined.

A vital step for the enteropathogens to initiate infection is through adherence to host cells, allowing localization and subsequent colonization of the appropriate target tissues [Finlay and Falkow, 1997; Scoglio *et al.*, 2001]. Colonization is therefore essential to cause gastrointestinal disease, *via* either toxin production or host cell invasion, or both [Knutton, *et al.* 1987]. Knowledge of these may help to identify strains which pose a public health risk.

The Caco-2 cells are derived from a human carcinoma of the colon, they exhibit structural and differentiation patterns characteristic of mature enterocytes and are being increasingly used as a substitute for human intestinal cells to study the adhesion of enteric pathogens [Pinto *et al.*, 1983; Russel and Blake, 1994 in Delie and Rubas, 1997].

The work reported here was therefore conducted on those cells as a preliminary investigation to determine, if *Aeromonas* present in several human environments, from food industry (slaughterhouse, cheese factory and supermarkets) to water treatment facilities (EPAL), in Portugal, may pose a threat to public health.

24 strains were chosen as representatives of the diversity present in a set of *Aeromonas* isolates obtained by the team of Doctor Teresa Semedo-Lemsaddek at the Faculty of Veterinary Medicine, University of Lisbon, according to the results of clusters diversity, presence of virulence factors and resistance to antibiotics.

The specific aims that were pursuit were:

- ✓ To implement assays for adherence, invasion and cytotoxicity for bacteria in mammal cells, particularly the Caco-2 cell line;
- ✓ To evaluate eventual cytotoxicity of bacterial culture medium to the intestinal Caco-2 cell line, in undifferentiated cells (UC);
- ✓ To characterize the ability of the different bacterial strains to adhere and to invade the intestinal Caco-2 cell line, in both undifferentiated (UC) and differentiated cells (DC);
- ✓ To compare the phenotype profile determined in this study with the genetic profile determined by Barroco (2013).

To our knowledge, this study is the first carried out on *Aeromonas* strains isolated from Portugal for adhesive and invasive properties.

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Chapter 3   Materials and Methods

3.1. *Aeromonas* culture and growth study

24 previously isolated *Aeromonas spp.* strains, identified by phenotypic and genomic typing, were used in this study and their source and characterization carried out to date by Barroco (2013) are listed in Table 1 in Annex. The strains were stored at -20ºC in brain heart infusion broth (BHI, AES Laboratories) containing 45% (v/v) glycerol (Hi-media Laboratories).

Escherichia coli *K-12 C600* (No. 426, DSMZ; Braunschweig, Germany), a non-pathogenic strain, and *Pseudomonas aeruginosa PAO1* (No. 19880, DSMZ; Braunschweig, Germany), an adherent, invasive and cell-contact cytotoxic strain, were included as negative and positive controls, respectively. *Aeromona hydrophila* subsp. *hydrophila* (No. 30187t, DSMZ; Braunschweig, Germany), an adherent, invasive and extracellular cytotoxic strain, was included as a reference of the *Aeromonas* genus.

Optical density was used to estimate colony forming units (CFU) in a bacterial suspension which was possible after taking some basic precautions to control the following: type of media, growth phase of microorganisms, nature and condition of the equipment [Scott, 2011]. Therefore the spectrophotometer (Ultraspec 20100 pro) used was calibrated for the linear range of absorption *vs* CFU relevant values, using the bacterial suspension under the specific conditions of the future assays.

Bacterial strains were subcultured on BHI agar (BHI broth with 15 g/L agar, Scharlau) plates and incubated at 30ºC overnight, then passed to new plates of BHI agar and incubated at 37ºC overnight. The bacterial were then washed by harvesting and suspending in BHI broth, centrifuged at 10.000x*g* for 10 minutes and resuspended in fresh BHI broth.

Bacterial suspensions were adjusted to 0.400 optical density at 600 nm wave-length (OD_{600}) and twofold serial dilutions were performed in BHI broth (0.400 to 0.005). Spectrophotometer readings of the dilutions, stored on ice, were taken in triplicate before performing serial dilutions of the bacterial suspension in peptone water (Merck Millipore) and plated by the pour plate method, which consists in suspending 1 mL of the dilutions in a Petri-dish using molten Luria-Bertani agar (LBA, Bioreagentes Fisher) cooled to approximately 48°C (just above the point of solidification to minimize heat-induced cell death), after the medium solidifies the plates were inverted and incubated for 24-48 hours at 37ºC.

Each dilution was plated in duplicate and the viable counting was performed in the dilutions that have between 30 and 300 colonies. The results were expressed in colony forming unit (CFU) per mL by using the following formula:

$$\text{Average of colonies} \times (1 / \text{dilution factor}) \times (1 / \text{dilution volume})$$

The calibration curves obtained with this method are represented in Figure A1A, 2.A and 3.A in Annex.

The same cultures were adjusted to 0.005 OD_{600} and incubated at 37ºC for 10 hours with 150 rpm. Spectrophotometer readings were taken from 30 to 30 minutes for 8 hours in triplicate, dilutions of the samples in BHI broth were carried out when the absorbance of the culture exceed 0.400 OD_{600}.

The growth curves obtained with this method are represented in Figure 1.B, 2.B and 3.B in Annex.

3.2. Caco-2 cells culture and growth study

Human colon carcinoma Caco-2 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). They were stored at -80ºC in fetal bovine serum (FBS, Gibco) containing 5% (v/v) dimethyl sulfoxide (DMSO, Sigma) at a concentration of $2x10^6$ cells/mL in passage 30. The cells were routinely grown in 75 cm^2 plastic tissue culture flasks (Falcon) containing Dulbecco's modified Eagle minimum essential medium (DMEM, Gibco) supplemented with 10% (v/v) FBS and 1% (v/v) nonessential amino acids (NAAS, Gibco) at 37ºC in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2. The culture medium was changed every 48 hours and passed before reaching confluence (once a week).

For the cytotoxicity, adhesion and invasion assays, the cells were expanded in 175 cm^2 plastic tissue culture flasks (Nunc) and when reaching 70-90% confluence, the cells were trypsinized with 0.25% (v/v) trypsin (Gibco) for 5 minutes and adjusted to a concentration of $2x10^5$ cells/mL in culture medium. Depending on the assay, 4.8 mL, 1 mL or 0.16 mL of cell suspension was dispensed into each well of a 6, 24 or 96-well tissue culture plate (Falcon), respectively, to attain $1x10^5$ cells/cm^2 and incubated at 37ºC in 5% (v/v) CO_2 to obtain, 3 days later, undifferentiated semi-monolayers (± 80% confluent), or 4 days later, confluent undifferentiated cells (UC) monolayers and, after 18 days or more, differentiated cells (DC) monolayers. The culture medium was changed every 48 hours. The Caco-2 cells used for those assays were at passage numbers between 35 and 45.

The existence of Caco-2 cell lines maintained in different laboratories and/or of different clonal origin, in addition to the effects of different culture protocols may result in variations in growth rate of the cell line. For this reason a growth study of Caco-2 cell line was undertaken in the in-house conditions of the assays to be performed in order to estimate growth kinetics and cell concentration.

Semi-confluent cell monolayers were trypsinized and adjusted to a concentration of $1x10^5$ cells/mL ($5x10^4$ cells/cm^2) in culture medium and then 1 mL cell suspension was dispensed into each 2 cm^2 well ($2x10^4$ cells/cm^2) of a 24-well tissue culture plate was visually evaluated in an inverted microscope (OlymPus CKX41) every day of incubation. The cells of 3 wells were trypsinized and counted using a Fuchs-Rosenthal Counting Chamber (EMS #63512-10) and the cell viability was evaluated by diluting the cell suspension in Dulbecco's phosphate-buffered saline (DPBS, Gibco) containing 0.1% (v/v) trypan blue (Gibco) exclusion dye to establish the growth curves.

The results were expressed in cells per cm^2 by using the following formula:

(Average of viable cells x (1 / dilution factor) x (1 / volume per quadrant)) / surface growth

The calibration and growth curve obtained with this method are represented in Figure 4 in Annex.

3.3. Bacterial adhesion and invasion evaluation on Caco-2 cell line

3.3.1. Adherence and invasion quantitative assays

Bacterial strains were subcultured on BHI agar plates and incubated at 30ºC overnight, then passed to new plates of BHI agar and further incubated at 37ºC overnight. The bacterial were then washed as described previously. Bacterial suspensions were adjusted to approximately $5x10^7$ CFU/mL by measuring 0.115 OD_{600} (0.080 and 0.085 OD_{600} for K-12 and PAO1 strains, respectively), according to the calibration curves previously established (Figures 1, 2 and 3 in Annex), and kept on ice until use; the number of bacteria of each strain was confirmed by CFU determination immediately after use as described previously.

In the adherence assay, differentiated and undifferentiated Caco-2 cells monolayers plated in 24-well plates and containing 400 µL of fresh culture medium, were incubated at 37ºC in 5% CO_2 for 90 minutes with 100 µL of bacterial suspension to give a MOI of 10 bacteria / Caco-2 cell (approximately $5x10^5$ epithelial cells per mL to $5x10^6$ bacteria per mL, according to the calibration curve presented in Figure 4 in Annex. To remove non-adherent bacteria, the monolayers were washed gently three times with 1 mL of DPBS and then the cells were lysed by adding 0.5 mL of DMEM containing 2% (v/v) Triton X-100 (Sigma-Aldrich) for 30 minutes at 37ºC. To ensure full lysis and release of all the bacteria, including those eventually internalized, the lysate was pipetted up and down several times, pointing the tip of the pipette directly at the surface of the well,

For the invasion assay, performed similarly and in the same plate as the adhesion test, after the period of infection, the cultures were replaced by 1 mL of a 300 µg/mL Gentamicin solution and incubated for 1 hour at 37ºC to kill extracellular bacteria. To remove the antibiotic, the monolayers were washed gently 2 times with 1 mL of DPBS and then the intracellular bacteria were released by lysing the cells as described above.

Lastly, 0.5 mL of cold DMEM was added to 0.5 mL of each lysate, while keeping them on ice, for the determination of the number of adherent and invasive bacteria by CFU as described previously. The results are expressed in percentage of bacteria recovered in comparison to other wells where the monolayers were infected under the same conditions but untreated, the culture was kept on ice rather than discarded after the period of infection and the cell lysate was subsequently added. This step is performed because the bacteria in the inoculum can sometimes grow much faster than the bacteria in the presence of cells, skewing the size of the inoculum by comparison with the real total number of bacteria in wells with cells. The percentage of adherent/invasive bacteria can then be calculated by dividing the number of CFU of adherent/invasive bacteria by the addition of the number of CFU of adherent/invasive and non- adherent/non-invasive bacteria. All tests were performed in duplicate and in two separate experiments for each isolate. The variation is expressed as propagated error.

Isolates which show adhered bacterial numbers up to 3 times the negative control were considered as non-adherent. Due to a higher error associated with invasion assays in this case, only the isolates that did show invasion number greater than 10 times the negative control were considered as invasive.

3.3.2. Adherence patterns assays

In this adhesion assays were used monolayers of cells plated on sterilized glass coverslips (20x20 mm, Marienfeld) in 6-well plates with 80% of confluence (3 days old cells) and containing 1 mL of fresh culture medium which were then incubated at 37ºC in 5 % CO_2 for 90 minutes with 2 mL of bacterial suspension to give a MOI of 100 bacteria / Caco-2 cell. To remove non-adherent bacteria, the monolayers were washed gently 3 times with 2 mL of DPBS and then the remaining adherent bacteria and monolayers were fixed with 2 mL of 99.8% (v/v) methanol (J. T. Backer) for 5 minutes. Methanol was removed by washing the cells with 2 mL of PBS (Calbiochem) and then the cells were stained for 45 minutes with 2 mL of 10% (v/v) Giemsa stain (Sigma) prepared in Giemsa buffer (Sigma). The coverslips were removed from wells, washed in distilled water, air dried, mounted on glass slides and examined by oil immersion under a light microscope (Zeiss Imager A2 with Zeiss AxioCam MRm) at x1000 magnification.

Isolates which show a uniform distribution on the cell surface, were characterized as having a diffuse adhesion (DA) pattern; those which show a localized distribution, were characterized as having a localized adhesion (LA) pattern; and those which show a "stacked-brick" appearance on the cell surface, were characterized as having a aggregative adhesion (AA) pattern.

3.4. Bacterial cytotoxicity evaluation on Caco-2 cell line

In vitro cytotoxicity assays may make use of various cell lines and the quantitative assessment of cytotoxicity generally relies on the visual counting of cells [Balaji *et al.*, 2004; Ghatak *et al.*, 2006; Castilho *et al.*, 2009], leading to low reproducibility of these approaches and consequently conferring significant limitations. Previous studies suggest that the MTS assay *in vitro* cytotoxicity assay combines all features of a good measurement system in terms of ease of use, precision, rapid [Berg *et al.*, 1994], as well as sensitive and specific indication of toxicity whose performance is very competitive to other toxicological test systems [Gregor *et al.*, 1997]. The MTS assay is based on the conversion of MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt into a colored, aqueous soluble formazan product by mitochondrial activity of viable cells at 37°C. The amount of formazan produced by dehydrogenase enzymes is directly proportional to the number of living cells in culture and can be measured at 490 nm [Barltrop *et al.*, 1991].

3.4.1. Cytotoxicity induced by culture supernatants assays

Bacterial strains were subcultured on BHI agar plates and incubated at 30ºC overnight, the bacterial were then washed as described previously. Bacterial suspensions were adjusted to 0,005 OD_{600} and incubated at 37ºC for 8 hours with 150 rpm, while the growth was at the beginning of the stationary phase, see Chapter 3.1. Subsequently, the bacterial supernatants were carefully collected in sterile tubes after centrifugation at 10.000x*g* for 10 minutes and filter sterilized through 0.45 µm pore size sterilized nylon syringe filter (VWR). Finally the crude protein suspensions (cell-free filtrates, CFS) were stored at -20ºC until further use which occurred not longer than one week after. The sterility of each preparation was checked by inoculation 100 µL in BHI broth and incubation at 37ºC for 48h.

Undifferentiated Caco-2 cells monolayers, grown in 96-well plates, were incubated at 37ºC in 5% CO_2 for 4 and 24 hours with 100 μL of twofold serial dilutions in culture medium with only 0,5% FBS (1:2 to 1:2048 v/v) of CFS of *Aeromonas spp.* strains, non-pathogenic *E. coli* K-12 and *P. aeruginosa* PAO1. Morphological changes were evaluated in an inverted microscope over the first 1-4 hours period. Afterwards the monolayers were washed gently with 200 μL DPBS and the viability of the cells was assessed by MTS assay. For this assay, 100 μL of DMEM containing 2% (v/v) of the colorimetric reagent MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Sigma) were added in each well and incubated for 1-4 hours at 37ºC. The medium with the colorimetric reagent was transferred to a new 96-well plate and were perform measurements of the absorbance at 490 nm in a BioTek™ Power Wave XS microplate reader. For the isolates that shown to be positive a sample of the same supernatant was pre-heated to 65ºC for 20 minutes in a water bath thermostated and analyzed again.

The results are expressed in percentage of viable cells, using the following formula:

$$((OD_{\text{cell treatment}} - OD_{\text{negative control}}) / (OD_{\text{positive control}} - OD_{\text{negative control}})) \times 100,$$

where the negative control (total cellular damage) is culture medium alone and the positive control (no cellular damage) are cells that receive BHI broth instead of CFS. Assays were performed in duplicate and in two separate experiments for each isolate. The variation is expressed as propagated error. Cytotoxic titre was considered as the reciprocal of the highest dilution of the culture filtrate that caused destruction of 50% of the Caco-2 cells. The CFS preparations that induced cytopathic effect above to 1:2 dilution in 50% or more cells were recorded as cytotoxic positive isolates.

3.4.2. Cytotoxicity induced by cell-contact assays

Bacterial strains were subcultured on BHI agar plates and incubated at 30ºC overnight, then passed to new BHI agar plates and incubated again at 37ºC overnight. The bacterial were then washed by harvesting and suspending in BHI broth, centrifuged at 10.000x*g* for 10 minutes and resuspended in fresh BHI broth. Bacterial suspensions were adjusted to approximately 5×10^7 CFU/mL by measuring 0.115 OD_{600} (0.080 and 0.085 OD_{600} for K-12 and PAO1 strains, respectively), according to the calibration curves previously established (Figures 1, 2 and 3 in Annex) and kept on ice until use; the number of bacteria of each strain was confirmed by CFU determination immediately after use as described previously.

Undifferentiated Caco-2 cells monolayers grown in 96-well plates containing 100 μL of fresh culture medium were incubated at 37ºC in 5% CO_2 for 90 minutes with 160 μL of bacterial suspension to give a multiplicity of infection (MOI) of 100 bacteria / Caco-2 cell, meaning that 1×10^5 epithelial cells per mL were incubated with approximately 1×10^7 bacteria per mL, according to the calibration curve presented in Figure 4 in Annex) and with 160 μL of bacterial suspension 1:10 diluted in BHI broth to give a MOI of 10:1. Next, the bacteria were removed and replaced by 200 μL DMEM containing 300 μg/ml Gentamicin (Sigma-Aldrich) for 1 hour at 37ºC. After washing gently two-times with 200 μL DPBS, for bacteria removal, the viability of infected cells was assessed by MTS assay as described previously.

To test the importance of bacteria-host cell contact in cytotoxicity, co-cultures of the bacteria and Caco-2 cells at the same time and under the same conditions were performed but by using cells cultivated on 96-well plates with 0.45 µm pore size transwell inserts (Corming). Caco-2 cells were cultured in the lower chamber and the bacteria cells were added in the upper chamber, preventing bacterial contact with Caco-2 cells.

Extracellular cytotoxicity activity was determined using transwells, while cell-contact cytotoxicity activity, after the subtraction of the extracellular cytotoxicity was determined in the plain wells, after the subtraction of the extracellular cytotoxicity activity.

The results are expressed in percentage of cell damage, which is the reverse percentage of the viable cells and was calculated by using the following formula:

$$(1 - ((OD_{cell\ treatment} - OD_{negative\ control}) / (OD_{positive\ control} - OD_{negative\ control}))) \times 100,$$

where the negative control (total cellular damage) is medium alone and the positive control (without cellular damage) are cells that receive BHI broth instead of bacterial suspension. Assays were performed in duplicate for each isolate. The variation is expressed as propagated error.

Chapter 4   Results and Discussion

4.1. CFU determination by optical density

The species of each isolate had not yet been clearly established at the time of the experiments (ongoing work at the laboratory of Doctor Teresa Semedo-Lemsaddek at the Faculty of Veterinary Medicine, University of Lisbon), therefore, the *Aeromona hydrophila subsp. hydrophila* (DSM 30187t) strain and 5 isolates was used to obtain an average calibration curve to estimate the concentration of the remaining isolates, but it must be stressed that this calibration must be done for all isolates, or per species, if it were needed a more accurate determination for future assays.

4.2. *Aeromonas* adhesion and invasion evaluated on Caco-2 cell line

The observed percentage of bacteria recovered is often very dependent on the experimental set-up (and, to a lesser extent, on the analyst), particularly the MOI and the number of washes [Letourneau, 2011]. A method for determining adherence and cytotoxic activity of *Aeromonas* has not been standardized, and cautious comparisons of between published reports are advised.

Bacterial resistance or sensitivity in the presence of Triton X-100 and gentamicin for each strain was demonstrated in control experiments with equivalents numbers of bacteria and under the same assay conditions (concentration, temperature and duration). Cell viability was determined by visual observation of growth / no growth after inoculation in BHI broth. Complete lysis of monolayer Caco-2 cells in 2% Triton X-100 after 30 minutes incubation was confirmed by microscopy.

The CFU determination at the start (to determine the initial MOI) and at the end (to determine the total number of bacteria) of each assay proved that, after the 90 minutes infection period, bacterial multiplication, that is required for the adhesion process, occurred in all the strains.

4.2.1. *Aeromonas spp.* adherence activity

The clinical relevance of *in vitro* adhesion is sometimes contested, firstly because bacterial interaction with the intestinal mucosa is complex, and secondly because it cannot be assumed that tissue culture cells derived by cell transformation possess the same surface receptors for bacterial adherence as those found on human intestinal cells *in vivo* [Freter and Jones, 1983 in Delie and Rubas, 1997]. However, in several cases a correlation between *in vitro* adhesion and *in vivo* infectivity has been demonstrated [Mathewson *et al.*, 1985; Kelly *et al.*, 1993 in Delie and Rubas, 1997].

The adherent activity ranged between 1.7% ± 0.3% to 92% ± 14% and 2.1% ± 0.3% to 69% ± 14% of recovered cells at MOI 10:1 in 92% (22) and 79% (19) of the strains that revealed values three times above to that of nonpathogenic K-12 strain, negative control which showed 0.32% ± 0.03% and 0.3% ± 0.1% as recovered cells baseline, and were classified as adherent to UC and to DC, respectively, which 21% (5) and 25% (6) expressing the lowest adherence values, but only 8% (2) and 21% (5) were classified as non-adherent to UC and to DC, respectively (Table 4.1, Figure 5A and Figure 6A).

Table 4.1. Adhesion abilities of *Aeromonas* isolates to undifferentiated (4-6 days old) and differentiated (19-21 days old) Caco-2 cells, as described in Chapter 3.3.1. The results are average ± propagated error.

Isolates	Sources	Adherence % of bacteria associated with Caco-2 cells				Invasion (ID)		Adherence patterns	Cytotoxicity CCD / ECD / ECD after preheating		
		MOI 10:1				MOI 10:1		MOI 100:1	Titer at 24h		
		Undifferentiated cells (UC)	AD	Differentiated cells (DC)	AD	UC	DC		Undifferentiated cells (UC)		
K-12	*E. coli* K-12 C600	0.32% ± 0.03%	NA	0.3% ± 0.1%	NA	NI	NI	None	NC	1:2	
PAO1	*P. aeruginosa* PAO1	6% ± 1%	MA	8.9% ± 0.9%	MA	SI	SI	Aggregative	SC	1:2	
AR5	*A. hydrophila* 30187t	9% ± 1%	MA	5% ± 1%	WA	SI	MI	Aggregative	MC	1:8	1:2
A 5	surfaces (slaughterhouse)	16% ± 4%	SA	7% ± 2%	MA	MI	SI	Aggregative	MC	1:2	
A 11	surfaces (slaughterhouse)	16% ± 3%	SA	11% ± 3%	MA	MI	WI	Aggregative	MC	1:4	1:2
A 13	surfaces (slaughterhouse)	92% ± 14%	SA	69% ± 14%	SA	MI	WI	Aggregative	MC	1:64	1:2
A 26	food (slaughterhouse)	7% ± 2%	MA	3% ± 1%	WA	SI	SI	Diffuse	SC	1:2	
A 31	food (slaughterhouse)	1.7% ± 0.3%	WA	1.1% ± 0.2%	NA	NI	NI	Diffuse		1:1024	1:4
A 53	surfaces (supermarket)	0.9% ± 0.2%	NA	0.9% ± 0.1%	NA	NI	NI	None		1:2	
A 62	surfaces (supermarket)	4% ± 1%	MA	3% ± 1%	WA	NI	NI	Diffuse	LC	1:2	
A 78	food (supermarket)	48% ± 7%	SA	24% ± 4%	SA	SI	MI	Aggregative	SC	1:2	
A 92	surfaces (supermarket)	1.6% ± 0.3%	WA	1.3% ± 0.2%	NA	NI	NI	Diffuse		1:2	
A 97	surfaces (cheese factory)	2.5% ± 0.4%	WA	1.8% ± 0.7%	NA	WI	NI	Diffuse	NC	1:64	1:2
A 98	surfaces (cheese factory)	4.5% ± 0.8%	MA	3.5% ± 0.7%	WA	MI	WI	Diffuse	LC	1:512	1:2
A 99	surfaces (cheese factory)	14% ± 4%	SA	7% ± 2%	MA	NI	NI	Aggregative	LC	1:2	
A 101	surfaces (cheese factory)	14% ± 3%	SA	7% ± 3%	MA	SI	MI	Aggregative	MC	1:512	1:2
A 104	surfaces (cheese factory)	2.5% ± 0.4%	WA	2.1% ± 0.3%	WA	NI	NI	Diffuse	NC	1:2	
A 127	food	2.9% ± 0.7%	WA	3% ± 1%	WA	NI	NI	Diffuse	NC	1:256	1:2
A 172	clinic	1.0% ± 0.1%	NA	0.95% ± 0.09%	NA	NI	NI	None		1:256	1:8
A 255	clinic	10% ± 4%	MA	6.7% ± 0.7%	MA	NI	NI	Aggregative	LC	1:512	1:4
A 258	clinic	34% ± 6%	SA	17% ± 6%	SA	SI	SI	Aggregative	SC	1:2	
A 259	clinic	15% ± 2%	SA	8% ± 1%	MA	SI	SI	Aggregative	SC	1:2	
S 2	water (EPAL)	15% ± 5%	MA	6.7% ± 0.3%	MA	NI	NI	Aggregative	LC	1:2	
S 3	water (EPAL)	75% ± 13%	SA	36% ± 8%	SA	SI	MI	Aggregative	SC	1:2	
S 8	water (EPAL)	23% ± 4%	SA	16% ± 2%	SA	SI	SI	Aggregative	SC	1:2	
S 10	water (EPAL)	11% ± 2%	MA	7% ± 2%	MA	SI	WI	Aggregative	MC	1:2	
S 18	water (EPAL)	5% ± 2%	MA	2.3% ± 0.6%	WA	NI	NI	Aggregative	LC	1:2	
% of positive		92% (22)		79% (19)		54% (13)	50% (12)		71% (17)	41% (10)	17% (4)

AD: Adhesion degree: NA: no adhesion (< 3x K-12); WA: weak adhesion (3x to10x K-12); MA: moderate adhesion (10x to 30x K-12); SA: strong adhesion (> 30x K-12);

ID: Invasion degree: NI: no Invasion (< 10x K-12); WI: weak invasion; MA: moderate invasion (20x to 40x K-12); SA: strong invasion (> 40x K-12);

CCD: Cell-contact cytotoxic degree: NC: no cytotoxic; LC: low cytotoxicity (< 25%); MC: moderate cytotoxicity (25 to 45%); SC: strong cytotoxicity (> 45%);

ECD: Extracellular cytotoxicity degree: NC: no cytotoxic (100% in 1:2 titer); LC: low cytotoxicity (< 1:2 titer); MC: moderate cytotoxicity (1.2 to 1:64 titer); SC: strong cytotoxicity (> 1:128 titer).

The adherent activity in 71% (17) and 54% (13) of the strains revealed adherence values equal to or greater than the adherence values of the pathogenic PAO1 strain to UC and to DC, respectively (Table 4.1). The highest adherent activity ranged between 14% ± 4% to 92% ± 14% and 16% ± 2% to 69% ± 14%, in 42% (10) and 21% (5) of the strains and only one of them is from clinical origin, where the pathogenic PAO1 strain only rated 6% ± 1% and 8,9% ± 0,9% in UD and DC, respectively (Table 4.1). In this study was observed that adherence of *Aeromonas spp.* strains were significantly higher in UC than in DC and that 3 weak adherent isolates to UC were not considered adherent to DC.

In Caco-2 cells, the time-course of the differentiation process, with UC exponentially dividing cells which differentiate when the cells stop dividing, closely mimics the situation found in the small intestine [Zweibaum *et al.*, 1991]. A major tropism for UC or DC is related to the species of microorganism, for example, *Salmonella typhimurium* and enteropathogenic *Escherichia coli* were found to have a more efficient adherence to brush borders of DC, while *Yersinia pseudotuberculosis* and *Listeria monocytogenes* presented an optimum adherence to the borders of UC [Coconnier *et al.*, 1993].

A decrease of adherence with the stage of differentiation of the cells was observed in all adherent *Aeromonas* strains and it is proportional; strains expressing very high levels of adherence in UC had consistently high levels of adherence in DC, and conversely, the adherence values of the PAO1 strain increased with the age of the cells.

These results indicate that *Aeromonas* interacts optimally with cultured human intestinal cells at cellular sites expressed in the brush border early in the differentiation process. These results also indicates a difference between the adhesion mechanism of the *Aeromonas* strains and the one belongs to the *Pseudomonas aeruginosa* PAO1 strain, whose adherence were significantly higher in DC than in UC, interacting optimally at cellular sites expressed late in the differentiation process,

All strains originated from water samples are adherent. 80% (4/5) have moderate to high levels of adhesion (Table 4.2), which may be related to a need of these strains have to form biofilms in their niche. This was similar to what was observed in the clinical strains that showed 75% (3/4) with the same levels of adhesion (Table 4.2). This similarity supports the thesis that the major cause of gastrointestinal infections by *Aeromonas spp.* is from ingesting infected water [Statner and George, 1987; Holmberg *et al.*, 1986], especially considering that only 25% (1/4) of the strains originated from food samples and 45% (5/11) of the strains originated from food processing surfaces have shown the same levels of adhesion (Table 4.2).

Table 4.2. Distribution of adherent *Aeromonas* isolates by source and level ability levels.

Adhesion Degree (Differentiated cells)	Strain Sources (%)				
	Surfaces	Food	Water	Clinic	Total
SA; strong adhesion (> 30x K-12)	9% (1)	25% (1)	40% (2)	25% (1)	21% (5)
MA; moderate adhesion (10x to 30x K-12)	36% (4)	0% (0)	40% (2)	50% (2)	33% (8)
WA; weak adhesion (3x to 10x K-12)	27% (3)	50% (2)	20% (1)	0% (0)	24% (6)
NA; no adhesion (< 3x K-12)	27% (3)	25% (1)	0% (0)	25% (1)	21% (5)
Total strains	**11**	**4**	**5**	**4**	**24**

Barroco (2013) determined the presence of *flaA/flaB* genes in the strains used in this study (Table 4.3), they encode two subunits of flagellin that compose the complex filament of the polar flagellum, that allow the colonization of different niches, including the colonization of host tissues [Kirov, 2003]. Its expression is highly regulated by a number of environmental factors, but the molecular inhibition mechanism is not known [Merino *et al.,* 2006].

The presence of *flaA/flaB* genes was detected in the genome of 2 strains, A53 and A172, considered nonadherent and in other 2 strains, A31 and A97, which expressed low adherence in UC and were considered nonadherent in DC. These results indicate the existence of a mechanism or genetic flaw in these strains that is preventing the polar flagellum to be expressed, at assay conditions, and the absence on the genome or no expression of other structures (e.g.: lateral flagella or pilli) and proteins (e.g.: OMPs) that can act as adhesins, allowing or facilitating adhesion to human epithelial cells *in vitro*.

The *Aeromona hydrophila* insertional *flaH, flaJ*, mutant and *flaA/flaB* double mutant resulted in the complete loss of motility, showing lateral flagella, absence of polar flagella, and a dramatic reduction in adhesion to HEp-2 cells and in ability to form biofilms [Canals *et al.,* 2006].

The presence of *flaA/flaB* genes were not detected in the genome of 8 strains with moderate to high levels of adhesion, including all adherent clinical strains and in other 2 strains (A92 and A127) with low levels of adhesion to UC. These results show that the polar flagellum of this genus, although important to acquire maximum adherence in some *Aeromonas* strains/species, as described in some literature, is not essential for adhesion to human epithelial cells *in vitro* in other strains, indicating the existence of other main adhesion mechanism which allows to obtain a level of adhesion capacity in these strains that competes with recognized pathogens, such as PAO1 strain. These strains are important for further analysis in order to determine the adhesion mechanism, especially in clinical strains.

Table 4.3. Comparison of the phenotype and genotype characterization of *Aeromonas* isolates carried out to date by Barroco (2013) and the phenotype characterization carried out in this study.

Isolates	Sources	Virulence genes									Phenotype				Adhesion (AD) / Invasion (ID)			Cytotoxicity CCD / ECD / ECD after preheating		
		ASC	AHH	ALT	AEXT	ELA	LIP	FLA	AST	ACT	LIPA	HEM	GEL	Dnase	Undifferentiated cells			MOI 100:1	Titer at 24h	
AR5	*A. hydrophila* 30187t	+	+	+	-	+	+	+	+	+	+	+	+	+	Aggregative	MA	SI	MC	1:8	1:2
A 5	surfaces (slaughterhouse)	-	-	+	-	+	+	+	-	-	+	+	+	+	Aggregative	SA	MI	MC	1:2	
A 11	surfaces (slaughterhouse)	-	-	-	-	+	-	+	-	-	+	+	+	+	Aggregative	SA	MI	MC	1:4	1:2
A 13	surfaces (slaughterhouse)	+	-	-	+	-	-	+	-	+	+	+	+	+	Aggregative	SA	MI	MC	1:64	1:2
A 26	food (slaughterhouse)	-	-	-	-	-	+	+	-	+	+	+	+	+	Diffuse	MA	SI	SC	1:2	
A 31	food (slaughterhouse)	-	+	+	-	+	-	+	-	+	+	+	+	+	Diffuse	WA	NI		1:1024	1:4
A 53	surfaces (supermarket)	-	-	-	-	+	-	+	-	+	+	+	+	+	None	NA	NI		1:2	
A 62	surfaces (supermarket)	-	+	-	-	+	+	-	-	+	+	+	+	+	Diffuse	MA	NI	LC	1:2	
A 78	food (supermarket)	-	-	+	-	-	+	+	-	+	-	+	+	+	Aggregative	SA	SI	SC	1:2	
A 92	surfaces (supermarket)	-	-	-	-	-	-	+	-	+	+	+	+	+	Diffuse	WA	NI		1:2	
A 97	surfaces (cheese factory)	+	+	+	+	+	+	+	+	+	-	+	+	+	Diffuse	WA	WI	NC	1:64	1:2
A 98	surfaces (cheese factory)	-	+	+	-	+	+	-	+	-	+	+	+	+	Diffuse	MA	MI	LC	1:512	1:2
A 99	surfaces (cheese factory)	-	-	-	-	+	+	+	-	+	+	+	+	+	Aggregative	SA	NI	LC	1:2	
A 101	surfaces (cheese factory)	-	+	+	-	+	+	+	+	+	+	+	+	+	Aggregative	SA	SI	MC	1:512	1:2
A 104	surfaces (cheese factory)	-	-	+	-	+	+	-	-	-	+	+	+	+	Diffuse	WA	NI	NC	1:2	
A 127	food	-	-	-	-	-	-	-	-	-	+	+	-	-	Diffuse	WA	NI	NC	1:256	1:2
A 172	clinic	-	+	+	-	+	+	+	-	+	-	+	-	+	None	NA	NI		1:256	1:8
A 255	clinic	-	+	+	-	+	+	-	+	+	-	+	+	+	Aggregative	MA	NI	LC	1:512	1:4
A 258	clinic	-	-	+	-	+	+	-	-	+	+	+	+	+	Aggregative	SA	SI	SC	1:2	
A 259	clinic	+	-	-	-	-	-	-	-	-	-	+	+	+	Aggregative	SA	SI	SC	1:2	
S 2	water (EPAL)	-	-	+	-	+	-	-	-	+	-	+	+	+	Aggregative	MA	NI	LC	1:2	
S 3	water (EPAL)	-	-	-	-	-	-	-	-	+	+	+	+	+	Aggregative	SA	SI	SC	1:2	
S 8	water (EPAL)	-	-	-	-	+	+	-	-	+	+	+	+	+	Aggregative	SA	SI	SC	1:2	
S 10	water (EPAL)	-	-	+	-	+	+	+	-	-	+	+	+	-	Aggregative	MA	SI	MC	1:2	
S 18	water (EPAL)	-	-	-	-	+	+	-	-	+	+	+	+	+	Aggregative	MA	NI	LC	1:2	
% of positive (> 5%)		13%	30%	50%	8%	71%	63%	58%	17%	66%	75%	100%	92%	92%		92%	54%	71%	42%	17%

Virulence genes: ASC (TTSS structural protein - *ascV*); AHH (β-hemolisina - *hlyA*); ALT (Aeromona heat-labile cytotonic enterotoxin- *alt*); AEXT (TTSS effector protein - ADP-Ribosyltransferase - *aexT*); ELA (elastase - *ahyB*); LIP (lipases and phospholipases - *pla/lip/lipH3/alp-1*); FLA (flagellin A and B - *flaA/B*); AST (*Aeromonas* heat-stable cytotonic enterotoxina - *ast*); ACT (*Aeromonas* cytotoxic enterotoxina - *act*). Phenotype: LIPA (lipase); HEM (hemolysin); GEL (gelatinase); DNases.

ECD: Extracellular cytotoxicity degree: NC: no cytotoxic (100% in 1:2 titer); LC: low cytotoxicity (< 1:2 titer); MC: moderate cytotoxicity (1.2 to 1:64 titer); SC: strong cytotoxicity (> 1:128 titer);
CCD: Cell-contact cytotoxic degree: NC: no cytotoxic; LC: low cytotoxicity (< 25%); MC: moderate cytotoxicity (25 to 45%); SC: strong cytotoxicity (> 45%);
AD: Adhesion degree: NA: no adhesion (< 3x K-12); WA: weak adhesion (3x to10x K-12); MA: moderate adhesion (10x to 30x K-12); SA: strong adhesion (> 30x K-12);
ID: Invasion degree: NI: no Invasion (< 10x K-12); WI: weak invasion; MA: moderate invasion (20x to 40x K-12); SA: strong invasion (> 40x K-12).

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4.2.2. *Aeromonas spp.* **adherence patterns**

Microscopic examination of the adhesive process indicated aggregative adhesion patterns similar to those of the stacked brick appearance noted for members of the *Enterobacteriaceae* [Knutton *et al.*, 1987; Gunzburg *et al.*, 1993] (Figure 4.1) in 54% (13) of the strains investigated here (Table 4.4), that include the PAO1 strain (Table 4.1), all clinical adherent strains and from source water (Table 4.4), and they are among the isolates that showed the highest adhesive capacity (Table 4.1). These results is another common feature between clinical and water strains that supports the thesis above-mentioned, that the major cause of gastrointestinal infections by *Aeromonas spp.* is from ingesting infected water [Statner and George, 1987; Holmberg *et al.*, 1986]

The remaining 9 adherent isolates (38%) showed a diffuse adherence pattern (Table 4.4), characterized by a uniform distribution of bacteria on the cell surface (Figure 4.1) that generally containing fewer bacteria compared to the aggregative patterns and they are among the isolates that showed the lowest adhesive capacity (Table 4.1).

These results indicate that the mode of arrangement of bacteria on the surface of human cell culture is related to the levels of adhesion obtained, being a good phenotypic trait to characterize strains, gathering all clinical strains in one group.

Table 4.4. Distribution of adherence patterns of *Aeromonas* isolates by source.

Adherence Patterns (Undifferentiated cells)	Strain Sources (%)				
	Surfaces	**Food**	**Water**	**Clinic**	**Total**
Aggregative pattern	36% (4)	25% (1)	100% (5)	75% (3)	54% (13)
Diffuse pattern	55% (6)	75% (3)	0% (0)	0% (0)	38% (9)
None	9% (1)	0% (0)	0% (0)	25% (1)	8% (2)
Total strains	**11**	**4**	**5**	**4**	**24**

The similarities of adhesive patterns suggest similarities between the pathogenic mechanisms of these isolates with other recognized pathogens [Parsot, 2005; Mitache *et al.*, 2009; Mora *et al.*, 2009].

One aggregative adherent pathogen that is well studied is enteroaggregative *Escherichia coli* (EAEC). Colonization by these bacteria induces damage in the intestinal epithelium characterized by shortening of the villi, hemorrhagic necrosis of the villous tips, and a mild inflammatory response of the submucosa, causing diarrhoea that is often watery and can be accompanied by mucus or blood [Nataro, 1998].

The colonization of diffusely adherent *Escherichia coli* (DAEC), on the other hand, induces only the effacement of the brush border microvilli [Berger, 2004]; these type of lesions disrupt several brush border enzymes that are involved in intestinal secretion and absorption, which may contribute to diarrhea [Servin, 2005].
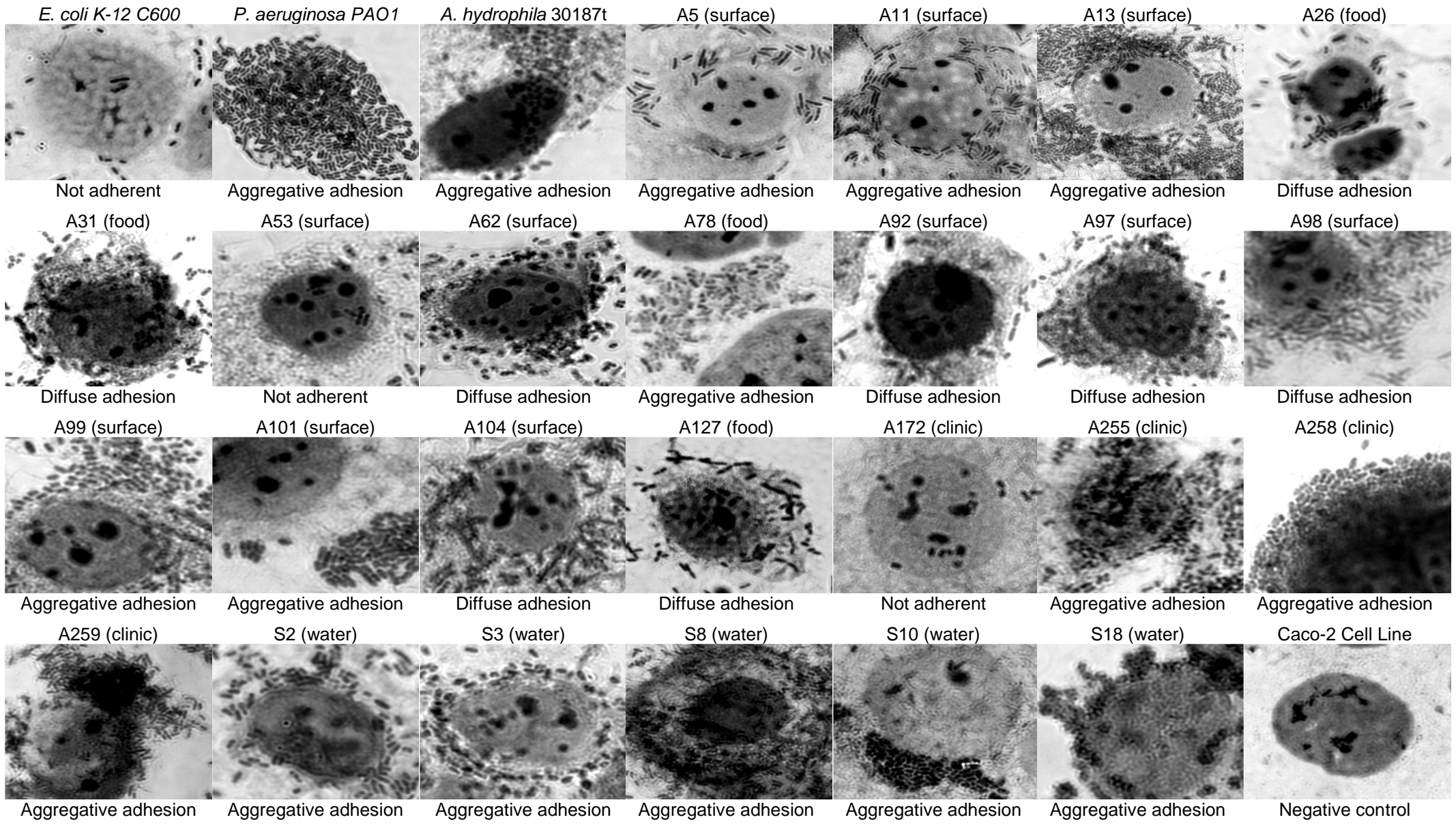
Figure 4.1. Optic microscopy observation showing the adherence ability and patterns of the *Aeromonas* isolates to undifferentiated (4-6 days old) Caco-2 cells, as described in Chapter 3.3.2. Giemsa stain: magnification (x1000).

4.2.3. *Aeromonas spp.* invasion activity

The ability to adhere and penetrate the epithelial cell barriers has been recognized as an important step in the pathogenesis of most infections in man and animals [Finlay and Falkow, 1997]. It has been reported that there is a direct correlation between the isolation of *Aeromonas spp.* strains from dysentery-like illness and the *in vitro* adhesion and the posterior invasion [Lawson *et al.*, 1985; Watson *et al.*, 1985].

The invasion activity ranged between 0.015% ± 0.002% to 0,49% ± 0,06% and 0.020% ± 0.004% to 0.32% ± 0,09% of recovered cells at MOI 10:1 in 54% (13) and 50% (12) of the strains that revealed values tenfold above to that of nonpathogenic K-12 strain, negative control which showed 0.0007% ± 0.0003% and 0.0010% ± 0.0004% as recovered cells baseline, and were classified as invasive to UC and to DC, respectively, which 17% (4) expressing the lowest values of invasion, but only 45% (11) and 50% (12) of the strains were classified as non-invasive to UC and to DC, respectively (Table 4.5, Figure 5A and Figure 6A).

Pseudomonas aeruginosa is an opportunistic pathogen, in immunocompromised hosts; it frequently causes severe septicemia, due to its invasiveness and subsequent passage to the bloodstream, arising from their own endogenous intestinal flora [Bryan, *et al*, 1983; Dick *et al*, 1988; Hirakata *et al*, 1991; Hirakata *et al*, 1993 in Hirakata, 1998]. In this study 8% (2) of the strains revealed values of cell invasion equal to or greater than the values of the PAO1 strain to DC and UC (Table 4.5). The highest invasion activity ranged between 0.054% ± 0,009% to 0,49% ± 0,06% and 0.10% ± 0.02% to 0.036% ± 0,009%, in 21% (5) and 25% (6) and only two of them is from clinical origin, where the pathogenic PAO1 strain rated 0,36% ± 0,05% and 0,31% ± 0,08% in UD and DC, respectively (Table 4.5).

A decrease of invasion with the stage of differentiation of the cells was observed in some adherent *Aeromonas* strains (4/12), which can be associated with the decrease in the number of bacteria in association with differentiated cells, but most (7/12) keep their values within the estimated error, similarly to PAO1 strain (Table 4.5), which indicates the existence of an invasion mechanism not specific to DC or to UC. Still one of the strains, the A5 strain, did increases its invasiveness from 0.025% ± 0.006% in UC to 0.11% ± 0.03% in DC (Table 4.5), which indicates the existence of a different invasion mechanism from the others *Aeromonas* strains, one more specific to cellular sites expressed in the brush border late in the differentiation process.

Table 4.5. Invasion abilities of *Aeromonas* isolates to undifferentiated (4-6 days old) and differentiated (19-21 days old) Caco-2 cells, as described in Chapter 3.3.1. The results are average ± propagated error.

Isolates	Sources	Invasion % of intracellular bacteria in Caco-2 cells — MOI 10:1 — Undifferentiated cells (UC)	ID	Differentiated Cells (DC)	ID	Adherence (AD) MOI 10:1 UC	DC	Adherence patterns MOI 100:1	Cytotoxicity CCD / ECD / ECD after preheating — Titer at 24h — Undifferentiated cells (UC) CCD	ECD	ECD after preheating
K-12	*E. coli* K-12 C600	0.0007% ± 0.0003%	NI	0.0010% ± 0.0004%	NI	NA	NA	None	NC	1:2	
PAO1	*P. aeruginosa* PAO1	0.36% ± 0.05%	SI	0.31% ± 0.08%	SI	MA	MA	Aggregative	SC	1:2	
AR5	*A. hydrophila* 30187t	0.046% ± 0.008%	SI	0.03% ± 0.01%	MI	MA	MA	Aggregative	MC	1:8	1:2
A 5	surfaces (slaughterhouse)	0.025% ± 0.006%	MI	0.11% ± 0.03%	SI	SA	MA	Aggregative	MC	1:2	
A 11	surfaces (slaughterhouse)	0.035% ± 0.005%	MI	0.024% ± 0.008%	WI	SA	MA	Aggregative	MC	1:4	1:2
A 13	surfaces (slaughterhouse)	0.029% ± 0.007%	MI	0.020% ± 0.004%	WI	SA	SA	Aggregative	MC	1:64	1:2
A 26	food (slaughterhouse)	0.28% ± 0.06%	SI	0.26% ± 0.07%	SI	MA	WA	Diffuse	SC	1:2	
A 31	food (slaughterhouse)	0.004% ± 0.002%	NI	0.004% ± 0.001%	NI	WA	NA	Diffuse		1:1024	1:4
A 53	surfaces (supermarket)	0.0006% ± 0.0003%	NI	0.005% ± 0.002%	NI	NA	NA	None		1:2	
A 62	surfaces (supermarket)	0.0021% ± 0.0006%	NI	0.0043% ± 0.0005%	NI	MA	WA	Diffuse	LC	1:2	
A 78	food (supermarket)	0.054% ± 0.009%	SI	0.046% ± 0. 009%	MI	SA	SA	Aggregative	SC	1:2	
A 92	surfaces (supermarket)	0.0061% ± 0.0009%	NI	0.0023% ± 0.0008%	NI	WA	NA	Diffuse		1:2	
A 97	surfaces (cheese factory)	0.015% ± 0.002%	WI	0.013% ± 0.005%	NI	WA	NA	Diffuse	NC	1:64	1:2
A 98	surfaces (cheese factory)	0.030% ± 0.005%	MI	0.020% ± 0.004%	WI	MA	WA	Diffuse	LC	1:512	1:2
A 99	surfaces (cheese factory)	0.004% ± 0.002%	NI	0.005% ± 0.002%	NI	SA	MA	Aggregative	LC	1:2	
A 101	surfaces (cheese factory)	0.11% ± 0.02%	SI	0.06% ± 0.03%	MI	SA	MA	Aggregative	MC	1:512	1:2
A 104	surfaces (cheese factory)	0.0048% ± 0.0007%	NI	0.0057% ± 0.008%	NI	WA	WA	Diffuse	NC	1:2	
A 127	food	0.0057% ± 0.0009%	NI	0.0011% ± 0.0005%	NI	WA	WA	Diffuse	NC	1:256	1:2
A 172	clinic	0.0059% ± 0.0008%	NI	0.0007% ± 0.0002%	NI	NA	NA	None		1:256	1:8
A 255	clinic	0.007% ± 0.003%	NI	0.0013% ± 0.0003%	NI	MA	MA	Aggregative	LC	1:512	1:4
A 258	clinic	0.21% ± 0.04%	SI	0.10% ± 0.02%	SI	SA	SA	Aggregative	SC	1:2	
A 259	clinic	0.49% ± 0.06%	SI	0.32% ± 0.09%	SI	SA	MA	Aggregative	SC	1:2	
S 2	water (EPAL)	0.0012% ± 0.0002%	NI	0.0017% ± 0.0007%	NI	MA	MA	Aggregative	LC	1:2	
S 3	water (EPAL)	0.05% ± 0.01%	MA	0.043% ± 0.009%	MI	SA	SA	Aggregative	SC	1:2	
S 8	water (EPAL)	0.14% ± 0.02%	SI	0.12% ± 0.03%	SI	SA	SA	Aggregative	SC	1:2	
S 10	water (EPAL)	0.035% ± 0.007%	MA	0.028% ± 0.008%	WI	MA	MA	Aggregative	MC	1:2	
S 18	water (EPAL)	0.003% ± 0.001%	NI	0.002% ± 0.001	NI	MA	WA	Aggregative	LC	1:2	
% of positive		**54% (13)**		**50% (12)**		**92% (22)**	**79% (19)**		**71% (17)**	**41% (10)**	**17% (4)**

ID: Invasion degree: NI: no Invasion (< 10x K-12); WI: weak invasion; MA: moderate invasion (20x to 40x K-12); SA: strong invasion (> 40x K-12);
AD: Adhesion degree: NA: no adhesion (< 3x K-12); WA: weak adhesion (3x to10x K-12); MA: moderate adhesion (10x to 30x K-12); SA: strong adhesion (> 30x K-12);
CCD: Cell-contact cytotoxic degree: NC: no cytotoxic; LC: low cytotoxicity (< 25%); MC: moderate cytotoxicity (25 to 45%); SC: strong cytotoxicity (> 45%);
ECD: Extracellular cytotoxicity degree: NC: no cytotoxic (100% in 1:2 titer); LC: low cytotoxicity (< 1:2 titer); MC: moderate cytotoxicity (1.2 to 1:64 titer); SC: strong cytotoxicity (> 1:128 titer).

50% of the strains originated from food samples showed moderate to high ability for cell invasion. The same was observed in 50% of the clinical strains and in 40% of the strains originated from water samples (Table 4.6). These results indicate that both water and food have the ability to serve as vehicles of potentially pathogenic strains.

Table 4.6. Distribution of invasive *Aeromonas* isolates by source and ability levels.

Invasion Degree (Differentiated cells)	Strain Sources (%)				
	Surfaces	**Food**	**Water**	**Clinic**	**Total**
SI; strong Invasion (>40x K-12)	9% (1)	25% (1)	20% (1)	50% (2)	21% (5)
MI; moderate Invasion (20x to 40x K-12)	9% (1)	25% (1)	20% (1)	0% (0)	13% (3)
WI; weak Invasion (10x to 20x K-12)	27% (3)	0% (0)	20% (1)	0% (0)	16% (4)
NI; no Invasion (<10x K-12)	55% (6)	50% (2)	40% (2)	50% (2)	50% (12)
Total strains	11	4	5	4	24

4.3. *Aeromonas* cytotoxicity evaluation on Caco-2 cell line

The data in both assays of cytotoxicity establishes a clear difference between cytotoxic and non-cytotoxic isolates, revealing high sensitivity and producing more accurate results than other viability assays, providing slightly differences between cytotoxicity levels and without the bias of human interpretation related to methods based on visual determination.

4.3.1. *Aeromonas spp.* cell-contact cytotoxic activity

A quantitative assay was implemented to characterize cell-contact cytotoxic activity of the strains and the results showed that bacterial cells were able to lyse epithelial cells within 90 minutes of incubation only by contact (Table 4.7, Figure 7A and Figure 8A).

The cell-contact cytotoxic ranged between 5% ± 3% and 82% ± 4% of cell damage at MOI 100:1 in 71% (17) of the strains that revealed values above to that of nonpathogenic K-12 strain, negative control which showed -1% ± 3% of cell damage baseline (Table 4.7 and Figure 7A). The highest cell-contact cytotoxic ranged between 61% ± 4% to 82% ± 4% of cell damage in 25% (6) of the strains and only two of them is from clinical origin, where the pathogenic PAO1 strain rated 49% ± 4% of cell damage in UD (Table 4.7).

Only low cytotoxicity, below 25% of damaged cells, could be observed when bacterial cells were not allowed contact with epithelial cells (Table 4.7 and Figure 7A). The extracellular activity ranged from 7% ± 3% to 20% ± 3% at MOI 100:1 in 25% (6) of the strains and the highest level of activity within the range (above 10%) was observed for 4 (17%) of the isolates (Table 4.7), which suggests that these strains produced extracellular toxins.

At MOI 10:1 there was a significant lowering of cell-contact cytotoxicity (without extracellular cytotoxicity), but still ranged between 6% ± 4% to 25% ± 3% in 41% (10) of the strains (Table 4.7 and Figure 8A). For this reason the MOI 10:1 was used to determine the ability of adhesion / invasion of each strain to prevent high tissue damage to the Caco-2 cells monolayers during the assays.

Table 4.7. Cell-Contact cytotoxicity ability of *Aeromonas* isolates to undifferentiated (4-6 days old) Caco-2 cells, as described in Chapter 3.4.2. The results are average ± propagated error.

Isolates	Sources	Cytotoxicity of Bacterial Cell-Contact						Cytotoxicity ECD / ECD after preheating		Adherence patterns	Adhesion (AD)	Invasion (ID)
		MOI 100:1			MOI 10:1			Undifferentiated cells				
		Extracellular	Cell-Contact	CCD	Extracellular	Cell-Contact	CCD	Titer at 24h		MOI 100:1	MOI 10:1	
K-12	*E. coli* K-12 C600	-1% ± 2%	-1% ± 3%	NC	-1% ± 3%	0% ± 4%	NC	1:2		None	NA	NI
PAO1	*P. aeruginosa* PAO1	0% ± 3%	49% ± 4%	SC	-1% ± 3%	13% ± 4%	LC	1:2		Aggregative	MA	SI
AR5	*A. hydrophila* 30187t	8% ± 3%	28% ± 4%	MC	1% ± 3%	7% ± 3%	NC	1:8	1:2	Aggregative	MA	SI
A 5	surface (slaughterhouse)	-1% ± 3%	30% ± 3%	MC	-1% ± 3%	9% ± 3%	LC	1:2		Diffuse	SA	MI
A 11	surface (slaughterhouse)	1% ± 3%	27% ± 4%	MC	0% ± 3%	8% ± 4%	LC	1:4	1:2	Diffuse	SA	MI
A 13	surface (slaughterhouse)	7% ± 3%	31% ± 3%	MC	3% ± 2%	9% ± 3%	LC	1:64	1:2	None	SA	MI
A 26	food (slaughterhouse)	-1% ± 3%	75% ± 4%	SC	0% ± 2%	21% ± 3%	LC	1:2		Diffuse	MA	SI
A 31	food (slaughterhouse)							1:1024	1:4	Aggregative	WA	NI
A 53	surface (supermarket)							1:2		Diffuse	NA	NI
A 62	surface (supermarket)	-1% ± 3%	6% ± 3%	LC	-1% ± 3%	0% ± 4%	NC	1:2		Diffuse	MA	NI
A 78	food (supermarket)	0% ± 2%	69% ± 3%	SC	-1% ± 3%	18% ± 4%	LC	1:2		Diffuse	SA	SI
A 92	surface (supermarket)							1:2		Aggregative	WA	NI
A 97	surface (cheese factory)	9% ± 3%	0% ± 3%	NC	0% ± 3%	0% ± 4%	NC	1:64	1:2	Aggregative	WA	WI
A 98	surface (cheese factory)	20% ± 3%	18% ± 4%	LC	-1% ± 2%	4% ± 3%	LC	1:512	1:2	Diffuse	MA	MI
A 99	surface (cheese factory)	-2% ± 4%	5% ± 4%	LC	-1% ± 3%	0% ± 5%	NC	1:2		Diffuse	SA	NI
A 101	surface (cheese factory)	20% ± 3%	40% ± 4%	MC	1% ± 3%	9% ± 3%	LC	1:512	1:2	None	SA	SI
A 104	surface (cheese factory)	0% ± 3%	0% ± 3%	NC	0% ± 2%	0% ± 3%	NC	1:2		Aggregative	WA	NI
A 127	food	15% ± 3%	1% ± 4%	NC	1% ± 2%	-1% ± 3%	NC	1:256	1:2	Aggregative	WA	NI
A 172	clinic							1:256	1:8	Aggregative	NA	NI
A 255	clinic	19% ± 4%	12% ± 5%	LC	2% ± 3%	-2% ± 4%	NC	1:512	1:4	Aggregative	MA	NI
A 258	clinic	-2% ± 4%	82% ± 4%	SC	-2% ± 3%	25% ± 3%	MC	1:2		Aggregative	SA	SI
A 259	clinic	0% ± 2%	69% ± 3%	SC	0% ± 3%	18% ± 3%	LC	1:2		Aggregative	SA	SI
S 2	water (EPAL)	0% ± 3%	10% ± 4%	LC	-1% ± 2%	-1% ± 3%	NC	1:2		Aggregative	MA	NI
S 3	water (EPAL)	-1% ± 3%	52% ± 4%	SC	0% ± 3%	12% ± 4%	LC	1:2		Aggregative	SA	SI
S 8	water (EPAL)	-1% ± 2%	61% ± 4%	SC	-1% ± 3%	16% ± 3%	LC	1:2		Aggregative	SA	SI
S 10	water (EPAL)	0% ± 3%	29% ± 4%	MC	-1% ± 3%	6% ± 4%	LC	1:2		Aggregative	MA	SI
S 18	water (EPAL)	-1% ± 3%	5% ± 3%	LC	-1% ± 2%	0% ± 3%	NC	1:2		Aggregative	MA	NI
% of positive (> 5%)		30% (6)	71% (17)		0% (0)	50% (12)		42% (10)	17% (4)		92% (22)	54% (13)

CCD: Cell-contact cytotoxic degree: NC: no cytotoxic; LC: low cytotoxicity (< 25%); MC: moderate cytotoxicity (25 to 45%); SC: strong cytotoxicity (> 45%);

ECD: Extracellular cytotoxicity degree: NC: no cytotoxic (100% in 1:2 titer); LC: low cytotoxicity (< 1:2 titer); MC: moderate cytotoxicity (1.2 to 1:64 titer); SC: strong cytotoxicity (> 1:128 titer);

AD: Adhesion degree: NA: no adhesion (< 3x K-12); WA: weak adhesion (3x to10x K-12); MA: moderate adhesion (10x to 30x K-12); SA: strong adhesion (>30x K-12);

ID: Invasion degree: NI: no Invasion (< 10x K-12); WI: weak invasion; MA: moderate invasion (20x to 40x K-12); SA: strong invasion (>40x K-12).

All strains tested were able to adhere to differentiated Caco-2 cells, but 25% (5) of the strains did not expressed cell-contact dependent cytotoxicity (Table 4.7), 3 environmental strains (A97, A104 e A127) that showed low adhesion capacity as 2 other environmental strains (A62 and A99) that showed moderate and strong adhesion capacity to DC, respectively (Table 4.1). These results indicates that the ability to adhere can be a prerequisite for colonization, as described in the literature, but alone does not lead to subsequent infection, characteristics of the pathogenic strains.

Most of the cell-contact cytotoxic strains, 76% (13/17), were able to invade UD (Table 4.5), as expected since invasion causes cell lysis, however, cellular damage is not restricted to toxin production and bacterial invasion, the induction of apoptosis and the disruption of normal cellular functions can all cause lysis of host epithelial cells and contributing to tissue damage which is necessary for bacterial invasion and a more rapid translocation of bacteria through the intestinal barrier to other sites within the host. The results suggest that close contact of the strains with host cells is a prerequisite to cause high levels of cytotoxicity and indicate the presence of another cell lysis mechanism than invasion, mainly because of 2 low cell-contact cytotoxic strains, S2 environmental strain and A255 clinical strain, that did not show invasive ability (Table 4.5) and those with highest invasion ability are not the only strains that cause the highest cell-contact damage; for example, A78 with 0.054% ± 0.009% recovered cells (Table 4.5) causes 69% ± 23% cell damage (Table 4.7), while S8 with 0.14% ± 0.02% recovered cells (Table 4.5) causes 61% ± 2% cell damage (Table 4.7), under the same conditions and incubation times. These results indicate that the strain A78 is a good candidate for further analysis to determine this cell lysis mechanism which is mediated by direct contact between bacteria and host cell.

60% of the strains originated from water samples have moderate to high levels of cell-contact cytotoxic. The same was observed in 50% of the clinical strains and 50% of the strains originated from food samples (Table 4.8). These results contribute to the previous conclusion that the water and food have the ability to serve as vehicles of potentially pathogenic strains.

Table 4.8. Distribution of cell-contact cytotoxic *Aeromonas* isolates by source and ability levels.

Cell-contact Cytotoxicity Degree (MOI 100:1)	Strain Sources (%)				
	Surfaces	Food	Water	Clinic	Total
SC; strong cytotoxicity (> 40%)	0% (0)	50% (2)	40% (2)	50% (2)	25% (6)
MC; moderate cytotoxicity (20% to 40%)	36% (4)	0% (0)	20% (1)	0% (0)	21% (5)
LC; low cytotoxicity (5% to 20%)	27% (3)	0% (0)	40% (2)	25% (1)	25% (6)
NC; no cytotoxicity (0% to 5%)	36% (4)	50% (2)	0% (0)	25% (1)	29% (7)
Total strains	11	4	5	4	24

Barroco (2013) determined the presence of the *ascV* gene in the strains used in this study (Table 4.3), that encode a conserved structural protein of secretion system used as a marker for the presence of T3SS [Stuber *et al.* 2003] and the presence of the *aexT* gene (Table 4.3), that encode one of the most studied proteins secreted by T3SS in *Aeromonas*, *Aeromonas salmonicida* bifunctional toxin [Sha *et al.,* 2007; Khajanchi *et al.,* 2010], when active, have cytotoxic activity, catalyzing the depolymerization of the actin cytoskeleton, which results in cell morphological changes, leading to host cell death [Litvak

et al., 2007 in Sha *et al.,* 2007], has been associated with the inhibition of phagocytosis of polarized epithelial cells and macrophages [Garrity-Ryan *et al.,* 2000 in Sha *et al.,* 2007], and have ADP-ribosyltransferase activity [Braun *et al.,* 2002 in Sha *et al.,* 2007], as *Pseudomonas aeruginosa* toxins, Exos and Exot, which have high homology with AexT, leading to apoptosis of host cells [Kaufman *et al.,* 2000; Jia *et al.,* 2007 in Sha *et al.,* 2007].

The presence of both genes, *aexT* and *ascV,* was detected in 2 environmental strains, A13 and A97. The A97 strain did not express adhesiveness in UC, indicating the inability of this strain to express cytotoxicity via T3SS, since an initial attachment is required to stabilize interactions between strain and host cells, in order to insert the T3SS through the cell membrane and then inject cytotoxic properties. The A13 strain, on the other hand, express the higher valor of adherence to UC, did express a moderate valor of cell invasion, 0.029% ± 0.007% recovered cells, and cell-contact cytotoxicity, 31% ± 3% cell damage. The A98 strain, in comparison, also express a moderate valor of cell invasion, 0.030% ± 0.005% recovered cells, but express a low valor of cell-contact cytotoxicity, 18% ± 4% cell damage, demonstrating a significant difference in cytotoxicity express by the A13 strain. This indicates the possibility of the T3SS and the AexT toxin are being expressed in A13 strain or other cytotoxic properties that are injected by this system.

The presence of *ascV* gene was detected in 2 strains, AR5 and A259, that express moderate and high levels of cell-contact cytotoxicity, respectively, but also express moderate and high levels of cell invasion, respectively, not demonstrating a significant difference in cytotoxicity when compared with the other strains. This indicates that the T3SS is not being expressed in these strains or they not have or not expressed cytotoxic properties that are injected by this system.

4.3.2. *Aeromonas spp.* extracellular cytotoxic activity

The *Aeromonas* produce a variety of biologically active extracellular products similar to those of enteropathogenic bacteria [Von Gravaenitz, 2007; Janda and Abbott, 2010], therefore for a better discrimination of the cytotoxic extracellular abilities other quantitative assay was performed, this time with supernatant that includes all products produced during the growth phase and early stationary phase, keeping the cell lysis and thereby endotoxin release in the culture broth to a minimum.

Some studies reported a correlation between the higher toxic activities from *Aeromonas* isolates and its potential to induce diarrhoea [Albert *et al.*, 2000; Sha *et al.*, 2002; Chang *et al.*, 2008] and both adhesins and enterotoxins were necessary for production of diarrhoea in volunteers fed orally with Enterotoxigenic *Escherichia coli* (ETEC) [Sattherwhite, 1978]. In the present study 6 (25%) *Aeromonas* isolates were found to express both adherence to DC (Table 4.1) and extracellular cytotoxicity with titers greater than 2 after 24 hours of incubation (Table 4.9 and Figure 10A), and only one of those strains come from clinical origin, demonstrating that environmental strains also have the ability to express these putative virulence properties at least at human body temperature.

Table 4.9. Extracellular cytotoxicity ability of *Aeromonas* isolates to undifferentiated (4-6 days old) Caco-2 cells, as described in Chapter 3.4.1. The results are average ± propagated error.

Isolates	Sources	Cytotoxicity of bacterial culture supernatants						Cytotoxicity (CCD)	Adherence patterns	Adhesion (AD)	Invasion (ID)
		4 hours		24 hours		24 hours after preheating		Undifferentiated cells (UC)			
		ECD	% Cell viability	ECD	% Cell viability	ECD	% Cell viability	MOI 100:1		MOI 10:1	
K-12	*E. coli* K-12 C600	1:2	100% ± 1%	1:2	99 ± 1%			NC	None	NA	NI
PAO1	*P. aeruginosa* PAO1	1:2	89% ± 2%	1:2	81 ± 1%			SC	Aggressive	MA	SI
AR5	*A. hydrophila* 30187t	1:2	62% ± 2%	1:8	43 ± 2%	1:2	90% ± 1%	MC	Aggressive	MA	SI
A 5	surface (slaughterhouse)	1:2	82% ± 2%	1:2	73 ± 1%			MC	Aggressive	SA	MI
A 11	surface (slaughterhouse)	1:2	51% ± 1%	1:4	40 ± 1%	1:2	84% ± 1%	MC	Aggressive	SA	MI
A 13	surface (slaughterhouse)	1:8	30% ± 1%	1:64	20 ± 2%	1:2	86% ± 2%	MC	Aggressive	SA	MI
A 26	food (slaughterhouse)	1:2	95% ± 1%	1:2	83 ± 2%			SC	Diffuse	MA	SI
A 31	food (slaughterhouse)	1:512	35% ± 2%	1:1024	38 ± 1%	1:4	40% ± 2%		Diffuse	WA	NI
A 53	surface (supermarket)	1:2	80% ± 2%	1:2	74 ± 1%				None	NA	NI
A 62	surface (supermarket)	1:2	88% ± 1%	1:2	78 ± 1%			LC	Diffuse	MA	NI
A 78	food (supermarket)	1:2	81% ± 1%	1:2	70 ± 1%			SC	Aggressive	SA	SI
A 92	surface (supermarket)	1:2	81% ± 1%	1:2	69 ± 1%				Diffuse	WA	NI
A 97	surface (cheese factory)	1:16	30% ± 1%	1:64	18 ± 1%	1:2	26% ± 2%	NC	Diffuse	WA	WI
A 98	surface (cheese factory)	1:256	32% ± 1%	1:512	31 ± 2%	1:2	60% ± 3%	LC	Diffuse	MA	MI
A 99	surface (cheese factory)	1:2	86% ± 1%	1:2	77 ± 1%			LC	Aggressive	SA	NI
A 101	surface (cheese factory)	1:256	30% ± 2%	1:512	40 ± 2%	1:2	88% ± 3%	MC	Aggressive	SA	SI
A 104	surface (cheese factory)	1:2	90% ± 1%	1:2	55 ± 2%			NC	Diffuse	WA	NI
A 127	food	1:128	40% ± 2%	1:256	29 ± 2%	1:2	87% ± 1%	NC	Diffuse	WA	NI
A 172	clinic	1:64	31% ± 2%	1:256	26 ± 1%	1:8	43% ± 1%		None	NA	NI
A 255	clinic	1:256	47% ± 2%	1:512	25 ± 1%	1:4	46% ± 3%	LC	Aggressive	MA	NI
A 258	clinic	1:2	80% ± 2%	1:2	74 ± 1%			SC	Aggressive	SA	SI
A 259	clinic	1:2	92% ± 1%	1:2	58 ± 1%			SC	Aggressive	SA	SI
S 2	water (EPAL)	1:2	88% ± 2%	1:2	55 ± 1%			LC	Aggressive	MA	NI
S 3	water (EPAL)	1:2	79% ± 1%	1:2	45 ± 2%			SC	Aggressive	SA	SI
S 8	water (EPAL)	1:2	88% ± 1%	1:2	55 ± 2%			SC	Aggressive	SA	SI
S 10	water (EPAL)	1:2	91% ± 1%	1:2	54 ± 1%			MC	Aggressive	MA	SI
S 18	water (EPAL)	1:2	93% ± 1%	1:2	60 ± 1%			LC	Aggressive	MA	NI
% of positive (> 1:2 titre)		33% (8)		42% (10)		17% (4)		71% (17)		92% (22)	54% (13)

ECD: Extracellular cytotoxicity degree: NC: no cytotoxic (100% in 1:2 titer); LC: low cytotoxicity (< 1:2 titer); MC: moderate cytotoxicity (1.2 to 1:64 titer); SC: strong cytotoxicity (> 1:128 titer);

CCD: Cell-contact cytotoxic degree: NC: no cytotoxic; LC: low cytotoxicity (< 25%); MC: moderate cytotoxicity (25 to 45%); SC: strong cytotoxicity (> 45%);

AD: Adhesion degree: NA: no adhesion (< 3x K-12); WA: weak adhesion (3x to10x K-12); MA: moderate adhesion (10x to 30x K-12); SA: strong adhesion (>30x K-12);

ID: Invasion degree: NI: no Invasion (< 10x K-12); WI: weak invasion; MA: moderate invasion (20x to 40x K-12); SA: strong invasion (>40x K-12).

The highest activity of the toxins was observed at 24h of incubation and all of the strains supernatants were found to have the ability to cause damage to Caco-2 cells above nonpathogenic K-12 strain that reached 100% ± 1%, but 63% (15) of the strains showed low cytotoxic activity, producing titers lower than 2 (Table 4.9). The highest cytotoxic titre ranging from 1024 to 64 was observed for 33% (8) of the strains (Table 4.9) and in these strains was observed morphological alteration within 30 minutes of incubation and cellular destruction within 1h, which was followed by the complete destruction of cell line within 4h (Table 4.9 and Figure 10A), but only 17% (4) of these strains also exhibit the capacity of cell adhesion in DC, including the A13 strain that showed the highest adherent (Table 4.1). Preheating (56ºC for 20 minutes) of the culture supernatants caused a decrease in the activity, not showing titers greater than 8, that suggested production of extracellular heat-stable toxins in 17% (4) of the strains that show positive titers (Table 4.9 and Figure 11A), and only one of those, A255 clinical strain, showed adherent activity to DC (Table 4.1).

50% of the strains originated from food samples have moderate to high levels of cell-contact cytotoxicity. The same was observed in 50% of the clinical strains and 45% of the strains originated from food processing surfaces (Table 4.10).

These results reinforce the previous conclusion that food has the ability to serve as vehicles of potentially pathogenic strains.

Table 4.10. Distribution of extracellular cytotoxic *Aeromonas* isolates by source and ability levels.

Extracellular Cytotoxicity Degree (24h incubation)	Strain Sources (%)				
	Surfaces	Food	Water	Clinic	Total
SC; strong cytotoxicity (> 1:128)	18% (2)	50% (2)	0% (0)	25% (1)	21% (5)
MC; moderate cytotoxicity (1:2 to 1:64)	27% (3)	0% (0)	20% (1)	25% (1)	21% (5)
LC; low cytotoxicity (< 1:2)	55% (6)	50% (2)	80% (4)	50% (2)	58% (14)
NC; no cytotoxicity (100% in 1:2)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
Total strains	11	4	5	4	24

Certain enterotoxins, with different functions, have been associated with the pathogenic potential in the genus *Aeromonas*. Barroco (2013) determined the presence of two enterotoxins with cytotonic activity - Alt (*Aeromonas* heat-labile cytotonic enterotoxin) and Ast (*Aeromonas* heat-stable cytotonic enterotoxin) (Table 4.3) - and two enterotoxins with cytotoxic activity – Act (*Aeromonas* cytotoxic enterotoxin) and HlyA (β-hemolisina) (Table 4.3).

In this study was not determined the expression of cytotonic activity since the cytotonic toxins do not cause cell lysis of Caco-2 cells, but produce a secretory response in intestinal cells. The expression of these toxins can be determined in the presence of CHO cells (Chinese Hamster Ovarian cells) where there is good correlation between the Alt and Ast cytotonic enterotoxins expression and elongation of CHO cells [Chopra *et al.,* 1994], but previous data show that Act is the major enterotoxin contributing to fluid secretory response, followed by Alt and Ast in *Aeromonas hydrophila* [Sha *et al.,* 2002], the presence of these three enterotoxin genes in various combinations may act synergistically [Albert *et al.,* 2000; Sha *et al.,* 2002] and the presence of all three genes is rare [Albert *et al.,* 2000]. In the

Barroco (2013) study, 50% (12), 17% (4) and 66% (15) of the strains used in this study showed the presence of *alt, ast* and *act* genes, respectively. The combinations were *alt* and *act* genes in 21% (5) of the strains, 3 environmental strains (A31, A78 and S2) and 2 clinic strain (A172 and A258), a*lt* and *ast* genes in A98 strain and the combination of all three genes in 13% (3) of the strains, 2 environmental strains (A101 and A97) and 1 clinic strain (A255) as the *Aeromonas hydrophila* reference strain.

Barroco (2013) determined, regarding cytotoxins with hemolytic activity, that 30% (7) of the strains used in this study showed *hlyA* gene and that 66% of the strains showed *act* gene (Table 4.3), but also that all strains expressed hemolytic activity, including at least 7 strains expressing other hemolysins whose genes were not surveyed. Barroco (2013) also determined that 25% (6) of the strains expressed phospholipase activity, including at least 7 strains expressing other lipases/ phospholipases whose genes were not surveyed. These results may explain the ability of culture supernatants obtained by all the strains have the ability to lyse Caco-2 cells to a certain extent. Also, the molecular level identification of genus involved the detection of two targets genes, rRNA16S and *gcat* gene, both amplified with primers designed for conserved sequences between *Aeromonas* species [Barroco, 2013]. The fact that the product of *gcat* gene, the GCAT phospholipase is associated with lysis of erythrocytes, may be contributing to the hemolytic phenotypes observed [Barroco, 2013].

The majority, 80% (8/10) of the strains, that showed cytotoxic titer after 24 hours of incubation showed at least one of cytotoxic genes searched, *act* and *hlyA* genes. In 50% (5/10) of the cytotoxic strains, including *Aeromonas hydrophila* reference strain and A255 clinic strain, showed both genes and exhibit a strong cytotoxic capacity. Moreover, the A13 (1:64 titer) and A98 (1:512 titer) strains showed only one of the genes, *act* and *hlyA,* respectively, but continue to demonstrate the same high levels of extracellular cytotoxic. These results indicate that the combined capacity of both toxins is not required to achieve high cytotoxic ability, but the individual expression of both toxins was not determined as the presence of other toxins that may be contributing to cytotoxic ability of these strains.

The A127 strain (1:256 titer) and A11 (1:4) strain supports this hypothesis, because they do not have these two genes in the genome, but their strong and moderate cytotoxic abilities respectively are due to the presence of other toxins whose genes has not been searched. These results indicate that the A127 and A11 strains are good candidates for further analysis to determine other toxin genes or putative genes.

On the other hand, the A62 strain has both genes in the genome, but their expression was not detected in the extracellular cytotoxicity assay, showing a titer of less than 2, as in 56% (10/18) of the strains that exhibit the *act* gene. These results indicate that there is an abstraction for the expression and release of Act toxin in most strains, may be related to the absence of the T2SS system where this toxin is secreted.

Chapter 5   Conclusions

A proportional decrease of adherence with the stage of differentiation of the Caco-2 cells was observed in all adherent *Aeromonas* strains, 22 (92%) and 19 (79%) *Aeromonas* isolates were found to be adhesive to undifferentiated and differentiated Caco-2 cells, respectively. These results indicate that the genus *Aeromonas* interacts optimally with cultured human intestinal cells at cellular sites expressed in the brush border early in the differentiation process, and that the *Aeromonas* strains adhesion mechanism is different from the one present in the *Pseudomonas aeruginosa* PAO1 strain, whose adherence were significantly higher in differentiated Caco-2 cells. In 13 (54%) strains investigated here showed aggregative adhesion patterns similar to those of the stacked brick appearance noted for members of the *Enterobacteriaceae* [Knutton *et al.*, 1987; Gunzburg *et al.*, 1993], including all clinical adherent strains and source water.

The invasion activity was show by 13 (54%) and 12 (50%) strains to undifferentiated and differentiated Caco-2 cells, respectively. A decrease in invasion was also observed in some adherent *Aeromonas* strains, which can be associated with the decrease in the number of bacteria in association with differentiated cells, but most keep their values, similarly to PAO1 strain, which indicates the existence of an invasion mechanism not specific to both undifferentiated and differentiated Caco-2 cells. Still the A5 strain did increases its invasiveness in differentiated Caco-2 cells, which indicates the existence of a different invasion mechanism from the others *Aeromonas* strains, one more specific to cellular sites expressed in the brush border late in the differentiation process.

The extracellular cytotoxicity was show in 6 (25%) *Aeromonas* isolates adherents to differentiated Caco-2 cells, with titers greater than 2 after 24 hours of incubation. Preheating the culture supernatants of these strains caused a decrease in the activity, only showing titer in the A255 clinical strain, which suggest production of extracellular heat-stable toxins in this strain.

The results also show that one of the important mechanisms of *Aeromonas spp.* cytotoxicity may be cell-contact mediated, that expressed cell damage greater than the extracellular properties expressed by the same strains. In this study 17 (71%) *Aeromonas* isolates were found to express this type of cytotoxicity to differentiated Caco-2 cells, but only 13 were able to invade undifferentiated Caco-2 cells and those with highest values of invasion are not the only strains that cause the greatest cell-contact damage, indicating the presence of another cell lysis mechanism than invasion, not yet determined.

The A255 clinical strain showed strong adherence and extracellular cytotoxicity heat-stable and the two other clinical strains, A258 and A259, showed to be very adherent and invasive, demonstrating the higher levels of cell damage. However, the A172 clinical strain did not show the ability to adhere to Caco-2 cells, but showed a high level of cytotoxicity, like the A31 environmental strain, which showed the highest level of extracellular cytotoxicity of all strains, but showed no ability to adhere to undifferentiated Caco-2 cells. Previous studies suggest that putative virulence associated or colonization factors of pathogenic strains can be lost upon *in vitro* passage [Morgan *et al.*, 1985]. Another explanation for this could be that the patient with gastroenteritis was a healthy carrier of *Aeromonas spp.* and the real etiological agents of the disease were not identified.

Overall, some *Aeromonas* strains isolated in this study from environmental, food and food processing surfaces showed adhesive, invasive and extracellular cytotoxic to Caco-2 cells similar or grater to those of clinical strains. These findings indicate that environmental *Aeromonas spp.* have the potential to cause human illness at 37ºC, human body temperature, consubstantiating the potential of food and water as vehicles for *Aeromonas* diseases.

One of the most important strains of environmental origin found in this study was the A26 strain, which showed the highest ability to invade cell and to cause cell damage by contact to Caco-2 cells. The A78 strain is the second environmental strain expressing the highest ability to cause cell-contact damage to Caco-2 cells, but causing only a low level of invasion, indicating the presence of a different cytotoxicity mechanism. Another is the A101 strain that showed high levels of adhesion, invasion and extracellular cytotoxicity to Caco-2 cells. Finally the A13 strain that showed the highest ability to adhere to Caco-2 cells, a high level of invasion and a moderate level of extracellular cytotoxicity.

These strains, as many enteropathogens, express a variety of virulence factors involved in the infection process, showing the ability to damage host tissues as well as to evade the host defense system. However, it is important to note that infections by potentially pathogenic *Aeromonas* may not always lead to disease, due to host factors such as susceptibility or immune status and infectious dose of the microorganism. For these reasons, gastroenteritis caused by *Aeromonas spp.* in humans remains relatively rare, but they are potential waterborne and foodborne pathogens and it is important further evaluation, research and regulatory consideration, which includes risk assessment.

When looking at Caco-2 cell cultures microscopically, it is evident even by visual inspection that the cells are heterogeneous. As a result, the existence of Caco-2 cell lines maintained in different laboratories and/or of different clonal origin, in addition to the effects of different culture protocols, have then diverged significantly, which makes the comparison of results particularly difficult across laboratories. Carrello *et al.* (1988) found that clinical strains were more adhesive on cells lines than environmental strains, while the opposite was found by Sechi *et al.* (2002). For this reason, the conclusions of the present study may not be directly comparable to those of other published studies.

The results presented in this thesis are an import contribution to knowledge on the pathogenicity of *Aeromonas* and when joined with other data gathered in the overall project will allow developments in food and water microbial quality control as well as in the area of study of virulence of species of clinical relevance.

Chapter 7   Future Work

One major problem in *Aeromonas* identification relies on the fact that some species are phenotypically very similar (for example: *Aeromonas caviae and Aeromonas media, Aeromonas veronii* and *Aeromonas sobria*). A valid alternative to conventional methods of bacterial identification and classification, that is based on the characterization of biomarker molecules, but definitely more rapid and reliable is the mass spectrometry technique [Fenselau, 2001]. Is in progress a preliminary investigation on the potential of fingerprinting data obtained by matrix-assisted laser desorption ionization – time of flight - mass spectrometry (MALDI-TOF-MS) to identification of the most virulent strains.

Further investigations are in progress to confirm the enteropathogenicity and clarify the mechanisms of adhesion, because the adhesion to Caco-2 cells does support the diarrhoeagenicity of the *Aeromonas* organisms. However, the importance of this process in human gastrointestinal disease has yet to be verified under competitive conditions of the gut and in the presence of mucus.

A set of assays can be suggested to provide further insights into these processes:

- Assessment of *Aeromonas* adherence in the presence of a competitive ecosystem

Adhesion of probiotic bacteria to the intestinal mucosa is considered important for the protection against pathogens [Coconnier *et al.*, 1993a, 1993b], transient intestinal colonization [Morelli *et al.*, 2006], modulation of the immune system [Schiffrin *et al.*, 1997], and enhanced healing of damaged intestinal mucosa [Elliott *et al.*, 1998]. Adhesion assays in the presence of an intestinal microbial community can be performed using samples taken from the descending colon of an informed donor or from a Simulator of the Human Intestinal Microbial Ecosystem (SHIME) [Molly *et al.*, 1993; Van den Abbeele *et al.*, 2010] and would be particularly interesting to evaluate *Aeromonas* performance in a competitive environment.

- Assessment of *Aeromonas* adherence in the presence a mucus layer

Intestinal epithelial cells are covered by a relatively thick (up to 400 μm) mucus layer consisting of mucin, a 2-megadalton (MDa) gel-forming glycoprotein, and a large number of smaller glycoproteins, proteins, glycolipids, and lipids [Allan, 1981; Kim *et al.*, 1984 in Neutra and Forstner, 1987]. The mucus layer itself is in a dynamic state constantly being synthesized and secreted by specialized goblet cells as well as degraded to a large extent by indigenous intestinal microbiota [Hoskins, 1984 in Neutra and Forstner, 1987] Bacterial enteropathogens must traverse the mucus layer in order to approach and adhere to intestinal epithelial cells. The mucus layer has been implicated in interacting with bacteria in a number of ways: as an initial site for bacterial adhesion, as a protective barrier which the bacteria must penetrate, and as a source of nutrients and matrix for bacterial replication, colonization, and infection [Hoskins, 1984 in Neutra and Forstner, 1987]. Adhesion assays with mucus can be performed by inclusion of HT29 cells in the Caco-2 cell model. HT29 cells are a human colonic adenocarcinoma cell line that forms a multilayer of UC in culture media containing glucose and serum. When glucose deprived, however, HT29 cells differentiate into a monolayer of cells with phenotypical

characteristics of enterocytes and mucin-secreting goblet cells [Zweibaum *et al.*, 1982 in Neutra and Forstner, 1987].

-   Translocation tests

It is not yet clear whether gastrointestinal disorders and gut-associated septicaemia are caused by ingesting environmental strains exhibiting the aforementioned pathogenic properties. In normal functioning gastrointestinal tracts, very few species of bacteria are capable of translocating to extra-intestinal sites. However, under certain conditions, some bacteria can cross gut epithelia and appear in mesenteric lymph nodes or other normally sterile sites to cause septicaemia, a process termed bacterial translocation [Berg, 1999]. Translocation mechanisms vary among bacteria and are dependent on bacterial interactions with the gut mucosal epithelium [Cruz *et al.*, 1994] and its inherent ability to translocate [Lunghdahl *et al.*, 2000]. For a translocation assays the differentiated Caco-2 cells are cultured in inserts with a 0.8 μm pore diameter filter containing and the bacteria were inoculated into each inner chamber and incubated, samples are collected, after certain periods of time, from each outer chamber.

Chapter 8   References

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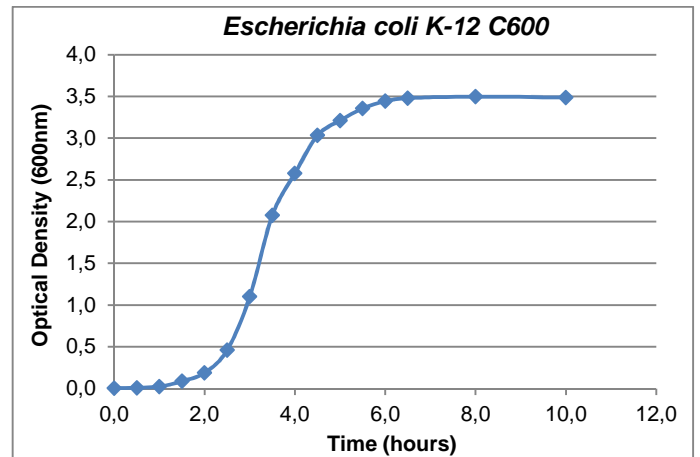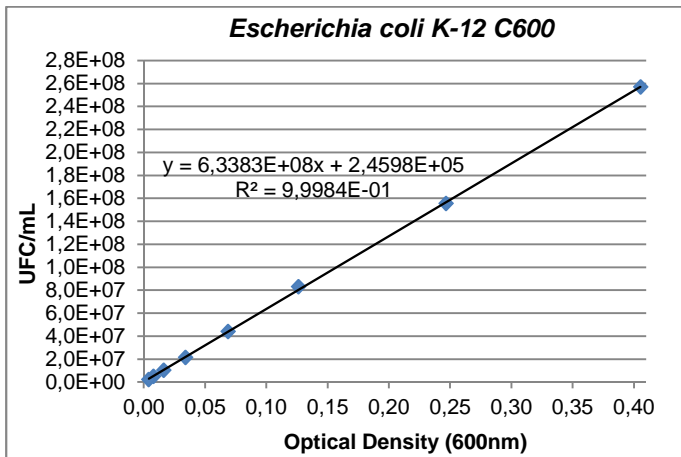
ANNEX

Figure A1. Calibration curve (A) and growth curve (B) of *Escherichia coli* K-12 C600 strain, performed as described in Chapter 3.1.
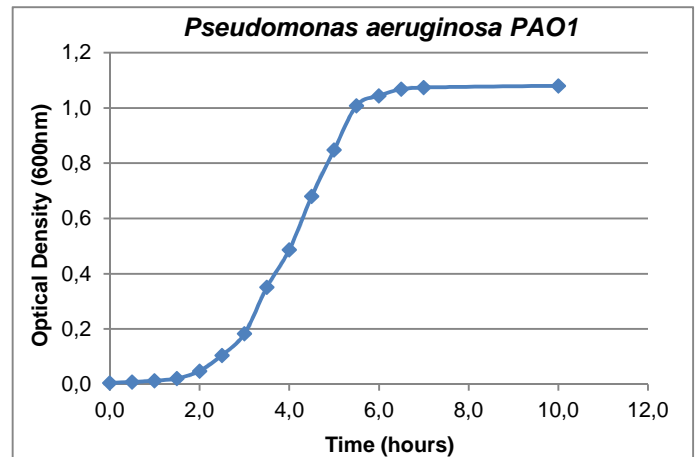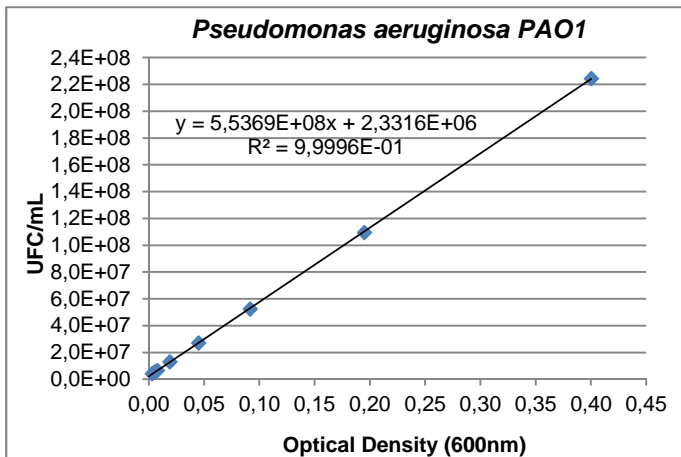

Figure A2. Calibration curve (A) and growth curve (B) of *Pseudomonas aeruginosa* PAO1 strain, performed as described in Chapter 3.1.
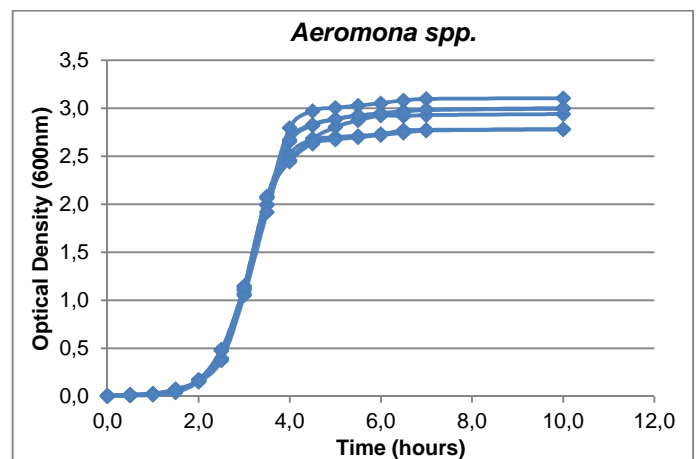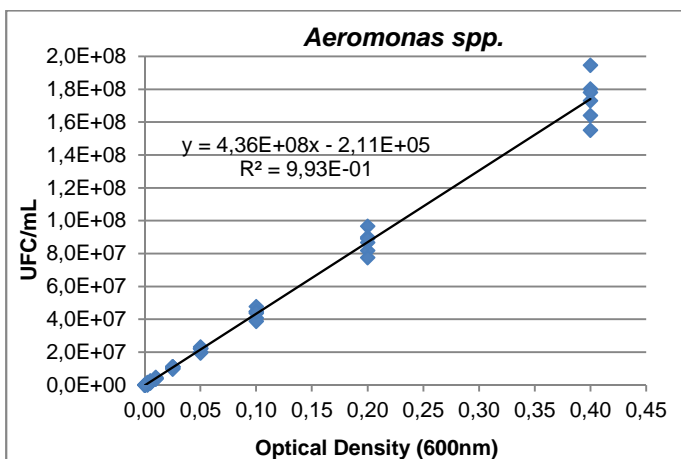

Figure A3. Average calibration curve (A) and growth curve (B) of *Aeromona hydrophila subsp. hydrophila* (DSM 30187t) strain and 5 *Aeromonas* isolates, performed as described in Chapter 3.1.
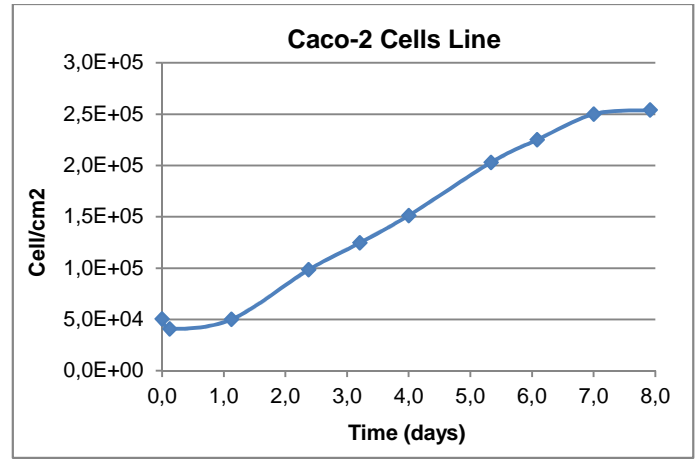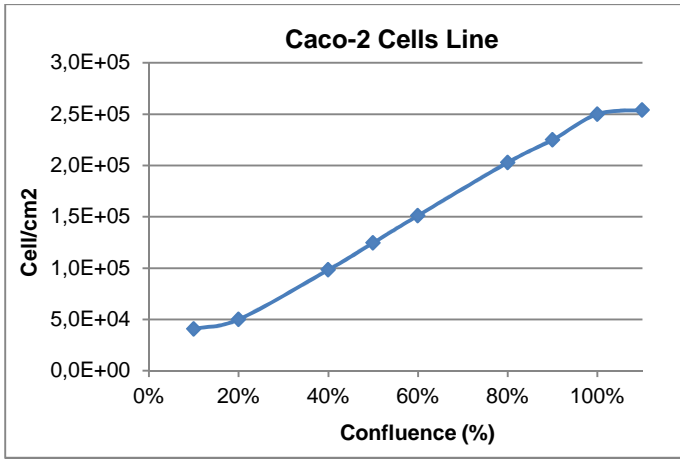
Figure A4. Growth curves of Caco-2 cells line at passage 35, performed as described in Chapter 3.2.

Figure A5. Adhesion and Invasion abilities of *Aeromonas* isolates to undifferentiated (4-6 days old) Caco-2 cells, as described in Chapter 3.3.1. The results are average ± propagated error.

Figure A6. Adhesion and Invasion abilities of *Aeromonas* isolates to differentiated (19-21 days old) Caco-2 cells, as described in Chapter 3.3.1. The results are average ± propagated error.
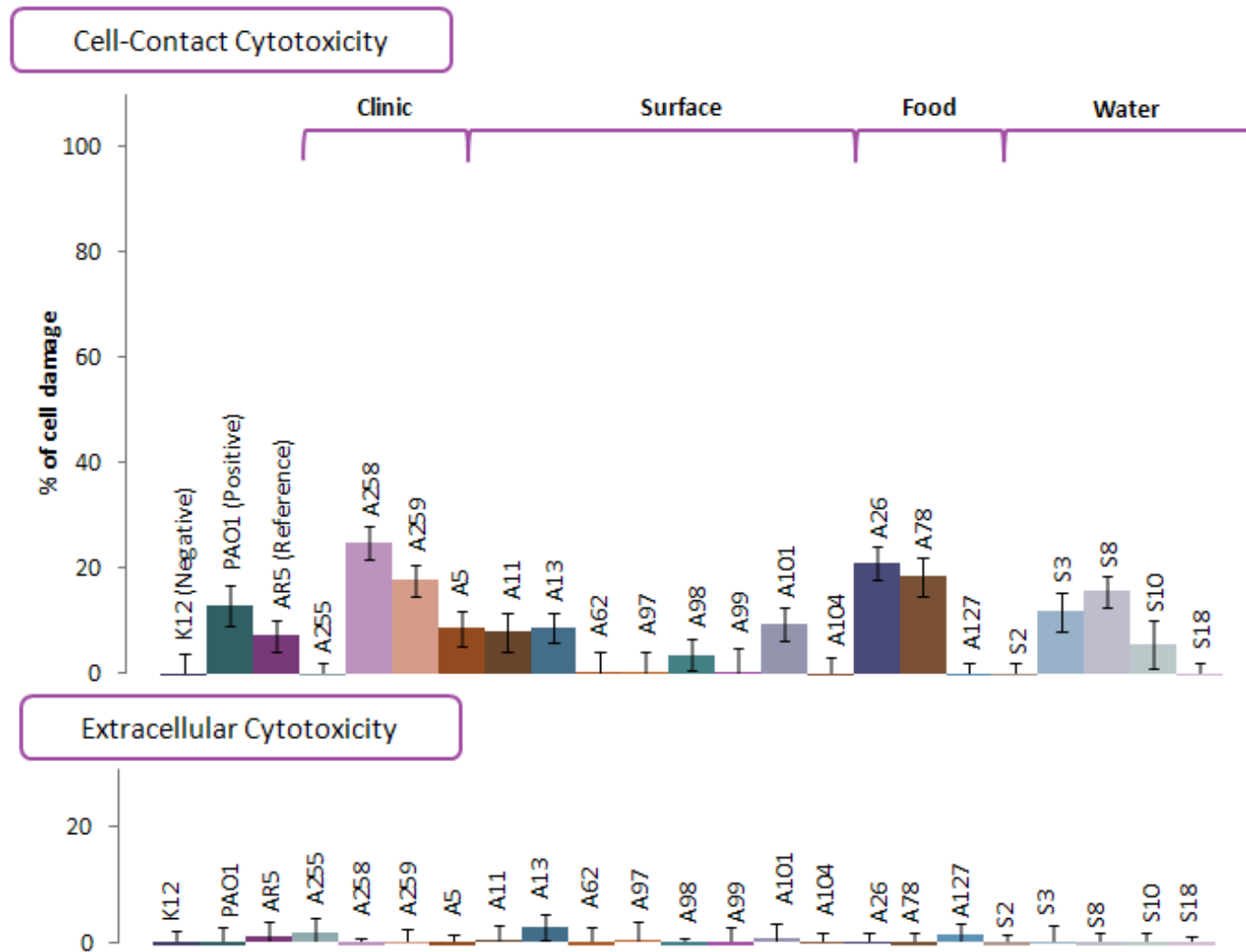
Figure A7. Cell-Contact cytotoxicity ability of *Aeromonas* isolates to undifferentiated (4-6 days old) Caco-2 cells at MOI 100:1, as described in Chapter 3.4.2. The results are average ± propagated error.
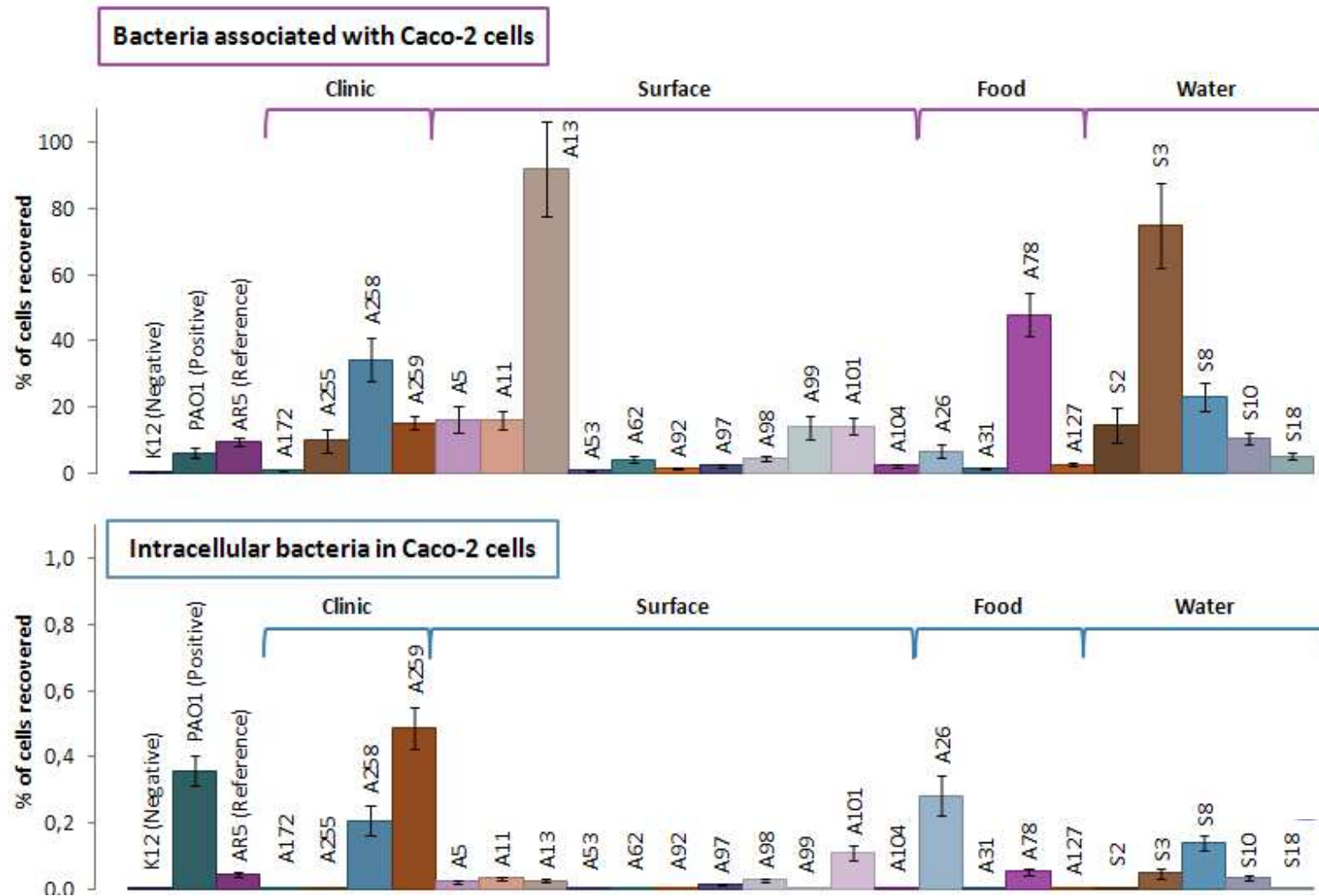
Figure A8. Cell-Contact cytotoxicity ability of *Aeromonas* isolates to undifferentiated (4-6 days old) Caco-2 cells at MOI 10:1, as described in Chapter 3.4.2. The results are average ± propagated error.
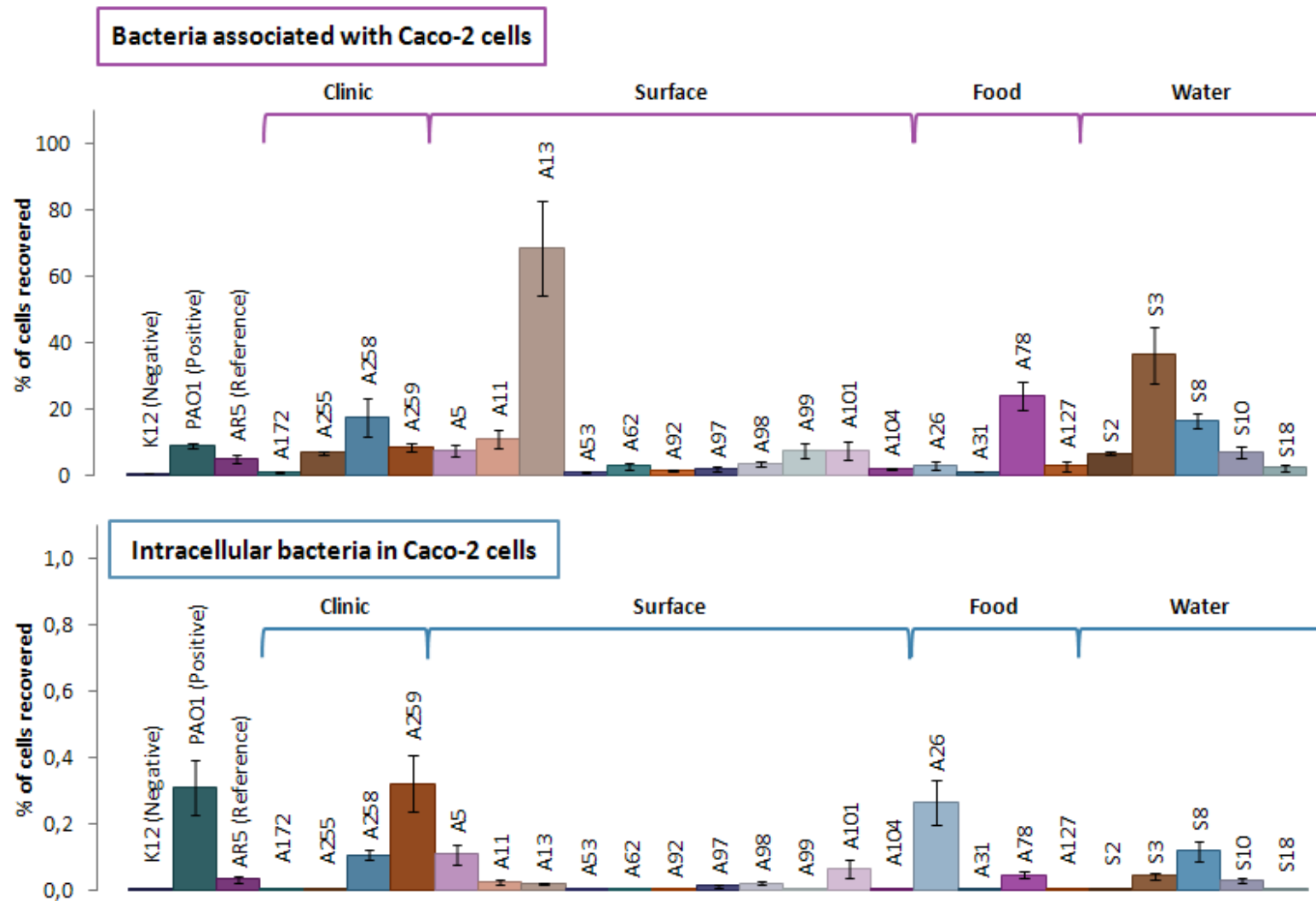
Figure A9. Extracellular cytotoxicity ability of *Aeromonas* isolates to undifferentiated (4-6 days old) Caco-2 cells at 4 hours of incubation, as described in Chapter 3.4.1. The results are average ± propagated error.

Figure A10. Extracellular cytotoxicity ability of *Aeromonas* isolates to undifferentiated (4-6 days old) Caco-2 cells at 24 hours of incubation, as described in Chapter 3.4.1. The results are average ± propagated error.
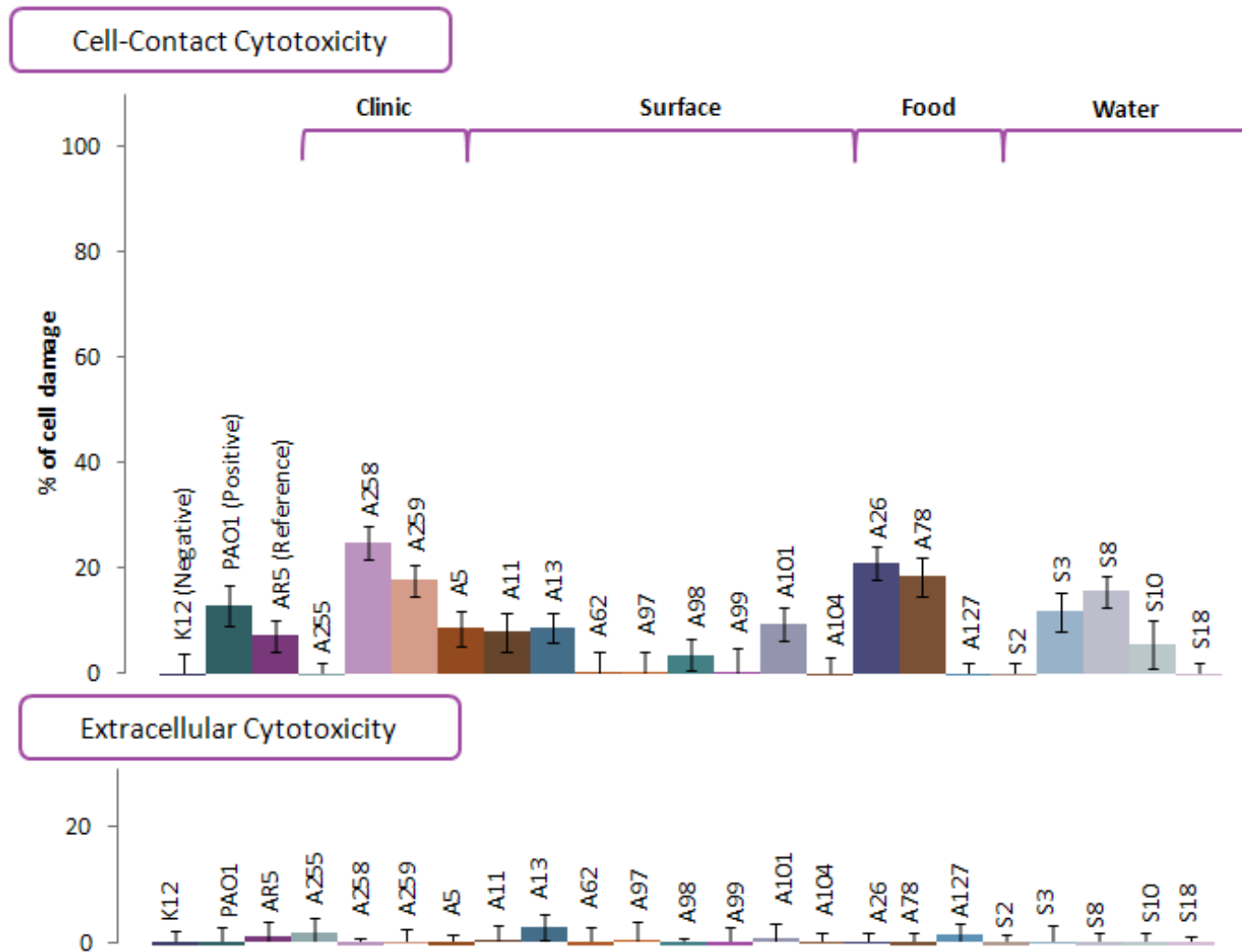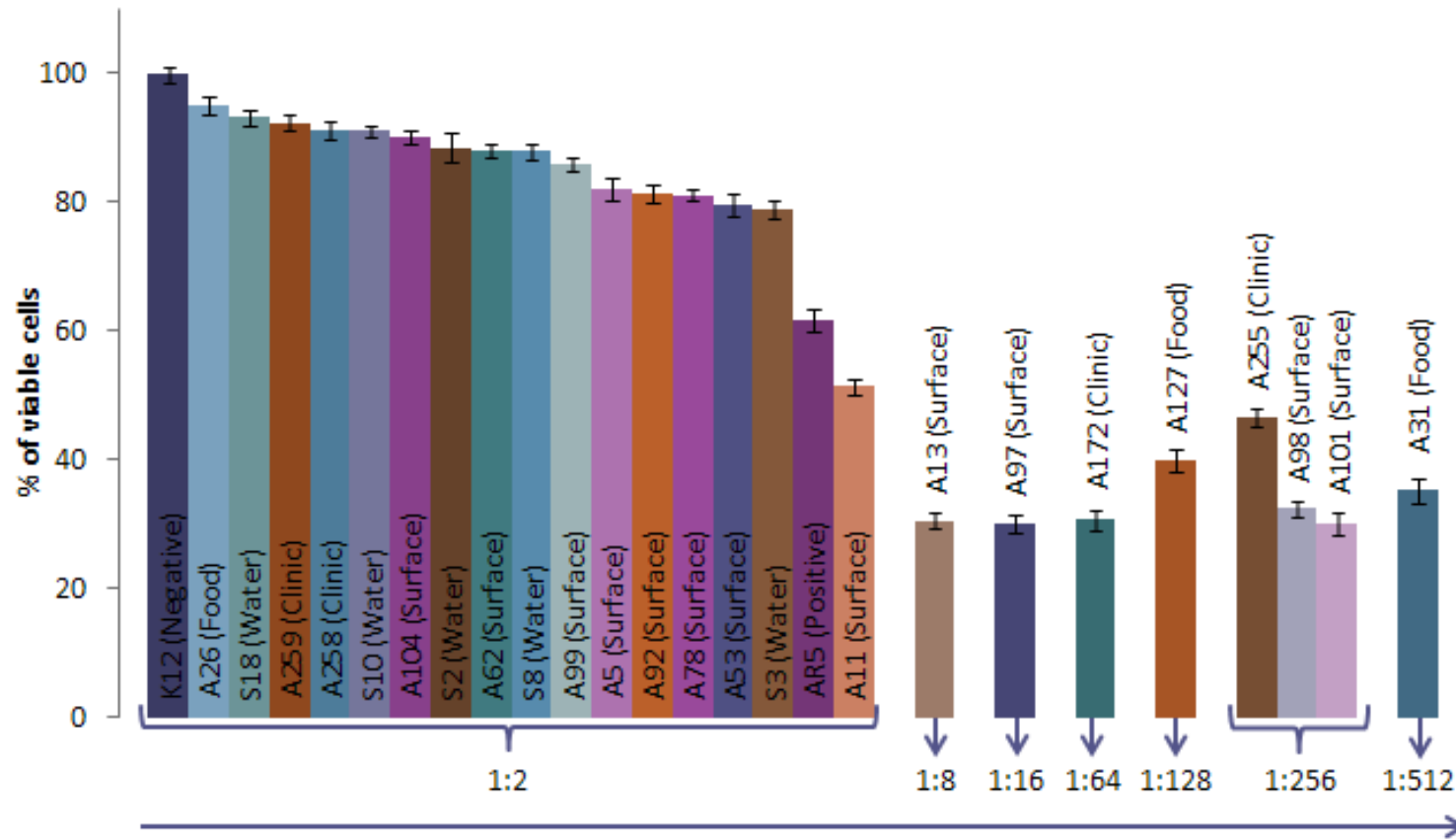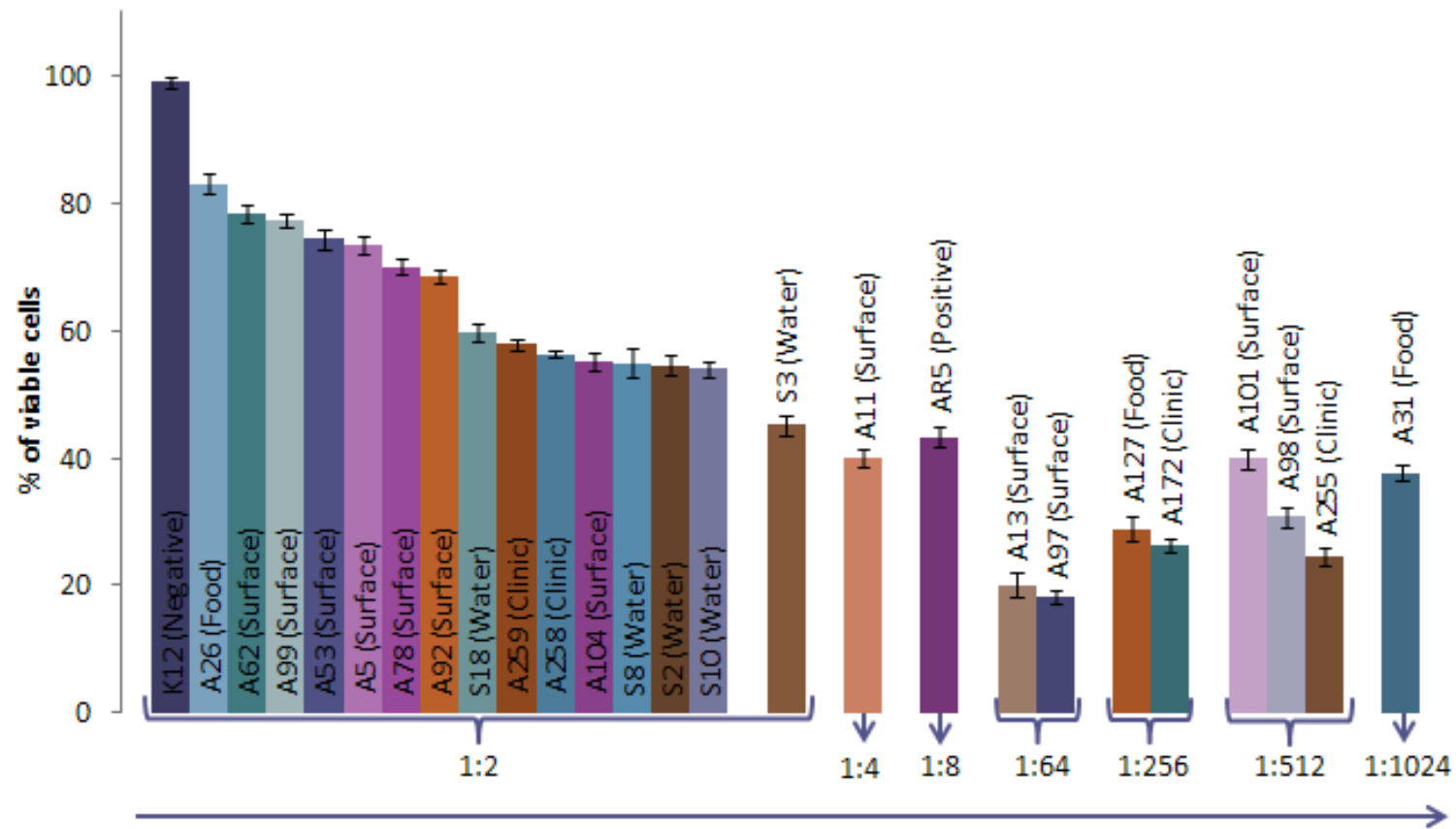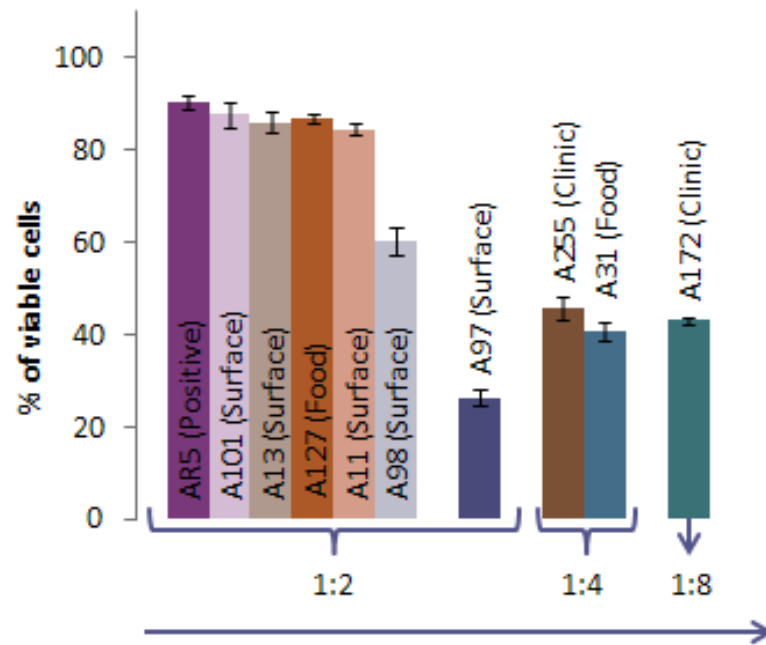
Figure A11. Extracellular cytotoxicity ability of *Aeromonas* isolates to undifferentiated (4-6 days old) Caco-2 cells at 24 hours of incubation after preheating, as described in Chapter 3.4.1. The results are average ± propagated error.

Table A1. Characterization of *Aeromonas* isolates carried out to date by Barroco (2013) at the Faculty of Veterinary Medicine, University of Lisbon.

Isolates	Sources	Virulence genes									Phenotype				Antibiograms															
		ASC	AHH	ALT	AEXT	ELA	LIP	FLA	AST	ACT	LIPA	HEM	GEL	Dnase	AMC	CTX	CAZ	CRO	ETP	IPM	ATM	AK	CN	TE	NA	CIP	LEV	NOR	C	STX
AR5	*A. hydrophila* 30187t	+	+	+	-	+	+	+	+	+	+	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A 5	surfaces (slaughterhouse)	-	-	+	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 11	surfaces (slaughterhouse)	-	-	-	-	+	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
A 13	surfaces (slaughterhouse)	+	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
A 26	food (slaughterhouse)	-	-	-	-	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 31	food (slaughterhouse)	-	+	+	-	+	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 53	surfaces (supermarket)	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 62	surfaces (supermarket)	-	+	-	-	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 78	food (supermarket)	-	-	+	-	-	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
A 92	surfaces (supermarket)	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 97	surfaces (cheese factory)	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 98	surfaces (cheese factory)	-	+	+	-	-	+	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 99	surfaces (cheese factory)	-	-	-	-	+	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 101	surfaces (cheese factory)	-	+	+	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 104	surfaces (cheese factory)	-	-	+	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 127	food	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 172	clinic	-	+	+	-	+	+	+	-	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 255	clinic	-	+	+	-	+	+	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 258	clinic	-	-	+	-	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A 259	clinic	+	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
S 2	water (EPAL)	-	-	+	-	+	-	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S 3	water (EPAL)	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
S 8	water (EPAL)	-	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	+	-	+
S 10	water (EPAL)	-	-	+	-	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-
S 18	water (EPAL)	-	-	-	-	-	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-

Virulence genes: ASC (TTSS structural protein - *ascV*); AHH (β-hemolisina - *hlyA*); ALT (Aeromona heat-labile cytotonic enterotoxin- *alt*); AEXT (TTSS effector protein - ADP-Ribosyltransferase - *aexT*); ELA (elastase - *ahyB*); LIP (lipases and phospholipases - *pla/lip/lipH3/alp-1*); FLA (flagellin A and B - *flaA/B*); AST (*Aeromonas* heat-stable cytotonic enterotoxina - *ast*); ACT (*Aeromonas* cytotoxic enterotoxina - *act*). Phenotype: LIPA (lipase); HEM (hemolysin); GEL (gelatinase); DNases. Antibiograms: AK (amicacina); AMC (amoxicilina/clavulanic acid); ATM (aztreonam); C (cloranfenicol); CAZ (ceftazidima); CIP (ciprofloxacina); CN (gentamicina); CRO (ceftriaxona); CTX (cefotaxima); ETP (ertapenem); IMP (imipenem); LEV (levofloxacina); NA (nalidixic acid); NOR (norfloxacina); STX (trimetoprim/sulfametoxazol); TE (tetraciclina).

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