UNIVERSIDAD AUTÓNOMA DE AGUASCALIENTES.
CENTRO DE CIENCIAS BÁSICAS.
DOCTORADO EN CIENCIAS BIOLÓGICAS.

“PATHOGENS IN BIOFILMS IN FARM DRINKING WATER IN AGUASCALIENTES”.

Thesis submitted for obtaining the degree of "Ph.D. in Biological Sciences"
for the student

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Aguascalientes, Ags., June, 2014.

UNIVERSIDAD AUTÓNOMA DE AGUASCALIENTES.

CENTRO DE CIENCIAS BÁSICAS.

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TESIS.

PATÓGENOS ASOCIADOS A BIOPELÍCULAS EN AGUA DE CONSUMO EN GRANJAS PORCÍCOLAS EN EL ESTADO DE AGUASCALIENTES.

PRESENTA.

Abraham Loera Muro.

PARA OBTENER EL GRADO DE DOCTORADO EN CIENCIAS BIOLÓGICAS.

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UNIVERSIDAD AUTONOMA DE AGUASCALIENTES

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BIOL. ABRAHAM LOERA MURO,
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P R E S E N T E .

Estimado (a) alumno (a) Loera:

Por medio de este conducto me permito comunicar a Usted que habiendo recibido los votos aprobatorios de los revisores de su trabajo de tesis y/o caso práctico titulado: **"PATÓGENOS ASOCIADOS A BIOPELÍCULAS EN AGUA DE CONSUMO EN GRANJAS PORCÍCOLAS EN EL ESTADO DE AGUASCALIENTES"**, hago de su conocimiento que puede imprimir dicho documento y continuar con los trámites para la presentación de su examen de grado.

Sin otro particular me permito saludarle muy afectuosamente.

A T E N T A M E N T E
Aguascalientes, Ags., 13 de junio de 2014
"SE LUMEN PROFERRE"
EL DECANO

M. en C. JOSÉ DE JESÚS RUIZ GALLEGOS

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P r e s e n t e

Por medio de este conducto hacemos constar que el Biólogo Abraham Loera Muro ha cumplido con todos los requisitos de escritura y revisión de tesis para optar por el grado de Doctor en Ciencia Biológicas con la tesis: "Patógenos Asociados a Biopeliculas en Agua de Consumo en Granjas Porcicolas en el Estado de Aguascalientes."

Después de revisar y corregir su escrito hacemos constar que el Biol. Loera Muro, incorporó todas las recomendaciones realizadas por el comité tutorial y brindamos nuestro voto aprobatorio para que proceda a los trámites correspondientes para la impresión de tesis y la obtención de grado.

ATENTAMENTE
"SE LUMEN PROFERRE"
Aguascalientes, Ags., 3 de junio de 2014.

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Dedicated to Sandra Hernández Camacho, my friend, my wife and my love, for all the support and confidence that she always had me, and because without all the support and unconditionally love, I could have never have finished this Ph.D. My parents Victor Manuel Loera Solís and María Lourdes Muro Lizárraga, for all teaching and support that they have given me throughout my life, and because they have taught me to work with honesty and perseverance to achieve any objetive in life. My brother Victor Manuel Loera Muro, for all the moments of joy that we shared and for being a role model as a brother.

"As the richest soil it can not bear fruit unless it is cultivated, the mind without cultivation can not produce."

Seneca.

“In scientific matters, the authority of a thousand is not merit against the humble reasoning of a single individual. "

Galileo Galilei.

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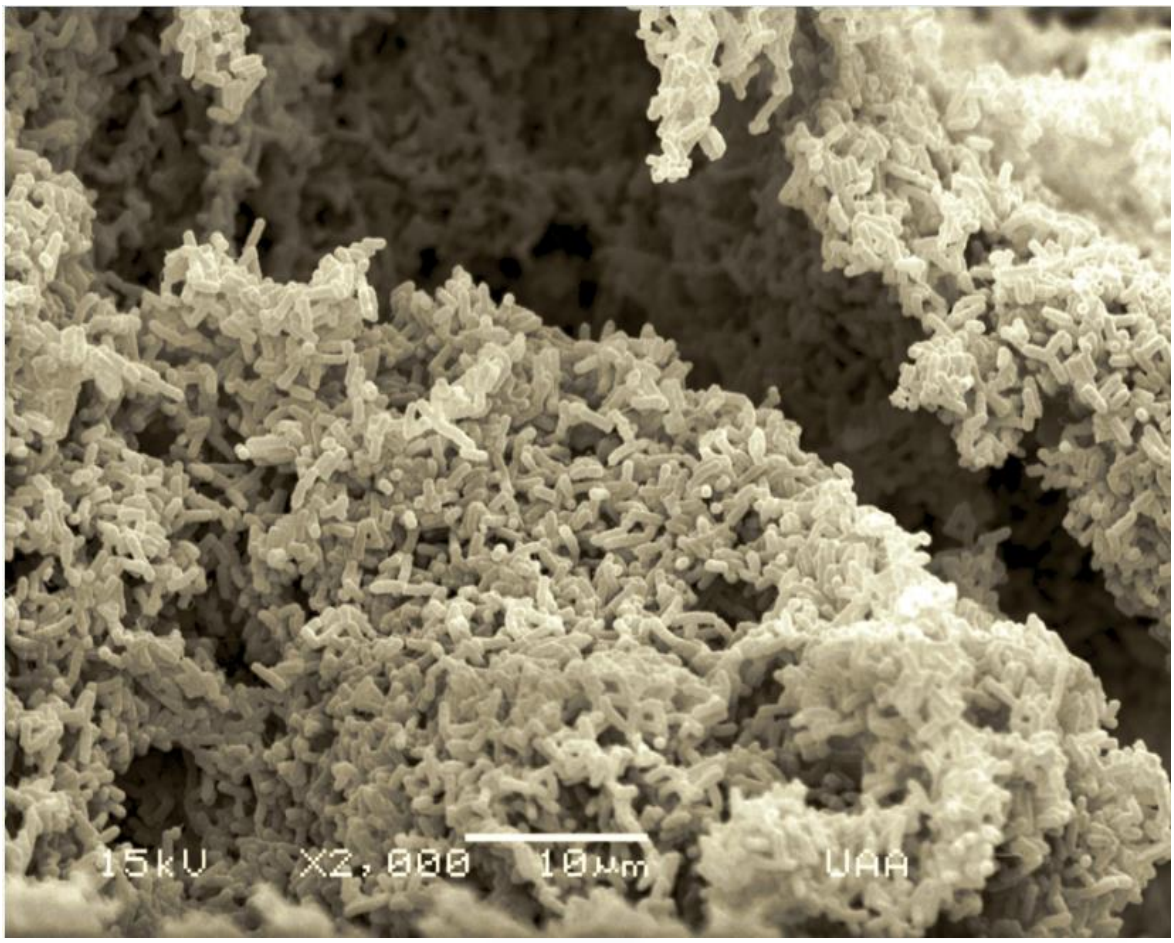
Dedicada a Sandra Hernández Camacho, mi compañera, mi esposa y mi amor, por todo el apoyo y confianza que siempre me tuvo, y porque sin todo ese apoyo y amor que incondicionalmente me entrega a diario, jamás hubiera podido haber terminado este doctorado. A mis padres Victor Manuel Loera Solís y María Lourdes Muro Lizárraga, por todas las enseñanzas y el apoyo que me han otorgado durante toda mi vida, y porque me han enseñado a trabajar con honestidad y constancia para lograr cualquier objetivo en la vida. A mi hermano Victor Manuel Loera Muro, por todos los momentos de alegría que hemos compartido y por ser un ejemplo a seguir como hermano.

"Como el suelo por más rico que sea, no puede dar fruto si no se cultiva; la mente sin cultivo tampoco puede producir".

Séneca.

"En cuestiones científicas, la autoridad de un millar no es mérito frente al humilde razonamiento de un solo individuo".

Galileo Galilei.



Winner photography of the fourth place in the National Scientific Photography Contest 2013 organized by CONACYT.

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ABSTRACT.

Respiratory diseases in pigs are one of the most important health problems in these animals. Due to the multifactorial nature of these diseases are considered as a porcine respiratory disease complex (PRDC). The PRDC is the term used to describe pneumonic diseases caused by multiple infectious agents that cause weight loss in animals or death. In the PRDC multiple pathogens (bacteria and/or viruses) work in combination to induce this respiratory disease. Within this complex, the bacteria *Actinobacillus pleuropneumoniae, Streptococcus suis, Pasteurella multocida, Bordetella bronchiseptica, Haemophilus parasuis* and *Mycoplasma hyopneumoniae* are the main bacterial pathogens involved in causing great economic losses to the swine industry. In this research project, one objective was to estimate the presence of *A. pleuropneumoniae, S. suis, P. multocida, B. bronchiseptica, H. parasuis* and *M. hyopneumoniae* in the upper respiratory tract of asymptomatic pigs in representative swine farms in Aguascalientes state in Mexico using PCR technique. The study was performed in 14 swine farms. We obtained a total of 162 nasal swabs. Twenty point thirty-seven percent of samples were positive for *A. pleuropneumoniae* (located in the 78.57% of farms), 18.52% were positive for *S. suis* (in 85.71% of farms), of these, corresponding to 3.09% *S. suis* serotype 2, 30.86% were positive for *H. parasuis* (92.86% of farms), 28.4% of the samples to *P. multocida* (in 78.57% of farms), and 22.84% to *M. hyopneumoniae* (in 64.29% of farms). We did not detect the presence of *B. bronchiseptica* in this study. With the first part of this study we observed that bacterial pathogens of PRDC were present in the upper respiratory tract of pigs in all farms studied; therefore, these pathogens are widely disseminated in pig farms of Aguascalientes, Mexico.

In the other hand, *A. pleuropneumoniae* is the etiologic agent of porcine contagious pleuropneumonia that causes great economic losses in the pig industry. Fifteen serotypes of *A. pleuropneumoniae* have been described based on capsular antigens and two biotypes described based on nicotinamide adenosine dinocleotide (NAD) requeriments. All serotype are obligate pathogens, but differ in virulence and regional distribution. In Mexico

serotypes of biotypes 2 has yet to be reported. *A. pleuropneumoniae* has traditionally been considered an obligate pathogen of pigs. However the presence of *A. pleuropneumoniae* in the environment has yet to be thoroughly investigated. Here was detected *A. pleuropneumoniae*, found in biofilms isolated from drinking water and directly in the drinkers of swine farms. Samples of drinking water were taken directly from the watering places in the swine farm, aseptically to avoid contamination. *A. pleuropneumoniae* detection was performed by PCR analysis using primers again *apxIV* toxin gene. Detection of *A. pleuropneumoniae* in drinking water was achieved in 5 farms; and from 3 of them were able to obtain 20 positives samples for *A. pleuropneumoniae*. In all samples, *A. pleuropneumoniae* growth independent of NAD in BHI media. Also, in these samples were detected for 16S rDNA *Stenotrophomonas maltophilia, Acinetobacter schindleri* and *Escherichia coli*. All samples had the ability to form biofilms in the liquid-air interface *in vitro* and *A. pleuropneumoniae* biofilm formation was observed *in vivo* by FISH assay. The samples were subjected to antimicrobial susceptibility testing. In conclusion, our data suggest that *A. pleuropneumoniae* has the ability to survive in aquatic environments using biofilms to survive outside its host, and could be surviving in association with other bacteria in a multi-species biofilm.

Likewise, *A. pleuropneumoniae* has the ability to form biofilms *in vitro*. Several respiratory tract infections are associated with biofilm formation, and *A. pleuropneumoniae* has the ability to form biofilms *in vitro*. Biofilms are structured communities of bacterial cells enclosed in a self-produced polymer matrix that are attached to an abiotic or biotic surface. The ability to form biofilms is now considered a universal trait of microorganisms, and multispecies biofilms are the most common form of microbial growth in nature. The goal of this part of the study was to determine the ability of *A. pleuropneumoniae* to form multi-species biofilms with other swine bacterial pathogens in the absence of pyridine compounds (nicotinamide mononucleotide [NMN], nicotinamide riboside [NR] or nicotinamide adenine dinucleotide [NAD]) that are essential for the growth of *A. pleuropneumoniae*. For the biofilm assay, strain 719, a field isolate of *A. pleuropneumoniae* serotype 1, was mixed with swine isolates of *Streptococcus suis, Bordetella bronchiseptica, Pasteurella multocida, Staphylococcus aureus* or *Escherichia coli*, and deposited in 96-well

microtiter plates. Based on the crystal violet and CFU results, *A. pleuropneumoniae* was able to grow with every species tested in the absence of pyridine compounds. Interestingly, *A. pleuropneumoniae* was also able to form strong biofilms, according with their thickness, when mixed with *S. suis, B. bronchiseptica*, *P. multocida* or *S. aureus.* In the presence of *E. coli*, *A. pleuropneumoniae* only formed a weak biofilm. The live and dead populations, and the matrix composition of multi-species biofilms were also characterized using fluorescent markers and enzyme treatments. The results indicated that poly-*N*-acetylglucosamine remains the primary component responsible for the biofilm structure. In conclusion, *A. pleuropneumoniae* is able to acquire pyridine compounds from other swine pathogens, which enables *A. pleuropneumoniae* to grow and form multi-species biofilms.

In conclusion, our data suggest that *A. pleuropneumoniae* has the ability to survive in aquatic environments and use multi-species biofilms as mean to survive outside its host. Finally, if this capacity for form multi-species biofilms is involved in the persistence and virulence in its host, should be investigated in more detail.

RESUMEN.

Las enfermedades respiratorias en cerdos son uno de los más importantes problemas de salud en este animal. Debido a la naturaleza multifactorial de estas enfermedades son conocidas como parte del complejo respiratorio porcino (CRP). El CRP es el término usado para describir enfermedades de tipo pneumónicas causadas por múltiples agentes infecciosos que causan desde pérdida de peso a los animals hasta la muerte. En el CRP, múltiples patógenos (bacterias y/o virus) trabajan en combinación para inducir estas enfermedades respiratorias. Dentro de este complejo, las bacterias *Actinobacillus pleuropneumoniae, Streptococcus suis, Pasteurella multocida, Bordetella bronchiseptica, Haemophilus parasuis* y *Mycoplasma hyopneumoniae,* son las principals bacterias patógenas involucradas en causar grandes pérdidas económicas a la industria porcícola. En este trabajo de investigación, uno de los primeros objetivos fue estimar la presencia de *A. pleuropneumoniae, S. suis, P. multocida, B. bronchiseptica, H. parasuis* y *M. hyopneumoniae* en el tracto respiratorio superior de cerdos asintomáticos en granjas porcícolas del Estado de Aguascalientes, México, usando la técnica de PCR. El estudio fué realizado en 14 granjas porcícolas. Se obtuvieron un total de 162 muestras de exudado nasal. Veinte punto treinta y siete porciento de las muestras fueron positvas para *A. pleuropneumoniae* (localizadas en el 78.57% de las granjas), el 18.52% fueron positivas a *S. suis* (en el 85.71% de las granjas), de las cuales, el 3.09% del total corresponden a *S. suis* serotipo 2; el 30.86% fueron positivas a *H. parasuis* (en el 92.86% de las granjas), el 28.2% de las muestras a *P. multocida* (en el 78.57% de las granjas), y el 22.84% a *M. hyopneumoniae* (en el 64.29% de las granjas). No se logró la detección de *B. bronchiseptica* en este estudio. Con esta primera parte, se puede observer que las bacterias patógenas del CRP están presentes en el tracto respiratorio superior de los cerdos en todas las granjas estudiadas; así mismo, que estos patógenos están ampliamente diseminados en las granjas porcícolas en el Estado de Aguascalientes.

A. pleuropneumoniae es el agente etiológico de la pleropneumoniae porcina contagiosa, la cual, causa grandes pérdidas económicas en la industria porcícola. Quince

serotipos de *A. pleuropneumoniae* han sido descritos basados en sus antigenos capsulares y dos biotipos en base a la dependencia de nicotinamida adenosina dinucleotido (NAD). Todos los serotipos son patógenos obligados, pero difieren en su virulencia y en su distribución. En México, serotipos pertenecientes al biotipo 2 no han sido reportados. *A. pleuropneumoniae* ha sido tradicionalmente considerado un patógeno obligado de cerdos. Sin embargo, su presencia en el ambiente no ha sido fuertemente investigada. Aquí, nosotros detectamos a *A. pleuropneumoniae* en biopelículas aisladas de agua de consume y directamente de bebederos de granjas porcícolas. Muestras de agua de consumo fueron tomadas directamente de los bebederos en las granjas de manera aséptica para evitar contaminación. La detección de *A. pleuropneumoniae* fue realizada por PCR mediante el uso de oligos contra el gen de la toxina ApxIV. La detección de este patógeno en agua de consume fue llevada a cabo en 5 granjas; dondede tres de estas granjas fueron obtenidas 20 muestras positivas para *A. pleuropneumoniae*. En todas las muestras, *A. pleuropneumoniae* creció de manera independiente de NAD en medio BHI. Además, en estas muestras fueron detectadas por ADNr 16S las bacterias *Stenotrophomonas maltophilia, Acinetobacter schindleri* y *Escherichia coli*. Todas las muestras tuvieron la habilidad de formar biopelículas *in vitro* en la interface líquiod-aire y la formación de biopelículas por parte de *A. pleuropneumoniae* fue también observada *in vivo* mediante hibridación fluorescente *in situ*. Las muestras fueron sometidas a ensayos de susceptibilidad antimicrobial. Con lo anterior, nuestros datos sugieren que *A. pleuropneumoniae* tiene la habilidad de sobrevivir en ambientes acuáticos usando biopelículas para su supervivencia fuera de su huésped, y que podría estar sobreviviendo gracias a asociaciones con otras bacterias formando biopelículas multi-especies.

A. pleuropneumoniae tiene la habilidad de formar biopelículas *in vitro*. Así mismo, se conoce que varias enfermedades respiratorias infecciosas son asociadas con la formación de biopelículas. Las biopelículas son comunidades de bacterias encerradas en una matriz de polímeros que ellas mismas producen y que están adheridas a una superficie viva o inerte. La habilidad de formar biopelículas es ahora considerada un atributo universal de todos los microorganismos y la formación de biopelículas de multiples especies es la manera más común en que los microorganismos crecen en la naturaleza. Por lo anterior, el objetivo de

esta parte de la investigación fue determinar la capacidad que el patógeno respiratorio porcino, *A. pleuropneumoniae*, tiene para formar biopelículas multi-especies con otros patógenos de cerdo en la ausencia de compuestos de piridina (nicotinamida mononucleotido [NMN], nicotinamida ribosida [NR] o nicotinamida adenina dinucleotido [NAD]) que son escenciales para el crecimiento de *A. pleuropneumoniae*. Para esta parte del estudio, fué utilizado un aislado de cerdo de *A. pleuropneumoniae* perteneciente al serotipo 1, cepa 719. *A. pleuropneumoniae* fue mezclado con los aislados de cerdo *Streptococcus suis*, *Bordetella bronchiseptica*, *Pasteurella multocida*, *Staphylococcus aureus* o *Escherichia coli*, y depositados en microplacas de 96 posillos. Basados en los resultados de los ensayos decristal violeta y de las UFC, *A. pleuropneumoniae* fue capaz de crecer con todas las especies usadas en ausencia de compuestos de piridina. Interesantemente, *A. pleuropneumoniae* fué capaz de formar una gruesa biopelícula en presencia de *S. suis*, *B. bronchiseptica*, *P. multocida* o *S. aureus*. En presencia de *E. coli*, *A. pleuropneumoniae* unicamente formó una biopelícula débil. Así mismo, fueron analizados los cambios que ocurrieron en la composición de la matriz extracelular y en el número de bacterias vivas o muertas en las biopelículas multi-especies con la ayuda de marcaje fluorsecente y tratamientos enzimáticos. Los resultados indican que la poli-*N*-acetilglucosamina permanence como el principal componente responsable de la estructura de la matriz. En conclusión, *A. pleuropneumoniae* fué capaz de adquirir compuestos de piridina de otros patógenos porcinos, lo que le permite a *A. pleuropneumoniae* crecer y formar biopelículas multi-especies.

En conclusión, todos nuestros datos sugieren que *A. pleuropneumoniae* tiene la habilidad de sobrevivir en ambientes acuáticos y que podría utilizar biopelíulas multi-especies como su principal forma de sobrevivir fuera de su huésped. Finalmente, si esta capacidad para formar biopelículas multi-especies esta involucrada en su persistencia y virulencia en su huésped, debe ser investigada con mayor detalle.

1. ANTECEDENTS.

1.1 Bacterial Biofilms.

Bacteria generally they operate in complex associations, communities or consortia called biofilms. These associations are responsible for the maintenance of the biosphere biogeochemical and in other cases to cause serious illness. Currently, the ability to form biofilms is considered a universal attribute of all microorganisms (Jacques *et al*., 2010; Burmølle *et al*., 2014). Biofilms are bacterial community attached an inert or living tissue surface and embedded in an exopolysaccharide matrix. This matrix may be composed of polysaccharides, nucleic acids and proteins, which can also coexist different bacterial species (Lasa, 2005; Chiers *et al*., 2010; Jacques *et al*., 2010; Almeida *et al*., 2011; Berck *et al*., 2012; Fröls, 2013; Orell *et al*., 2013; Scherr *et al*., 2014). The term biofilm, which refers to bacterial aggregation, was introduced in 1981 by Costerton, but in environmental microbiology, bacterial aggregation has been observed and regarded important for community function for a much longer time. The classic example is the observation of aggregated bacteria in the 'scurf of the teeth' by Anthony van Leeuwenhoek (published in 1684), which refers to the plaque development later described by many dentists (Bjarnshol *et al*., 2013). Donlan (2002) defined to biofilms as a sessile microbial community characterized by cells that are irreversibly adhered to a substrate or interface, or joined together enclosed in a matrix of extracellular polymeric substances (EPS) synthesized by them and exhibiting an altered phenotype relative to and growth rate of gene transcription. It is postulated that 80% of the microbial world is forming biofilms. Likewise, it is estimated that 75% of human diseases are caused by bacteria in this state (Jacques *et al*., 2010). Biofilms have a very dynamic structure, in which they develop a multitude of metabolic interactions between neighboring cells (Ramadam, 2006). Microorganisms in these communities interact physically, via adhesin-receptor mediated attachment, and metabolically, via cross-feeding, collective degradation of host macromolecules and exchange of metabolic signals (Biyikoglu *et al*., 2012). The solid-liquid interface between a surface and an aqueous medium provides an ideal environment for the establishment and

growth of microorganisms, therefore, biofilms are ubiquitous in nature and are found in virtually all natural body of water in the world. Bacterial biofilms represent an ancient prokaryotic survival strategy (Stoodley *et al*., 2002). This is because the bacteria are able to provide significant advantages biofilm protection against environmental fluctuations of humidity, temperature and pH (Jacques *et al*., 2010; Almeida *et al*., 2011; Ganguly & Mitchell, 2011; Trappetti *et al*., 2011; Berk *et al*., 2012). Fossil records show that made in prokaryotes have been living in biofilms for more than three billions of years (Gorbushina, 2007).

Cells in biofilms are physiologically active, metabolically coordinated and very resistant to physical attacks. This structure allows bacteria population develop in a protected microenvironment suitable for growth and reproduction, also functions as an energy reserve, provides structural stability, allowing the diffusion of substances, as well as the adsorption of metal ions and nutrients (Nijland *et al*., 2010; Bowen & Koo, 2011). This matrix also contributes to antibiotics resistance and allows the biofilm cells to escape attack by host defenses (Pereira *et al*., 2010; Almeida *et al*., 2011). In this structure may be any bacteria; photosynthetic, chemosynthetic, chemolithotrophic or chemoorganotrophic (Labrie *et al*., 2010).

1.2 Development of Biofilms.

Biofilms, especially multi-specie biofilms, are the most common form to microbial growth in nature (Bridier *et al*., 2010; Dominiak *et al*., 2011; Yang *et al*., 2011; Fröls, 2013; Orell *et al*., 2013; Burmølle *et al*., 2014). Biofilms formation is a complex event that could involve many bacterial species and several factors (Pereira *et al*., 2010). Biofilm biology focuses on the life cycle and interactions with the environment. The life cycle is a dynamic process that involves several stages.

The initial stage corresponds to the bacterial cell adhesion to a substrate live or dead. The best substrates are rough, porous, hydrophobic and non polar. In hydrophilic surface and non porous the attachment is difficult. The appendices as flagella, fimbriae or

pili (Gram negative), help to move and adhesion to substrates (An *et al*., 2006; Pereira *et al*., 2010; Orell *et al*., 2013). In Gram positive, proteins associated with the cell wall, called adhesins, play an important role in bacterial adhesion and coaggregation (Yang *et al*., 2011). Adhesion and biofilm formation are coordinated elements. For both, bacterial secreted small chemicals substances of low molecular weight and rapid diffusion that can modulate the activities of neighboring cells. These molecules are called **"**quorum sensing" or autoinducers (Bordi & Bentzmann, 2011). Quorum sensing is a population-density-dependent regulating mechanism used by bacteria to control gene expression. In was first described for the bioluminescent marine bacteria *Vibrio fischeri* and *Vibrio harveyi* (Rashid *et al*., 2011). In Gram negative bacteria the system is dependent to acyl-homoserine-lactone (AHL) (Davis *et al*., 2010). In Gram positive there are several modified oligopeptides. Two autoinducer molecule ribose type S-4, 5-dihydroxy-2, 3-pentadione (AI-2) is used both Gram negative and Gram positive. In bacterial adhesion may also influence variations in flow rate, water temperature and nutrient concentration. It has been found that an increase in the concentration of various cations (sodium, calcium, iron) affects the adhesion of *Pseudomonas* spp. to glass surfaces (Nazar, 2007).

Early in the process adhere primary and early settlers to the surface, forming multiply these microcolonies that eventually accordance with microenvironmental conditions, colonized completely and cover the surface, facilitating the arrived of colonizers secondary or later inducing development multi-species consortia (Bowen & Koo, 2011). This step is marked by cell division and expansion of the daughter cells surrounding the site of primary adhesion. Is secreted the biofilm matrix: an exopolysaccharide, which can be composed of *N*-acetylglucosamine, alginate, cellulose, glucose, galactose, among other (Trappetti *et al*., 2011). The composition of this matrix depends on the bacteria strains involved and the environmental conditions. For *A. pleuropneumoniae, A. actinomycetemcomitans, Staphylococcus aureus, S. epidermidis, Yersinia pesti, Bordetella* spp. and *Escherichia coli*, has identified the poly-*N*-acetylglucosamine as the major component of adhesion in the biofilm formed (Izano *et al*., 2007; Chiers *et al*., 2010; Jacques *et al*., 2010), likewise, has shown the importance of the binding histone-like protein (H-NS) in the formation of the same biofilms in the case of *A.*

pleuropneumoniae (Chiers *et al*., 2010). Moreover, the cellulose is used by some strains of *E. coli, Salmonella, Citrobacter, Enterobacter* and *Pseudomonas. P. aeruginosa* used alginate as main component of its extracellular matrix, which is a polymer of junctions β-1-4 of mannuronic acid and guluronic acid (Jacques *et al*., 2010). In *A. pleuropneumoniae*, pathogen of pigs, the formation and growth of the biofilm is inhibited by the addition of zinc to the medium, however, other metals do not affect their growth (Ca, Cu, Mg and Mn) (Labrie *et al*., 2010); Pereira *et al*. (2010) reported the same effect of zinc on biofilm formation of *E. coli*.

The last step is the detachment or separation of the biofilm bacterial cells. At this stage, some bacteria are released from the biofilm matrix, alone or in bacterial clusters, and begin to colonize new environments. This release can be for: the lack of the exopolysaccharide synthesis for some bacteria (*Staphylococcus aureus*), for the enzymes synthesis (alginate lyases, dispersin B), which degrades the exopolysaccharide matrix, allowing bacterial release (*Aggregatibacter -Actinobacillus- actinomycetemcomitans* and *Pseudomonas aeruginosa*) (Loera-Muro *et al*., 2008), or for bacteriophages activity within the biofilm. Other mechanisms involved in this phenomenon are: a) erosion or sliding: continuous removal of small parts of the biofilm; b) separation: rapid and massive remotion; and c) abrasion: liberation by collisions with particle in the fluid with the biofilm (Rickard *et al*., 2003; Nazar, 2007; Bjarnshol *et al*., 2013).

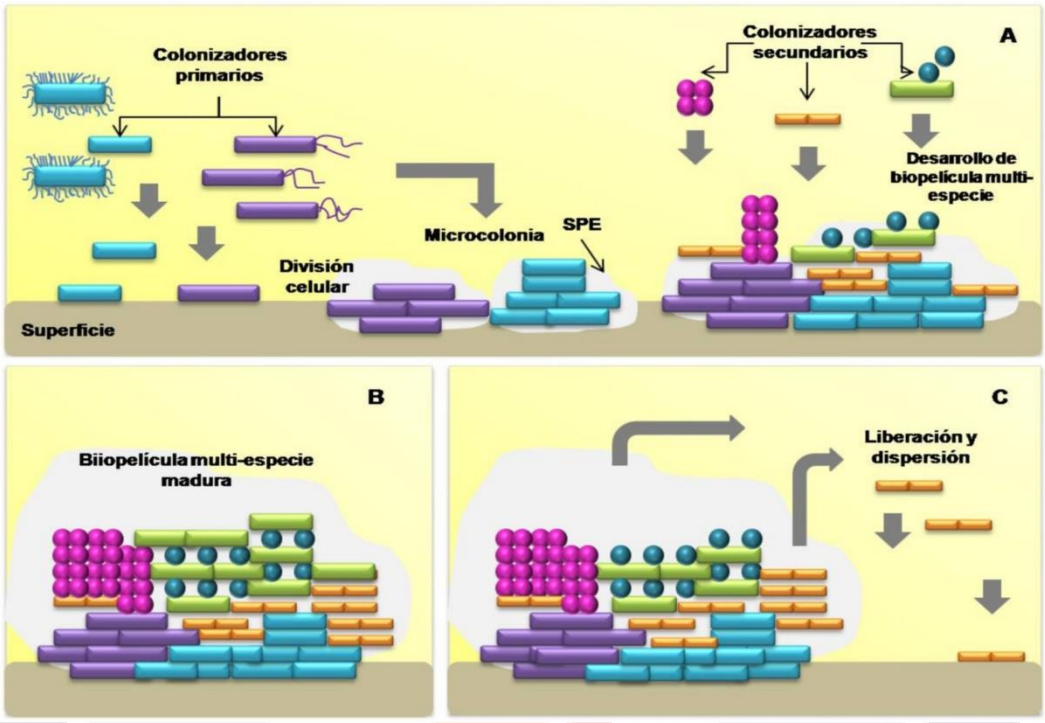


Figure 1: Formation of a multi-species biofilm: A) The primary colonizers adhere and cover a surface (live or inert), promoting growth, cell proliferation, extracellular matrix production and microcolony formation, secondary colonizers arrived and adherence to the biofilms to promote B) the growth and maturation of the multi-species biofilm, C) promote cell dispersal and planktonic cells to change cell biofilm (Loera-Muro *et al*., 2012).

1.3 Structure of Biofilm.

Each bacteria species form biofilms with different structure. All biofilms consist of sessile bacteria (15-20%), attached to an extracellular matrix and may contain up to 97% to water (Donlan, 2002). The matrix is formed for exopolysaccharides, which constitute its fundamental component, produced by the own microorganism. Others components such as proteins, DNA, surfactants, lipids, glycolipids, membrane vesicles and ions (Ca^{2+}) have been identified in the matrices of biofilm (Jacques *et al*., 2010). The groups of exopolysaccharides, nucleic acids, proteins, etc., are called extracellular polymeric substances (EPS). The DNA that is part of the extracellular matrix, in addition to helping microbial adhesion, it also helps to increase the genetic versatility of the consortium, allowing greater horizontal gene transfer (Almeida *et al*., 2011; Ganguly & Mitchell, 2011; Trappetti *et al*., 2011). In this matrix also can be exist no bacterial matter, such as minerals,

sediments, or blood components, as the environment in which the biofilm develops. In addition, EPS may be associated with metal ions and divalent cations. EPS may have neutral or polyanionic charge, depending on the type of exopolysaccharide, allowing them to interact with different antimicrobials, so that they can be trapped in the matrix without capacity to act on the bacteria (Post *et al*., 2004).

The biofilm structure is not strong. Bacteria living in biofilms on cell towers that extends in three dimensions from the surface to which they are attached. These towers are composed for bacterial microcolonies of different cells, both aerobic and anaerobic enclosed by exopolysaccharides, and separated from each other by interstitial void spaces, called water channels or waterways, that allow the flow of fluid and act as a primitive circulatory system for transport and diffusion of nutrients and oxygen to the bacteria located on the inside, even those located in deeper areas of the biofilm. They also provide a mechanism for remotion of metabolic waste products (Donlan, 2002; Ganguly & Mitchell, 2011). These channel no preventing that inside of the biofilm exist zones with different concentration of nutrients, pH or oxygen. For this reason, bacteria located on the up are more active than the bacteria located on the inside. In the latter, the bacteria must adapt to reduced oxygen availability (Sanderson, 2006).

These exopolysaccharides can make up a crucial part of the extracellular polymeric substance (EPS) associated with biofilm development that serves to cement whole bacterial populations to a surface rather than enclosing individual cells. While surface-associated exopolysaccharides and capsules play a role in both extracellular and intracellular adherence during the conversion from planktonic to biofilm growth. Human pathogens associated with biofilm development include species of *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. These ‘‘ESKAPE’’ pathogens are the leading causes of nosocomial infections and are so-named to emphasize their ability to ‘‘escape’’ the effects of antimicrobial treatment due to acquisition of resistance genes as well as formation of biofilms. Bales *et al*. (2013), made a glycosyl composition analysis where found a high total mannose content across all strains tested with *P. aeruginosa* and *A. baumannii*

exopolysaccharides comprised of 80 – 90% mannose, *K. pneumoniae* and *S. epidermidis* strains containing 40 – 50% mannose, and *E. coli* with 10% mannose. Galactose and glucose were also present in all eight strains, usually as the second and third most abundant carbohydrates. N-acetylglucosamine and galacturonic acid were found in 6 of 8 strains, while arabinose, fucose, rhamnose, and xylose were found in 5 of 8 strains.

One of the most common and most extensively studied matrix exopolysaccharides is a polymer of β-1, 6-Nacetyl-D-glucosamine called poly-glucosamine (PGA), poly-N-acetylglucosamine (PNAG) or polysaccharide intercellular adhesin (PIA). Several bacterial species such as *E. coli*, *S. aureus*, *Staphylococcus epidermidis*, *Yersinia pestis*, *Actinobacillus* spp., *Aggregatibacter actinomycetemcomitans* and *Bordetella* spp. produce PGA in their biofilm matrices. Another exopolysaccharides that is commonly found in biofilm matrices is cellulose, a linear polymer of β-1-4-linked glucose. Cellulose is found in some strains of *E. coli*, *Salmonella*, *Citrobacter*, *Enterobacter* and *Pseudomonas*. Alginate, a polymer of β-1-4-linked mannuronic acid and guluronic acid, is found in *P. aeruginosa* (Jacques *et al*., 2010).

Extracellular DNA (eDNA) has recently been shown to be an abundant component of many single and multi-species cultured biofilms (Dominiak *et al*., 2011; Ali Mohammed *et al*., 2012; Jakubovics *et al*., 2013; Tang *et al.*, 2013). eDNA is also present in natural environments and engineered systems in considerable amounts where it can form a significant source of organic nutrients. In marine sediments, the concentration can be 2 mg per g-L dry soil, and eDNA can comprise more than 70% of the total DNA pool. Another example is the top of deep sea sediments; it can contain an estimated 0.45 gigatons of eDNA, constituting approximately 50% of the total phosphorous pool for the resident micro-organisms (Dominiak *et al*., 2011; Jakubovics *et al*., 2013). The highest amount of eDNA was found in and around the microcolonies of denitrifiers belonging to the genera *Curvibacter* and *Thauera*, the ammonium-oxidizing *Nitrosomonas* and the nitriteoxidizing *Nitrospira*. Also, in others biofilms from *P. aeruginosa*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Staphylococcus epidermidis*, *Shewanella* sp., *Acinetobacter calcoaceticus* and *Bacillus subtilis* was found eDNA in the matrix. Some authors have concluded that

eDNA in *Staphylococcus* biofilms primarily originated from cell lysis (microbial fraticide), and was thus a natural and inevitable part of biofilm development. Recent experiments furthermore demonstrated that *cidA*-controlled cell lysis plays a significant role during development of *S. aureus* biofilms and that released genomic DNA is an important structural component. However, the discovery of eDNA in young *Pseudomonas* biofilms, where lysis is not a dominant process, suggests that lysis is most probably only part of the answer. In *P. aeruginosa* several biochemical pathways leading to eDNA production have been reported, such as the excretion of double-stranded DNA from living cells, release of vesicles containing DNA from living cells, and prophage-mediated lysis of a sub-population of cells. The production of eDNA in *P. aeruginosa* and other bacteria has also been linked to quorum sensing signals (Dominiak *et al*., 2011; Tang *et al*., 2011). The structure of this eDNA is reported to be double-stranded and largely similar to chromosomal DNA of the organism. It is also possible that eDNA differs chemically from DNA inside cells, for example by having different patterns of methylation. Clearly, there are many unanswered questions about the structure of eDNA within biofilms (Dominiak *et al*., 2011**;** Jakubovics *et al*., 2013). The role of eDNA in biofilms appears to be many. Studies of *P. aeruginosa* have documented the importance of eDNA for surface attachment and biofilm strengthening. Similar discoveries have been made for other bacteria, e.g. *S. epidermidis*, *Streptococcus*, *Bacillus cereus* and marine photosynthetic bacterium *Rhodovulum* sp. Extracelular DNA can also act as a nutrient source during starvation periods, indispensable link in phosphorus cycling in sea sediments and in natural DNA transformation in single-species biofilms of *Acinetobacter calcoaceticus* and *B. subtilis*. Extracellular DNA may also be a source of genes in the horizontal gene transfer (Dominiak *et al*., 2011**;** Ali Mohammed *et al*., 2012; Jakubovics *et al*., 2013; Tang *et al.*, 2013). Many bacteria produce extracellular deoxyribonuclease (DNase) enzymes that are apparently tightly regulated to avoid excessive degradation of the biofilm matrix. Interfering with these control mechanisms, or adding exogenous DNases, could prove a potent strategy for controlling biofilm growth. For example, characterization of biofilm-inhibiting compounds from marine bacteria identified a potent anti-biofilm molecule produced by an isolate of *Bacillus licheniformis* recovered from the surface of seaweed (Tang *et al*., 2010). After fractionation, the active agent was shown to be the DNase enzyme NucB. NucB dispersed

biofilms formed by *Micrococcus luteus*, *E. coli*, *B. subtilis* or *B. licheniformis*, indicating that eDNA is critical for stabilizing biofilms of each of these organisms (Jakubovics *et al*., 2013).

Biofilms provide an excellent environment for DNA exchange because cells are in close proximity and DNA can be trapped within the extracellular matrix. Indeed, horizontal gene transfer between oral streptococci in biofilm communities has been reported, and many genera of oral bacteria, including *Actinomyces*, *Bifidobacterium*, *Fusobacterium*, *Haemophilus*, *Peptostreptococcus*, *Streptococcus* and *Veillonella*, contain conjugative transposons that facilitate the DNA transfer between bacteria through conjugation (cell–cell mating). Analysis of the genomes of sequenced oral bacteria suggests that past horizontal gene transfer events account for between 5% and 45% of genes in different species. The transfer of DNA between different strains of *P. gingivalis*, and between *P. gingivalis* and *E. coli*, seems to occur by conjugation (Kolenbrander *et al*., 2010).

The proteins are other main component by the matrix biofilms. For example; small curli subunits (CsgA) are secreted to the extracellular space where they polymerize into the amyloid and contribute the major proteinaceous component of the *E. coli* and *Salmonella enterica* serotypes Typhimurium biofilm matrix. Curli are crucial in these biofilms and mediate initial surface attachment and provide a scaffold for the community. Whilst highly stable models of bacterial amyloids have been proposed, the molecular details that underlie these processes are poorly understood (Garnett & Matthews, 2012). Dispersin B is an extracellular enzyme (PDB: 1YHT) secreted by *A. actinomycetemcomitans* and can degrade matrix polysaccharides. This is a classic example of enzymatic disruption of the biofilm matrix (Jacques *et al*., 2010; Garnett & Matthews, 2012). In *Vibrio cholerae* biofilm formation involves the production of Vibrio polysaccharide (VPS) and three matrix proteins (RbmA, RbmC, and Bap1) predicted to contain carbohydrate-binding domains (Berk *et al*., 2012). Wu *et al*. (2013) and Tremblay *et al.* (2013), reported that the biofilm matrix of *A. pleuropneumoniae*, *S. suis* and different species of *Staphylococcus*, are composed of proteins.

1.4 Interactions in Biofilms.

For binding of microorganisms to a surface and subsequent formation of a biofilm, the bacteria need make sure they have made contact. For can do this, bacteria requires coordinated chemical signals that allow them to communicate with each other. The development of cell-to-cell interactions is facilitated by the close proximity between bacteria in the biofilm. This relationship, called ***quorum sensing***, benefits allow the bacteria to sense the presence of microorganisms neighbors, determine the density of the existing population and respond to any changing conditions. The ***quorum sensing*** process works because each bacterium that binds to a surface produces a signal molecule, called autoinducer, so that the more bacteria attach, increasing the local concentration of this signal. Once this is achieved, different phenomena are induced in the bacteria, eventually triggering a wide variety of biological process, like bioluminance production, biofilm formation, virulence factors expression, etc. Its purpose is to coordinate certain behaviors or actions mediate a wide range of intra and interspecific interactions census the population density between microorganisms (Yang *et al.*, 2011; Bordi & Bentzmann, 2011). The principal molecules used to communicate with other bacteria (autoinducers) are acyl-homoserine lactones (AHL), which predominate in Gram-negative bacteria; modified oligopeptides while prevalent in Gram-positive bacteria. In some plant pathogens, AHL-negative mutants show defects in pathogenicity, so it is expected that disrupting or manipulating quorum sensing signals could inhibit the expression of virulence and infection of host cell (Rashid *et al*., 2011). Bacteria also have a receptor that can specifically detect the respective self-inductor. When it binds to the receptor activates transcription of certain genes, including those for the synthesis of the inducer (Thomas & Nakaishi, 2006). Using the model system of marine *V. harveyi*, Bassler and coworkers led to identification of autoinducer-1 (AI-1)-dependent intra-species communication pathway and AI-2-dependent interspecies communication system (Cao *et al*., 2011). For the latter, *luxS* gene product was determined to catalyze the last committed step of AI-2 biosynthetic pathway. No less than 55 bacterial species were suggested to harbor *luxS* orthologs, some of which exhibited AI-2-like activities. The receptor of AI-2 is the kinase of LuxPQ two component system in *Vibrio* species, whereas it is a ABC-type transporter, Lsr in *E. coli* and *S. typhimurium*.

Interestingly, LuxS/AI-2 systems seemed to play multiple/varied roles in different bacterial species: 1) it regulates growth of *Neisseria meningitidis*; 2) it is associated with biofilm formation in *Streptococcus gordonii*, *Streptococcus mutans*, *Salmonella enterica* serotype Typhimurium, *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, *Vibrio cholera*, *Helicobacter pylor*i and *Klebsiella pneumoniae*; 3) it contributed to virulence in *Streptococcus pneumoniae*, *E. coli* 0157:H7 (EHEC) and *Serratia marcescens* ATCC 274; 4) it controls antibiotics susceptibility in *Streptococcus anginosus*; and 5) it is involved in motility in *Campylobacter jejuni* and *Helicobacter pylori*. Recently, presence of a *luxS* homologue was suggested in strain H19801 of *S. suis* 2, and AI-2 activity was also observed (Donlan, 2002; Cao *et al*., 2011).

Moreover, we can find other ways to regulate the interactions and communication between microorganisms that form biofilms, which are: 1) two component system (TCS), 2) extracytoplasmic function (ECF), and 3) other molecules of small molecular weight, which can include the c-di-GMP (Jonas *et al.*, 2009; Bordi & Bentzmann, 2011).

Two-component system (TCS) and extracytoplasmic function (ECF) signaling pathways are the major signaling mechanisms used by bacteria to monitor external and internal stimuli (e.g., nutrients, ions, temperature, redox states) and translate these signals into adaptive responses. The TCS pathways (Figure 2A) include two proteins: a histidine kinase (HK) protein, called "sensor," and a cognate partner, called "response regulator" (RR). Upon detection of the stimulus, the HK is activated and auto-phosphorylates on a conserved histidine residue. The phosphoryl group is then transferred onto a conserved aspartate residue on the cognate RR (Stock *et al*., 2000; Bordi & Bentzmann, 2011). Phosphorylation results in RR activation, which is most frequently a transcriptional regulator. As an example, the GacS (HK)/GacA (RR) TCS is one of the major systems involved in the control of *P. aeruginosa* biofilm formation (Brenci *et al*., 2009; Bordi & Bentzmann, 2011).

The second major signaling mechanism used by bacteria and probably underestimated is the ECF signaling pathway, which involves an alternative sigma factor,

an anti-sigma factor located preferentially in the cytoplasmic membrane, sequestering and inhibiting its cognate sigma factor and one or several periplasmic or outer membrane proteins required for the activation of the pathway. Upon perception of the extracellular signal by the periplasmic or outer membrane proteins, degradation of the anti-sigma factor induces releasing of the sigma factor, which can promote the transcription of a specific set of target genes (Bordi & Betzmann, 2011). In *P. aeruginosa*, for example, AlgU ECF sigma factor controls production of the EPS alginate, which further impacts biofilm architecture (Hay *et al*., 2009). The AlgU sigma factor functions with the antisigma MucA, which C-terminal periplasmic domain is cleaved by the AlgW protease in response to an unknown signal (Figure 2B) (Cezairliyan & Sauer, 2009; Bordi & Bentzmann, 2011).

Finally, among signaling molecules is the intracellular second messenger cyclic-dimeric guanosine monophosphate (c-di-GMP) (Figure 2D). An intracellular second messenger unique to bacteria, c-di-GMP, has gained appreciation as a key player in adaptation and virulence strategies, such as biofilm formation, persistence, and cytotoxicity. C-di-GMP is synthesized from two GTP molecules by diguanylate cyclase (DGC) enzymes containing GGDEF domains consisting of approximately 170 amino acids. Conversely, c-di-GMP is degraded by phosphodiesterase (PDE) enzymes containing EAL or HD-GYP domains that are approximately 250 amino acids in length. In general, increased intracellular c-di-GMP levels resulting from higher diguanylate cyclase activity lead to enhanced biofilm formation and inhibit flagellar and pilus-mediated motility. Conversely, low levels of the nucleotide associated with active phosphodiesterase catalysis suppress the maintenance of extracellular adhesins and promote biofilm dispersion and bacterial virulence (Bordi & Bentzmann, 2011; Krasteva *et al*., 2012; Srivastava & Waters, 2012).

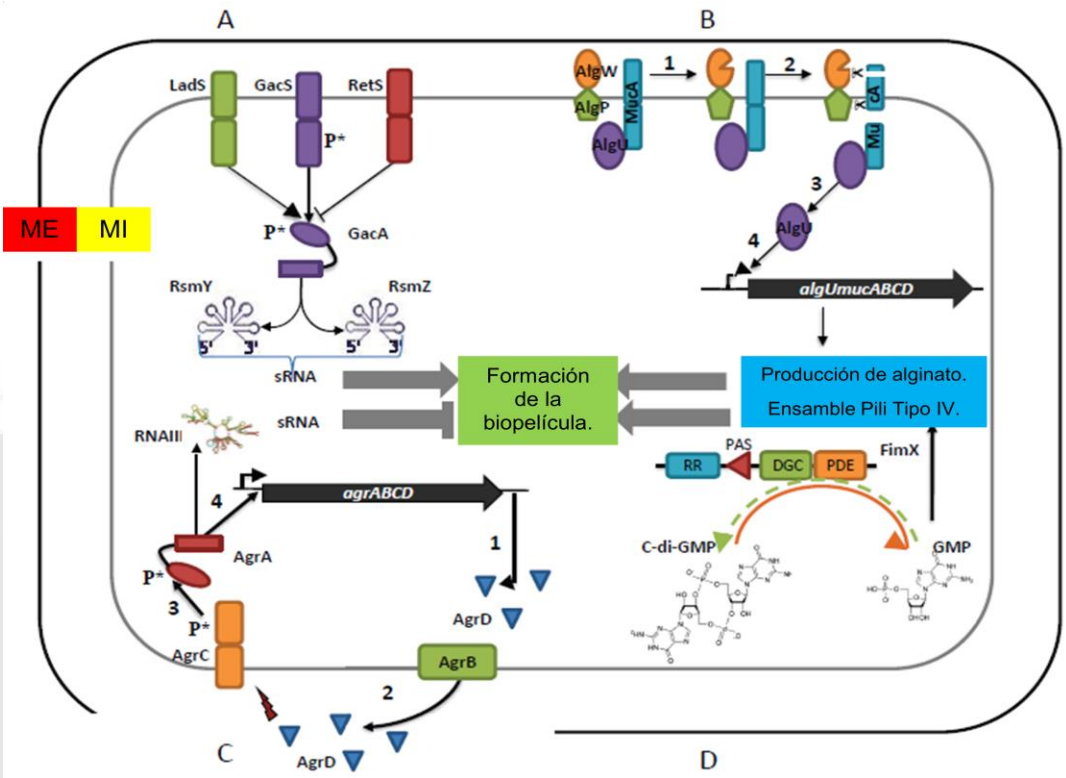


Figure 2: Regulatory networks controlling transition between planktonic and biofilm lifestyle. The external lines represent the inner membrane (IM) and outer membrane (ME) according to the Gram positive bacteria (C) and Gram negative (A, B and D). A) Control of biofilm formation in *P. aeruginosa* through the TCS GacS (HK)/GacA (RR). B) Control of EPS alginate in *P. aeruginosa*, which further impacts biofilm architecture by the system ECF sigma factor AlgU - anti-sigma MucA - AlgP (IM)-AlgW (periplasmic) complex. C) Control of biofilm formation in *S. aureus* through QS. D) Control of biofilm formation in *P. aeruginosa* through the second messenger pathway of c-di-GMP (Bordi & Betzmann, 2011; Loera-Muro *et al*., 2012).

1.5 Associations in Multi-species Biofilms.

Regularly biofilms are a multi-species microbial society, with its own rules and behavior patterns that favor the success of the biofilm (Parsek & Greenberg, 2005). The cooperative associations are highly extended within these microbial consortia are essential for their survival. Many of these associations are involved in the degradation and bioremediation processes such as denitrification through the *Nitrosomonas* and *Nitrobacter*. Moreover, these associations typically allow a spatial organization of different species in the multi-species biofilm, which ensures efficient dissemination of organic compounds (Yang *et al*., 2011). Also, the associations in multi-species biofilms may promote resistance

to antimicrobial agents. For example, *Candida albicans* has been reported to induce resistance in *S. aureus* to vancomycin during multi-species biofilms formed by these two species (Harriott & Noverr, 2010). There may also be competitive relationships within multi-species biofilms (Rendueles *et al*., 2011). The microorganisms competing for nutrients and attempt to inhibit the growth of other species. For this, many microbial species secrete toxins that kill or inhibit the growth of other (Yang *et al*., 2011). For example, *E. coli* secrete substances during the formation of the biofilm inhibiting the growth of *S. aureus* and other Gram positive bacteria, but not Gram negative bacteria (Rendueles *et al*., 2011).

1.6 Advantages of Biofilms.

The bacteria biofilm have a gene expression differences from their planktonic counterparts, bacteria causing phenotypically distinct with respect to the latter. It has been found that up to 30% of genes may be expressed differently among the same conditions developed in planktonic or bacteria biofilm. Biofilms have a very dynamic environment, where genetic material is exchanged as plasmids, transposons, or other molecules such as enzymes. For example, recent studies postulate that the matrix of biofilms of *P. aeruginosa, Bordetella* ssp., *Enterococcus, Listeria, Staphylococcus* and *Bacillus,* among other, contain deoxyribonucleic acid (eDNA) as main constituent during different stages of biofilm (Thomas *et al*., 2008; Nijland *et al*., 2010; Conover *et al*., 2011). These studies combined with others show a rate of gene transfer mediated by plasmids is greatly increased among bacteria in biofilms. This suggested that genes redistribution among these is a continuous process with important implications for evolutionary adaptation (Almeida *et al*., 2011; Ganguly & Mitchell, 2011; Trappetti *et al*., 2011).

As mentioned above, biofilms help increase resistance to antibiotics by bacteria. These bacterial biofilms found in natural and pathogenic conditions are formed in the presence of multiple species and genetically distinct strains (Pereira *et al*., 2010). In general, the increased resistance of bacteria is due to: 1) Difficulty of diffusion of antibiotics into them and electrostatic charge of the exopolysaccharides which attract

oppositely charged antibiotics. 2) A low growth rate. 3) Changes in phenotype acquired by bacteria forming biofilms. 4) Inactivation of antibiotics by polymers and enzymes secreted by bacteria. They are also resistant to antibodies, bactericidal enzymes and disinfectants such as hypochlorite and glutaraldehyde (Loera-Muro *et al*., 2008; Loera-Muro *et al*., 2012; Jolivet-Gougeon & Bonnaure-Mallet, 2014).

1) Difficulty of diffusion of antibiotics into the biofilms: The bacteria in biofilms are embedded in a matrix consisting of exopolysaccharide, extracellular DNA, and protein. This matrix limits antibiotic diffusion. The negatively charged exopolysaccharide can effectively prevent positively charged aminoglycoside molecules from penetrating by binding the antimicrobial (Maddox, 2011). Ganeshnarayan *et al*. (2009) menttioned that the poly-*N*-acetylglucosamine, the major biofilm matrix componentin the biofilms of *A. pleuropneumoniae* and other bacteria, contribute with the biocide resistance. However, many studies have surprisingly shown that the penetration of antibiotics is not limited in bacterial biofilms (Ciofu & Tolker-Nielsen, 2011; Bordi & De Bentzmann, 2011). For example, fluoroquinolones diffuse rapidly within *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* biofilms, tetracycline diffuses rapidly in *Escherichia coli* biofilms, and vancomycin diffuses rapidly in *Staphylococcus epidermidis* biofilms (Bordi & De Bentzmann, 2011). The above suggests that low metabolic activity and oxigen limitation appear to be more relevant to biofilm tolerance than restricted antimicrobial penetration (Ciofu & Tolker-Nielsen, 2011; Maddox, 2011).

2) Low growth rate: The available evidence suggests that the differential physiological activity seen in biofilms is caused by limited oxygen and nutrient penetration through the biofilm due to bacterial consumption (Ciofu & Tolker-Nielsen, 2011). To be fully effective, many antimicrobial agents (such as some cephalosporins, aminoglycosides and fluoroquinolones) require that the target bacteria be undergoing growth, as they preferentially act on cells undergoing division and multiplication. Consequently, the bacteria in such regions will be less affected by these specific antimicrobials as they are undergoing reduced cell division (Maddox, 2011; Marcinkiewicz *et al*., 2013).

3) Changes in phenotype acquired by bacteria forming biofilms: Differentiation in biofilm development has been explored in increasing detail since the 1980s. Studies from this period have resulted in various models that characterize biofilm development as a process of adaptation and changing genetic regulation (Stoodley *et al*., 2002). A further mechanism speculated to play a role in the increased resistance seen in bacteria in biofilms is the development of so-called "persister" bacteria (Maddox, 2011; Ciofu & Tolker-Nielsen, 2011). The pathways leading to the formation of persister cells are not known, but it has been proposed that they are connected to bacterial toxin/antitoxin systems in *E. coli*. Evidence has been provided that increased expression of toxins may block cell metabolism, so that random fluctuations in the expression of toxin and antitoxin genes can lead to the formation of persister subpopulations (Ciofu & Tolker-Nielsen, 2011; Bordi & De Bentzmann, 2011). Many toxin genes, activated upon degradation of the cognate antitoxin, encode mRNases that rapidly degrade mRNA, stopping translation and replication, thereby inducing antibiotic tolerance. Recently, several suggested pathways for persister formation implicate a role for oxidative stress in persister formation and survival. Oxidative stress is one of several signals that results in induction of the SOS response, which has been shown in *E. coli* to induce both β-lactam and fluoroquinolone antibiotic tolerance. More recently, the small molecule indole, induced by oxidative stress, has been implicated in persister formation (Grant & Hung, 2013). Finally, the upregulation of efflux pomp proteins and activation of quorum-sensing systems reduces and neutralizes incoming antimicrobial agents (Marcinkiewicz *et al*., 2013). A *P. aeruginosa* efflux pump that is expressed only during the biofilm mode of growth and mediates resistance to tobramycin, gentamicin, and ciprofloxacin in *P. aeruginosa* PA14 has been reported (Ciofu & Tolker-Nielsen, 2011).

4) Inactivation of antibiotics by polymers and enzymes secreted by bacteria: Certain gene products that are produced specifically in biofilms may exert unique functions that enhance the antibiotic tolerance of the biofilm. One example of a biofilm specific factor is the *ndvB* gene of *P. aeruginosa* PA14. This gene evidently encodes an enzyme involved in the synthesis of periplasmic glucans that binds tobramycin and prevents cell death most likely by sequestering the antibiotic (Ciofu & Tolker-Nielsen, 2011; Bordi & De Bentzmann, 2011). The *pmr*-operon encodes a system that adds aminoarabinose to LPS

thereby changing the charge and preventing the interaction between colistin and the surface of the bacteria (Ciofu & Tolker-Nielsen, 2011). The antibiotic degrading enzymes that play a role in the resistance of *P. aeruginosa* to antibiotics are the aminoglycoside-degrading enzymes and the β-lactamases. In biofilms that contain β-lactamase producing bacteria, penetration of β-lactam antimicrobials such as ampicillin can be restricted as the enzyme will degrade the antimicrobial molecule (Martínez *et al*., 2005; Ciofu & Tolker-Nielsen, 2011; Maddox, 2011).

Finally, a very important factor to be considered is the horizontal gene tranfer. The horizontal gene transfer is one of the main mechanisms of microbial adaptation and is responsible for the transfer of many different phenotypic traits including metabolic capabilities, virulence factors, heavy metal resistance and antibiotic resistance (Hannan *et al*., 2010). Biofilms are uniquely suited for horizontal gene trasnfer because they sustain high bacterial density and provide a stable physical environment with cell–cell contact (Hannan *et al*., 2010; Burmølle *et al*., 2014). Horizontal gene transfer through conjugation occurs in biofilms. This potentially results in new genetic combinations that could become problematic for human health. Biofilms also facilitate horizontal gene transfer by transformation. The large amount of extracellular DNA (eDNA) in biofilms is likely to be an important common source of usable genetic information for members of the biofilm community (Jakubovics *et al*., 2013; Burmølle *et al*., 2014). For example, the tetracycline resistance determinant (TetM) has been shown to be spread among different bacteria within oral biofilms by transformation (Burmølle *et al*., 2014).

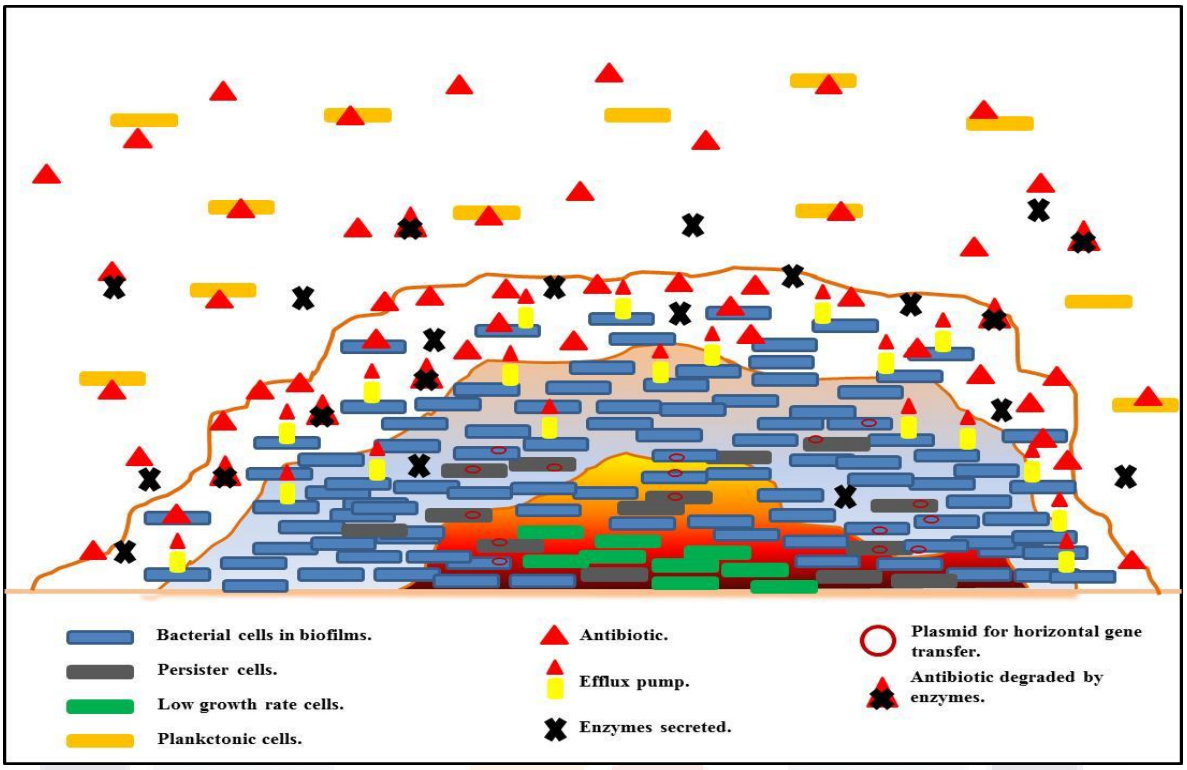


Figure 3: Antimicrobial resistance in biofilms. Resistance to antimicrobial agents is the most important cause of noneffective therapy of biofilm-associated infections. The increased resistance of bacteria is due to: 1) Difficulty of diffusion of antibiotics into them and electrostatic charge of the exopolysaccharides which attract oppositely charged antibiotics. 2) A low growth rate. 3) Changes in phenotype acquired by bacteria forming biofilms. 4) Inactivation of antibiotics by polymers and enzymes secreted by bacteria (Ramírez *et al*., in press).

In the last 10 years due to its overwhelming prevalence, biofilms have been increasingly recognized as important factors in the pathogenesis of many human persistent infections, including dental plaque, caries, periodontal infection, pneumonia in cystic fibrosis, chronic cystitis, endocarditis bacterial osteomyelitis and chronic prostatitis (Chen & Wen, 2011). In addition, biofilms allow the survival and spread of virus (Scraber *et al*., 2007). Some of these viruses tend to accumulate in biofilms belong to the genera caliciviruses and nodovirus (Loera-Muro *et al*., 2008).

Biofilms have been studied for decades using various *in vitro* models, but it remains debatable whether such *in vitro* biofilms actually resemble *in vivo* biofilms in chronic infections. *In vivo* biofilms share several structural characteristics that differ from most *in vitro* biofilms (Bjarnshol *et al*., 2013).

1.7 Porcine Respiratory Disease Complex (PRDC).

Respiratory disease in pigs is common in modern pork production worldwide and is often referred to as porcine respiratory disease complex (PRDC). PRDC is polymicrobial in nature, and results from infection with various combinations of primary and secondary respiratory pathogens. As a true multifactorial disease, environmental conditions, population size, management strategies and pig-specific factors such as age and genetics also play critical roles in the outcome of PRDC. Pathogens involved in respiratory disease in pigs vary significantly among farms, production sites, regions and countries, making generalizations about porcine respiratory disease complex (PRDC) treatment and control difficult. The interactions that occur on the cellular and molecular levels during concurrent infection of pigs with two or more respiratory pathogens are multifaceted and complex. Morbidity rates associated with PRDC may range from 30 to 70% and mortality rates between 4 and 6% or even higher in affected farms (Opriessnig *et al*., 2011).

There are a variety of viral and bacterial pathogens commonly associated with PRDC. The main associated bacteria are *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Mycoplasma hyopneumoniae* and *Haemophillus pasaruis*. Respiratory pathogens can be further divided into primary pathogens (*A. pleuropneumoniae*, *B. bronchiseptica* and *M. hyopneumoniae*), capable of inducing severe lesions in respiratory tissues as a result of their own virulence, and secondary or opportunistic infectious pathogens that typically need help from other co-infecting pathogens or cofactors to induce substantial lesions in the respiratory system (*S. suis*, *P. multocida* and *H. parasuis*) (Bochev, 2007; Opriessnig *et al*., 2011).

1.8 *Actinobacillus pleuropneumoniae.*

A. pleuropneumoniae is the etiologic agent of porcine contagious pleuropneumonia, an infectious respiratory disease of swine, which causes important worldwide economic losses in the pig industry (Negrete-Abascal *et al.*, 2003; Ramjeet *et al.*, 2008; Auger *et al.*, 2009; Gouré *et al.*, 2009; Deslandes *et al*., 2010; Ohba *et al.*, 2010; Buettner *et al.*, 2011; Li

et al., 2011; Li *et al*., 2012; Sadilkova *et al.*, 2012). This disease is highly contagious and deadly, causing great economic losses (Buettner *et al*., 2011). The administration of antimicrobial agents is the most common and effective method to treat and control this deadly disease (Yang *et al*., 2010). Injuries caused by these bacteria in the lung are characterized by hemorrhage and necrosis (Buettner *et al*., 2011). This pathogen is commonly isolated from the nasal cavity, middle ear cavity and lugs of infected animals, as well as liver, bone, pericardium and tendons (Ohba *et al*., 2010; Heegaard *et al*., 2011).

A. pleuropneumoniae is a Gram negative coccobacillus bacteria, pleomorphic, facultative anaerobic, non-spore-forming, encapsulated and belonging to the family *Pasteurellaceae* (Enríquez *et al*., 2003; Xu *et al*., 2008; Labrie *et al*., 2010; Ohba *et al*., 2010; Klitgaard *et al*., 2012). Two biotypes have been described based on dependence of nicotinamide adenine dinucleotide (NAD). Similarly, fifteen serotypes have been recognized by the capsular antigens compositions with distribution in different countries (Yang *et al*., 2010; Perry *et al*., 2011). Serotypes 1 to 12 and 15 usually belong to biotype 1, which contain NAD-dependent strains and are commonly found in pneumonias. Serotypes 13 and 14 are usually biotype 2 and are independent of NAD (Jacques, 2004; Serrano *et al.*, 2008; Gouré *et al.*, 2009; Deslandes *et al.*, 2010). However, variants have been reported belonging to biotype 2 serotype 2, 4, 7, 9 and 11 (Gouré *et al.*, 2009; Maldonano *et al.*, 2009; Deslandes *et al.*, 2010; Perry *et al.*, 2011). All serotypes are obligate pathogens, but have differences in virulence and geographic distribution. Serotypes 1, 5 and 7, are found predominantly in North America, the serotype 2 is the most commonly found in Europe and serotypes 1, 3, 4, 5 and 7 are typically isolated in China (Jacques, 2004; Xu *et al.*, 2008; Buettner *et al.*, 2011). Moreover, atypical *A. pleuropneumoniae* serotype 13 have been described in North America, Canada and USA, these strains being dependent NAD (Biotype 1) and phenotypically and antigenically different to those found in Europe, including reference strain (Perry *et al.*, 2011). With respect to Mexico, the serotypes 1a, 3, 5a, 5b and 7 belonging to biotype 1 are generally found (Williams *et al.*, 2000; Serrano *et al.*, 2008) and subtype 1a belongs to serotype 1 has been associated with several cases of acute infections. In Mexico, serotypes belonging to biotype 2 have not been reported yet (Serrano *et al.*, 2008).

Many virulence factors have been reported in *A. pleuropneumoniae* including lipopolysaccharide (LPS), exotoxins (Apx), capsule polysaccharide, proteases (Negrete-Abascal *et al.*, 1998; García-González *et al.*, 2004; Serrano *et al.*, 2008; Li *et al*., 2012), urease, iron acquisition systems and enzymes involved in anaerobic respiration also contribute to the disease (Lone *et al.*, 2009; Chiers *et al.*, 2010; Li *et al.*, 2011). Recently, some adhesion structures as type IV pilus (Stevenson *et al.*, 2003; Boekema *et al.*, 2004; Li *et al.*, 2011), Flp pilus (Auger *et al*., 2009, Li *et al*., 2011), autotransporters of adhesins (Baltes & Gerlach, 2004, Li *et al*., 2011) and biofilm formation (Auger *et al*., 2009, Labrie *et al*., 2010; Li *et al*., 2011) were also found to be associated with infection processes. However, Apx toxin, are major virulence factors involved in pathogenesis pleuropneumoniae (Bossé *et al.*, 2002; Chen *et al*., 2011). Furthermore, Apx toxins are primarily responsible for the lesions observed in the lungs (Park *et al*., 2009; Chiers *et al*., 2010), being ApxI and ApxII toxins the most damaging. Therefore, it is known serotypes 1, 5, 9 and 11 are among the more virulent because they exhibit both toxins (Auger *et al*., 2009).

The Apx toxin belonging to RTX toxin family ("repeat in toxin"). These toxins are widely distributed in Gram negative bacteria. These toxins are called Apx by "*A. pleuropneumoniae* RTX toxin"; existing four of them, the first, ApxI is strongly hemolytic and cytotoxic, the second ApxII is weakly hemolytic and moderately cytotoxic, the third ApxIII, is not hemolytic but is strongly cytotoxic, and the fourth, the ApxIV, is weakly hemolytic but cytotoxic. It has been shown that the latter toxin ApxIV, is only expressed in animal infections but not *in vitro*. Different serotypes secrete different set of toxins causing variations in hemolytic and cytotoxic activity. These toxins are encoded by *apx* operon which consists of four genes usually arranged as continuous *apxCABD* (Xu *et al*., 2008; Weia *et al*., 2012).

Moreover, it is known that the major component of the extracellular matrix of the biofilm that forms *A. pleuropneumoniae* is poly-*N*-acetylglucosamine (PGA). However, recent studies have shown the existence of protein and eDNA in the matrix of this pathogen (Wu *et al*., 2013). The PGA is dependent biosynthesis of proteins encoded within the

pgaABCD operon (Kaplan *et al*., 2004). This operon can be regulated by various proteins, for example, H-NS regulates synthesis operon by repression of *pga*. Also, the sigma factor σ^E positively regulates expression of this operon, which indicates that the formation of biofilms in *A. pleuropneumoniae* is part of an extracytoplasmic stress response (Bosée *et al*., 2010). Other genes associated with biofilm formation in *A. pleuropneumoniae* are: *luxS* (Li *et al*., 2008), the two-component system ArcAB (Buettner *et al*., 2008) and a serine protease autotransporter (Grastreau *et al*., 2011). It has also been seen that when *A. pleuropneumoniae* biofilm is formed, has a greater resistance to different antibiotics. Archambault *et al*. (2011) reports that cells of this pathogen in biofilms presented from 100 to 30,000 times more resistant than their planktonic antimicrobial.

A. pleuropneumoniae has traditionally been considered a pathogen of pigs (Buettner *et al*., 2011) and their presence in the environment has been little studied. However, it is important to know whether this bacterium is able to persist only in the respiratory tract of pigs or if possible their survival in the environment, above, in order to improve control strategies and vaccination for this disease. Assavacheep & Rycroft (2012), investigated under controlled laboratory conditions the survival of *A. pleuropneumoniae* outside the pig. They found, which was capable of surviving 3-4 days in an aqueous suspension in the presence of NaCl and mucin; prolonging survival at low temperature and in dry conditions. Loera-Muro *et al*. (2013) reported the presence of bacteria *A. pleuropneumoniae* ApxIV positive in samples of drinking water in pig farms in the State of Aguascalientes, Mexico, and also infecting pigs in the same farm. This bacterium was found in a viable and cultivable state, suggesting, that can survive in the environment, specifically in the drinking water in pig farms located in Mexico, and that might be using the formation of biofilms for survival. This because, scanning electron microscopy, was detected the presence of biofilm on these samples and presenting the structure suggested the presence of bacteria surviving on them.

The possible presence of this pathogen in water samples from swine farms survive in biofilms is a major health problem to consider, because that could be a route of transmission of the disease among pigs. This type of transmission through water for

drinking by surviving bacteria in biofilms has already been reported for various species of bacteria belonging to the genus *Mycobacterium* (Vaerewijck *et al*., 2005) as well as *Helicobacter pylori*, for which, has been considered thus transmitted as one of the most important in developed countries (Giao *et al*., 2008). Likewise, Giao *et al.* (2010) reported that biofilms of *H. pylori* can survive the addition of low concentrations of chlorine, which is one of the most prevalent forms of drinking water treatment. Also reported the presence of bacteria belonging to the genus *Pseudomonas* in different water samples, which includes drinking water, where the same were resistant to several antibiotics (Vaz-Moreira *et al*., 2012). Moreover, it is known that the pathogen *Campylobacter jejuni* has the ability to form biofilm in the drinkers of the animals and that these biofilms may continue to serve as inoculum for animals. Likewise, environmental isolates obtained had a higher ability to adhere and form biofilms, but had a poor ability to colonize cells (Jacques *et al*., 2010).

Besides the above, another important factor to consider is that within biofilms may coexist different types of bacteria and viruses. Giao *et al*. (2010) mentioned that *H. pylori* was able to enter and survive in biofilms formed on samples previously drinking water treated with chlorine, which represents a health hazard. For *A. pleuropneumoniae,* has identified the PGA as the major component of adhesion in biofilms formed, and this is also used by other bacteria with same ability to form biofilms and can survive in the environment, such as *S. aureus, S. epidermidis, Yersinia pesti, Bordetella* spp. and *E. coli* (Izano *et al.*, 2007, Jacques *et al*., 2010), this could indicate that these biofilms found in samples of drinking water in pig farms could be formed by several species of bacteria, allowing them to both survival in adverse conditions, as in the case of *A. pleuropneumoniae*, which is considered an obligate pathogen, increasing the potential danger of pathogenic species (Almeida *et al*., 2011).

In the laboratory (unpublished data) found other bacterial species (*Pasteurella multocida* and *Streptococcus suis*) as well as virus like the porcine respiratory syndrome virus (PRRSV) and swine influenza virus (SIV) H1N2 and H3N2 types associated respiratory diseases survived in the environment in pig farms in the State of Aguascalientes. Some of these species are pathogenic for pigs only, but others, can also

affect humans. This marks the importance of multi-species biofilms in the environment of pig farms. Found pathogens such as *A. pleuropneumoniae* (in drinking water, food, soil and air), *Streptococcus suis* (water and soil) and *Pasteurella multocida* (water, food, soil, air and urine) in different parts of the environment surrounding the pig. The results so far suggest that the environment serves as a vehicle for dispersal these pathogens and is a niche in the future should be considered to reduce their prevalence. In order to demonstrate the pathogenicity of the species found in the environment of swine farms are corroborated the presence of *A. pleuropneumoniae, P. multocida, Haemophilus parasuis, S. suis* and *Mycoplasma hyopneumoniae* in nasal swabs. Additionally, also in nasal swabs were identified Virus reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV) (Loera-Muro *et al*., submitted).

Finally, Kaplan & Mulks (2005) show that biofilm formation is a prevalent phenotype among *A. pleuropneumoniae* field isolates, and that this phenotype may have been previously overlooked because of its tendency to be lost upon subculturing in broth. Biofilm formation may have relevance to the colonization, pathogenesis and transmission of this bacterium. Important to mention that all the strains that formed biofilms in microtiter plates also formed thick biofilms at the air–liquid interface when cultured in glass tubes with agitation.

1.9 *Streptococcus suis*.

Streptococcus suis is a Gram positive bacteria, facultative anaerobic, endemic to most countries, and is recognized as one of the leading pig respiratory pathogens and emerging zoonotic agent (Gu *et al.*, 2009; Wertheim *et al*., 2009; Hao *et al*., 2011; Kim *et al*., 2011; Li *et al*., 2012; Gottschalk *et al*., 2013). This pathogen is responsible for great economic losses in the swine industry, in addition, there are records of outbreaks in humans in different parts of the planet, through contact with pigs or their products (Feng *et al.*, 2010; Kim *et al.*, 2011). Also, this pathogen can be isolated from other ruminant animals such as cats, dogs, goats and horses (Wertheim *et al.*, 2009). *S. suis* infections are associated with different clinical conditions such as, encephalitis, meningitis, arthritis,

sepsis, endocarditis and abortions in pigs, and septicemia and meningitis in humans (Li *et al.*, 2012), can cause a rapid and progressive damage that can lead to death (Pachirat *et al*., 2012). In the event that *S. suis* fails to cause acute fatal septicemia, bacteria are able to reach the central nervous system via mechanisms that are only partially elucidated (Lachance *et al*., 2013). Thirty-five serotypes have been described (1-34 and 1/2), based on its capsular antigen (Gottschalk *et al*., 2012). However, few serotypes are responsible for infections in pigs, which include serotypes 1-9 and 14, where the serotype 2 is considered the most pathogenic for pigs and humans (Blume *et al*., 2009; Wertheim *et al.*, 2009; Hao *et al*., 2011; Calzas *et al*., 2013). In addition to serotype 2, *S. suis* serotype 14 has been described as being an important swine pathogen and an emerging zoonotic agent (Lachance *et al*., 2013; Van Calsteren *et al*., 2013). Serotypes 1, 2, 7, 9 and 14 are most commonly isolated from diseased pigs in Europe and serotypes 3 and 8 in North America (Li *et al*., 2012). In Asia, are commonly found serotypes 1/2, 2, 3, 4 and 7 (Wang *et al*., 2012). In fact, it has been shown that *S. suis* is the primary cause of adult meningitis in Vietnam, the secondary cause in Thailand and the tertiary cause in Hong Kong. Two deadly human outbreaks of *S. suis* occurred in China within the last years, with the atypical characteristic of most patients presenting a streptococcal toxic shock-like syndrome (STSLS) that had rarely been reported beforehand. Both outbreaks were caused by the same clonal epidemic *S. suis* strain, characterized as sequence type (ST) 7 by multilocus sequence typing (MLST), which is different from the classical highly virulent ST1 usually isolated in Europe (Lachance *et al*., 2013).

Little is known about the virulence factors of this pathogen, these include the capsular polysaccharide (CPS), the toxin suilysin (SLY), muramidase protein (MRP), extracellular proteins (EF), adhesins, extracellular proteins and associated cell wall, fibronectin and fibronectin binding proteins (FBP), serum opacity factor and arginine deiminase system (Gu *et al*., 2009). Some factors that are encoded by the *mrp* gene for MRP, *epf* encodes an EF and *sly* to cytotoxin (hemolysin) SLY (Onishi *et al*., 2012). This hemolysin (suilysin) seems to protect bacteria against complement-mediated uptake and killing by neutrophils, macrophages and dendritic cells (Lachance *et al*., 2013). The CPS, which defines the serotype, is considered as the major virulence factor (Calzas *et al*., 2013;

Van Calsteren *et al*., 2013). The structures of types 2 and 14 *S. suis* CPSs are composed of the monosaccharides glucose, galactose, *N*-acetylglucosamine, and rhamnose (for type 2 only) arranged into a unique repeating unit that also contains a side chain terminated by sialic acid. In fact, these streptococci are the sole Gram-positive bacteria possessing sialic acid in their capsules. Sialic acid forms an α-2,6 linkage with the adjacent galactose in *S. suis.* Also, sialic acid of bacterial polysaccharides has been suggested to be involved in immune evasion via several mechanisms (Calzas *et al*., 2013). Therefore, the importance of this pathogen pig plus it can cause severe economic losses in this sector and in recent times appears as a major public health problem, especially in countries in Southeast and East Asia, such as China, Vietnam and Thailand, and in some European countries (Wertheim *et al.*, 2009; Fittipaldi *et al*., 2010; Hao *et al*., 2011; Hao *et al*., 2011; Kim *et al*., 2011; Nga *et al*., 2011). In Mexico, Talavera *et al*. (2001) reports on a study of workers of different slaughterhouse in the center of the country had isolated serotypes 2 and 27 of these workers.

Recently have been studied the ability by this pathogen to form biofilms, observing that this property is restricted to a few strains. Additionally, this ability was induced by fibrinogen, but not by other mammalian protein, presenting an increase in resistance to antibiotics (Bonifait *et al*., 2008; Bonifait *et al*., 2010). Likewise, it has been shown *luxS* gene involvement in biofilm formation in this species. Wang *et al*. (2011) reported that *luxS* mutant exhibited a significant reduction in their ability to form biofilms, cell adhesion, hemolytic activity and transcript levels of several virulence factors. Moreover, it is known that the capsular polysaccharide (CPS) is a vital component of the extracellular matrix responsible for the formation of the biofilm, besides being an important virulence factor. Finally, it is known that bacteria in the biofilm are embedded in an matrix composed of a exopolysaccharide highly hydrated called glycocalyx and the capsule of *S. suis* serotype 2 is composed of glucose, galactose, *N*-acetylglucosamine, rhamnose and sialic acid (Wang *et al*., 2011).

Quorum sensing is a widespread chemical communication in response to fluctuation of bacterial population density, and has been implicated into bacterial biofilm formation

and regulation of expression of virulence factors. The *luxS* gene product, S-ribosylhomocysteinase, catalizes the last committed step in biosynthetic pathway of autoinducer 2 (AI-2), a signaling molecule for inter-species quorum sensing. A *luxS* homologue in 05ZYH33, an epidemic strain of *S. suis* serotype 2 (SS2) from China, was found. LuxS was determined to be required for AI-2 production in 05ZYH33 strain of *S. suis* serotype 2 (SS2). Inactivation of *luxS* gene led to a wide range of phenotypic changes including thinner capsular walls, increased tolerance to H_2O_2, reduced adherence capacity to epithelial cells, etc. In particular, loss of LuxS impaired dramatically its full virulence of SS2 in experimental model of piglets, and functional complementation restored it nearly to the level of parent strain. Genome-wide transcriptome analyses suggested that some known virulence factors such as CPS are down-regulated in the Δ*luxS* mutant, which might in part explain virulence attenuation by *luxS* deletion. Similarly, 29 of 71 genes with different expression level were proposed to be targets candidate regulated by LuxS/AI-2-dependent quorum sensing (Cao *et al*., 2011).

1.10 *Pasteurella multocida*.

Pasteurella multocida is a Gram negative coccobacillus belonging to the family *Pasteurellaceae*, facultative anaerobic and immobile, and can be alone, in pairs or as short chains (Chomnawang *et al*., 2009; Hotchkiss *et al*., 2011). There are three subspecies (ssp.) *P. multocida* ssp. *multocida*, *P. multocida* ssp. *gallicida* and *P. multocida* ssp. *septica*, being the subspecies *multocida*, the most commonly found in pigs (Chomnawang *et al*., 2009; Stahel *et al*., 2009). *P. multocida* is a pathogen associated with a variety of animal species. In pigs, induces pneumonia and progressive atrophic rhinitis (PAR) (Kim *et al.*, 2012). The most obvious symptoms of this disease include: shortening and twisting of the mouth, tears and dark spots usually appear at 8-12 weeks of age (Susan, 2000; Lee *et al*., 2012). Atrophic rhinitis rarely causes death, but it have economic importance because it significantly reduces the growth of infected animals (Harper *et al*., 2006; Kim *et al*., 2012). Anyway, the protection of animals with an efficient vaccination has been considered the most important and attractive method of controlling this disease (Lee *et al*., 2012).

The strains of this organism may be separated into groups A, B, D, E and F (cap genes) based on the antigenicity of the capsules, and based on their lipopolysaccharide antigens on serotypes 1 to 16 (Harper *et al*., 2006; Chomnawang *et al*., 2009; Campuzano *et al*., 2011; Liu *et al*., 2012). The composition and structure of capsular material found in subgroups A, D and F are similar to those found in mammalian glycosaminoglycans (Tang *et al*., 2009). Atrophic rhinitis in pigs is usually caused by a strain of *P. multocida* expressing a toxin (PMT) and typically belong to serogroup D and in general, it is known that strains have capsules are more virulent than those variants that do not possess (Kim *et al*., 2012; Lee *et al*., 2012; Liu *et al*., 2012). PMT toxin is a 146 KDa monomeric protein and is encoded by the gene *toxA*. This toxin is dermonecrotic and possesses usually weakly antigenic (Kim *et al*., 2012; Lee *et al*., 2012).

Toxin PMT is an AB-type toxin that causes pleiotropic effects in intoxicated host cells. PMT protein has four functionally defined regions: a N-terminal region that contains the receptor-binding/translocation domains (residues 1–568) and a C-terminal region that contains a C1 domain (residues 569–719) for membrane localization; a C2 domain (residues 720–1104) of unknown function; and a catalytic C3 domain (residues 1105–1284) with Ga-protein-deamidase activity (Brothers *et al*., 2013).

PMT has many properties that mark it out as a potential carcinogen. PMT is a highly potent mitogen and has been demonstrated to block apoptosis. PMT modifies and activates members of three of the four families of heterotrimeric G-proteins, all of which have potential roles in carcinogenesis. Many signalling components downstream of these G-proteins are known proto-oncogenes and have been shown to be activated by PMT. On the evidence so far, it would appear unlikely that PMT contributes significantly to human cancer. It is of interest that some human respiratory infections with *P. multocida* are suggested to be chronic, as this is a further characteristic that is shared by many carcinogenic agents (Lax, 2012).

P. multocida isolates are known to possess type IV fimbriae (pili) as one of the major virulence factor. Type IV fimbriae, encoded by the gene *ptfA* (~435 bp), is one of

major surface components of *P. multocida* known to frequently mediate colonization of host surfaces by adhesion. Fimbriae is composed of highly conserved 21-amino acid N-terminal sequences with repeated subunits of 15–20 kDa which has been previously isolated and sequenced from different *P. multocida* serotypes (A, B, D and F), indicated significant variation among strains. Multiple sequence alignment revealed highly conserved N-terminus α-1 helix region and heterogeneous C terminus (68–137 aa) comprised of β-strand regions (β1, β2, β3, β4) with conserved two pairs of cysteine residues (Shivachandra *et al*., 2013).

Although the biofilm mode could possibly be one of its virulence factors for survival inside host (Rajagopal *et al*., 2013), there are not many studies on biofilm formation in *P. multocida*. Olson *et al*. (2002) report that their formation was necessary to add fetal bovine serum medium and, to be tested with different antibiotics, did not show much difference from their planktonic counterparts, showing a greater tolerance only trimethoprim sulphadoxine.

1.11 *Bordetella bronchiseptica.*

Bordetella bronchiseptica is a Gram negative bacteria pathogen of respiratory tract and can infect a wide range of mammals (Irie *et al*., 2006; Kaut *et al*., 2011). *B. bronchiseptica* is also being increasingly isolated from humans mainly from immunocompromised patients (Nicholson *et al*., 2012). It is considered the main causative agent of atrophic rhinitis in pigs (Dugal *et al*., 1990; Register & DeJogn, 2006; MacArthur *et al.*, 2011) along with *P. multocida* (Posá *et al*., 2011). Infections with this microorganism are transmitted by aerosol and bacteria can be localized in epithelial cells invade the respiratory tract without invading the tissue (Kaut *et al*., 2011). Clearly is described that the damage to the respiratory tract for the disease is fundamentally caused by dermonecrotic toxins (Posá *et al*., 2011).

A majority of its virulence determinants are controlled by a two-component signal transduction system, BvgAS (for *Bordetella* virulence gene) (Irie *et al*., 2006). This locus

comprises a sensor kinase protein, BvgS, and a DNA-binding response-regulator protein, BvgA. In response to environmental cues, BvgAS controls the expression of a spectrum of phenotypic phases transitioning between a virulent (Bvg^+) phase and a non-virulent (Bvg^- phase), a process referred to as phenotypic modulation (Nicholson *et al*., 2012). In the virulent Bvg^+ phase, the response regulator BvgA becomes highly phosphorylated, and the transcription of various Bvg-activated genes confers on the bacterium its virulent phenotype. Virulence factors expressed in the Bvg^+ phase include adhesins such as filamentous hemagglutinin (FHA) and fimbriae as well as toxins/toxin delivery systems such as adenylate cyclase/hemolysin (CyaA) and type III secretion system. Conversely, BvgAS is inactive during the Bvg^- phase, resulting in the maximal expression of motility loci, virulence repressed genes (vrg), and genes required for the production of urease (Irie *et al*., 2006**;** Nicholson *et al*., 2012).

Recently studies showns that these bacteria are capable of living as biofilms on a number of abiotic surfaces (Parise *et al*., 2007). FHA is required for *B. bronchiseptica* biofilm formation and proposed that the expression of CyaA suppresses biofilm via its association with FHA. The biofilm phenotype being predominantly expressed only when *B. bronchiseptica* is grown in the Bvg^i phase but not in the Bvg^+ or Bvg^- phase (Irie *et al*., 2006). Flagella are necessary and enhance the initial cell-surface interactions, thereby providing mechanistic information on the initial stages of biofilm development for *B. bronchiseptica*. Biofilm formation by *B. bronchiseptica* involves the production of both Bvg-activated and Bvg-repressed factors followed by the repression of factors that inhibit formation of mature biofilms (Nicholson *et al*., 2012). Finally, Sisti *et al*. (2013) shows the presence of c-di-GMP regulatory signalling in *B. bronchiseptica*.

The extracellular components of *B. bronchiseptica* biofilm matrix revealed that the major sugar component in the matrix was xylose, and linkage analysis indicated a majority of it to be in a 4-linked polymeric form. The production of xylose was independent of Bvg regulation but instead was dependent on bacterial growth phase. In addition, *N*-acetylglucosamine (called Bps polysaccharide in *B. bronchiseptica*) in the matrix was found to be important for the initial development of the biofilm. These results suggest that

B. bronchiseptica biofilm formation is growth phase dependent in addition to being regulated by the Bvg virulence system (Irie *et al*., 2006). *Bordetella* Bps polysaccharide encoded by the *bpsABCD* locus is critical for the stability and maintenance of three-dimensional structures of biofilms. Bps is essential for the formation of efficient nasal biofilms and is required for the colonization of the nose. Likewise, the Bps polysaccharide has an essential role in the biofilm lifestyle for *Bordetella* in mammalian respiratory tracts and in the persistence of the nares (Sloan *et al*., 2007; Conover *et al*., 2012). *bpsA* is predicted to encode an outer membrane protein with four transmembrane domains, suggesting that it might mediate translocation and/or docking of Bps to the cell surface. BpsB contains a polysaccharide *N*-deacetylase domain and is homologous to IcaB and PgaB, which are involved in the deacetylation of PIA/PNAG and PGA, respectively. IcaB has crucial roles in biofilm formation, immune evasion, and virulence. BpsC is homologous to the glycosyltransferase 2 family of proteins and is predicted to encode a processive glycosyltransferase. IcaC of *S. epidermidis* and PgaC of *E. coli* are required for the complete synthesis of the longer oligomeric chains of PIA. Finally, IcaD and PgaD are required for optimal production of the PIA and PGA polysaccharides, respectively. *bpsD* does not show any significant similarity to either *icaD* or *pgaD* (Conover *et al*., 2012).

Like Bps polysaccharide, DNA is a significant component of *B. bronchiseptica* biofilm matrix. Addition of DNase I at the initiation of biofilm growth inhibited biofilm formation. Treatment of pre-established mature biofilms formed under both static and flow conditions with DNase I led to a disruption of the biofilm biomass. DNase I treatment of nasal biofilms caused considerable dissolution in biofilms formed in the mouse respiratory tract. These results suggest that eDNA is a crucial structural matrix component of both *in vitro* and *in vivo* formed *B. bronchiseptica* biofilms (Conover *et al*., 2011).

1.12 *Haemophilus parasuis.*

Haemophilus parasuis is a commensal bacterium that inhabits the airways of pigs, also belongs to the family *Pasteurellaceae* and like another member in the same family, *A. pleuropneumoniae*, its growth requires V factor (nicotinamide adenine dinucleotide, NAD)

but not X factor (hemin) (Jin *et al*., 2006; Blanco *et al*., 2008; Mullins *et al*., 2011; Zhang *et al*., 2012; Zhuo *et al*., 2012; Zou *et al*., 2013). Under certain circumstances becomes an opportunistic pathogen (Zhang *et al*., 2012). *H. parasuis* is a Gram negative bacterium that causes Glässer's disease or poliserositis, can also cause septicemia, arthritis, meningitis and pneumonia (Chu *et al*., 2011). Fifteen serotypes have been described, but above 25% of isolates are usually not established (Whang *et al*., 2012; Zhuo *et al*., 2012). Anyway, there is no clear correlation between virulence and serotypes. Little is known of the specific virulence factors of *H. parasuis* and recent studies are just beginning to reveal components involved in the mechanisms that cause the disease. Numerous genes have been identified that may be involved in virulence, including a variety of carriers, metabolic and biosynthetic enzymes, surface membrane proteins and some homologous genes apparently expressed by other members of the family *Pasteurellaceae* (Zhou *et al*., 2012). Vaccine immunity confers limited crossserotype protection (Jin *et al*., 2006).

H. parasuis can form biofilms and the relationship between biofilm formation and pathogenesis or persistent *H. parasuis* infection is unknown (Jin *et al*., 2006). Jin *et al*. (2006) tested the ability to form biofilms for a total of 80 field isolates and 15 reference strains of *H. parasuis*, by glass tube and polystyrene microtiter plate assays. A total 43% of field isolates, including strains representing 13 serotypes (except serotypes 3 and 8) and non-typable strains, exhibited the ability to form biofilms at different levels via polystyrene microtiter plate assays. Among the reference strains representing 15 serotypes, only serotypes 2, 9, 12, 13 and 15 could not form biofilms on the polystyrene surface. A total of 85% of the strains forming biofilms at air–liquid interfaces in glass tubes also formed biofilms on polystyrene surfaces. Generally, non-virulent serotypes showed a higher degree of biofilm formation than virulent serotypes.

Genes *galU* and *galE* are known as an important virulence factors in a number of Gram-negative pathogens. GalU is a UDP-glucose pyrophosphorylase responsible for the synthesis of UDP-glucose from glucose 1-phosphate and UTP, whereas UDP-glucose 40-epimerase (GalE) converts UDP-glucose to UDP-galactose. As substrates, UDP-glucose and UDP-galactose could participate in the biosynthesis of capsular polysaccharide (CPS)

which could contribute to complement resistance or extracellular polysaccharide (EPS) which was proposed to be involved in the formation of biofilm. Lack of expression of GalU protein by the *galU* mutant increased its tendency to autoagglutinate. The results indicated that the *galU* plays a role in autoagglutination and biofilm formation, while *galE* may affect the biofilm production indirectly (Zou *et al*., 2013).

1.13 *Mycoplasma hyopneumoniae.*

The mycoplasmas are prokaryotic pathogens of humans and other animals, distinguished by the lack of a cell wall, diminutive size, and a limited genome. They are parasitic obligate scavengers of numerous host factors required for growth. Colonization occurs predominantly at the mucosal surfaces of the genital and respiratory tracts and is a prerequisite for infection (Bolland & Dybvig, 2012). In the past few years, reports of zoonotic human mycoplasmas infections have increased markedly. Strains of *M. suis*, *M. haemofelis* and *M. ovis* have been isolated from diseased humans. Additionally a novel mycoplasma species, termed *Candidatus M. haemohominis*, was recently reported in a human subject and was associated with clinical symptoms of pyrexia and hemolytic anemia (Sokoli *et al*., 2013).

M. hyopneumoniae is the primary cause of enzootic pneumonia in pigs, a disease that causes significant worldwide losses in the swine industry due to reduced performance and increased medication (Liu *et al*., 2011; Villareal *et al*., 2012; Vranckx *et al*., 2012). Also, is the pathogen that contributes more to the porcine respiratory disease complex (PRDC) (Strait *et al*., 2008; Siqueira *et al*., 2011). *M. hyopneumoniae* is very difficult to detect an organism and is difficult to isolate in pure culture (Strait *et al*., 2008). Like other mycoplasmas, has a small genome with limited biosynthetic potential (Siqueira *et al*., 2011).

Some mycoplasmas have been shown to form biofilms on glass and plastic surfaces, and it has been determined whether they form biofilms on the tracheal epithelium. Biofilms formed *in vitro* protect mycoplasmas from the lytic effects of not only complement but also

the small antimicrobial peptide gramicidin (Simmons & Dybvig, 2007). In *M. hyopneumoniae,* biofilm formation has not been well studied.

In other mycoplasmas, as *M. pulmonis*, the causative agent of murine respiratory mycoplasmosis (MRM) and which is among the most serious of naturally acquired diseases of rodent colonies (Bolland & Dybvig, 2012), Simmons & Dybvig (2007) observed the biofilms formed on the epithelium of trachea in tracheal organ culture and in experimentally infected mice and found similar structure and biological characteristics as biofilms formed *in vitro*. This tracheal organ-mounting system can be used to study interactions between biofilms formed by respiratory pathogens and the host epithelium and to identify the factors that contribute to biofilm formation *in vivo*.

M. pulmonis that has a known role in avoiding killing by complement is the Vsa (variable surface antigen) protein, a size- and phase-variable lipoprotein exposed at the cellular surface. Cells producing a long Vsa with 40–60 tandem repeats are resistant to complement, while cells producing a protein with five or fewer repeats are susceptible. Variability in the number of repeats in the Vsa protein has multi-faceted roles in immune avoidance, adherence and biofilm formation (Bolland *et al*., 2012).

Bacterial polysaccharides are often virulence factors that can contribute to immune modulation, immune evasion, biofilm formation, and cellular adherence (Bolland & Dybvig, 2012). The polysaccharide EPS-I of *M. pulmonis* is predicted to be a linear chain of alternating residues of glucose and galactose, with galactose being the terminal sugar. The *M. pulmonis* EPS-I mutants encased within a biofilm are protected from complement, affirming the role of mycoplasma biofilms in providing protection from innate immunity (Bolland *et al*., 2012).

M. pulmonis that produced a long Vsa protein was found to attach to epithelial cells less robustly than did mycoplasmas producing a short Vsa. Thus, the length of the Vsa protein has a similar effect on the adherence of the mycoplasmas to epithelial cells as it does on the ability of the mycoplasma to form a biofilm. These results are in contrast to the

effect of the EPS-I polysaccharide, which has a negative effect on the ability of the mycoplasma to form a biofilm on abiotic surfaces, but a positive effect on cytoadherence (Bolland & Dybvig, 2012). Cells encased within a biofilm of *M. pulmonis* producing a short form of the Vsa protein were more resistant to complement and gramicidin than mycoplasmas that were dispersed. The resistance appeared to be localized to those mycoplasmas within tower structures of the biofilms. Biofilm formation may be a mechanism that protects mycoplasmas from host immunity (Simmons & Dybvig, 2007).

M. gallisepticum is the causative agent of chronic respiratory disease in chickens and of infectious sinusitis in turkeys, chickens, game birds, pigeons, and passerine birds of all ages (Chen *et al*., 2012). Chen *et al*. (2012) tested eleven strains of *M. gallisepticum* for their ability to biofilm formation, which varied considerably. Biofilm formation was significantly inhibited by 5% sucrose and 5 Mmol/L EDTA. Compared with the planktonic mycoplasma, these biofilm-grown cultures were more resistant to tetracycline, gentamicin, and Triton X-100 treatments. The transcriptions of some genes in the biofilm-grown cells were markedly decreased, including *vlhA3.03*, *csmC*, *hatA*, *gapA*, neuraminidase, and *mgc2*. Those results will benefit further research on the persistence of *M. gallisepticum* infections (Chen *et al*., 2012).

Henrich *et al.* (2010) worked with *M. salivarium*, preferentially an inhabitant of the human oral cavity and has rarely been found in other locations associated with disease. In this work detected *M. salivarium*, together with *Candida glabrata*, in an occluded biliary stent of an icteric, cholestatic patient.

In pigs, acute *M. suis* infection (infectious anemia in pigs, or IAP) manifests as hemolytic anemia and hemorrhagic diathesis accompanied by immune modulation and coagulation dysfunction due to intravasal coagulation and subsequent consumption coagulopathy. *M. suis* formed biofilm-like microcolonies on the surface of endothelial cells, and may represent a putative persistence mechanism of *M. suis* (Sokoli *et al*., 2013).

2. JUSTIFICATION.

Biofilms represent an ancient prokaryotic survival strategy (Almeida *et al*., 2011). This is because the bacteria are able to provide significant benefits to environmental protection from fluctuations in humidity, temperature, pH, concentrating nutrients, facilitating the elimination of waste, and above all, increase resistance to antibiotics and horizontal gene transfer (Bowen & Koo, 2009). The ability to form biofilms seems not restricted to any specific group of microorganisms, and today it is considered that suitable environmental conditions the vast majority of bacteria, regardless of the species, can exist within biofilm attached to a surface (Ganguly & Mitchell, 2011). Also, these bacterial biofilms can be formed by multiple genetically distinct strains and species (Yang *et al*., 2011). Moreover, biofilms play an important role in the pathogenesis of many infectious diseases (Chen & Wen, 2011).

In the case of pigs, porcine respiratory disease complex is the description that has been done to the series of changes that occur as a result of infections in the respiratory tissues of the pig (Brogden & Guthmiller, 2002). This term is appropriate because the swine diseases are mostly cases result from combinations of environmental factors and infectious agents that work together (Susan, 2000). On the other hand, is a proven fact the phenomenon of association of pathogens, both work in the field, as in controlled laboratory conditions (Brogden & Guthmiller, 2002). Within this complex, the bacteria *A. pleuropneumoniae, S. suis* and *P. multocida*, have a great relevance, as are major bacterial pathogens involved, resulting in Mexico and globally, large economic losses to the swine industry, and in recent years, the last two, begin to be considered important zoonotic agents in several countries, with a significant increase in reported cases of infectious disease in humans, with consequences that can lead to death of the individual.

In this study, our main objective is to analyze the presence of pathogens associated with multi-species biofilms in drinking water of swine farms in the Aguascalientes State, with greater attention to other pathogens that may be surviving in these structures together

with *A. pleuropneumoniae*, as found in previous studies in different parts of the environment surrounding the pig for Loera *et al*. (submitted), *Streptococcus suis* and *Pasteurella multocida*, among other pathogenic bacteria, as well as in commensal bacteria, such as *Escherichia coli*, which could allow the formation of biofilms by the survival of these pathogens in the environment as well virus could also be associated with these structures enable their survival in the same. This, in order to find the possible associations that may be exist between these microorganisms and get a clearer idea of the involvement of these microbial consortia in the survival of these pathogens and the spread of the diseases they produce.

3. HYPHOTESIS.

A. pleuropneumoniae, S. suis, P. multocida and *E. coli*, and other swine respiratory pathogens and commensal bacteria, are associated with multi-species biofilms in drinking water in swine farms in the state of Aguascalientes.

4. OBJECTIVES.

4.1 General Objective.

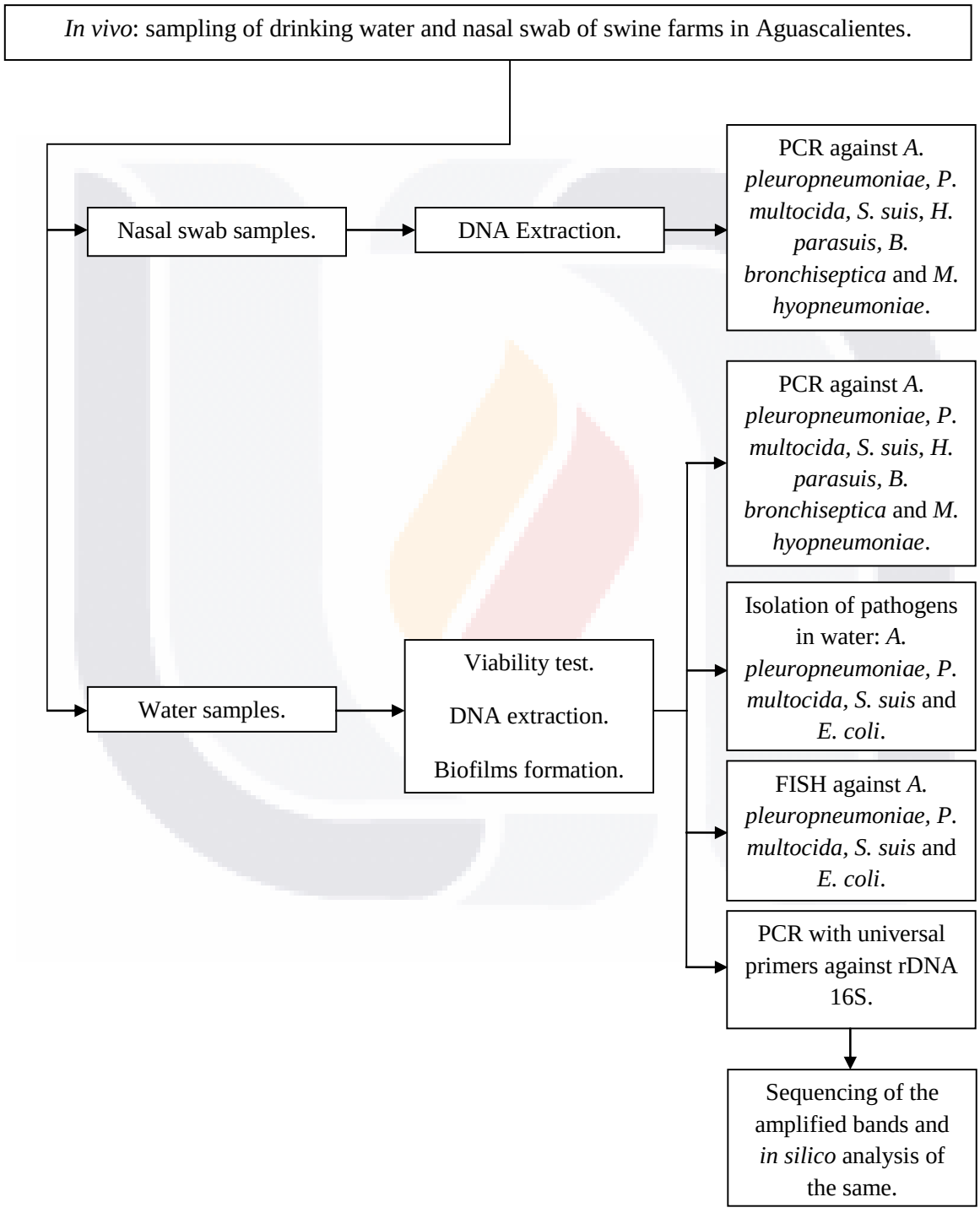
Evaluate the presence of pathogens associated with multi-species biofilms in drinking water in swine farms in the state of Aguascalientes.

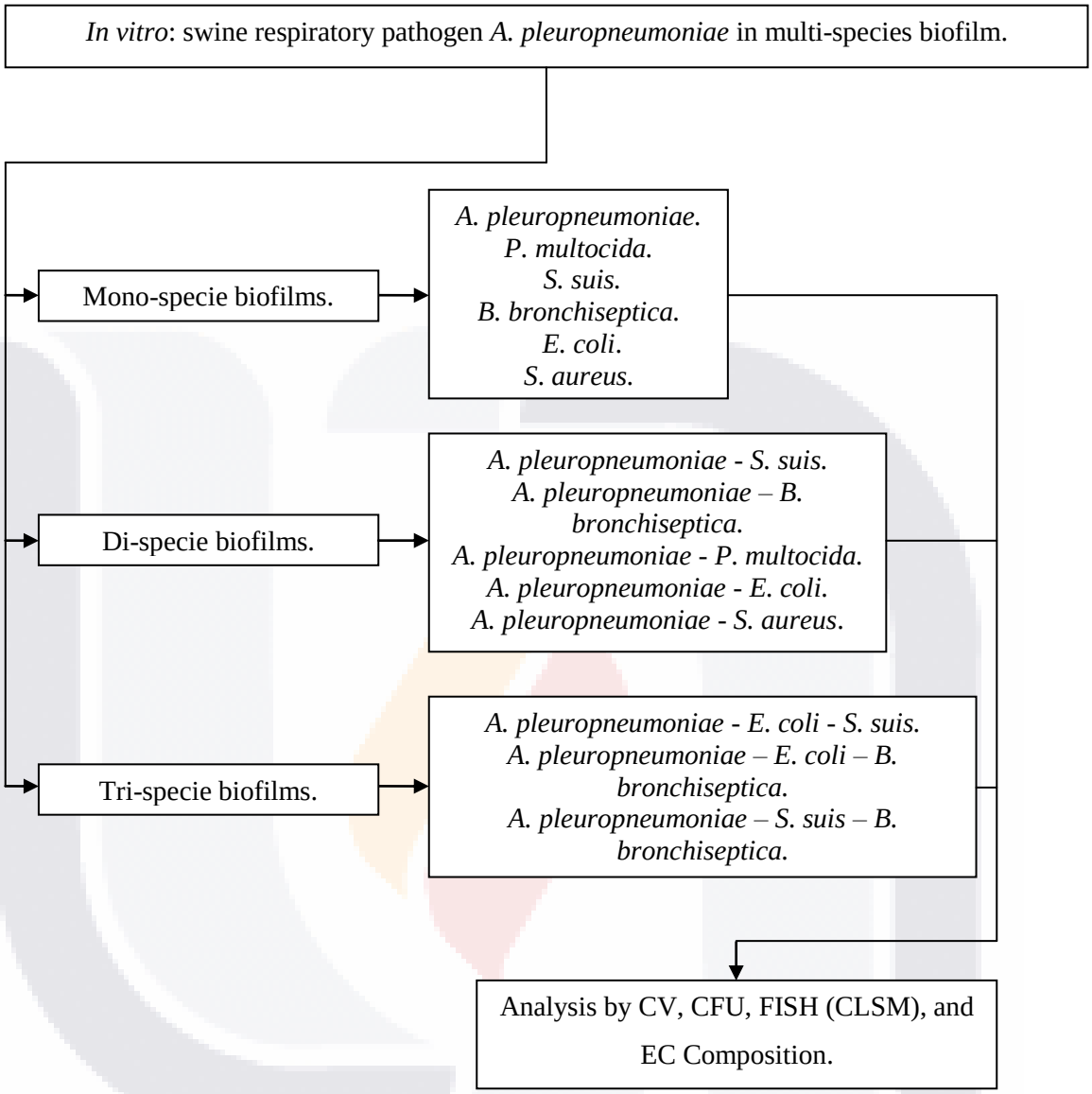
4.2 Specific Objectives.

- To identify bacterial pathogens in drinking water in swine farms using the technique of polymerase chain reaction (PCR).
- To identify porcine pathogens in multi-species biofilms present in drinking water from swine farms using techniques of fluorescence in situ hybridization (FISH).
- To identify non-pathogenic or commensal bacteria that could be related to multi-species biofilms in drinking water in swine farms by PCR.
- To analyze the associations of porcine pathogens and commensal bacterial in multi-species biofilms in drinking water in vitro and in vivo.

5. MATERIALS AND METHODS.

5.1 General scheme of work.





5.2 Protocol for sampling of swine farms.

Have been selected 14 farms producing pigs in the state of Aguascalientes, with the cooperation of the MVZ Hector Gutiérrez and owners thereof. The farms selected are shown in the Table 1 and their location can be seen in the Figure 3.

Table 1: Farms selected for analysis of associated pathogens in multi-species biofilms in drinking water in the state of Aguascalientes.

Number of Farm.	Localization.
1	Granja Fátima.
2	Granja Fátima.
3	Granja Fátima.
4	South of the Aguascalientes city.
5	La Concepción.
6	La Concepción.
7	San Carlos.
8	La Posta, UAA.
9	Los Arquitos.
10	Los Arquitos.
11	Hacienda Chichimecos.
12	San Antonio de los Arcones.
13	Jesus María.
14	Jesus María.

5.2.1 Sampling of swine farms:

First we scheduled the appointment (date and time of sampling) to the veterinarian or swine farm manager. The following material was prepared to visit and take samples: overalls or coat clean, clean boots, gloves, disinfectant, cooler, markers or markers to make signs, alcohol, cigarette lighter, vitafilm or parafilm, field notes, tubes with sterile swabs and vacuum tubes (50 mL) for water. Upon entering the farm, followed the orders of the farm managers and security (order, disinfection, use of overalls and boots, etc.). Samples were taken as follows:

Samples of water: the pacifiers were washed with an alcohol swab and sterilized with lighter, then the sample was collected in tubes of 50 mL after was left to run some water. We took about 25 mL per pacifier, to get as much cover as possible in the farm pacifiers. In farms without pacifiers, we took water from the drinkers where the pigs drink water (directly from the soil, of drinkers, etc.). The tubes were marked with the number of farm and tube number. In the laboratory, the tubes were stored at room temperature until processing.

Nasal swabs: the swab was introduced into the nasal cavity of the pig softly, turning to enter easily into the cavity, pulling in the same way, and left the sample in a sterile tube. The pigs were randomLy selected, ensuring the collection of samples from pigs of different ages and weights, covering best around the farm. Each tube is marked with the number of the farm followed by a period and the number of tube following a consecutive order (example: 1.1, 1.2, 1.3 ....). The tubes were kept in the cooler until it reaches the laboratory. In the laboratory, the tubes were stored at 4 ° C until processing. After each sampling, all the material was cleaned upon arrival at the laboratory.

5.2.2 Statistical calculation for sampling nasal swabs.

For calculated the sample size forthe detection of *A. pleuropneumoniae* was used according to Alvarez *et al*. (2004) for detect the presence of Aujeszky's disease virus

(pseudorabies) for a study in Yucatan, Mexico, where they analyzed the presence of three pathogens in the porcine respiratory disease complex, the swine flu, *A. pleuropneumoniae* and *M. hyopneumoniae*; briefly considered an expected prevalence of 8%, although in our studies we observed that the prevalence rate is higher for *A. pleuropneumoniae*, a confidence index of 95%, sampling within farms and random error 0.05. For this, the calculation was performed by the statistical formula is as follows.

$$\mathbf{N=}\ \frac{(Z_{\alpha/2}{}^2\ (pq)}{E^2} \qquad \mathbf{N=}\ \frac{(1.96)^2\ ((0.08)(0.92))}{(0.05)^2}\ \mathbf{113}$$

IC = 95%	q = 0.92
$Z_{\alpha/2}$ = 1.9	E = 0.05
p = 0.08	

N = 113, this is the minimum of all samples required to detect A. pleuropneumoniae, taking into account what was said by Alvarez *et al*. (2004).

Number of farms: 14.

Samples of nasal exudate farms: the 12 samples on average per farm beyond what is necessary according to the statistics.

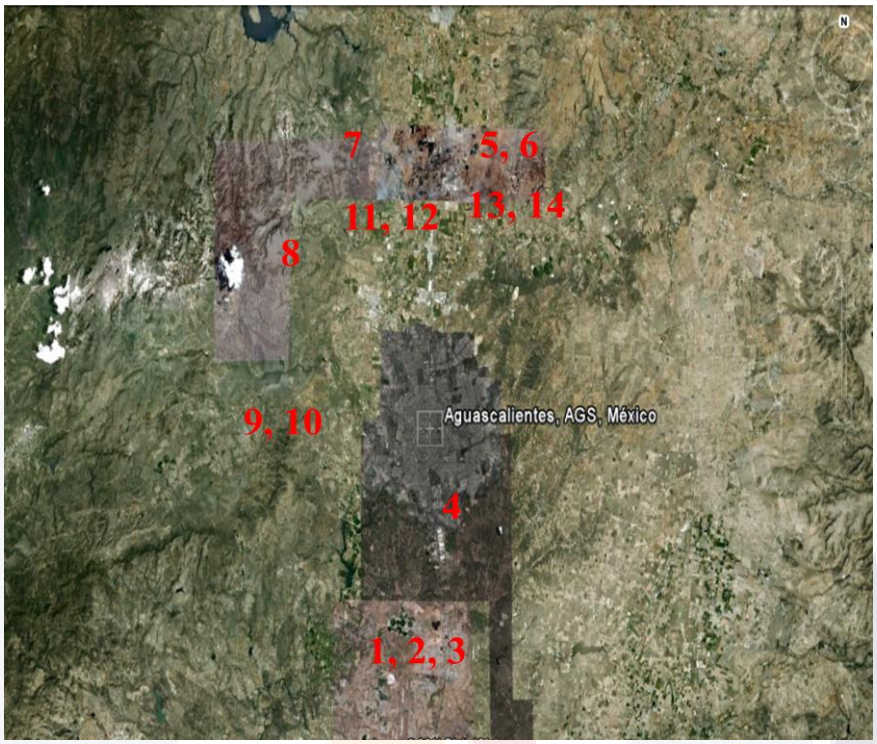


Figure 4: Localization of the farms selected for the study: 1, 2 and 3) Granjas Fátima 4) south of the city of Aguascalientes, 5 and 6) la Concepción 7) San Carlos, 8) La Posta, UAA, 9 and 10) Los Arquitos, 11) Hacienda Chichimecas 12) San Antonio de los Arcones, 13 and 14) Jesus María.

5.3 Bacterial Viability Live/Dead *Bac*Light Bacterial Viability Kit (Molecular Probes).

First the water sample was centrifuged selected consumption (on average 30 mL total, depending on the waste water) at 5000 rpm for 10 min in tubes cornell (two centrifugations of 15 mL each). To each tube was added 15 mL of each tube with 50 mL water to try to cover all the tubes from each farm. The pellet was resuspended in 1 mL of sterile distilled water. Subsequently combined in equal volumes of reagents A and B the Live/Dead Bac Light Bacterial Viability Kit (Molecular Probes) and mixed liberally (dependent on the total amounts of samples to perform). Three µL were added to this mixture to the sample and allowed to incubate for 15 minute period in the absence of light. The samples were mounted in Prolong gold (3 µL of Prolong-Gold + 3 µL of sample, ratio 1:1). We placed the drop of Prolong-gold on the slide and then placed on the side of the sample, mixed with the tip of the micropipette. Carefully the samples were covered with a

coverslip and sealed with nail polish. Filter for fluorescein-living (green) and rhodamine-dead (red) were used.

5.4 Extraction of DNA (Sambrook & Russell (2001), with modifications).

a) For samples inoculated into culture medium:

Directly to the microbiological plate were added 3 mL of lysis buffer, (15% sucrose, lysozyme (0.03 mg/mL USB Corporation), 0.05 M EDTA and 0.3 mM Tris, pH 8). Then the sample was resuspended and passed a 1 mL eppendorf tubes. For each 1 g. of sample were added 3 mL of lysis buffer, mixed vigorously and spent 1 mL in eppendorf tubes. Then, vortex 30 sec was given.

b) For water samples:

Thirty milliliters of water was centrifuged for 15 min at 5000 rpm (Universal 320R, Hettich). The supernatant was discarded and the pellet was resuspended in 1 mL of lysis buffer. Then, vortex 30 sec was given.

c) For nasal swabs:

Swabs were cut in a pre-labeled eppendorf tube with tweezers and scissors sterilized by flame. Then in the tubes with the swabs were directly added 1 mL of lysis buffer. Then, vortex 30 sec was given.

d) Liquid cultures:

Three milliliters of bacterial culture was centrifuged for 10 min at 5000 rpm (Universal 320R, Hettich). The supernatant was discarded and the pellet was resuspended in 1 mL of lysis buffer. Then, vortex 30 sec was given.

Subsequently, all samples are processed the same way. The samples were incubated for 30 min at 37 °C. Following incubation, to each sample were added 100 µL to SDS 10% (0.1 v / v), 100 µL NaCl 5 M (0.1 v/v) and 5 µL Proteinase K (0.4 mg/mL; USB Corporation). This mixture was stirred and incubated for 1 h at 50 °C. Five hundred

microliters of supernatant was taken and adds 200 µL of phenol-chloroform-isoamyl alcohol (24:24:1, Gibco BRL). Then, vortex and centrifuged for 5 min at 12000 rpm. The supernatant was recovered and 200 µL of ammonium acetate (7.5 M) and 450 µL absolute ethanol (1V) were added. The samples was stirred for investment for 5 min and allowed to precipitate overnight at 4 °C. Then, the samples were centrifuged at 4 °C for 15 min at 12000 rpm. The supernatant was discarded and the pellet was washed with 100 µL of ethanol 70%, centrifuged for 5 min at 12000 rpm at 4 °C. The pellet was dried at 37 °C and resuspended in 50 µL of sterile distilled H_2O. The DNA was stored at -20 °C until use.

5.5 Bacterial strains used for amplification, CAMP test, and growth conditions.

All strains used in this study as positive control belong from Dr. Mario Jacques laboratory, Université de Montréal. Strains controls were: *A. pleuropneumoniae* serotype 1 strain 4074, serotype 1 strain 719 (Dr. Slavic, Ontario Veterinary College, University of Guelph, ON), serotypes 3, 4 and 10, *S. suis* serotype 2 strain 735, *H. parasuis* serotype 5 strain Nagazaki, *P. multocida* 4 strain 4056 (type D, DNT +), *M. hyopneumoniae* ATCC 25095, *B. bronchiseptica* strain 276, *E. coli* ATCC 25922 and strain L17608, and *S. aureus* strain 154N. *A. pleuropneumoniae* serotype 1-4074, 1-719, 3, 4 and 10 were grown on brain heart infusion agar plates (BHI; Bioxon, Mexico) supplemented with 15 µg NAD mL^{-1} incubated at 37 °C overnight. A colony was transferred into 5 mL of BHI broth with 15 µg NAD mL^{-1} and incubated at 37 °C overnight with agitation. This culture was used for the DNA extraction, and serotype 1-4074 was used in the biofilms assay like positive control. For *S. aureus* was grown on BHI incubated at 37 °C overnight.

5.6 Protocol for detection of porcine respiratory pathogens by PCR (Polymerase chain reaction).

5.6.1 Protocol for PCR against *A. pleuropneumoniae apxIV* gene:

The PCR was performed using the method of Schaller *et al*. (2003), with modifications by MacInnes *et al*. (2008) and Loera-Muro *et al*. (2013). Amplification of the DNA was performed in 12.5 µl of a reaction mixture that contained were used: 1.25 µL of a mixture of primers at a concentration of 100 ng/µL each (ApxIVAN-1L: GGG GAC CGG TGA ACT GTA TT, ApxIVAN-1R: GCT CTC TTG CAC CGT CAA), 6.25 µL of Master Mix (Fermentas), 1 µL distilled H_2O and 4 µL of DNA problem. The amplification conditions were: 1 cycle for 1 min at 95 °C, 35 cycles of denaturation for 30 seconds at 94 ° C, 35 cycles of 30 seconds alignment at 54 °C, 35 cycles of 1.5 min to elongation at 72 °C and a final elongation cycle at 72 °C for 5 min. All amplifications were visualized in 1.5% agarose gel electrophoresis. Amplified band of 377 bp.

5.6.2 Protocol for PCR against toxigenic *P. multocida*:

The PCR against toxigenic strains of *P. multocida* was developed under the terms of Kamp *et al*. (1996). Amplification of the DNA was performed in 20 µl of a reaction mixture that contained: 2.5 µL 10X PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP's, 0.3 mM of each primers (TOXA Set 1-1F: GGT CAG ATG ATG CTA GAT ACT DC, TOXA Set 1-1R: CCA AAC AGG TTC ATA TGG GTT AC), 12.75 µL distilled H_2O, 2 µL of sample DNA and 0.25 µL Taq DNA polymerase (5U/µL). The amplification conditions were: 1 cycle for 1 min at 95 °C, 32 cycles of denaturation for 30 seconds at 95 °C, 32 cycles of 60 seconds to alignment to 65 °C, 32 cycles of 2.5 min to elongation at 72 ° C and a final elongation cycle at 72 °C for 20 min. All amplifications were visualized in 1.5% agarose gel electrophoresis. Amplified band of 338 bp.

5.6.3 Protocol for PCR for *S. suis*:

The multiplex PCR for identification of *S. suis* serotype 2 or any serotype was developed based on the method of Marois *et al*. (2004). Amplification of the DNA was performed in 20 µl of a reaction mixture that contained: 2.5 µL 10X PCR (NH_4) buffer, 2.5 mM $MgCl_2$, 600 µM of each dNTP's, 1.1 µM of each primers CPS2J (CPS2J-F: GTT GAG TCC TTA TAC ACC TGT T, CPS2J-R: CAG GTC AAA ATT ATT CAC CAT C), 600 nM of each primers 16S RNA (16S F: CAG CAT TAT CCG GGT TTA AGA TAT, 16S R: GTA AGA CAA TAC GTG CGT AGA A), 2.0 µL of sample DNA and 0.25 µL Taq DNA polymerase (5U/µL). The amplification conditions were: 1 cycle for 1 min at 95 °C, 40 cycles of denaturation for 30 seconds at 94 °C, 40 cycles of 30 seconds to alignment to 60 °C, 40 cycles of 1 min to elongation at 72 °C and a final elongation cycle at 72 °C for 10 min. All amplifications were visualized in 1.5% agarose gel electrophoresis. Amplified band of 294 for all serotypes and 459 bp for serotype 2.

5.6.4 Protocol for PCR against *B. bronchiseptica*:

The PCR was performed using the method Hozbor *et al.* (1999), and Resgister & DeJong (2006). Amplification of the DNA was performed in 20 µl of a reaction mixture that contained: 2.5 µL 10X PCR (KCl) buffer, 2.5 mM $MgCl_2$, 400 mM of each dNTP's, 1.0 mM of each primers (Fla 2: AGG CTC CCA AGA GAG AAA GGC TT, Fla 4: CTG CCC CGC TGG TAT C), 2 µL of sample DNA and 0.25 µL Taq DNA polymerase (5U/µL). The amplification conditions were: 1 cycle for 1 min at 95 °C, 35 cycles of denaturation for 60 seconds at 94 °C, 35 cycles of 30 seconds to alignment at 53 °C, 35 cycles of 20 seconds to elongation at 72 °C and a final elongation cycle at 72 °C for 5 min. All amplifications were visualized in 1.5% agarose gel electrophoresis. Amplified band of 237 bp.

5.6.5 Protocol for PCR against *M. hyopneumoniae*:

The PCR was developed using the method of Cai *et al*. (2007). Amplification of the DNA was performed in 20 µl of a reaction mixture that contained: 2.5 µL 10X PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 mM of each primers (MH649-F: GAG CCT TCA AGC TTC ACC AGG A, MH649-R: TGT AGT GTT GAC TGC CAC TTT C), 2.0 µL of sample DNA and 0.25 µL Taq DNA polymerase (5 U/µL). The amplification conditions were: 1 cycle for 1 min at 95 °C, 35 cycles of denaturation for 20 seconds at 94 °C, 35 cycles of 30 seconds to alignment to 60 °C, 35 cycles of 40 seconds to elongation at 72 °C and a final elongation cycle at 72 °C for 7 min. All amplifications were visualized by in 1.5% agarose gel electrophoresis. Amplified band of 237 bp.

5.6.6 Protocol for PCR against *H. parasuis*:

The PCR was developed based on the method of Oliveira *et al.* (2001), and MacInnes *et al.* (2008). Amplification of the DNA was performed in 22 µl of a reaction mixture that contained: 2.5 µL 10X PCR (KCl) buffer, 1.2 µL $MgCl_2$ 25 mM, 2 µL of dNTP's 2 mM, 2 µL of a mixture of primers to 100 ng/µL each one (HPS-F: ATG GTG AGG AAG GGT GGT GT, HPS-R: GGC GTC TTC ACC CTC TGT), 2 µL of DNA sample and 0.3 µL Taq DNA polymerase (5U/µL). The amplification conditions were: 1 cycle for 1 min at 95 °C, 35 cycles of denaturation for 30 seconds at 94 °C, 35 cycles of 30 seconds to alignment at 59 °C, 35 cycles of 2 min to elongation at 72 °C and a final elongation cycle at 72 °C for 5 min. All amplifications were visualized in 1.5% agarose gel electrophoresis. Amplified band of 821 bp.

5.6.7 Protocol for PCR with universal primers against 16S rDNA:

For detection of 16S rDNA from drinking water samples we use the primers described by Nadkarni *et al*. (2002). Amplification of the DNA was performed in 20 µl of a reaction mixture that contained: 12.5 µL Master Mix (Fermentas), 0.9 µM forward primer (16S rDNA F: TCC TAC GGG AGG CAG CAG T), 0.6 µM reverse primer (16S rDNA R:

GGA CTA CCA GGG TAT CTA ATC CTG TT) and 2 μL of DNA sample. The amplification conditions were: 1 cycle for 2 min at 50 °C, 1 cycle for 10 min at 95 °C, 40 cycles of denaturation for 15 seconds at 95 °C, 40 cycles of 1 minute to alignment at 60 °C, 40 cycles of 1 minute to elongation at 72 °C and 1 cycle of final elongation at 72 °C for 5 min. All amplifications were visualized in 1.5% agarose gel electrophoresis. The bands were purified by QIAquick Gel Extraction Kit under conditions recommended by the manufacturer for subsequent sequencing. Sequencing was performed at the National Laboratory of Agricultural Biotechnology, Medical and Environmental (LANBAMA), "Instituto Potosino de Investigación Científica y Tecnológica" (IPICYT), San Luis Potosi, Mexico.

5.6.8 PCRs again 16S rDNA and *apx* toxin genes:

PCR detection of 16S rDNA of samples isolated was doing in the Molecular Biology Diagnostic Laboratory of Veterinary Medicine Faculty of Université du Montréal. For detection of genes *apxIA, apxIB, apxII* and *apxIII* of the Apx toxins from *A. pleuropneumoniae* were used the methodology described by Rayamajhi *et al*. (2005) with modifications. The PCR run conditions were: 94°C for 5 min followed by 30 cycles of 30 seconds at 94°C, 60 seconds at 69°C for *apxIB* and *apxIII*, at 66°C for *apxII* and at 72°C for *apxIA*, and 3 min at 72°C with a final elongation step at 72°C for 10 min. The primer sequences are shown in Table 2.

Table 2: Primers sequences used again *apx* toxins genes in this study.

Name.	Sequence (5'-3').	Product size (bp).
ApxIAF	ATCGAAGTACATCGCTCGGA	723
ApxIAR	CGCTAATGCTACGACCGAAC	
ApxIBF	TTATCGCACTACCGGCACTT	811
ApxIBR	TGCAGTCACCGATTCCACTA	
ApxIIF	GAAGTATGGCGAGAAGAACG	965
ApxIIR	CGTAACACCAGCAACGATTA	
ApxIIIF	GCAATCAGTCCATTGGCGTT	396
ApxIIIR	GACGAGCATCATAGCCATTC	

5.7 Protocol for isolation of different pathogens in drinking water in swine farms.

Samples were taken only where *A. pleuropneumoniae* was detected. The isolation was according to as described below for each pathogen isolated:

5.7.1 Isolation of *A. pleuropneumoniae*:

The positive samples of *A. pleuropneumoniae* were performed as follows: in an eppendorf tube one pool was performed with equal amounts of water tubes previously agitated. All water samples from each farm were took. Dilutions were made: 1:10, 1:100, 1:1,000 and 1:10,000, and 100 µl water consumption of each one were taken and inoculated into BHI and BHI + NAD plates. dilutions were made of drinking water, and for the leachate the determination was performed according to the methodology set out in Standard Methods (APHA-AWWA-WPCF, 1998) and the Mexican Standard NMX-AA-42, both was plated in BHI supplemented with 15 µg NAD mL^{-1} and BHI without supplementation with NAD. The plates were incubated at 37 °C for 24 hr. Suspect bacteria were plated on blood agar (5% sheep blood) with nurse strain of *S. aureus*, were subsequently incubated at 37 °C for 24 hours anaerobically. Colonies that had satellitism, beta hemolysis and bright, were considered suspect colonies to *A. pleuropneumoniae* and plated again in BHI with NAD by streaking dilution to obtain a positive samples. Gram staining, Catalase, Oxidase and β-hemolytic test were performed on colonies obtained from the cAMP test and those suspected to represent *A. pleuropneumoniae* were grown in BHI-NAD. For confirmation of *A. pleuropneumoniae*, was extracted DNA for implementing the technique of PCR. To confirm its NAD-independent growth, once confirmed by PCR, were grown in BHI + NAD again and BHI alone for 24 hours at 37 °C. Likewise, positive samples were lyophilized and placed in 30% glycerol and stored at -20 °C.

CAMP Test:

Colonies candidates of *A. pleuropneumoniae* were plated on blood agar. Then two lines with *S. aureus* were made traversing the entire plate. The procedure was repeated but

performing the center line only once with *S. aureus*, to have a negative control and with *A. pleuropneumoniae* (S-1-4074) was performed for positive control. The plates were lefts 24 hours at 37 °C in an anaerobic conditions. Satellite colonies that grew round *S. aureus*, β-hemolytic (alo bleached) and clear color, were taken as candidates for *A. pleuropneumoniae*.

5.7.2 Isolation of *S. suis*:

A mixture was made of all tubes for each farm in question, in a corning tube (15 mL), and mixed gently. One hundred microliters of water from this mixture were plated with glass beads in streptococci selective agar (Bioxon, Mexico). The samples were plated in duplicate. Then, plates were incubated at 37 °C for 24 hours under aerobic conditions. Colonies were subsequently replated on blood agar (5%), supplemented with 10 µg colistin sulfate mL^{-1} (to inhibit the growth of Gram negative) and 15 µg nalidixic acid mL^{-1} (quinolone that helps to prevent the growth of other Gram positive) (Blume *et al*., 2009; Hao *et al*., 2011) and were incubated at 37 °C for 24 hrs under aerobic conditions. Colonies that grown on blood agar were selected: small colonies, translucent white with incomplete hemolysis or α-hemolysis (green shown), Gram positive and catalase negative (Blume *et al*., 2009; Muckle *et al*., 2010; Hao *et al*., 2011). Colonies with these characteristics were picked and grown in BHI broth and agar at 37 °C for 24 hours under aerobic conditions, for extracting the DNA first, and second, to be stored at 4 °C if they were PCR positive. Multiplex PCR was performed as described above.

5.7.3 Isolation of *P. multocida*:

A mixture was made of all tubes for each farm in question, in a corning tube (15 mL), and mixed gently. One hundred microliters from this mixture of water were plated with glass beads on blood agar (5%). The samples were seeded in duplicate and incubated at 37 °C for 24 hours under aerobic conditions. Colonies with the following characteristics were selected: smooth colonies 1-2 mm in diameter, a bright blue-gray, and sometimes non-hemolytic mucosal NAD independent. A colony with these characteristics were tested

for oxidase and catalase (positive) and Gram (negative, coccoid forms or as short rods or filamentous, which can occur singly or grouped in pairs or short chains) (Stahel *et al.*, 2011). The colonies with these characteristics were picked and grown again for dilution groove on blood agar (5%) and incubated at 37 °C for 24 hours under aerobic conditions. Colonies were tested for indole production (positive) and ornithine decarboxylation of (positive) in MIO media, and urease (negative) in Urea media. The colonies with these characteristics were grown in BHI broth and agar at 37 °C for 24 hours under aerobic conditions, the first for DNA extraction, and the second for storing at 4 °C. PCR was performed as described above.

5.7.4 Isolation of *E. coli*:

A mixture was made of all tubes for each farm in question, in a corning tube (15 mL), and mixed gently. One milliliter of water were seeded in EC medium. The samples were seeded in duplicate and incubated at 44 °C for 24 hours under aerobic conditions. From what grown in this media, were took a roasted and seeded by dilution streak in EMB and incubated at 37 °C for 24 hours under aerobic conditions. Flat colonies with metallic green and black center were selected. The colonies with these characteristics were grown on BHI agar at 37 °C for 24 hours under aerobic conditions. The following biochemical tests were performed: indole production, citrate and lactic acid fermentation and Gram. Positive colonies for these tests were considered as *E. coli* and replated on BHI agar for storage at 4 °C. Likewise, were lyophilized and placed in glycerol (30%) and stored at -20 °C.

5.7.4.1 Confirmation of *E. coli* by PCR.

For confirmation of isolates of *E. coli* by PCR and view the group that it belongs to, it was used the method described by Ramirez *et al*. (2013). Furthermore, the methodology described by Clermont *et al*. (2013), was performed for the identification of the phylogenetic group to which it belonged each isolated obtained from drinking water from swine farms. The sequences of the primers used are shown in Table 3.The PCR conditions

was for the *fyuA* gene: 94°C for 30 seconds followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 65°C and 45 seconds at 68°C with a final elongation step at 72°C for 10 min; for the *kpsMTII* gene: 94°C for 5 min followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 63°C and 30 seconds at 68°C with a final elongation step at 72°C for 7 min; and for the *papC* gene: 94°C for 4 min followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 57°C and 30 seconds at 72°C with a final elongation step at 72°C for 10 min. Finally, for the identification of the phylogroup (genes *arpA*, *chuA*, *yjaA*, TspE4.C2, and *trpAgpc*) the PCR run conditions were 94°C for 4 min followed by 30 cycles of 20 seconds at 94°C and 20 seconds at 57°C with a final elongation step at 72°C for 7 min.

Table 3: Primers sequences used for *E. coli* confirmation and phylogroup in this study.

Gen	Name.	Sequence (5'-3').	Product size (bp).
fyuA	FyuAf	TGATTAACCCCGCGACGGGAA	880
	FyuAr	CGCAGTAGGCACGATGTTGTA	
kpsMTII	KpsMIIf	GCGCATTTGCTGATACTGTTG	272
	KpsMIIr	CATCCAGACGATAAGCATGAGCA	
papC	papC-forward	GACGGCTGTACTGCAGGGTGTGGCG	350
	papC-reverse	ATATCCTTTCTGCAGGGATGCAATA	
arpA	AceK.f	AACGCTATTCGCCAGCTTGC	400
	ArpA1.r	TCTCCCCATACCGTACGCTA	
chuA	chuA.1b	ATGGTACCGGACGAACCAAC	288
	chuA.2	TGCCGCCAGTACCAAAGACA	
yjaA	yjaA.1b	CAAACGTGAAGTGTCAGGAG	211
	yjaA.2b	AATGCGTTCCTCAACCTGTG	
TspE4.C2	TspE4C2.1b	CACTATTCGTAAGGTCATCC	152
	TspE4C2.2b	AGTTTATCGCTGCGGGTCGC	
arpA	ArpAgpE.f	GATTCCATCTTGTCAAAATATGCC	301
	ArpAgpE.r	GAAAAGAAAAAGAATTCCCAAGAG	
trpAgpc	trpAgpC.1	AGTTTTATGCCCAGTGCGAG	219
	trpAgpC.2	TCTGCGCCGGTCACGCCC	

5.8 Antimicrobial Susceptibility Testing.

The antimicrobial susceptibility testing was performed according to the regulations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2011), and Clinical Laboratory Standard Institute (CLSI, 2011). *E. coli* ATCC 25922 and *A. pleuropneumoniae* serotype 1-4074 were used as quality control strains. The inoculum is

prepared from an agar plate culture BHI supplemented with 15 µg NAD mL^{-1} to 24 h. Colonies were selected and transferred to a tube containing 5 mL of culture medium Trypticase soy broth supplemented with 15 µg NAD mL^{-1} and incubated at 37 °C until reaching an OD of turbidity of a 0.08 to 0.13 at 625 nm (2-5 h). The turbidity of each culture was adjusted with saline or broth to a turbidity equivalent to 0.5 McFarland standard in sterile saline (0.85% NaCl) (625nm, equivalent to 0.08 to 0.13 units). For inoculation, sterile swab was dipped into a bacterial suspension inoculum removing excess pressing against the walls. The samples were inoculated into a Mueller Hinton agar plate supplemented with 15 µg NAD mL^{-1} streaking the swab over the entire agar surface. The plate is rotated approximately 60 degrees several times to ensure proper distribution of inoculum. Multi-antimicrobial test was placed with sterile forceps. Antibiotics tested were: 30 µg amikacin, 10 µg ampicillin, 100 µg carbenicillin, 30 µg cephalothin, 30 µg cefotaxime, 30 µg ceftriaxone, 30 µg chloramphenicol, 10 µg gentamicin, 30 µg netilmicin, 300 µg nitrofurantoin, 5 µg pefloxacin, and 25 µg trimethoprim-sulfamethoxazole (BIO-RAD, DF, Mexico). The discs were distributed so that the length was not less than 24 mm in each center. Plates were incubated at 37 °C for 16-18 h. Over time, the plate was examined to see if it was successfully inoculated inhibition zones, they must be uniformLy circular and must have a mushy zone of growth. The diameters of complete inhibition zones were measured, including the diameter of the disc. The diameters were measured in millimeters with clamps or sliding on the back of the inverted petri dish. The sizes of inhibition zones were interpreted according to the regulations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2011), and Clinical Laboratory Standard Institute (CLSI, 2010). Isolates were reported as susceptible, intermediate or resistant to different antibiotics tested.

5.8.1 Interpretation of Results.

The results of antimicrobial susceptibility testing were reported in three categories: sensitive (S), intermediate (I) and resistant (R). Interpretations followed the rules established by the CLSI and EUCAST (Table 3).

- Sensitive: When a bacterial isolate *in vitro* is inhibited by a concentration of an antimicrobial that is associated with a high probability therapeutic success.
- Intermediate: When a bacterial isolate *in vitro* is inhibited by a concentration of an antimicrobial that is associated with a therapeutic effect uncertain.
- Resistant: when a bacterial isolate is inhibited *in vitro* by a concentration of an antimicrobial that is associated with a high probability with treatment failure.
- Isolates resistant to two or more antibiotics are defined as multi-drug-resistant.

Table 4: Interpretation of results for antimicrobial susceptibility testing.

Antibiotic	Abbreviation	Quantity (µg)	Resistant (cm)	Intermediate (cm)	Sensitive (cm)
Amikacin	AK	30	≤14	15-16	≥17
Ampicillin	AM	10	≤13	14-16	≥17
Cephalotin	CF	30	≤14	15-16	≥18
Cefotaxime	CTX	30	≤14	15-22	≥23
Ceftriaxone	CRC	30	≤13	14-20	≥21
Chloranphenicol	CL	30	≤12	13-17	≥18
Gentamicin	GE	10	≤12	13-14	≥15
Netilmicin	NET	30	≤12	13-14	≥15
Nitrofurantoin	NF	300	≤14	15-16	≥17
Pefloxacina	PEF	5	≤14	15-22	≥23
Trimethoprim-sulfamethoxazole	SXT	25	≤10	11-15	≥16

5.9 Biofilms formation.

5.9.1 Biofilms formation assay for drinking water samples.

Three trials were performed to observe biofilm formation of drinking water samples. First, biofilm formation were screened using the glass tube biofilm assay as described previously for Dalai *et al.* (2009) and Jin *et al.* (2006). The second trial was *in vitro* biofilms formation as described by Loera-Muro *et al*. (2013). The fluorescent *in situ* hybridization (FISH) was performed as described by Lehtola *et al*. (2006) and Rodriguez-

Nuñez *et al*. (2012) with modifications. The last test was performed directly on the swine farm.

5.9.2 Biofilms formation for FISH assay for drinking water samples.

A slide was placed in a sterile petri dish, adding 25 mL of a bacterial suspension (25 mL BHI with NAD + 2.5 mL of the pathogen previously grown for 24 hours under stirring in liquid medium, dilution 1:10). Allowed to incubate for 24 hours at 37 °C. Once the time allowed to dry the slide at room temperature. In the case of samples of drinking water, was added 40 µl of drinking water on a slide in a sterile petri dish and allowed to dry at room temperature.

5.9.3 FISH assay for drinking water samples.

FISH assay was performed as described previously (Lehtola *et al.*, 2006; Rodriguez-Nuñez *et al.*, 2012) with modifications. The samples were treated with 80% ethanol for 20 min to fix the sample. Were subjected to a pretreatment with sodium citrate (1 mM) at a temperature of 95 °C for 5 min. Samples were washed with distilled water at 50 °C for 5 min, removed and allowed to dry in the incubator at 37 °C. Aliquots (30 µL) containing the following hybridization mixture were applied to each slide: 10 mM NaCl (J.T. Baker, Xalostoc, Mexico), 50 mM Tris-HCl (Invitrogen, Carlsbad, California, USA) (pH 7.5), 10% (w/vol) sodium dodecylsulfate (Sigma, Steinheim, Germany), 30 % (v/v) formamide (Pharmacia Biotech AB, Uppsala, Sweden), 0.1% (v/v) Triton X-100 (USB, OH, USA), and a fluorescent probe with a final concentration of 1 µM. Used the primers APXIVAN L (GGG GAC GTA ACT CGG TGA TT) and APXIVAN R (GCT CAC CAA CGT TTG CTC) labeled with fluorescein (FITC) or TAMRA in the N-terminus (5') (Alpha DNA Montreal, Canada) like probes. The slides were covered with cover glass and then placed in a preheated moisture chamber in the dark at 55°C for all night. After slides were washed in preheated washing buffer (5 mM Tris, 15 mM NaCl, 0.1% [v/v] Triton X-100 [pH 10]) at hybridization temperature for 30 min. Following a brief immersion in bi-distilled water, slides were air-dried and mounted with one drop of prolong Gold with 4'-6-Diamidino-2-

phenylindole (DAPI) (Invitrogen Oregon, USA). The samples were stored at -20 °C in dark until Laser Scanning Confocal Microscopy (LSCM) (Leica, Germany) or epifluorescence microscope (EM) (Leica, Germany) observation.

5.9.4 Biofilms formation for staining with Crystal Violet for drinking water samples.

The biofilm formation assay in microplates is a test used to analyze the early events that occur during the formation of a biofilm. This technique was performed according to Labrie *et al*. (2010). First all strains used for the experiment were grown 24 hours at 37 °C under agitation. Subsequently, in a sterile 96 well microplate was added a 1/100 dilution of the culture grown overnight before. Everything was done in triplicate and three different assays were performed. The incubation was performed for 5 to 24 hours (depending on the optimum times for each strain used to form their biofilm) at 37 °C and 5% CO_2. It was then washed by immersion in water and the excess was removed with a micropipette. The wells were stained with 100 µl of 0.1% crystal violet for 2 min at room temperature. Removed the crystal violet solution and subsequently washed first with water and allowed to dry for 30 min at 37 °C. Then 100 µl of ethanol 70% were added. Finally, the absorbance was measured at 590 nm using a spectrophotometer.

5.9.5 Biofilms formation directly in drinkers.

We designed a system for obtaining environmental biofilms of drinking water of swine farms. This study was done at the swine farm belonging to the Center of Agricultural Sciences at the Autonomous University of Aguascalientes, which was previously detected *A. pleuropneumoniae* in samples of drinking water. It took a stainless steel box of 2.7 x 2.7 x 0.8 inches, with circular openings on the sides and an open side which introduced a slide and covered with wire mesh with holes of 0.4 inches. The box was sterilized by autoclaving. The boxes were placed in the drinkers in the farm, took care that the water completely covering the case and to stay all the way. The boxes were left for a period of 7 days periodically checking that the box remain within the drinkers and that has not been turned. Over time, the boxes were removed carefully and stored in a cooler immediately

sealed, taking care that there is less pollution as possible. In the laboratory, the slides were removed from the boxes and dried at 37 °C. We performed FISH to the samples as described previously.

5.10 Scanning Electron Microscopy (SEM).

Bacterial biofilm was performed as described above *in vitro* in petri dish. Subsequently centrifuged at low speed (4,000 rpm) at room temperature for 10 min. to obtain a pellet of the biofilm. Washing was performed with 1X PBS and then add 1 mL of glutaraldehyde. This was followed by several steps to dehydrate the sample with increasing alcohol dilutions (60 - 100%), this step is known as the critical point. Excess moisture was removed with liquid CO_2 in a camera Tousimis. Samples were coated with gold using a special camera (Chamber Desk II). Samples were observed with the scanning electron microscope (Jeol MF-5900) and images were captured with the software of the microscope.

5.11 Swine respiratory pathogen *A. pleuropneumoniae* in multi-species biofilm.

5.11.1 Bacterial strain.

Bacterial strains selected for this present study were as follows. *A. pleuropneumoniae* serotype 1 strain 719, previously reported that form a strong biofilm, and strain 4074. Three bacteria belonging to the porcine respiratory disease complex (PRDC): *S. suis* serotype 2 strain 735, *B. bronchiseptica* strain 276, and *P. multocida* D strain 1703. Positive control was used *S. aureus* strain 154N, a non-pathogenic nasal isolated from pig. Negative control was used *E. coli* strain ECL17608, a porcine intestinal Enterotoxigenic (ETEC). All bacteria were grown on brain heart infusion agar plates (BHI; Oxoid Ltd, Basingstoke, Hampshire, UK) with supplementation of 15 μg/mL nicotinamide adenine dinucleotide (NAD) for *A. pleuropneumoniae* and only BHI for all the others bacteria. Plates were incubated overnight at 37ºC with 5% CO_2. A colony was transferred into 5 mL BHI (Oxoid Ltd, Basingstoke, Hampshire, UK) with 5 μg/mL NAD or without

this supplementation, and incubated at 37 °C overnight with agitation. This culture was used for the biofilms assays.

5.11.2 Multi-specie biofilms assay.

Multi-species biofilms assay was performed as described previously Labrie *et al*. (2010) for mono-species and multi-species biofilms with modifications. Briefly, overnight cultures of *A. pleuropneumoniae*, *S. suis*, *B. bronchiseptica*, *P. multocida*, *E. coli* or *S. aureus* were diluted 1/100 in BHI broth with and without supplementation of NAD and a volume (200 µL) was aliquoted in triplicate in wells of a sterile 96-well microtiter plate (Costa3599, Corning, NY, USA) in the following order: 100 µL *A. pleuropneumoniae* in BHI-NAD + 100 µL *S. suis* in BHI-NAD, and 100 µL *A. pleuropneumoniae* in BHI + 100 µL *S. suis* in BHI. The same order was followed for mixtures: *A. pleuropneumoniae* - *B. bronchiseptica*, *A. pleuropneumoniae* - *P. multocida*, *A. pleuropneumoniae* - *E. coli*, and *A. pleuropneumoniae* - *S. aureus*. For the tri-specie biofilms *A. pleuropneumoniae* - *S. suis* - *B. bronchiseptica*, *A. pleuropneumoniae* - *S. suis* - *E. coli*, and *A. pleuropneumoniae* - *B. bronchiseptica* - *E. coli*, 50 µL were used of each one. Wells containing 100 µL *A. pleuropneumoniae* in BHI-NAD + 100 µL of BHI-NAD, and 100 µL *A. pleuropneumoniae* in BHI + 100 µL of BHI, were used as control for mono-species biofilms for *A. pleuropneumoniae*. The above was repeated with the other bacteria used in this study. Wells containing sterile broth were used as negative control. Following an incubation of 24 h at 37 °C, the wells were washed by immersion in water and excess water was removed by inverting plates onto a paper towel. Multi-species biofilms were then stained with 0.1% (w/v) crystal violet for 2 min. Multi-species biofilms were washed once with distilled water, dried at 37°C for 30 min, and then 100 µL of ethanol (70%) were added to the wells. Absorbance was measured at 590 nm using a spectrophotometer (Powerwave, BioTek Instruments, Winooski, VT, USA).

On the other hand, di-species biofilms for *A. pleuropneumoniae* with *S. suis* or *E. coli* were made in *S. suis* or *E. coli* conditions that described Wu *et al* (2013). Overnight cultures of *A. pleuropneumoniae*, *S. suis* or *E. coli* were diluted 1/100 in Basal Broth Media

(BBM) with fibrinogen (5 mg/mL) for the di-specie biofilm *A. pleuropneumoniae - S. suis* or in M9 minimal medium for the di-specie biofilm *A. pleuropneumoniae - E. coli*, and a volume (100 µL) was aliquoted in triplicate in wells of a sterile 96-well microtiter plate (Costa3599, Corning, NY, USA) in the following order: 100 µL *A. pleuropneumoniae* in BBM with fibrinogen + 100 µL *S. suis* in BBM with fibrinogen, or 100 µL *A. pleuropneumoniae* M9 minimal medium + 100 µL *E. coli* M9 minimal medium. Wells containing sterile broth were used as negative control. Following an incubation of 24 h at 37 °C for *A. pleuropneumoniae-S. suis* and 24 h at 30°C for *A. pleuropneumoniae-E. coli*. Di-species biofilms were then stained with 0.1% (w/v) crystal violet and for colony forming unit assay as as previously described.

Also, the capacity of *A.pleuropneumoniae* for incorporate in a pre-formed biofilms was tested. Biofilms of *S. aureus, S. suis* and *E.coli* were made like described Wu *et al* (2013). Overnight cultures of *S. aureus, S. suis* or *E. coli* were diluted 1/100 in BHI for *S. aureus* biofilms, Basal Broth Media (BBM) with fibrinogen (5 mg/mL) for *S. suis* biofilm or in M9 minimal medium for *E. coli* biofilm, and a volume (100 µL) was aliquoted in triplicate in wells of a sterile 96-well microtiter plate (Costa3599, Corning, NY, USA). After 24 h of incubation, was added *A.pleuropneumoniae* 100 µL of overnight culture in the specific media previously said (BHI or BBM or M9), and incubate 24 h more in the conditions for each bacteria. Wells containing sterile broth were used as negative control. The incorporation of *A. pleuropneumoniae* was tested by stained with 0.1% (w/v) crystal violet and for colony forming unit assay as as previously described.

Finally, the ability to form multi-species biofilms from *A. pleuropneumoniae* was performed with the 10 isolated of *E. coli* obtained from drinking water of swine farms in ideal conditions for the formation of isolated biofilms, the which were in LB + 30% glycerol at 30 °C. The assay was carried out as previously described.

5.11.3 Colony Forming Units Assay (CFU).

Colony forming units (CFU) of *A. pleuropneumoniae* and the other bacteria were enumerated. Multi-specie biofilms were prepared as described above. Multi-specie biofilms samples were washed with sterile MilliQ water (200 µL), then all the biofilms were disrupted with micropipette for microbial segregation. Samples were then serially diluted in NaCl (0.85%) and plated on BHI, BHI-NAD, BHI-NAD-crystal violet (1 µg/mL), blood agar and blood agar-NAD (15 µg/mL) (Oxoid Ltd, Basingstoke, Hampshire, UK), and mannitol salt agar (Oxoid Ltd, Basingstoke, Hampshire, UK). Plates were incubated overnight at 37ºC with 5% CO_2. All the bacteria were identified in the mixed biofilms samples by colony morphology.

5.11.4 Fluorescent in situ hybridization (FISH).

FISH protocol was performed as described Jensen *et al*. (2010) and Loera *et al.* (2013) with modifications. Mono-specie biofilms of *A. pleuropneumoniae* strain 719 was used as positive controls. Mono-specie biofilm of *S. suis* strain 735 was used as a specificity control and was made as described Wu *et al* (2013). Multi-specie biofilms were formed on glass slides with flexiPERM® slide with eight wells (Sarstedt, Nümbrecht, Germany) by placing a glass slide in a Petri dish containing 300 µl diltion 1/100 of *A. pleuropneumoniae* or *S. suis* culture, and 150 µl dilution 1/100 of *A. pleuropneumoniae* and *S. suis* or *B. bronchiseptica* culture for the mix biofilms. The Petri dish was incubated for 24 h at 37 °C with 5% CO_2. The slides were air-dried (1 h at 37 °C) and gently flamed. The slides were dehydrated in 100% alcohol for 30 min before hybridization. The hybridization was carried out at 45 °C with 40 mL of hybridization buffer (100 mM Tris-HCl [pH 7.2], 0.9 M NaCl, 0.1% sodium dodecyl sulfate) and 200 ng of each probe (APXIVAN-Forward and APXIVAN-Reverse labelled with 633 Alexa Fluor) for 16 h in a slide rack. The samples were then washed one time in prewarmed (45 °C) hybridization buffer for 15 min and subsequently one time in prewarmed (45 °C) washing solution (100 mM Tris-HCl [pH 7.2], 0.9 M NaCl). Every sample was then washed with water for 5 min. After hybridization, multi-species biofilms were stained with FilmTracerTM FM® 1-43

fluorescent marker (Molecular Probes, Eugene, OR, USA) according to manufacturer's instructions. Every biofilm was incubated for 30 min at room temperature in the dark. Every sample was then washed with water for 10 min. The samples were covered with ProLong Gold antifade reagent (Invitrogen). The labelled bacteria were visualized using a confocal microscope (FV1000 IX81; Olympus, Markham, ON, Canada) and images were acquired using Fluoview software (Olympus).

5.11.5 Confocal laser scanning microscopy (CLSM).

To determine the composition of the biofilm matrix, multi-species biofilms were stained with Wheat Germ Agglutinin (WGA-Oregon Green 488, Molecular Probes) (bind *N*-acetyl-d-glucosamine [PGA] and *N*-acetylneuraminic acid residues), FilmTracerTM SYPRO® Ruby biofilm matrix stain (Molecular Probes)(bind proteins) or BOBOTM-3 iodide (Molecular Probes) (label extracellular DNA or eDNA) as prescribed by the manufacturer. The same multi-species biofilm assay protocol was used as described previously in section 2.2. After at 30 min incubation at room temperature, the fluorescent marker solution was removed, multi-species biofilms were washed with water and the wells were then filled with 100 µL of water or PBS for WGA-stained multi-species biofilms. Likewise, biofilms were labeled with FilmTracer FM 1-43 (Invitrogen, Eugene, OR) and FilmTracer LIVE/DEAD Biofilm Viability Kit (Invitrogen) according to manufacturer's instructions. The stained multi-species biofilms were visualized by CLSM (FV1000 IX81; Olympus, Markham, ON, Canada) and images were acquired using Fluoview software (Olympus).

5.11.6 Enzymatic treatments of multi-species biofilms.

The enzymatic treatments assay was performed as described previously Izano *et al*. (2007) and Tremblay *et al*. (2013). Multi-species biofilms were prepared as described in section 2.2. After the desired incubation time, 50 µL of DNase I (500 µg/mL in 150 mM NaCl, 1 mM $CaCl_2$), or 50 µL of proteinase K (500 µg/mL in 50 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$) was added directly to the biofilms. Wells were incubated for 1 h at 37°C. For

dispersin B, 50 µL (100 µg/mL in PBS; Kane Biotech Inc., Winnipeg, MB, Canada) was added and wells were incubated for 5 min at 37°C. Control wells were treated with 50 µL of the buffer without the enzyme. Multi-species biofilms were then stained with crystal violet as described above.

5.11.7 Measurement of NAD production by the mono and multi-species biofilms.

The production of NAD in the supernatant by the mono and multi-species biofilms was quantificated by the NAD/NADH Extraction Kit (Sigma-Aldrich Co., St. Louis, MO, USA). Mono and multi-species biofilms were prepared as described above. After 24 h of incubation, the supernatants were taken and placed in new tubes. CV technique was performed to the plate to verify that there were biofilms formations. Then, all tubes were centrifugated at 2,000 rpm for 5 min. The supernatans were taken and filtered trough filter of 0.2 µm. From here we proceeded according to manufacturer's specifications.

5.11.8 *A. pleuropneumoniae* biofilm formation with crude cell-free supernatants.

Single species biofilm formation of *A. pleuropneumoniae* was performed with the crude cell-free supernatants of the other five different bacteria. To obtain the supernatants, were made of the mono-species biofilms of the bacteria *S. suis*, *B. bronchiseptica*, *P. multocida*, *S. aureus* and *E. coli*. Biofilms were performed as follows. Overnight cultures of *S. suis*, *B. bronchiseptica*, *P. multocida*, *E. coli* or *S. aureus* were diluted 1/10 in BHI broth (final volume of 25 mL) in plate. The plates were incubated for 24 h at 37 °C. After of incubation, the supernatants were taken and placed in new tubes. Then, all tubes were centrifugated at 2,000 rpm for 5 min. The supernatans were taken and filtered trough filter of 0.2 µm. After here, were performed the mono-species biofilms of *A. pluropneumoniae* with the supernatan at three different concentrations. Overnight culture of *A. pleuropneumoniae* were diluted as described below; 1) dilution 1/100 in the supernatants, and 2) dilution 1/100 in BHI. A volume (200 µL) was aliquoted in triplicate in wells of a sterile 96-well microtiter plate (Costa3599, Corning, NY, USA) in the following order: 1) 200 µL of dilution 1/100 in the supernatants (100%), 2) 100 µl dilution *A.*

pleuropneumoniae in BHI + 100 µl supernatant (50%), and 3) 150 µl dilution *A. pleuropneumoniae* in BHI + 50 µl supernatant (25%). The *A. pleuropneumoniae* biofilms formation with the crude cell-free supernatants of the other five different bacteria was tested by stained with 0.1% (w/v) crystal violet as previously described.

5.11.9 Scanning electron microscopy.

Biofilms formations by *A. pleuropneumoniae* with *E. coli* were scanning by electron microscopy (EM). Mono-specie biofilms of *A. pleuropneumoniae* and *E. coli* was used as positive controls and was made as described Wu *et al*. (2013). The two-species biofilms was prepared as described above. The samples were processed as described by Loera-Muro *et al*. (2013). Samples were observed with a Jeol LV-5900 scanning electron microscope. The sizes of the bacteria were measured with the microscope software. The shading in the figure to differentiate bacteria in multi-species biofilm, was used the program Adobe Photoshop CS.

5.12 Statistical analysis.

All the statistical significance (*p* value) analyses of differences in biofilm phenotypes were performed with the GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA).

6. RESULTS.

6.1 General screening of swine farms in the state of Aguascalientes.

A total of 14 farms producing pigs belonging to the State of Aguascalientes, Mexico, were selected for this study. This selection was made primarily for our convenience and the owners too (see figure 3 in materials and methods). We obtained a total of 162 nasal swabs, with about 12 per farm. Pigs were sampled at random, these had no apparent signs of illness and were in normal production process on the farms. Most pigs were between 1-2 years of age. Likewise, nurses also sampled females of different ages and stages of parenting. As a result of the general screening it was found that the 20.37% of the samples were positive for *A. pleuropneumoniae*, presenting a distribution in 78.57% of the farms; for *S. suis* 18.52% were positive, found in 85.71% of the farms, corresponding to 3.09% of the cases to *S. suis* serotype 2, distributed in the 28.57% of farms; 30.86% were positive for *H. parasuis*, with the largest distribution found on farms, for it was 92.86%; 28.40% were positive to *P. multocida*, with a distribution of 78.57% of the farms, and 22.84% for *M. hyopneumoniae*, distributed in 64.29% of the farms. We did not detect the presence of *B. bronchiseptica* in this study (Table 5, Figures 6 and 7).

Moreover, we had already 14 farms sampled in this study, 35.71% (5 farms) found the presence of 5 and 4 bacterial pathogens of PRCD, respectively, in 21.43% of cases were 3 pathogens on the same farm, in 7.14% with 2 pathogens and not found any farm affected by the 6 pathogens or free of them all in this study (Table 6 and Figure 8). Likewise, the pathogens found a greater number of times to affect the same farm were: *S. suis* and *H. parasuis* in 85.71% of cases (12 farms), and 71.43% of cases (10 farms): *A. pleuropneumoniae* and *H. parasuis*, *H. parasuis* and *P. multocida, P. multocida* and *S. suis* (Table 6 and Figure 8).

Figure 5: Images of some farms selected for this study. Nurses females are shown and drinkers where some of the samples obtained for this study.

Table 5: Total nasal swabs samples obtained in this study, total positive results (per farm and total) by PCR for each pathogen searched and the percentages observed in the same.

Farm	Nasal swabs samples	*A. pleuropneumoniae*	*S. suis.*	*S. suis* serotype 2	*H. parasuis.*	*P. multocida* DNT+.	*B. bronchiseptica.*	*M. hyopneumoniae.*
1	12	2	3	1	5	6	0	6
2	10	0	4	2	6	6	0	6
3	10	4	2	1	6	4	0	2
4	10	4	0	0	0	5	0	3
5	12	3	2	0	6	3	0	5
6	12	3	2	0	5	6	0	2
7	12	0	1	0	4	4	0	0
8	12	4	1	0	2	6	0	6
9	12	0	2	0	1	2	0	4
10	12	1	1	0	3	0	0	0
11	12	3	1	0	3	0	0	3
12	12	3	0	0	2	0	0	0
13	12	5	6	1	3	3	0	0
14	12	1	5	0	4	1	0	0
Total	**162**	**33**	**30**	**5**	**50**	**46**	**0**	**37**
Percentages		**20.37**	**18.5**	**3.09**	**30.86**	**28.40**	**0.00**	**22.84**
N. of infected farms		**11**	**12**	**4**	**13**	**11**	**0**	**9**
Distribution on farms		**78.57**	**85.7**	**28.57**	**92.86**	**78.57**	**0.00**	**64.29**

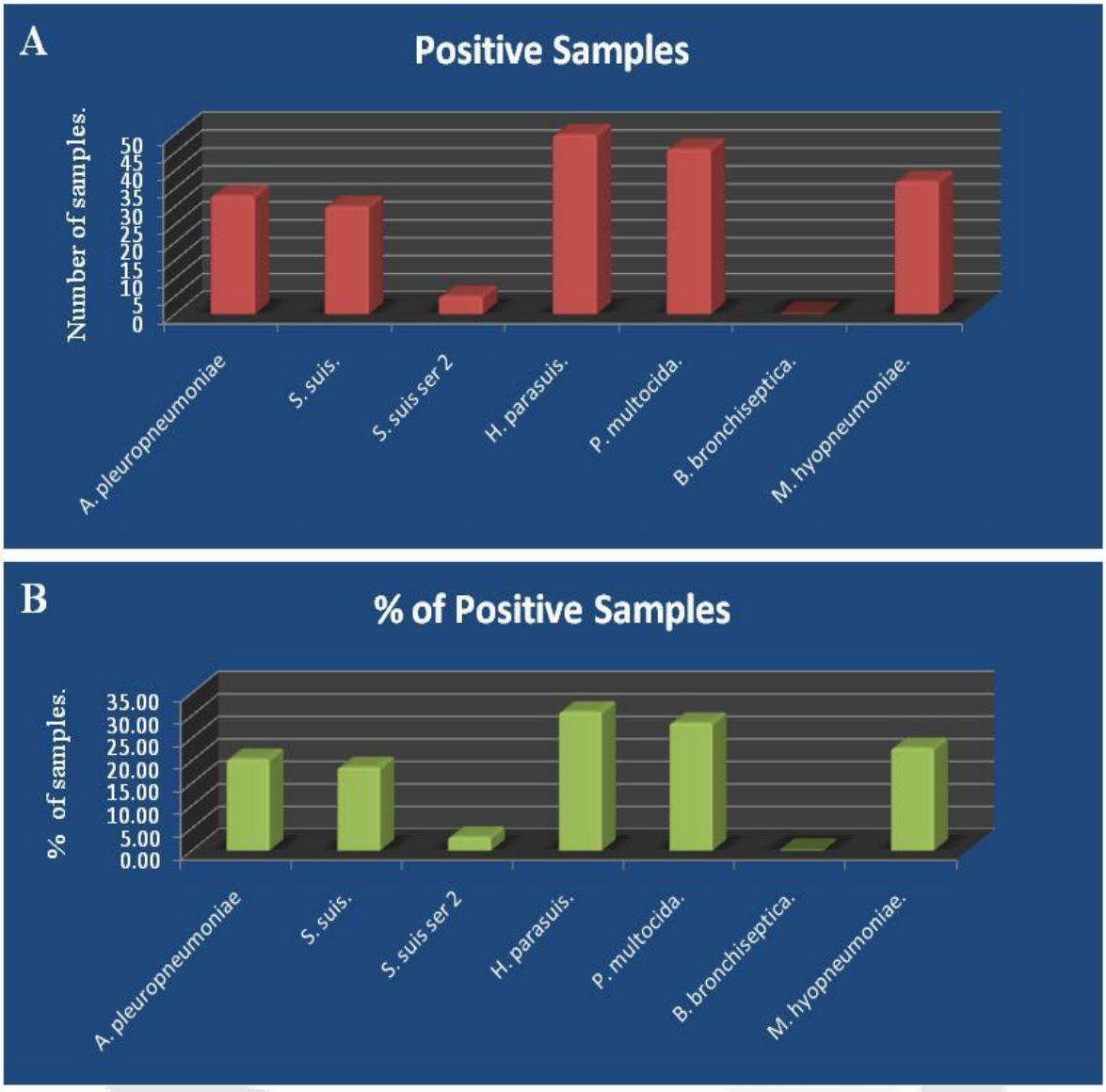


Figure 6: Graph of total positive samples of nasal swab for each pathogen selected in this study by PCR. A) Total of positive samples for each pathogen, and B) percentage of total positive samples for each pathogen.

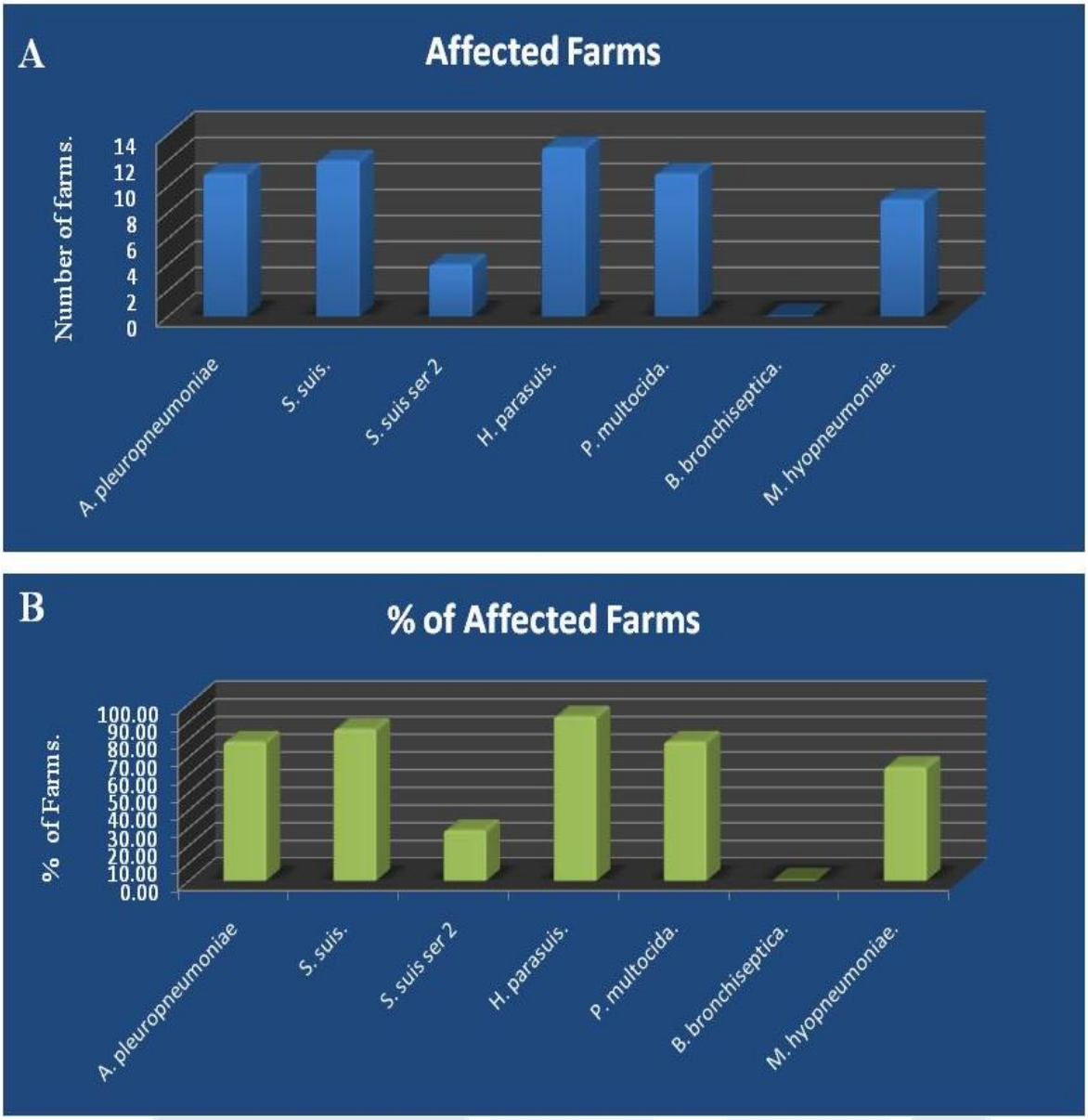


Figure 7: Graph which shows the number of farms affected by each pathogen selected in this study obtained according to the screening by PCR. A) Number the farms affected, and B) percentage of total farms that were affected.

Table 6: Number and percentage of pathogens species per farm sampled.

Number of Pathogens	6	5	4	3	2	1	0
Number of Farms	0	5	5	3	1	0	0
Percentage	0	35.71	35.71	21.43	7.14	0	0

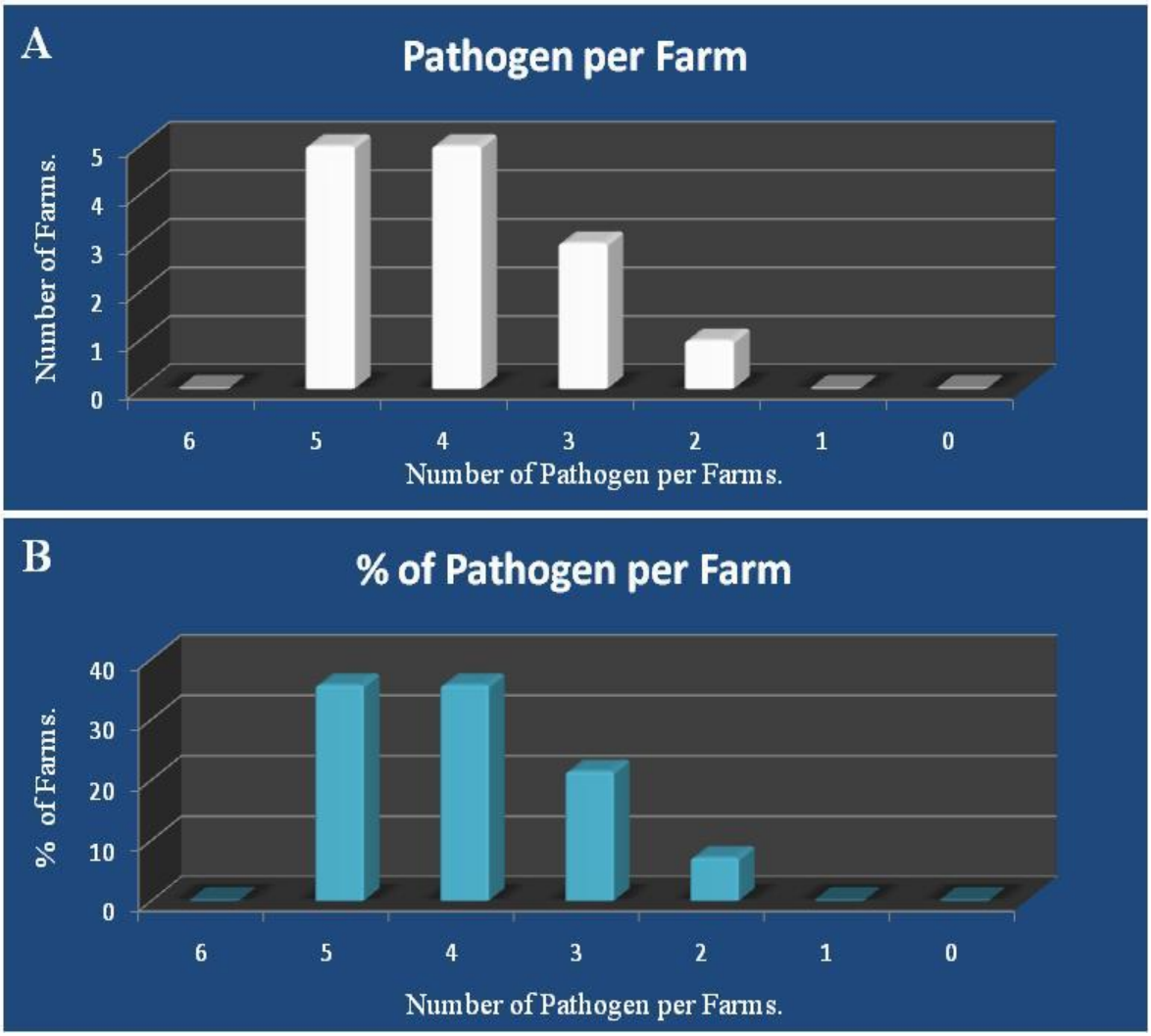


Figure 8: Graph which shows, A) the distribution the number of pathogens found by farm sampled and B) the percentage of number of pathogen per farm found in each one.

Table 7: Number and percentage of pathogens species that were found to affect the same farm.

Pathogens.	Number of Affected Farms.	% of Affected Farms.
Ss, Hp	12	85.71
Hp, Pm	10	71.43
Ss, Pm	10	71.43
App, Hp	10	71.43
App, Ss	9	64.29
App, Pm	8	57.14
App, Ss, Pm, Hp	3	21.43

App: *A. pleuropneumoniae*, Ss: *S. suis*, Hp: *H. parasuis*, Pm: *P. multocida*.

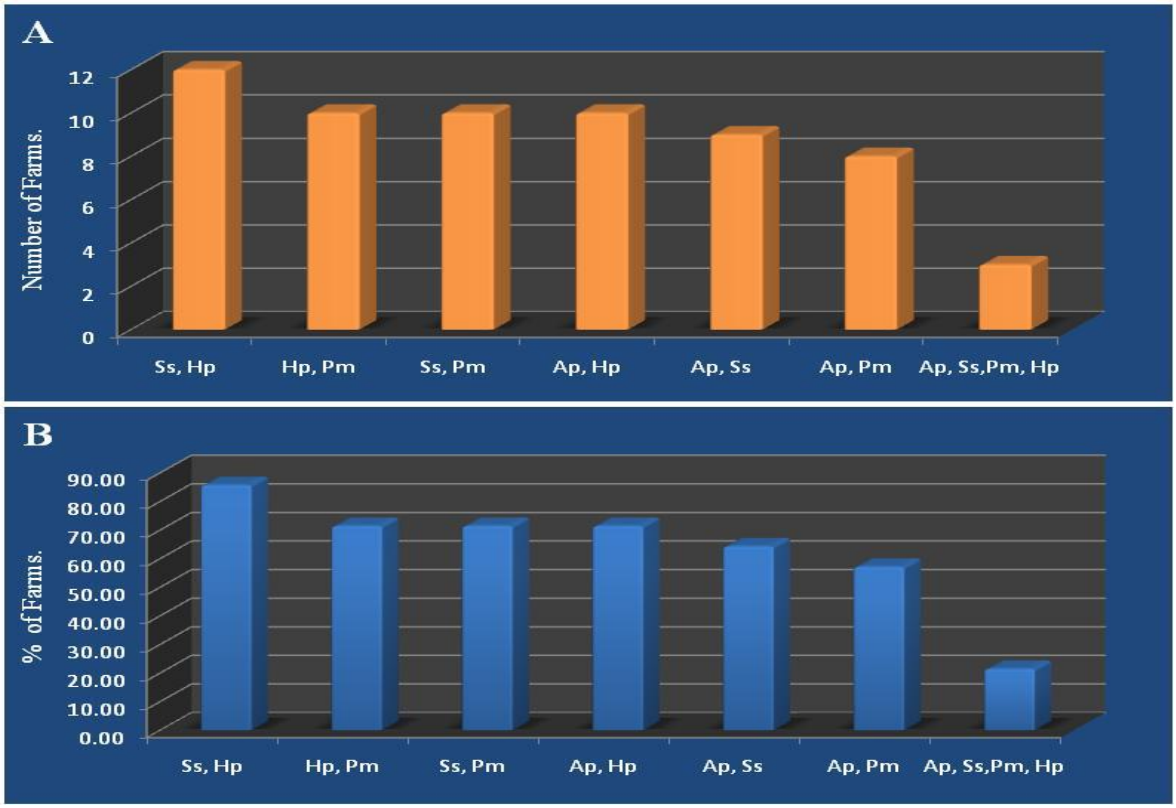


Figure 9: Shows the distribution of pathogens that were most frequently found to affect the same farm. A) Total of pathogens for farm, and B) the percentage the distribution of pathogens (App: *A. pleuropneumoniae*, Ss: *S. suis*, Hp: *H. parasuis*, Pm: *P. multocida*).

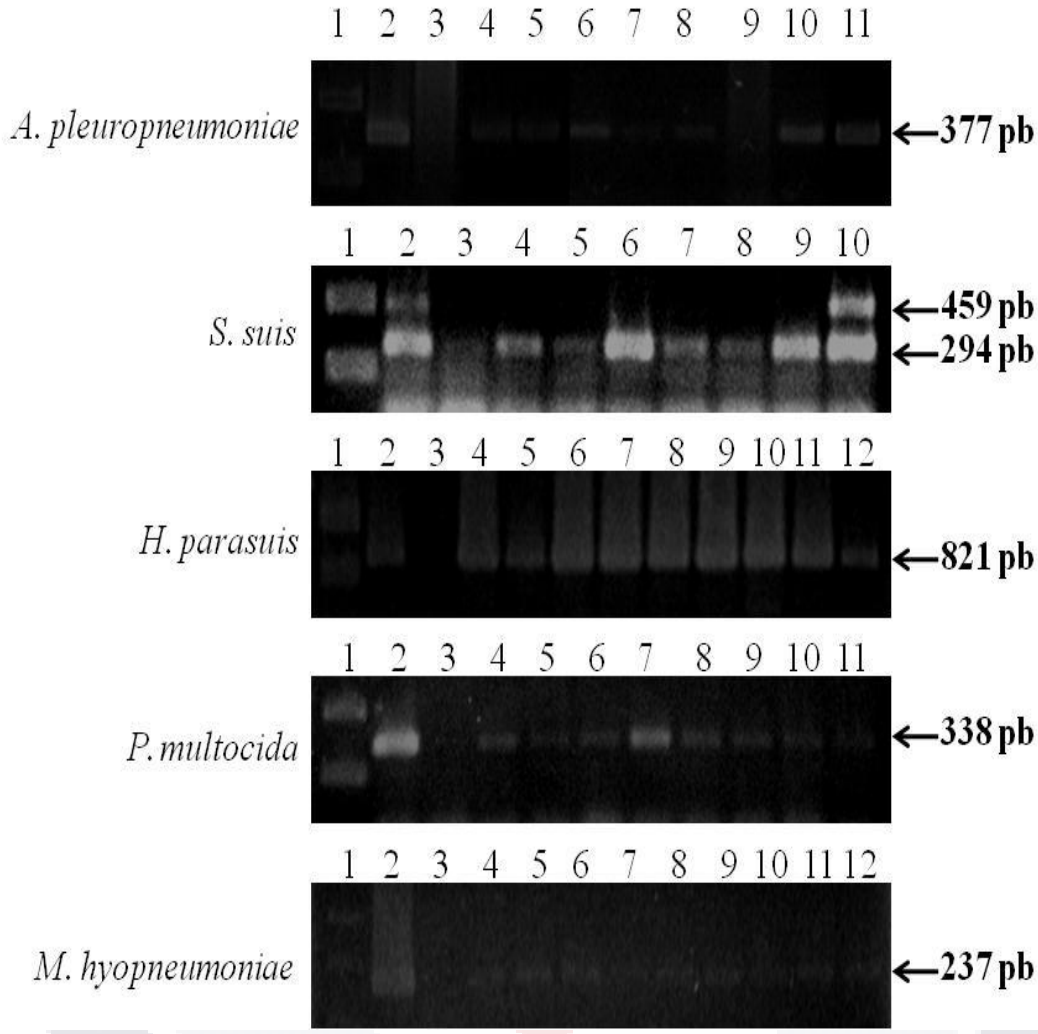


Figure 10: Amplifications obtained for different pathogens from PRCD: all the line 1 - molecular weight marker, line 2 - positive control for *A. pleuropneumoniae* S4074, *S. suis* 735 ser. 2, *H. parasuis* ser. 5 (Nagazaki), *P. multocida* 4-4056 (type D, DNT +), and *M. hyopneumoniae* ATCC 25095, line 3 - negative control using *E. coli* ATCC 25922 in all cases, line 4 onwards - nasal swabs. Not was detected the presence of *B. bronchiseptica* in this study.

6.2 Viability tests on samples of drinking water from swine farms.

One of the first steps that were performed on samples of drinking water were a viability test with the Live/Dead *Bac* Light Bacterial Viability Kit (Molecular Probes) at 84 samples from swine farms. This test was performed as already remarked above in Materials and Methods and initial showed interesting results. With this assay we observed that in all the water samples was a large number of bacteria in a viable state (and hopefully be culturable) to continue with the other analyzes of these water samples. In Figure 11 it can clearly see a large number of live bacteria (green) with the dead bacteria (red).

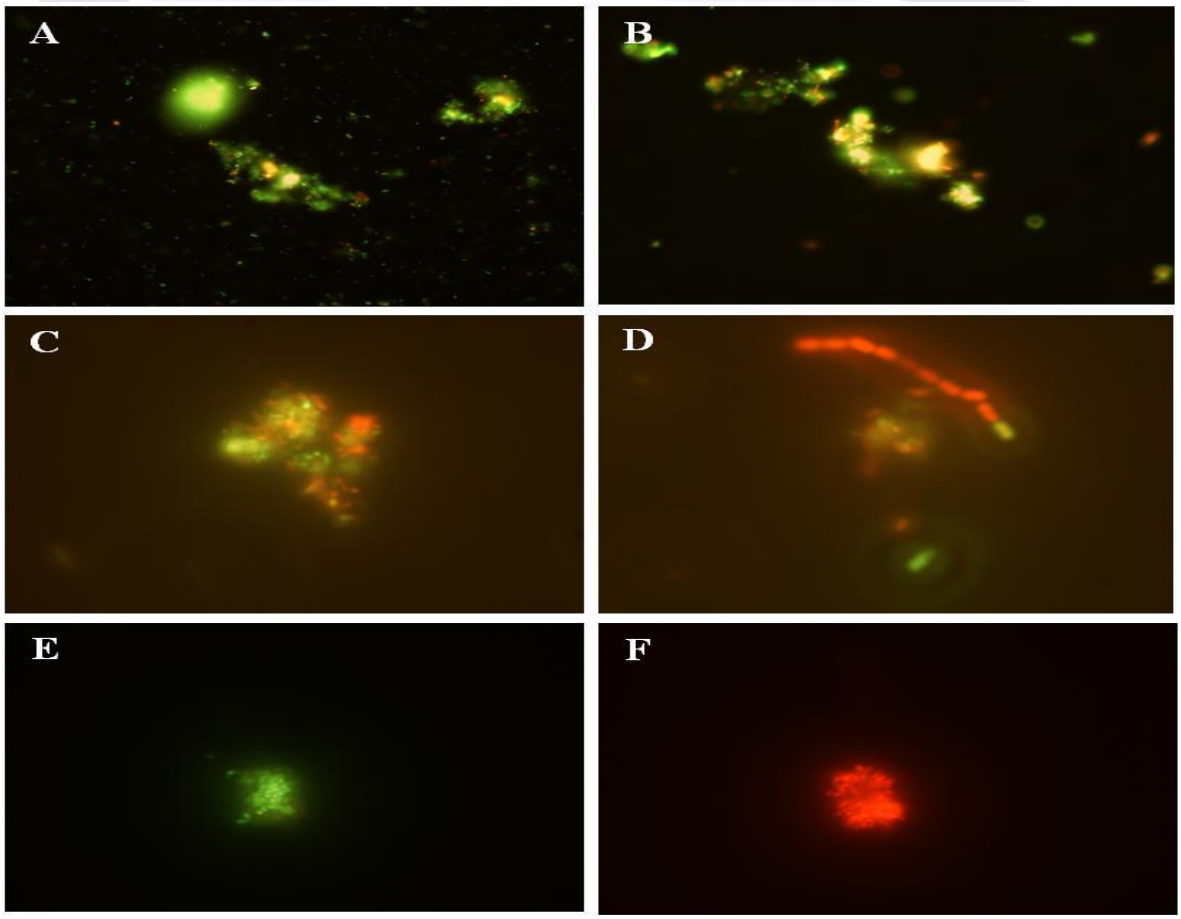


Figure 11: Viability assay with the Live/Dead *Bac* Light Bacterial Viability Kit (Molecular Probes) where one can clearly see a large number of live (green) and dead (red) bacteria in all samples of drinking water obtained from swine farms.

6.3 General scanning and isolation of pathogens from drinking water samples from swine farms.

With regard to the detection of these pathogens in samples of drinking water from swine farms, we obtained a total of 84 water samples from these 14 selected farms, averaging 6 samples per farm. These samples came from water from pacifiers or drinkers in the soil. Using the PCR technique, detection was achieved for *A. pleuropneumoniae* in drinking water at 5 farms. Also achieved detecting *H. parasuis* in 3 farms and *S. suis* in only one farm using the same technique (Figure 12). From here we proceeded to the isolation of these pathogens from water, where were able to obtain 20 isolates of *A. pleuropneumoniae* from 3 different farms. Not achieved the isolation of *S. suis* and *H. parasuis* in any water sample, even with the use of specific media, as was the case used to try to isolate the pathogen *S. suis* (Table 8).

Table 8: Total detections in the drinking water of swine farms and, the total and place of the isolates obtained of bacteria *A. pleuropneumoniae*, *S. suis* and *H. parasuis*.

Farm.	*A. pleuropneumoniae* in drinking water.	*A. pleuropneumoniae* isolates.	*S. suis* in drinking water.	*H. parasuis* in drinking water.
2	-	-	-	+
3	-	-	-	+
5	+	6	-	-
8	+	6	-	-
9	-	-	+	-
12	+	8	-	-
13	+	-	-	-
14	+	-	-	+
Total	5	20	1	3

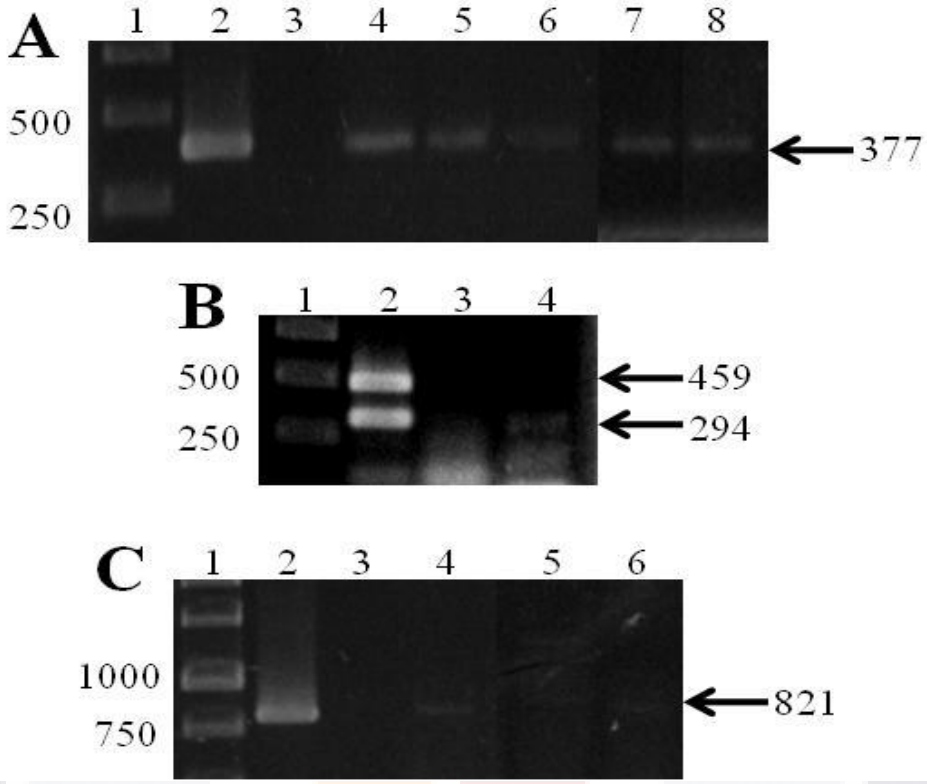


Figure 12: Samples of drinking water from swine farms. A) Positive samples of *A. pleuropneumoniae*: Line 1 - molecular weight marker, line 2 - *A. pleuropneumoniae* S4074, line 3 - *E. coli* ATCC 25922, line 4 onwards - positive water samples. B) Positive samples for *S. suis*: Line 1 - molecular weight marker, line 2 - *S. suis* 735 serotype 2, line 3 - *E. coli* ATCC 25922, line 4 - positive water sample. C) Samples positive for *H. parasuis*: Line 1 - molecular weight marker, line 2 - *H. parasuis* serotype 5 (Nagazaki), line 3 - *E. coli* ATCC 25922, line 4 onwards - positive water samples.

6.4 Positive samples of *A. pleuropneumoniae*.

Samples of drinking water were performed as previously described. Conventional microbiologic analysis of the drinking water samples (coupled with PCR and FISH tests) led the identification of 20 positive samples of *A. pleuropneumoniae* (Figure 13). These samples showed copious growth, β hemolysis, Gram negative, catalase positive, oxidase positive, and after overnight incubation NAD-independence. This preliminary identification of colonies with the ability to grow without NAD allowed us to know that these isolates belong to *A. pleuropneumoniae* biotype 2. All samples scored positive in CAMP test. All samples were confirmed by PCR as described in the methodology for *apxIV* gene, that encoding the ApxIV toxin, specific of *A. pleuropneumoniae*. Likewise, all samples were positive for FISH assay using probes labeled with fluorescein (Table 9, Figures 13).

Table 9: Characteristics of 20 samples positive of *A. pleuropneumoniae* from drinking water from swine farms in Mexico.

No.	SAMPLE	NAD IDEPENDENT	*apxIA*	*apxIB*	*apxII*	*apxIII*	*apxIV*	*apxIV* FISH	BIOFILMS
1	Ags5-I	+	-	+	-	-	+	+	††††
2	Ags5-II	+	-	+	+	-	+	+	††††
3	Ags5-III	+	-	+	-	-	+	+	††††
4	Ags5-IV	+	-	-	-	-	+	+	†
5	Ags5-V	+	-	-	-	-	+	+	†
6	Ags5-VI	+	-	-	-	-	+	+	†
7	Ags8-I	+	-	-	-	-	+	+	†
8	Ags8-II	+	-	-	-	-	+	+	†
9	Ags8-III	+	-	-	-	-	+	+	†
10	Ags8-IV	+	-	-	-	-	+	+	†
11	Ags8-V	+	-	-	-	-	+	+	†
12	Ags8-VI	+	-	-	+	-	+	+	†
13	Ags12-II	+	-	-	-	-	+	+	††
14	Ags12-III	+	-	-	-	-	+	+	††
15	Ags12-V	+	-	-	-	-	+	+	††
16	Ags12-VIII	+	-	-	-	-	+	+	††
17	Ags12-XII	+	-	-	-	-	+	+	††
18	Ags12-XIII	+	-	-	-	-	+	+	††
19	Ags12-XIV	+	-	-	-	-	+	+	††

20	Ags12-XVI	+	-	-	-	-	+	+	††

+ Positive. † Biofilms formation.

6.5 16S rDNA and Apx toxin sequencing of isolates of *A. pleuropneumoniae* ApxIV positive.

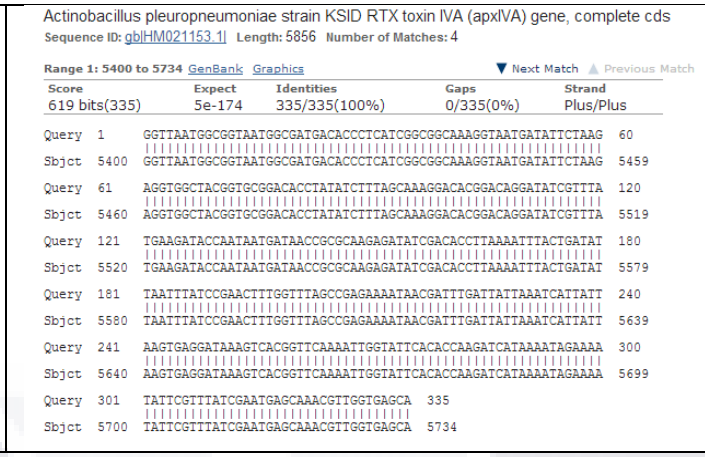
To verify exactly our isolates of *A. pleuropneumoniae* ApxIV toxin positive, was carried 16S rDNA sequencing and the amplified bands corresponding to the toxin ApxIV detected by PCR. However, the initial results of the sequencing of the 16S showed unexpected results; these sequences showed that we had two completely different species to *A. pleuropneumoniae*. The first species obtained with 99% sequence similarity (Table 10), was *Acinetobacter schindleri*, natural inhabitant of water bodies and now begins to be taken into account as an opportunistic pathogen in hospitals. This species was for the 3 isolates that showed high biofilm formation. The second species, *Stenotrophomonas maltophilia*, also with 99% similarity in their sequences (Table 10). This species is also a natural inhabitant of water bodies and opportunistic pathogen of the respiratory tract of pigs and humans. In this kind belong the 8 isolates were multiresistant according to the sequence of 16S. Also, in all these samples were detected *E. coli*. In the other nine, has not been able to confirm if have other co-isolated bacteria. It is noteworthy that the detections were made in pairs; *A. pleuropneumoniae* and *S. maltophilia* in some farms, and *A. pleuropneumoniae* and *A. schindleri* in other, always with *E. coli*.

However, all the samples were positive for *apxIV* gene, also we have the sequence corresponding to the amplified bands ApxIV toxin detected by PCR. These sequences showed a positive result of the sequence of toxin *A. pleuropneumoniae* ApxIV, corresponding from base 5400 to 5734. Likewise, there are no mutations or deletions in these sequences. Moreover, with respect to the detection of the others genes of the Apx toxins, only in 3 samples were positive for *apxIB* gene (Ags5-I, Ags5-II and Ags5-III), two samples were positive for *apxII* gene (Ags5-II and Ags8-VI), and any sample was positive for *apxIA* and *apxIII* genes (Table 9 and Figure 13). With this information we can only

know the one serotype of *A. pleuropneumoniae* found in the water samples, which belongs to serotype 7.

Table 10: Sequencing of 16S rDNA and the amplified band ApxIV toxin obtained from two isolates of *A. pleuropneumoniae* ApxIV positive.

Sample.	**Sequences and alignments.**	**Species.**	**Percent Similarity**
Ags5-I	Acinetobacter schindleri strain LUH5832 16S ribosomal RNA gene, partial sequence Sequence ID: refINR_025412.1| Length: 1466 Number of Matches: 1 Range 1: 8 to 484 GenBank Graphics Score: 802 bits(434) Expect: 0.0 Identities: 464/479(97%) Gaps: 6/479(1%) Strand: Plus/Plus Query 6 GGCTT-AC-CATGC-AGTCGAGCGGGG-NAGGTTGCTTCGGTAACTGACCTAGCGGCGGA 61 Sbjct 8 GGCTTAACACATGCAAGTCGAGCGGGGAAAGGTACCTT-GCTACCTGACCTAGCGGCGGA 66 Query 62 CGGGTGAGTAATGCTTANGAATCTGCCTATTAGTGGGGGACAACATTCCGAAAGGAATGC 121 Sbjct 67 CGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACGTTCCGAAAGGAACGC 126 Query 122 TAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGAT 181 Sbjct 127 TAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGAT 186 Query 182 GAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAG 241 Sbjct 187 GAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAG 246 Query 242 CGGGTCTGAGAGGATGATCCGCCACACTGGGTACTGAGACACGGCCCAGACTCCTACGGG 301 Sbjct 247 CGGGTCTGAGAGGATGATCCGCCACACT-GGG-ACTGAGACACGGCCCAGACTCCTACGGG 305 Query 302 AGGCAGCAGTGGGGAATATTGGACAATGGGCGGAAGCCTGATCCAGCCATGCCGCGTGTG 361 Sbjct 306 AGGCAGCAGTGGGGAATATTGGACAATGGGCGGAAGCCTGATCCAGCCATGCCGCGTGTG 365 Query 362 TGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTCCTTTAGTTAATA 421 Sbjct 366 TGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTCCTTTAGTTAATA 425 Query 422 CCTAAAGAGAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCNGCC 480 Sbjct 426 CCTAAAGAGAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCC 484	*Acinetobacter schindleri* 16S, from 8 to 484.	97%
Ags12-2	Stenotrophomonas maltophilia strain ATCC 19861 16S ribosomal RNA gene, complete sequence Sequence ID: refINR_040804.1| Length: 1517 Number of Matches: 1 Range 1: 33 to 501 GenBank Graphics Score: 817 bits(442) Expect: 0.0 Identities: 460/469(98%) Gaps: 2/469(0%) Strand: Plus/Plus Query 14 CATGC-ANTCG-ACGGCAGCACAGAGGAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGG 71 Sbjct 33 CATGCAAGTCGAACGGCAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGG 92 Query 72 TGAGGAATACATCGGAATCTACTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAAT 131 Sbjct 93 TGAGGAATACATCGGAATCTACTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAAT 152 Query 132 ACCGCATACGACCTACGGGTGAAAGCAGGGGATCTTCGGACCTTGCGCGATTGAATGAGC 191 Sbjct 153 ACCGCATACGACCTACGGGTGAAAGCAGGGGATCTTCGGACCTTGCGCGATTGAATGAGC 212 Query 192 CGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGG 251 Sbjct 213 CGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGG 272 Query 252 TCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA 311 Sbjct 273 TCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA 332 Query 312 GCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAG 371 Sbjct 333 GCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAG 392 Query 372 AAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCCGGCTAATACCTGG 431 Sbjct 393 AAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCTAATACCCGG 452 Query 432 TTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGC 480 Sbjct 453 TTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGC 501	*Stenotrophomonas maltophilia* 16S, from 33 to 501.	99%
Ags5-I	Actinobacillus pleuropneumoniae strain KSID RTX toxin IVA (apxIVA) gene, complete cds Sequence ID: gblHM021153.1| Length: 5856 Number of Matches: 4 Range 1: 5401 to 5734 GenBank Graphics Score: 606 bits(328) Expect: 4e-170 Identities: 332/334(99%) Gaps: 1/334(0%) Strand: Plus/Plus Query 1 GTT-ATGGCGGTAATGGCGATGACACCCTCATCGGCGGCAAAGGTAATGATATTCTAAGA 59 Sbjct 5401 GTTAATGGCGGTAATGGCGATGACACCCTCATCGGCGGCAAAGGTAATGATATTCTAAGA 5460 Query 60 SGTGGCTACGGTGCGGACACCTATATCTTTAGCAAAGGACACGGACAGGATATCGTTTAT 119 Sbjct 5461 GGTGGCTACGGTGCGGACACCTATATCTTTAGCAAAGGACACGGACAGGATATCGTTTAT 5520 Query 120 GAAGATACCAATAATGATAACCGCGCAAGAGATATCGACACCTTAAAATTTACTGATATT 179 Sbjct 5521 GAAGATACCAATAATGATAACCGCGCAAGAGATATCGACACCTTAAAATTTACTGATATT 5580 Query 180 AATTTATCCGAACTTTGGTTTAGCCGAGAAAATAACGATTTGATTATTAAATCATTATTA 239 Sbjct 5581 AATTTATCCGAACTTTGGTTTAGCCGAGAAAATAACGATTTGATTATTAAATCATTATTA 5640 Query 240 AGTGAGGATAAAGTCACGGTTCAAAATTGGTATTCACACCAAGATCATAAAATAGAAAAT 299 Sbjct 5641 AGTGAGGATAAAGTCACGGTTCAAAATTGGTATTCACACCAAGATCATAAAATAGAAAAT 5700 Query 300 ATTCGTTTATCGAATGAGCAAACGTTGGTGAGCA 333 Sbjct 5701 ATTCGTTTATCGAATGAGCAAACGTTGGTGAGCA 5734	*apxIV* toxin, from 5401 to 5734.	99%

Ags12-2		*apxIV* toxin, from 5400 to 5734.	100%

6.6 Detection of *A. pleuropneumoniae* in biofilms *in vitro* and *in vivo*.

Because biofilm formation is a strategy that allows bacteria survival in hostile environments besides increasing its pathogenicity because of the protection afforded to the action of different antimicrobial compounds because it allows a greater transfer of genes, we tested whether these samples had the ability to form this structure *in vitro*. The 20 positive samples of *A. pleuropneumoniae* obtained from drinking water in pig farms were able to generate biofilms in the interface liquid-air *in vitro* (Figure 15). All biofilms were positive by probes for *A. pleuropneumoniae* by FISH. In three of them, which was together to *A. schindleri*, generated a large amount of biofilm on the glass tube biofilm assay (Figure 15). By observing this ability that had all the isolates, we tested whether there was this structure of biofilms *in vivo* in environment surrounding the pigs mainly within the drinkers used by them. It was observed by probes for *A. pleuropneumoniae* by FISH testing biofilm formation *in vivo* in swine farms (Figure 16) and 4 to 100 samples tested. Here, found that apparently biofilms structures help to pathogen *A. pleuropneumoniae* to survive in the environment in the drinking water in the drinkers used by pigs on swine farms.

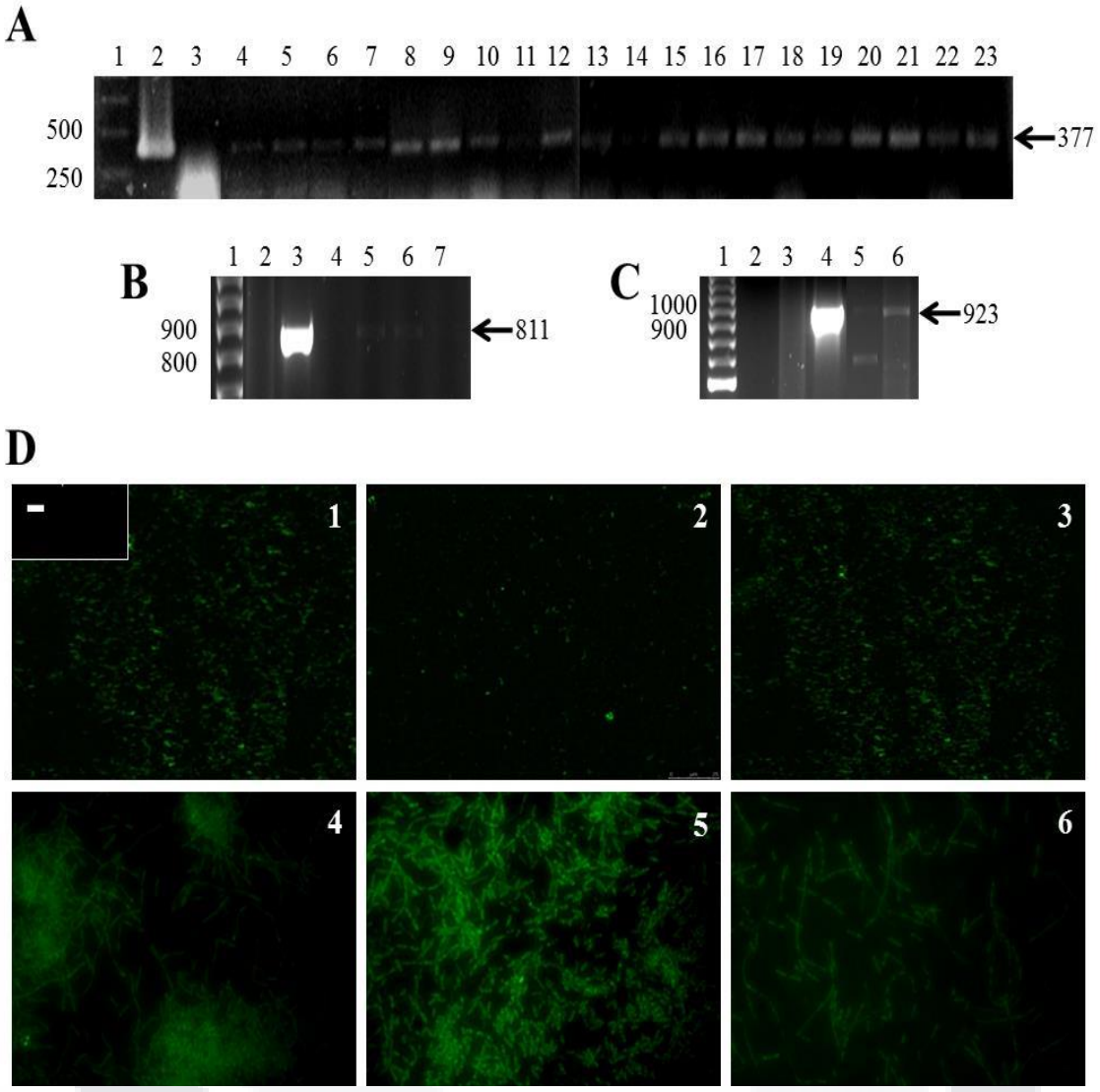


Figure 13: PCR performed on the 20 positive samples for of *A. pleuropneumoniae* obtained from drinking water from swine farms in three different farms. A) Lane 1 – molecular weight marker; Lane 2 – *A. pleuropneumoniae* 1-4074; Lane 3 – *E. coli* ATCC 25922; Lane 4 to 23, – isolates positives for *A. pleuropneumoniae apxIV* gene. B) Lane 1 – DNA ladder (Fermentas); Lane 2 – *A. pleuropneumoniae* serotype 3; Lane 3 – *A. pleuropneumoniae* 1-4074; Lane 4 – H_2O; Lane 5 to 7 – isolates positives for *A. pleuropneumoniae apxIB* gene. C) Lane 1 – DNA ladder (Fermentas); Lane 2 – H_2O; Lane 3 – *A. pleuropneumoniae* serotype 4; Lane 4 – *A. pleuropneumoniae* 1-4074; Lane 5 to 6 – isolates positives for *A. pleuropneumoniae apxII* gene. D) FISH images confirmation. Figures 1-3, CLSM. Figures 4-6, fluorescence microscope. 1) Positive control *A. pleuropneumoniae* S4074, 2-6) Samples of *A. pleuropneumoniae*. In the upper left of the first image shown negative control *E. coli* ATCC 22925.

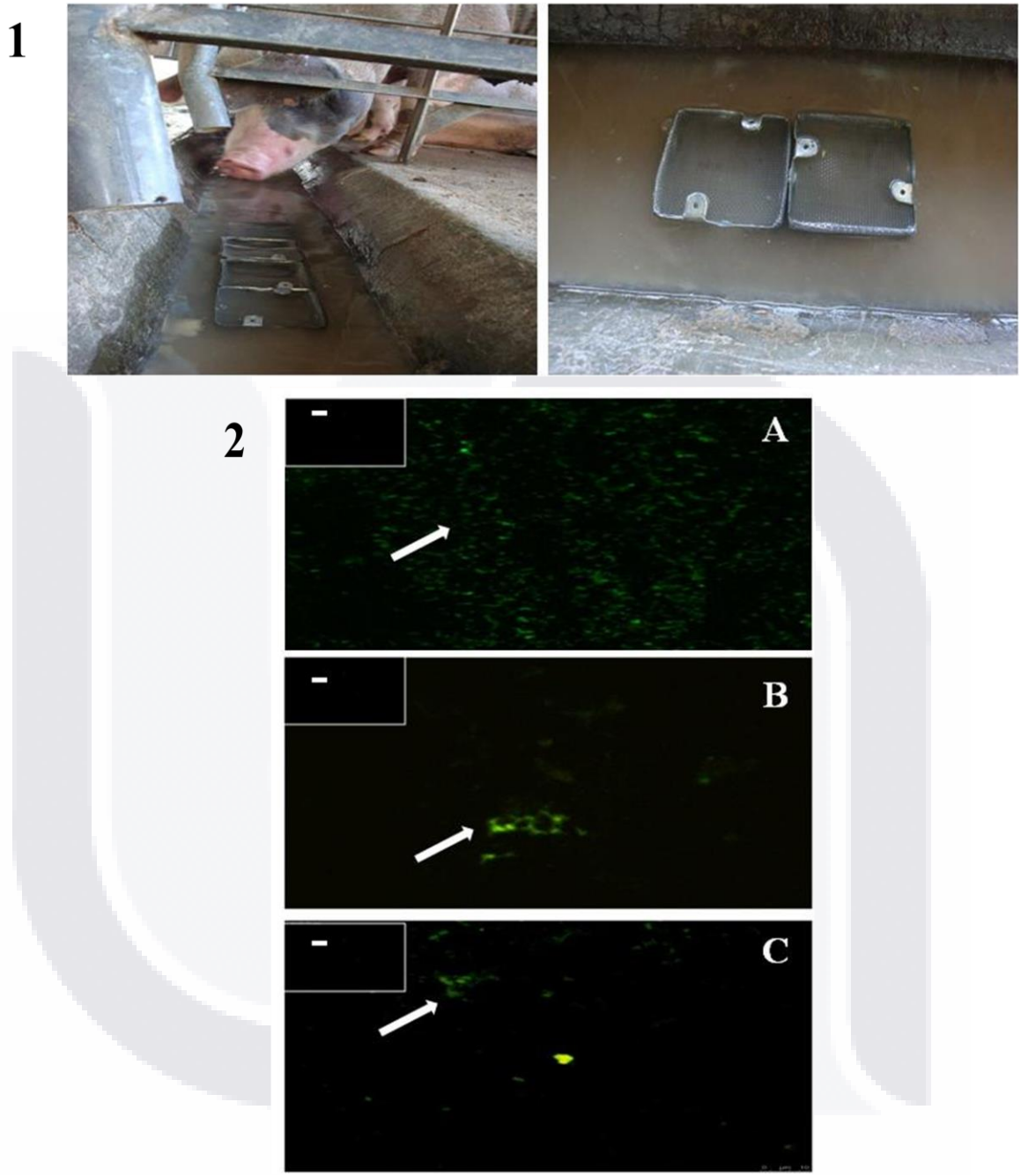


Figure 14: Detection by FISH of biofilms *in vivo* of *A. pleuropneumoniae*. 1) Shows the equipment designed for the production of biofilm directly in drinkers of pigs. 2) Analysis by FISH of samples, A) Positive control of *A. pleuropneumoniae* 1-4074, B and C) Biofilms views in drinkers in pigs from swine farms. In the upper left of all images shown negative control *E. coli* ATCC 22925.

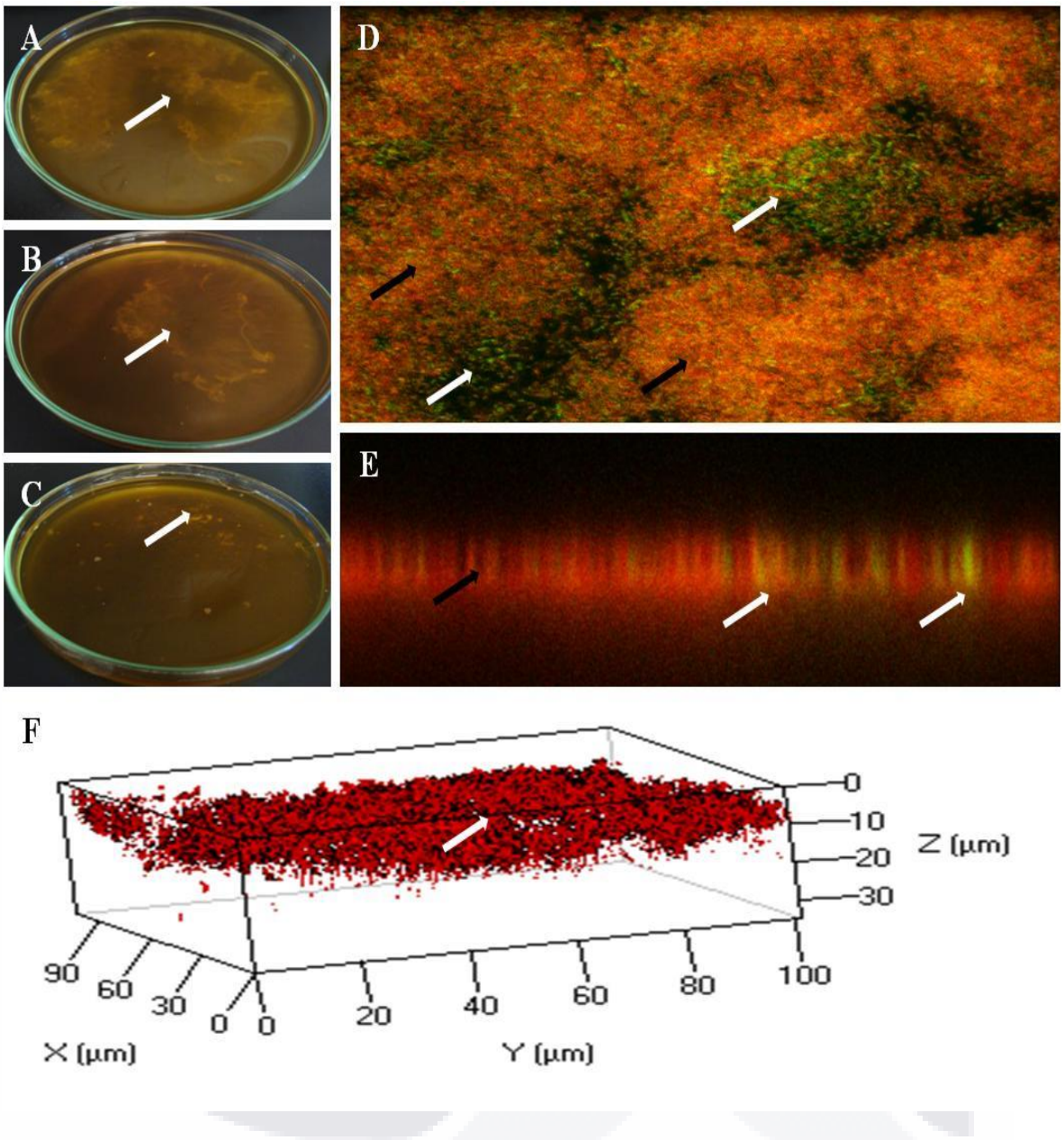


Figure 15: Biofilms assay to isolates of *A. pleuropneumoniae* from drinking water. A - C) Show *in vitro* biofilm formed in the interfaceliquid-air by the three isolates of *A. pleuropneumoniae* from drinking water from farm 5. D - E) CLSM photographs taken from one of the isolates from farm 5 (Ags5-II), can be seen to bacteria (green - labeled with fluorescein, white arrows) embedded in extracellular matrix (red – labeled with bromide ethidium, black arrow); F) Measurement of biofilm form for the isolate Ags5-II with CLSM. Can be observed where the biofilm had a thickness of 25 µm. D - E) Marked in green *A. pleuropneumoniae* with fluorescein and F) with TAMRA.

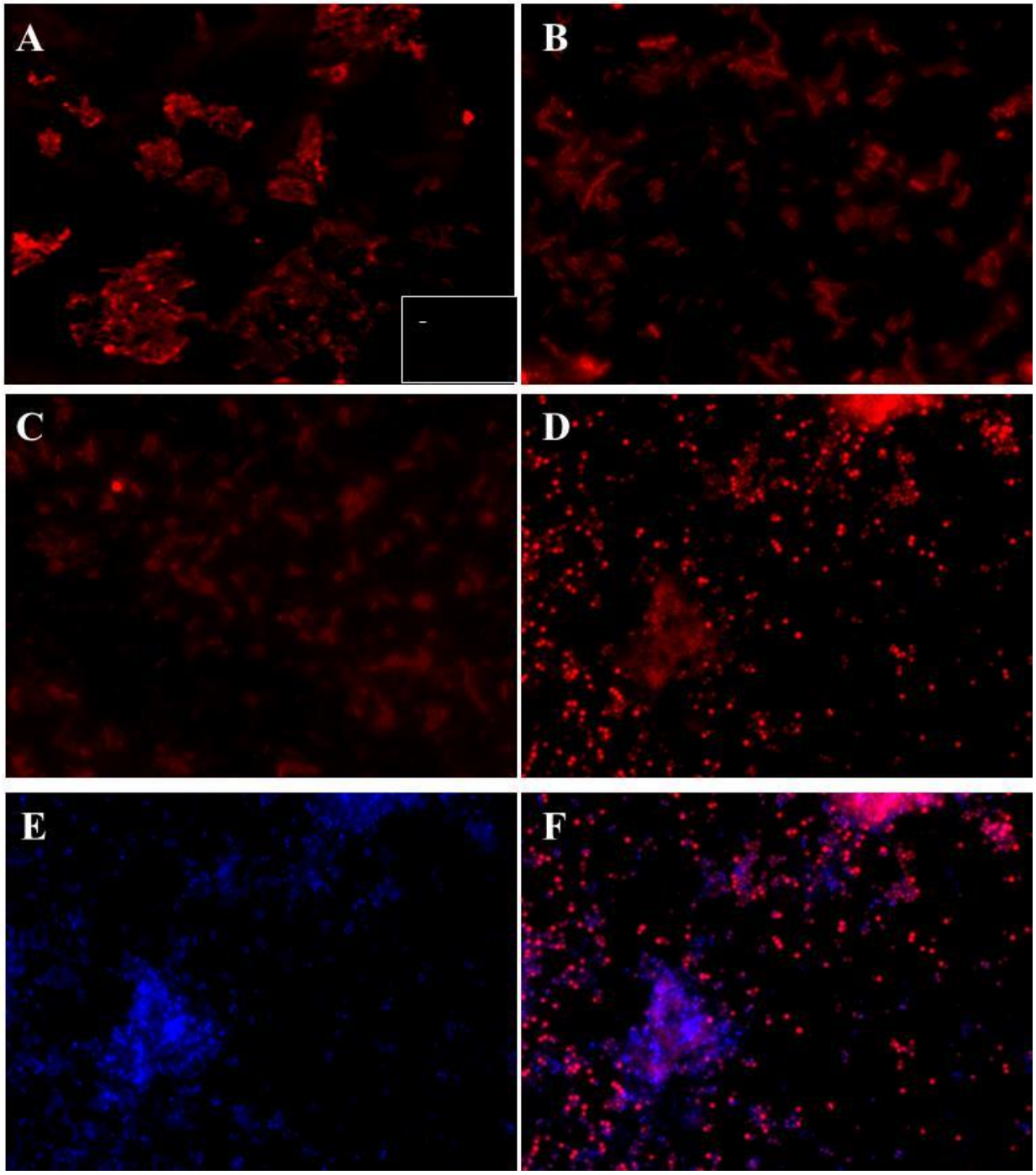


Figure 16: FISH of *A. pleuropneumoniae* in biofilms detected *in vivo* in drinking water in swine farms with the ApxIVAN-TAMRA probes. A-D) Epifluorescence microscopy pictures. A) *A. pleuropneumoniae* 1-4074 and in box in the lower right corner (-) Negative control. B-D) *A. pleuropneumoniae* positives samples from drinkers. E) General DAPI staining of microorganisms in the sample. F) Merge between D and E.

6.7Antimicrobial susceptibility testing.

Likewise, and as discussed above, biofilms increase resistance by microorganisms has antimicrobial agents. For this reason, all samples were subjected to Antimicrobial Susceptibility Testing where we tested 12 different antibiotic resistance, these antibiotics being used widely in the swine industry and in human disease. Antibiotics tested were: amikacin, ampicillin, carbenicillin, cephalothin, cefotaxime, ceftriaxone, chloramphenicol, gentamicin, netilmicin, nitrofurantoin, pefloxacin, and trimethoprim-sulfamethoxazole. The results are shown in Table 11. Resistance was observed against the antibiotics nitrofurantoin (40%), pefloxacin (20%), gentamicin (5%), cefotaxime (40%), amikacin (35%), ampicillin (40%), ceftriaxone (40%), chloramphenicol (40%), and cephalothin (40%). Intermediate resistance was observed to nitrofurantoin (10%), pefloxacin (5%), gentamicin (15%), cefotaxime (10%), amikacin and cefotaxime (5%), and chloramphenicol (15%). All isolates showed high biofilms formation was susceptible to all the antibiotics in planktonic phase (Table 11 and Figure 17). However, were able to detect 8 samples presented multiresistance to antibiotics, coming all from the same farm (Figure 18).

Table 11: Antimicrobial susceptibility testing performed at 20 samples from drinking water from hog farms.

	NF	CB	PEF	NET	GE	CTX	SXT	AK	AM	CRC	CL	CF
S	10	20	15	20	16	10	20	12	12	11	9	12
%	50	100	75	100	80	50	100	60	60	55	45	60
I	2	0	1	0	3	2	0	1	0	1	3	0
%	10	0	5	0	15	10	0	5	0	5	15	0
R	8	0	4	0	1	8	0	7	8	8	8	8
%	40	0	20	0	5	40	0	35	40	40	40	40

S: Susceptible; I: Intermediate; R: Resistant.
NF: Nitrofurantoin, CB: Carbenicillin, PEF: Pefloxacin, NET: Netilmicin, GE: Gentamicin, CTX: Cefotaxime, SXT: Trimethoprim-Sulfamethoxazole, AK: Amikacin, AM: Ampicillin, CRC: Ceftriaxone, CL: Chloramphenicol, CF: Cephalothin.

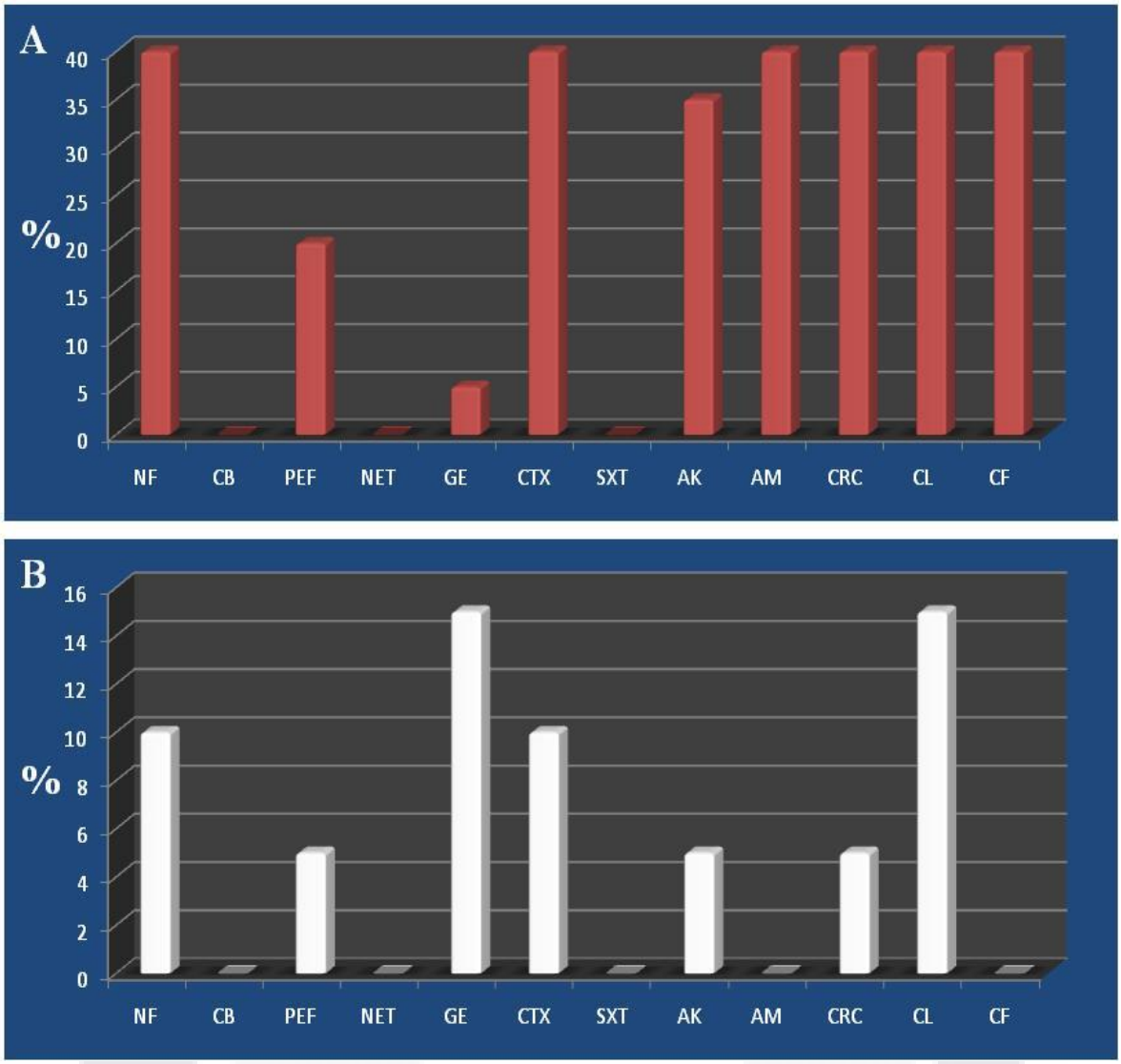


Figure 17: A) Percentage of isolated from drinking water from swine farms with antibiotics resistance. B) Percentage of isolated from drinking water from swine farms with intermediate resistance to antibiotics. NF: Nitrofurantoin, CB: Carbenicillin, PEF: Pefloxacin, NET: Netilmicin, GE: Gentamicin, CTX: Cefotaxime, SXT: Trimethoprim-Sulfamethoxazole, AK: Amikacin, AM: Ampicillin, CRC: Ceftriaxone, CL: Chloramphenicol, CF: Cephalothin.

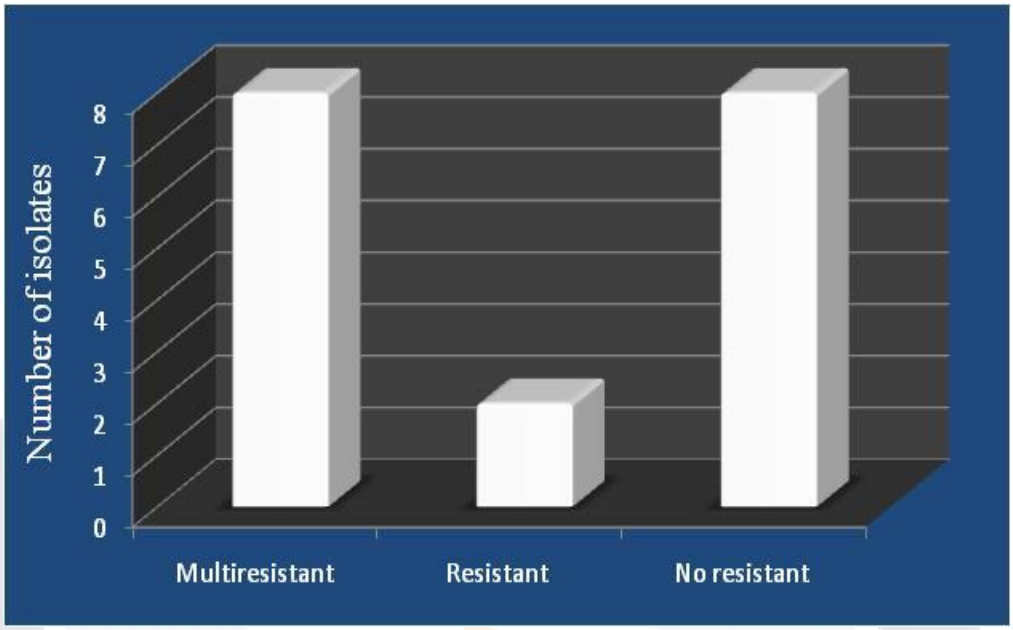


Figure 18: Graph showing the number of isolates in this study showed antimicrobial multiresistance, antibiotic resistance or no resistance to any of them.

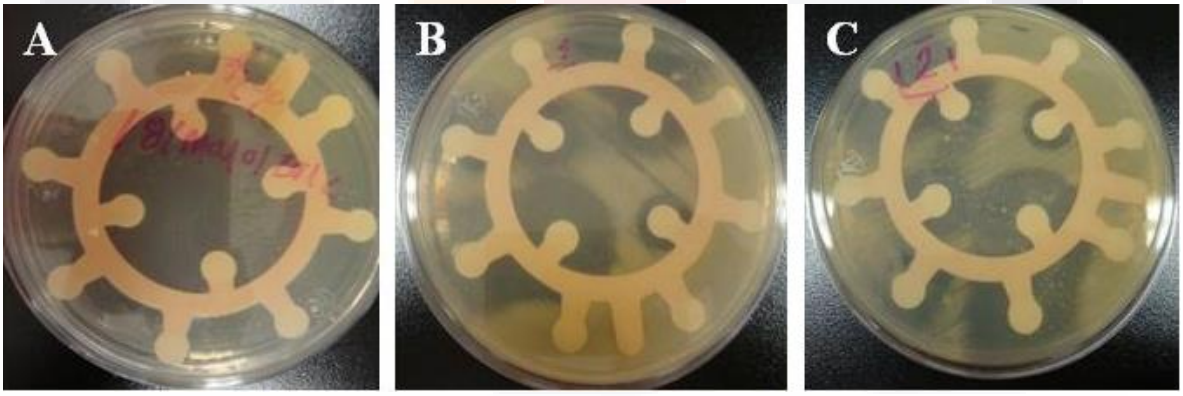


Figure 19: Antimicrobial susceptibility testing performed according to the CLSI (2011). A) Control strain *A. pleuropneumoniae* 1-4074, B and C) show two strains showing antimicrobial multiresistance.

6.8 EM analysis of biofilms formed by isolated from drinking water.

For analysis by electron microscopy three samples were chosen (Ags5-I, 5-II and 5-III). Isolates were growing, as mentioned previously in the methodology, until biofilms were obtained for further processing. Once the results obtained, it was possible to observe first, that these isolates have a morphology different from the control used (*A. pleuropneumoniae* 1-4074) (Figure 20). The isolates have form of coccobacillus although present, are larger and apparently more roughened surface when compared with the control. Moreover, in the biofilms of the samples can be readily appreciated that the extracellular matrix surrounding the cells to form the biofilm and structures, cell-cell adhesion in a greater number than in the control (Figure 20).

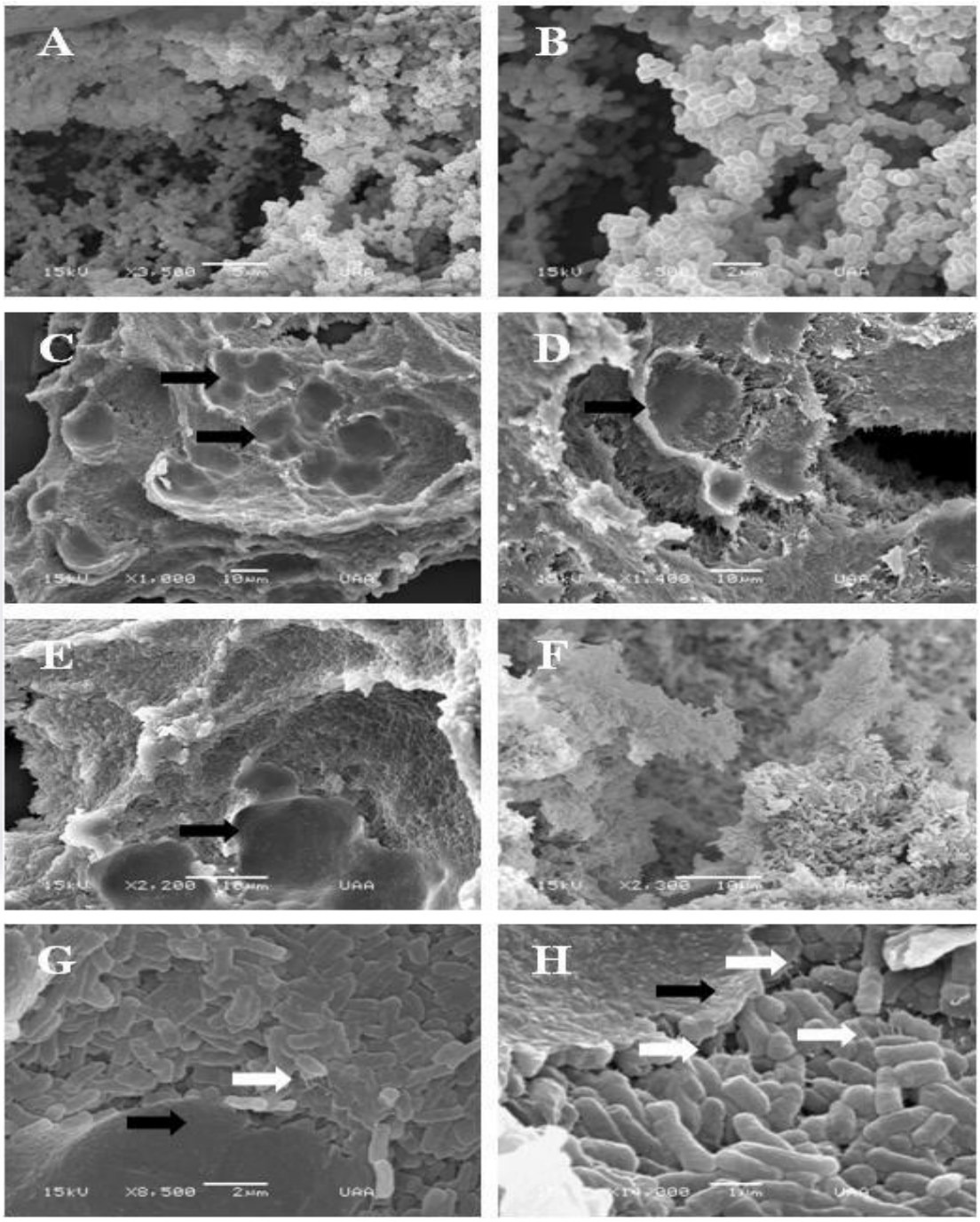


Figure 20: Photographs by EM of biofilms formed by three isolates of *A. pleuropneumoniae* ApxIV positive from drinking water from swine farms. A and B) Biofilm *A. pleuropneumoniae* S-1-4074, C - H) Biofilms of the isolated 5-I (C, E and H), 5-II (D and G) and 5-III (F) at different magnifications can be seen where the matrix where extracellular bacteria are immersed (black arrows) and some adhesion structures or extracellular matrix in development (white arrows). Increases 3,500; 6,500; 1,000; 1,400; 2,200; 2,300; 8,500 and 14.300 respectively.

6.9 Analysis nonpathogenic or commensal bacteria in drinking water of swine farms.

We performed the analysis of nonpathogenic or commensal bacteria that could be related to multi-species biofilms in drinking water in swine farms. At this point, first were obtained 10 isolates of *E. coli* from three farms where they had located *A. pleuropneumoniae* by PCR, as well as, a fourth farm were obtained isolates of *Citrobacter freundii* and *Enterobacter aerogenes* (Tables 12 and 13). Should be noted that all *E. coli* isolates belonging to the group of extraintestinal (ExPEC) and all had the ability to form a weak biofilm in the assay of 96-well microplate in LB + 30% glycerol, and also formed biofilms in the liquid-air interface. Only one of the farms, no bacteria was isolated by this method that helps us isolate mainly *E. coli* and other bacteria belonging to the *Enterobacteriaceae*. Likewise, were analyzed by sequencing of the ribosomal DNA (16S rDNA) samples of DNA extracted from samples of drinking water, in order to detect other bacterial species that could be related to multi-species biofilms where may be participating *A. pleuropneumoniae* or other pathogen analyzed in this study. This was done using universal primers as mentioned in the methodology. The samples for this test were selected in which only could be detected at *A. pleuropneumoniae* in drinking water (farms being 5, 8, 12, 13 and 14 only). In total 25 samples were sequenced, 5 per farm. This list of bacteria was obtained by *in silico* analysis performed with the software page of NCBI (National Center for Biotechnology Information), which include the genera: *Prevotella*, *Ideonella*, *Novosphingobium*, *Erythrobacter*, *Propionivibrio*, *Burkholderia*, *Pseudomonas* and *Enterobacter* (Table 14). In this analysis, a very important thing to note is that none of the species found, was found in more than one farm, that is, each farm has its bacterial population, at least dominant species, different from the other. Furthermore, we failed to detect *E. coli* by this analysis (although isolated from samples), and, also failed to detect any of the 2 bacteria species were obtained by analysis of 20 isolates 16S drinking water (*S. maltophilia* and *A. schindleri*). Moreover, it is worth mentioning that the primers used for this study were able to recognize and amplify the pathogen *A. pleuropneumoniae*, *P. multocida*, *B. bronchiseptica* and *M. hyopneumoniae*, something that had not been reported in the article. However, we failed to detect *E. coli* ATCC25922 with these universal primers (Figure 21).

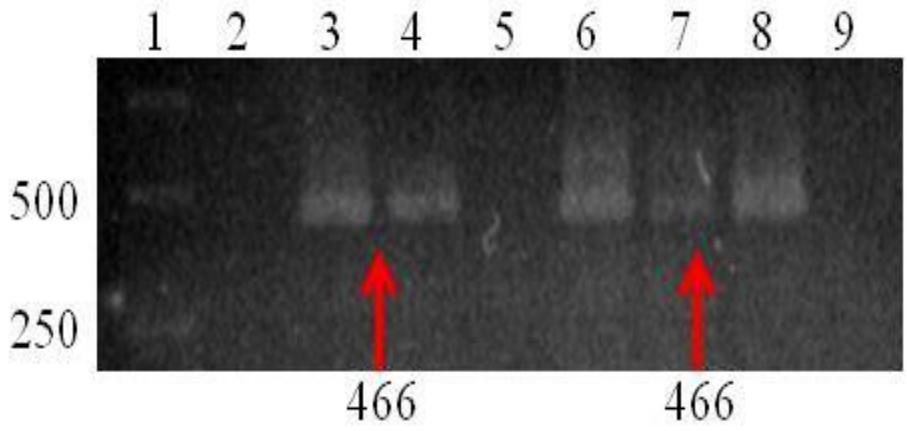


Figure 21: Ribosomal DNA amplification with universal primers: Line 1 - Molecular weight marker, line 2 - *E. coli* ATCC25922, line 3 and 4 - *A. pleuropneumoniae* S-4074 and serotype 15, line 5 - *S. suis* 735 serotype 2, line 6 - *P. multocida* 4-4056 (type D, DNT +), line 7 - *B. bronchiseptica* 276, line 8 - *M. hyopneumoniae* ATCC 25095and line 9 - H_2O.

Table 12: Farms where it was isolated *E. coli* from drinking water samples which achieved positive detection of *A. pleuropneumoniae*.

Farm	*E. coli* isolated.
1	X
2	X
3	X
4	X
5	-
6	X
7	X
8	+(4)
9	X
10	X
11	X
12	+*(1)
13	+(3)
14	+(3)
Total	10

+ Isolates of *E. coli*; + * isolation of *C. freundii* and *E. aerogenes*; () number of isolates per farm; X farms where they are held attempts to isolate *E. coli*, since it is not detected *A. pleuropneumoniae* there in.

Table13: Characteristics of *E. coli* isolated from drinking water in swine farms.

Isolated	*fyuA*	*kpsMTII*	*papC*	Group	*arp*	*chuA*	*yjaA*	TspE4.C2	*arpA* (goupE)	*trpA* (groupC)	Phylogroup	Biofilms.
8-1	+	+	+	ExPEC	+	+	-	-	-	NA	D	0.021± 0.009
8-2	+	+	+	ExPEC	+	+	-	-	-	NA	D	0.053± 0.013
8-3	+	+	+	ExPEC	+	+	-	-	-	NA	D	0.027± 0.009
8-4	+	+	+	ExPEC	+	+	-	-	-	NA	D	0.048± 0.016
13-1	+	+	+	ExPEC	+	-	-	+	NA	NA	B1	0.033± 0.018
13-2	+	+	+	ExPEC	+	-	-	+	NA	NA	B1	0.023± 0.009
13-5	+	+	+	ExPEC	+	-	+	-	NA	+	C	0.023± 0.008
14-1	+	+	+	ExPEC	+	-	-	-	NA	NA	A	0.003± 0.011
14-2	+	+	+	ExPEC	+	-	-	-	NA	NA	A	0.019± 0.01
14-5	+	+	+	ExPEC	+	+	-	-	+	NA	E	0.019± 0.014

+, positive sample; -, negative sample; NA, no screening for C-specific primer or for D-specific primer.

Table 14: Analysis of 16S rDNA of samples obtained from total DNA from drinking water of swine farms. Note that the bacterial community in this drinking water is composed of different bacterial species.

Farm.	Bacterial Species.
5	*Ideaonella dechloratans* (87%).
	Propionivibrio dicarboxylicus (86%).
	Methylococcus capsulatus (81%).
	Methanosarcina spp. (53%).
	Nocardiopsis spp. (39%).
	Halomonas spp. (34%).
8	*Prevotella paludivivens* (97%).
	Prevotella oulorum (95%).
	Prevotella albensis (95%).
	Prevotella micans (95%).
	Prevotella spp. (>95%).
12	*Novosphingobium tardaugens* (88%).
	Novosphingobium resinovorum (87%).
	Novosphingobium spp. (87%).
	Erythrobacter spp. (87%).
	Sphingomonadaceae spp. (87%).
	Sphingopyxis sp. (87%).
13	*Burkholderia ferrariae* (97%).
	Burkholderia spp. (97%).
	Methyloversatilis universalis (97%).

	Zoogloea caeni (96%).
	Thauera spp. (96%).
	Azospira restricta (96%).
14	*Dehalobacter restrictus* (96%).
	Pseudomonas flectens (89%).
	Enterobacter pulveris (84%).
	Halochromatium spp. (82%).
	Salmonella enterica (65%).
	Erwinia psidii (65%).

6.10 Multi-species biofilms analysis.

6.10.1 Multi-species biofilms formation with NAD supplementation.

Field isolate, *A. pleuropneumoniae* serotype 1, strain 719 and 4074, were used in a 96-well microtiter plate in multi-species biofilms assays. Under favorable growth conditions for *A. pleuropneumoniae* biofilms formation, this respiratory swine pathogen was able to form a strong two-species biofilms (OD 2.984 - 2.701) with bacteria belonging to PRCD *S. suis, B. bronchiseptica* and *P. multocida*, and with the non-pathogenic nasal isolated *S. aureus* (Figures 22, 23, 24 and 25). By contrast, with *E. coli* produced a considerable decrease on biofilms formation (OD 0.661), or even prevents their formation (Figure 26). In order to confirm that the other species were present in these biofilms, CFU of *A. pleuropneumoniae* and the other bacteria were counted as described in materials and methods (Figures 22 - 26). In all cases, *A. pleuropneumoniae* was not able to avoid growth of other species and vice-versa; hence, both species participated in biofilms formation. Moreover, the proportions of live/dead bacteria in the multi-species biofilms were observed too (Figures 30 - 37). In the two-species biofilms formed by *A. pleuropneumoniae* - *B. bronchiseptica* and *A. pleuropneumoniae* - *S. aureus*, there were appreciated an increase in the proportion of live bacteria in the multi-species biofilms, with respect to the mono-species biofilm of *A. pleuropneumoniae*. On the other hand, there were also changes in the proportion of dead bacteria in some multi-species biofilms. In the two-species biofilms

formed with *B. bronchiseptica* and *P. multocida*, there were appreciated a slight increase in dead bacteria. Otherwise, in all biofilms where *E. coli* was present, decreased the number of dead bacteria. Finally, in the biofilms formed by *A. pleuropneumoniae* - *S. aureus*, no dead bacteria were observed.

In the case of three-species biofilms, *A. pleuropneumoniae* was able to form strong biofilms with *S. suis* - *B. bronchiseptica* (2.505); and, as in all the biofilms involving *E. coli*, weak biofilms with *S. suis* - *E. coli* (0.673) and *B. bronchiseptica* - *E. coli* (0.717, Figure 27 - 29). With respect to the CFU counts, it was notable that *A. pleuropneumoniae* proportions were higher in the three-species biofilms with *B. bronchiseptica*, than in the two-species biofilms (Figures 27 - 29); while in three-species biofilms there were no differences between these two bacteria ($p \geq 0.0693$), in two-species biofilms *A. pleuropneumoniae* proportion was lesser than *B. bronchiseptica*.

In the case of *S. suis*, its CFU counts were lesser in the three-species biofilms with *A. pleuropneumoniae* and *B. bronchiseptica* ($p < 0.0001$), than in the respective two-species biofilms (Figures 22 and 27). *E. coli* prevented growth of *S. suis*, but *A. pleuropneumoniae* and *B. bronchiseptica* CFU counts were not affected. As previously observed in biofilms involving *E. coli*, this bacteria was the most predominant ($p \leq 0.0005$, Figures 28 and 29).

Finally, is very important to remark that, in the condition tested, *S. suis*, *B. bronchiseptica* and *P. multocida* only formed weak mono-specie biofilms. On the contrary, in two or three-species biofilms with *A. pleuropneumoniae*, they formed strong biofilms. These results suggests than interaction whit *A. pleuropneumoniae* favored the biofilms formation of these three bacteria.

6.10.2 Multi-species biofilms matrix composition with NAD supplementation.

To search for variations in the composition and structure of extracellular matrix in the two-species biofilms of *A. pleuropneumoniae,* with respect to the mono-specie biofilms, the main components were tested (Figures 30 - 37). Regarding with the polysaccharide PGA component, important structural differences were observed. In most of two-species biofilms it showed clusters or filament-like structures, while in *A. pleuropneumoniae* mono-species biofilms, the polysaccharide component presented a mainly homogeneous distribution.

In the case of eDNA, which was labeled with BOBO^{TM}-3 iodide, several differences were observed. With NAD supplementation, significant increases were observed in the two-species biofilms formed with *B. bronchiseptica*, *P. multocida*, *S. aureus* and *E. coli*; and also in the three-species biofilms with *S. suis* and *B. bronchiseptica* (Figures 30 - 37).

Regarding to proteins, no important variations were observed in most two-species biofilms, with respect to the mono-species biofilms of *A. pleuropneumoniae*. Only in the biofilms of *A. pleuropneumoniae* - *S. suis*, small increases in the proteins component were observed. Finally, in all biofilms with *E. coli* (two and three-species) the proteins composition was appreciated slightly lower.

The stain FilmTracer FM 1-43, which inserts into the surface membrane in all bacteria, helps to evaluate the morphology of the biofilms. Some differences were observed in the morphology of two-species biofilms, compared with the *A. pleuropneumoniae* mono-species biofilms. In the two-species biofilms formed with *B. bronchiseptica*, *S. aureus* and *E. coli*, slight variations were observed in the homogeneity of biofilms. Likewise, these changes were once more observed in all three-species biofilms in which was involving *E. coli*. Moreover, in the three-species biofilms formed by *A. pleuropneumoniae, S. suis* and *B. bronchiseptica*, there was an increase in the occurrence of clusters, clearly seen by fluorescence enhancement (Figures 30 - 37).

6.10.3 Effect of enzymatic treatment on multi-species biofilms with NAD supplementation.

To verify if the variations seen in the composition of extracellular matrix of these multi-species biofilms were related to changes in the structural function, enzymatic treatments of biofilms with proteinase K, DNase I and dispersin B were made (Figures 38 - 42). In the cases of proteinase K and DNAse I, which is used to observe the structural function of the proteins and eDNA in the biofilms, respectively, there were no changes with respect to the control of mono-species biofilms formed by *A. pleuropneumoniae*, and also with the biofilms without enzymatic treatment (Figures 38 - 41). Finally, the tests with dispersin B, which is known to have the ability to disperse the mono-species biofilms formed by *A. pleuropneumoniae,* showed significant increments in the resistance against the action of this enzyme in some multi-species biofilms. These increments were observed in the two-species biofilms with *S. aureus* (21%) and *E. coli* (24%), and also in all the three-species biofilms with *E. coli* (increased between 20 - 68%, Figure 42).

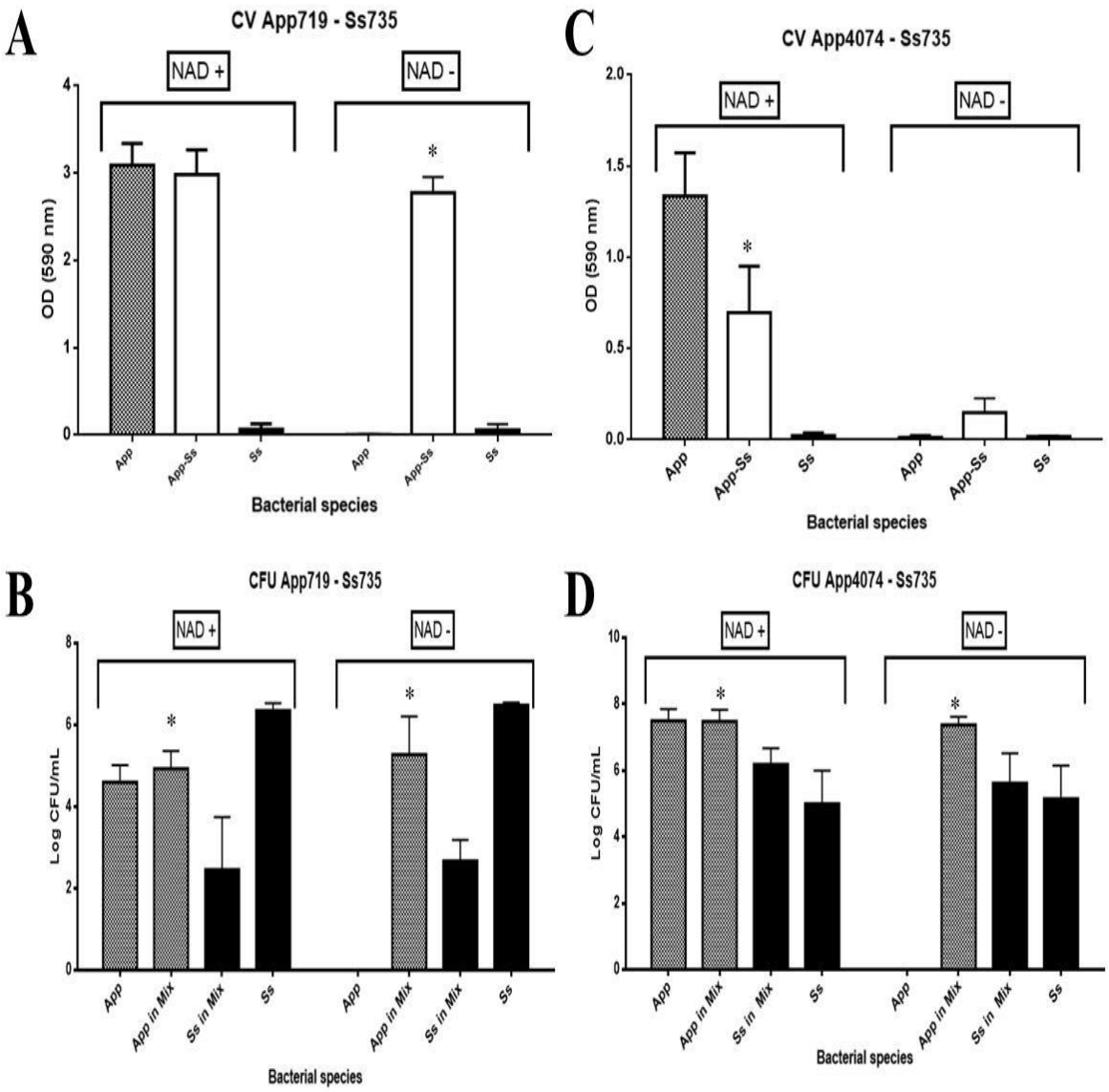


Figure 22: Di-specie biofilms between *A. pleuropneumoniae* 719 and 4074 with *S. suis* 735 in microplates in *A. pleuropneumoniae* conditions. A and C) Crystal Violet (CV) measuring of *A. pleuropneumoniae* - *S. suis* biofilms. B and D) Colony Forming Units (CFU) of *A. pleuropneumoniae* - *S. suis* biofilms. App: *A. pleuropneumoniae*; Ss: *S. suis* 735.

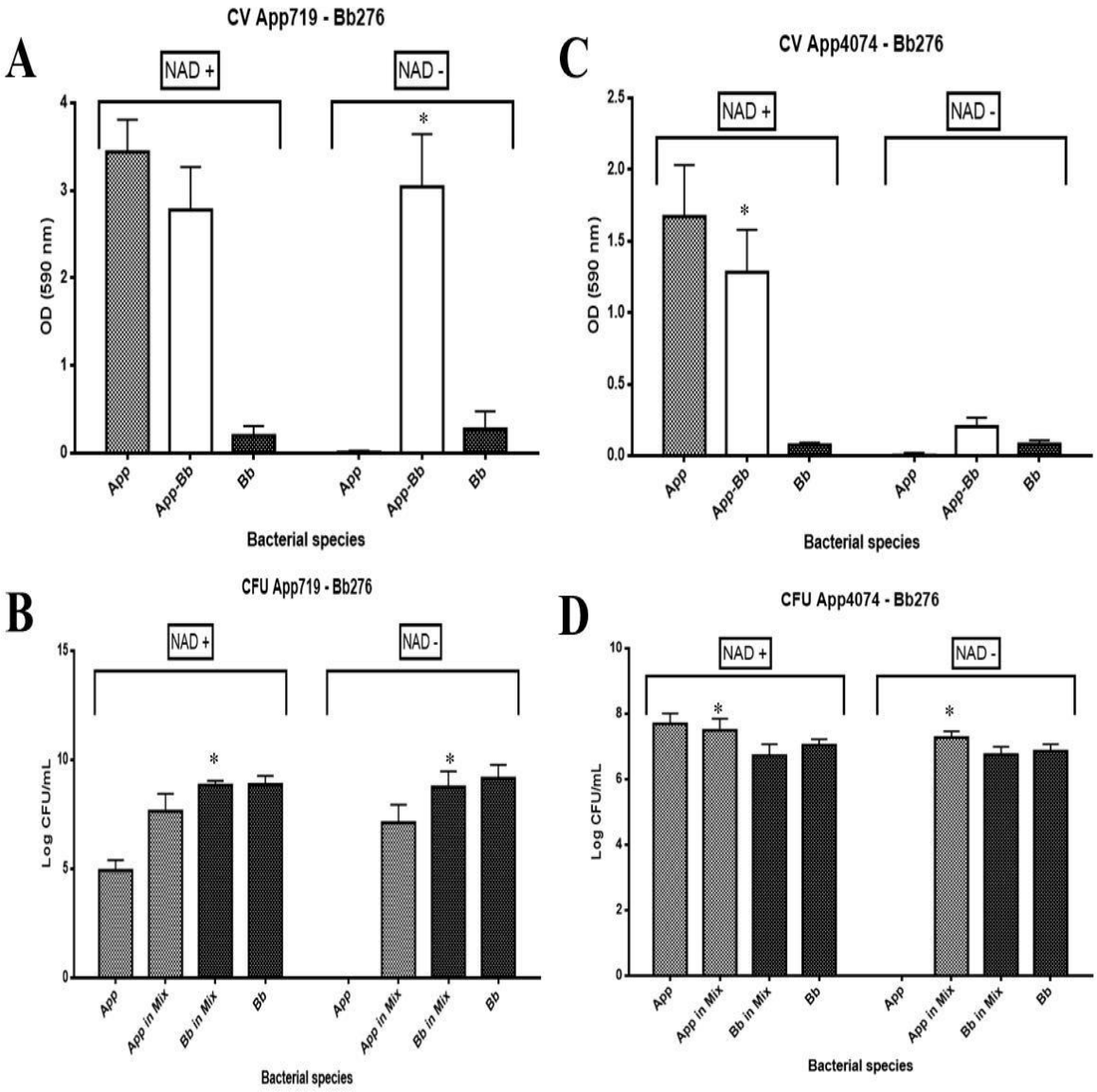


Figure 23: Di-specie biofilms between *A. pleuropneumoniae* 719 and 4074 with *B. bronchiseptica* 276 in microplates in *A. pleuropneumoniae* conditions. A and C) Crystal Violet (CV) measuring of *A. pleuropneumoniae* - *B. bronchiseptica* biofilms. B and D) Colony Forming Units (CFU) of *A. pleuropneumoniae* - *B. bronchiseptica* biofilms. App: *A. pleuropneumoniae*; Bb: *B. bronchiseptica* 276.

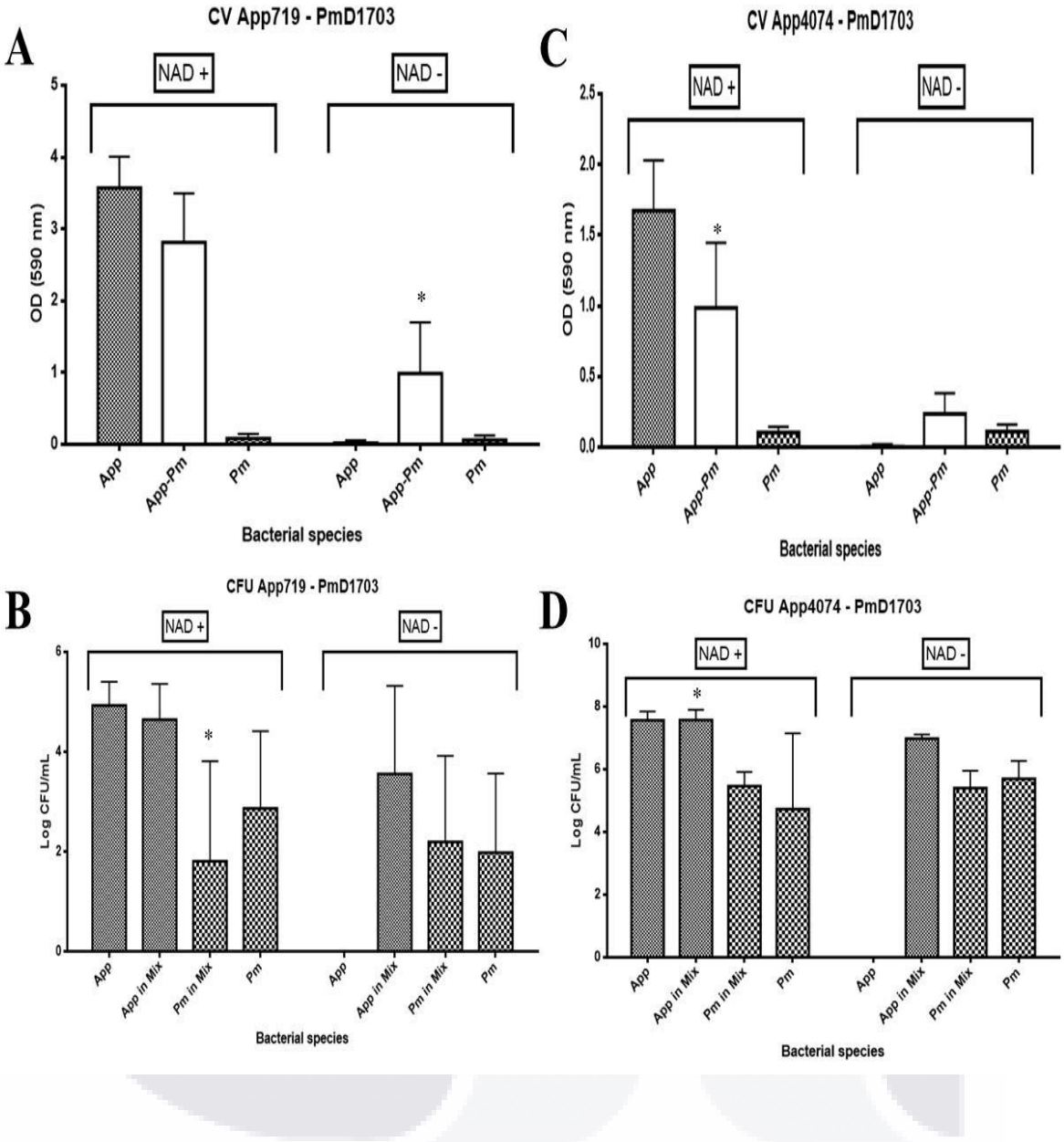


Figure 24: Di-specie biofilms between *A. pleuropneumoniae* 719 and 4074 with *P. multocida* D 1703in microplates in *A. pleuropneumoniae* conditions. A and C) Crystal Violet (CV) measuring of *A. pleuropneumoniae* - *P. multocida* biofilms. B and D) Colony Forming Units (CFU) of *A. pleuropneumoniae* - *P. multocida* biofilms. App: *A. pleuropneumoniae*; Pm: *P. multocida* D1703.

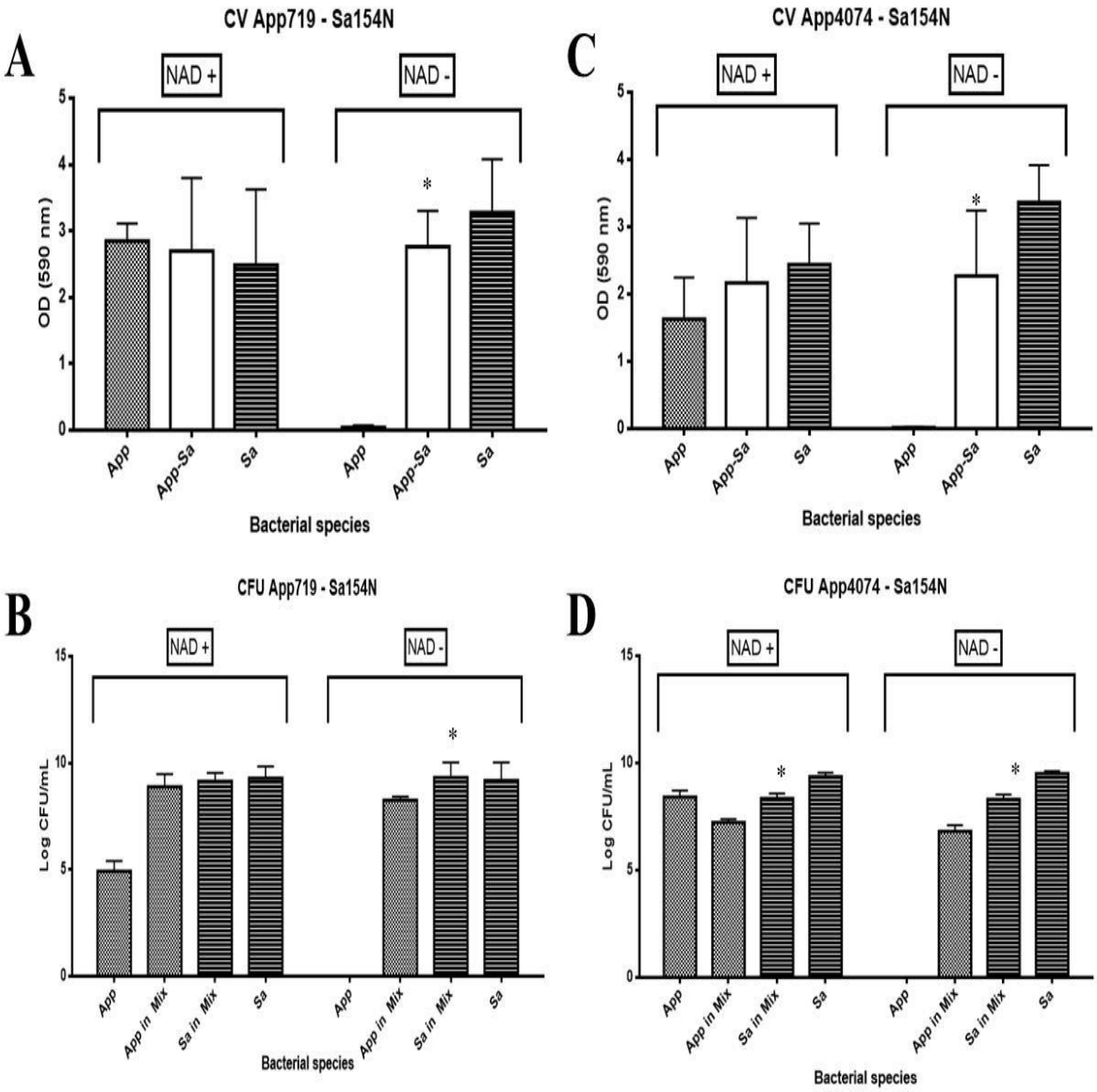


Figure 25: Di-specie biofilms between *A. pleuropneumoniae* 719 and 4074 with *S. aureus* N154 in microplates in *A. pleuropneumoniae* conditions. A and C) Crystal Violet (CV) measuring of *A. pleuropneumoniae* - *S. aureus* biofilms. B and D) Colony Forming Units (CFU) of *A. pleuropneumoniae* - *S. aureus* biofilms. App: *A. pleuropneumoniae*; Sa: *S. aureus* 154N.

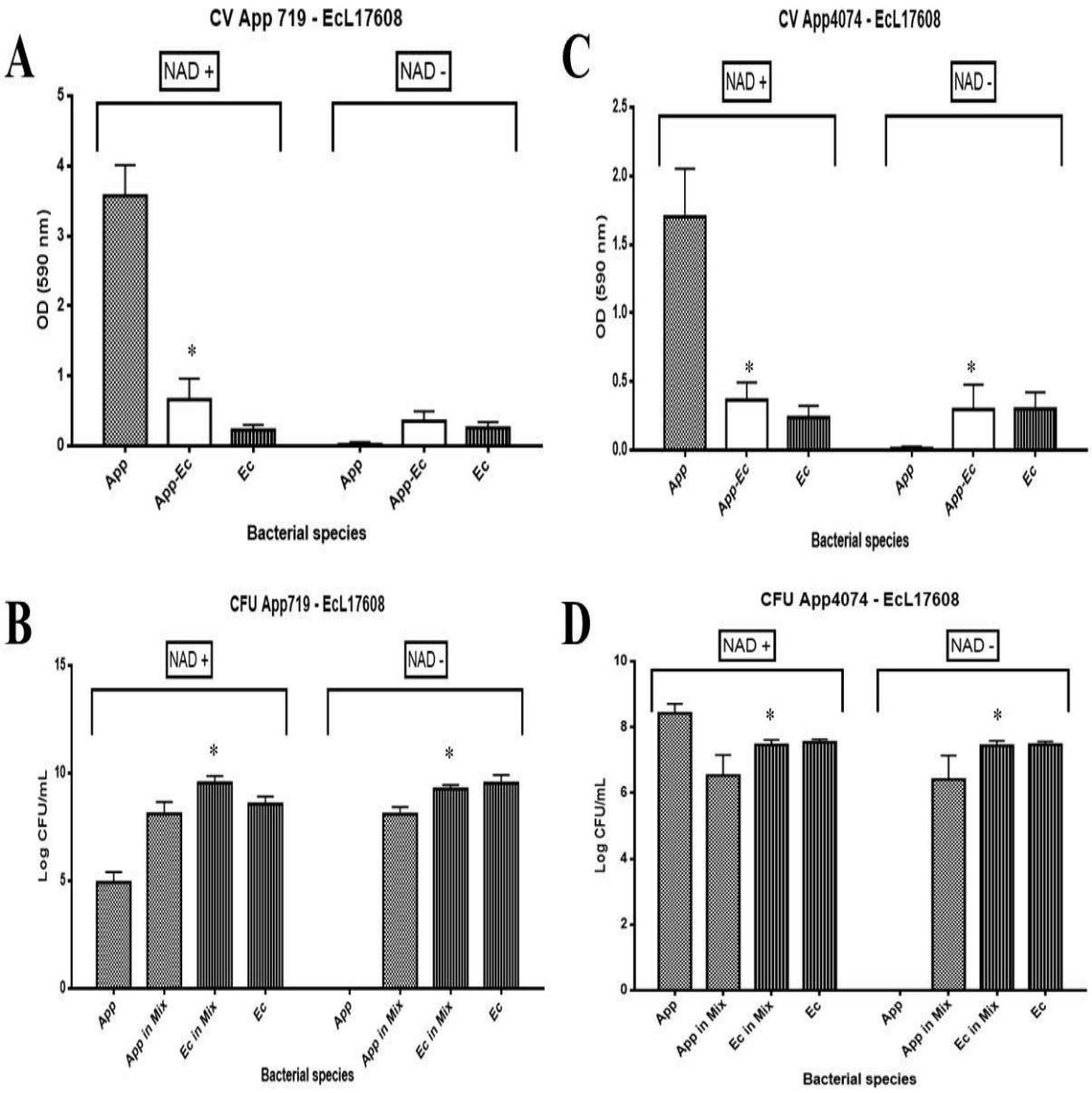


Figure 26: Di-specie biofilms between *A. pleuropneumoniae* 719 and 4074 with *E. coli* L17608 in microplates in *A. pleuropneumoniae* conditions. A and B) Crystal Violet (CV) measuring of *A. pleuropneumoniae* - *E. coli* biofilms. B and D) Colony Forming Units (CFU) of *A. pleuropneumoniae* - *E. coli* biofilms. App: *A. pleuropneumoniae*; Ec: *E. coli* L17608.

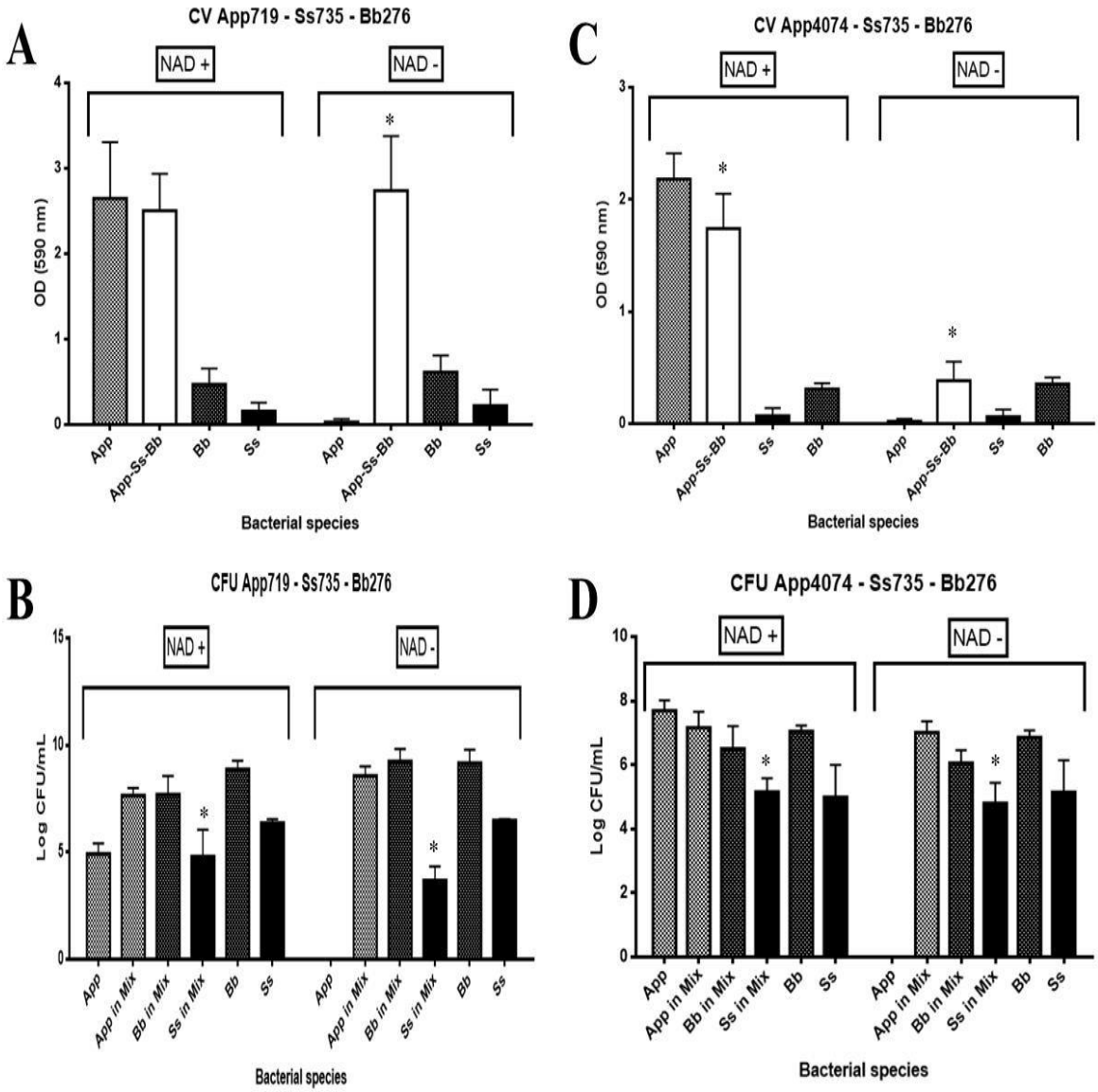


Figure 27: Tri-specie biofilms between *A. pleuropneumoniae* 719 and 4074 with *S. suis* 735 and *B. bronchiseptica* 276 in microplates in *A. pleuropneumoniae* conditions. A and C) Crystal Violet (CV) measuring of *A. pleuropneumoniae* - *S. suis* - *B. bronchiseptica* biofilms. B and D) Colony Forming Units (CFU) of *A. pleuropneumoniae* - *S. suis* - *B. bronchiseptica* biofilms. App: *A. pleuropneumoniae*; Ss: *S. suis* 735; Bb: *B. bronchiseptica* 276.

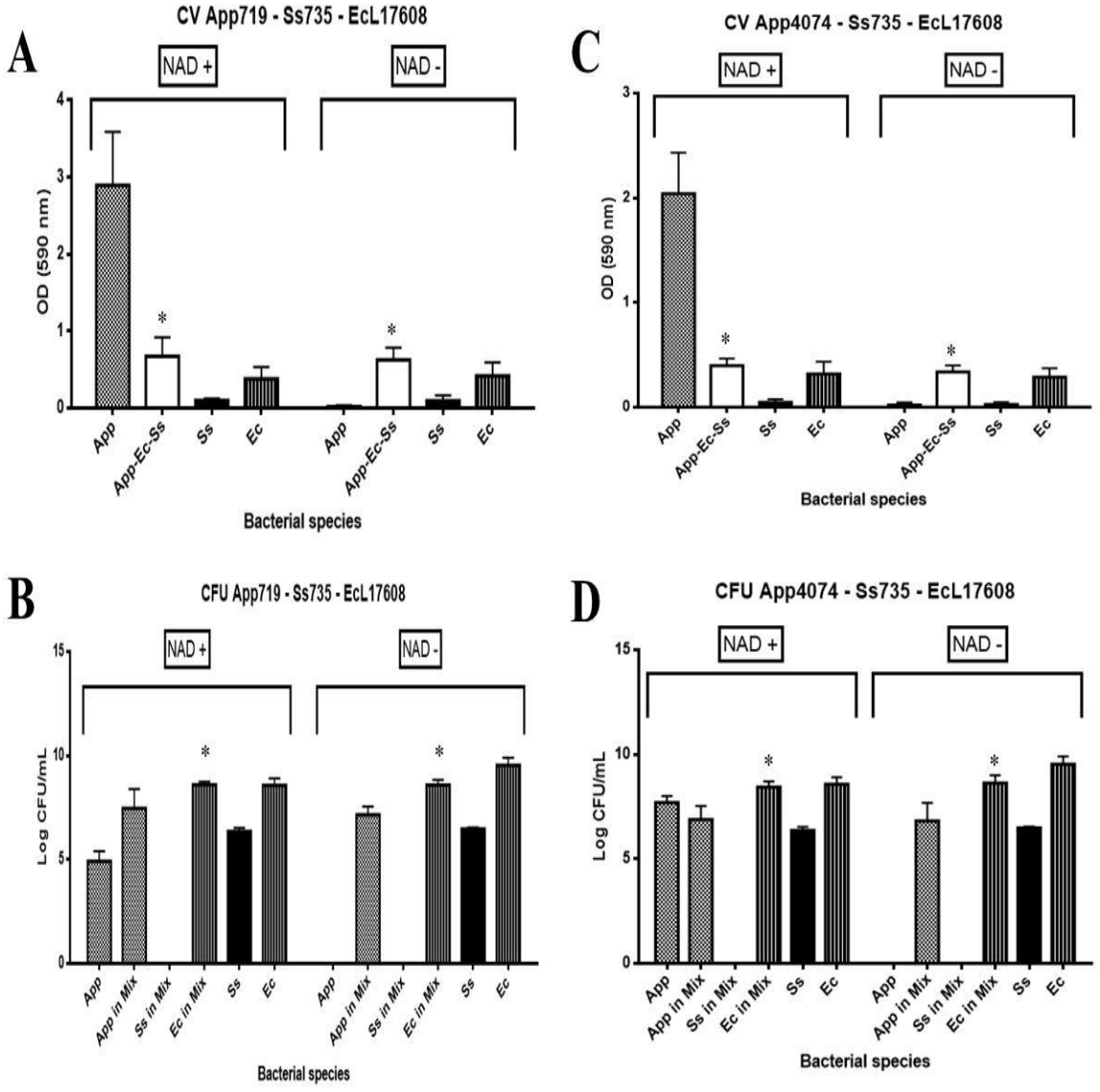


Figure 28: Tri-specie biofilms between *A. pleuropneumoniae* 719 and 4074 with *S. suis* 735 and *E. coli* L17608 in microplates in *A. pleuropneumoniae* conditions. A and C) Crystal Violet (CV) measuring of *A. pleuropneumoniae* - *S. suis* - *E. coli* biofilms. B and D) Colony Forming Units (CFU) of *A. pleuropneumoniae* - *S. suis* - *E. coli* biofilms. App: *A. pleuropneumoniae*; Ss: *S. suis* 735; Ec: *E. coli* L17608.

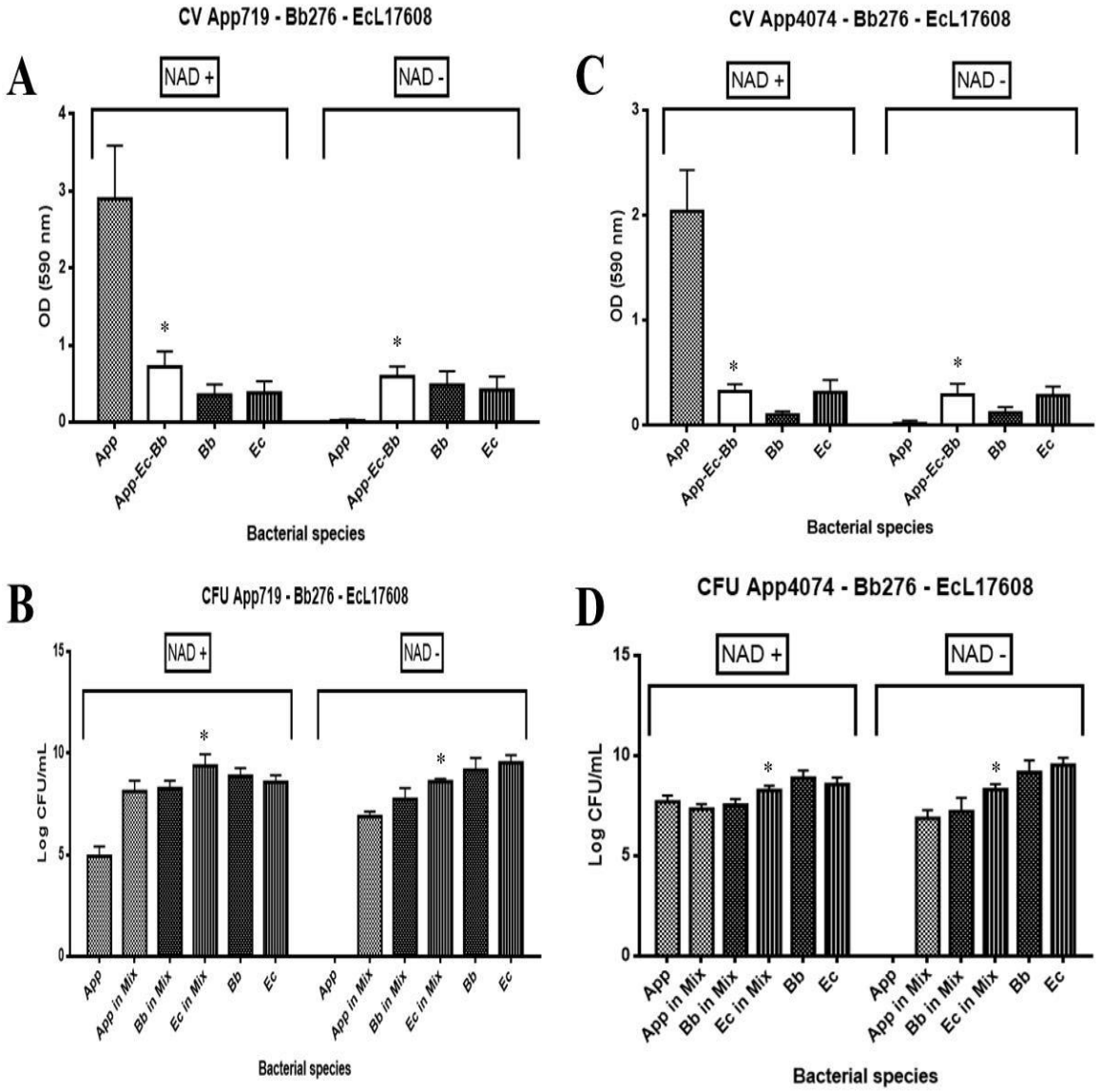


Figure 29: Tri-specie biofilms between *A. pleuropneumoniae* 719 and 4074 with *B. bronchiseptica* 276 and *E. coli* L17608 in microplates in *A. pleuropneumoniae* conditions. A and C) Crystal Violet (CV) measuring of *A. pleuropneumoniae* - *B. bronchiseptica* - *E. coli* biofilms. B and D) Colony Forming Units (CFU) of *A. pleuropneumoniae* - *B. bronchiseptica* - *E. coli* biofilms. App: *A. pleuropneumoniae*; Bb: *B. bronchiseptica* 276; Ec: *E. coli* L17608.

6.10.4 Multi-species biofilms formation without NAD supplementation.

For growth *in vitro*, *A. pleuropneumoniae* serotype 1 required the supplementation of NAD (a pyridine compounds) in the growth media. It is well established that *A. pleuropneumoniae* can acquire NAD from *S. aureus*. To test if *A. pleuropneumoniae* could acquire NAD or other pyridine compounds from other swine pathogen species and also form biofilms, the multi-species biofilms assays were also performed in the absence of NAD. Based on OD and CFU results, *A. pleuropneumoniae* was able to grow, form multi-species biofilms and acquire pyridine compounds (probably NAD) with every bacteria tested. This swine respiratory pathogen was able to form strong dual biofilms again with the swine respiratory pathogens *S. suis* and *B. bronchiseptica*, and with the non-pathogenic *S. aureus* (OD 3.042 - 2.762). *A. pleuropneumoniae* - *P. multocida* two-species biofilms were significant weaker (OD 0.987, $p < 0.001$) than with NAD supplementation (OD 2.811). With *E. coli*, similar results were observed that with NAD, forming also weak biofilms (OD 0.349). Regarding with the CFU counts, as observed with NAD supplementation, *A. pleuropneumoniae* was again the largest component (1000 - 2000 times) of the two-species biofilms formed with *S. suis*, and the lower component with *B. bronchiseptica* and *E. coli* (Figures 22, 23 and 26); so, the absence of NAD did not cause significant changes in these two-species biofilms. On the contrary, with *P. multocida* no significant differences were observed for two-species biofilms ($p = 0.7003$), and with *S. aureus*, *A. pleuropneumoniae* was significantly lesser than with NAD ($p = 0.0446$, Figure 25), suggesting that the absence of NAD was a limiting factor for *A. pleuropneumoniae* growth in these two-species biofilms, and so caused a different equilibrium between both species in the biofilms.

Moreover, as with the presence of NAD, the proportions of live/dead bacteria in the multi-species biofilms were observed. Again, in the two-species biofilms with *B. bronchiseptica* and *S. aureus*, there were an apparent increment in the proportion of live bacteria in the multi-specie biofilms. Likewise, in the two-species biofilms formed by *A. pleuropneumoniae* - *S. suis*, increase the proportion of live bacteria, with respect to the mono-species biofilm of *A. pleuropneumoniae*. With the proportion of dead bacteria, again

in all biofilms where *E. coli* was present, decreased the number. Notably, in the biofilms formed by *A. pleuropneumoniae* - *S. aureus*, like with NAD supplementation, no dead bacteria were observed.

Regarding with the three-species biofilms, again *A. pleuropneumoniae* was able to form strong biofilms with *S. suis* - *B. bronchiseptica* (2.737); and weak biofilms with *S. suis* - *E. coli* (0.625) and *B. bronchiseptica* - *E. coli* (0.594, Figure 1c). With respect to the CFU proportions, was observed the same situation that *A. pleuropneumoniae* presented with *B. bronchiseptica*, *S. suis* and *E. coli* mentioned previously in the three-species biofilms formed with NAD supplementation (Figures 27 - 29).

6.10.5 Multi-species biofilms matrix composition without NAD supplementation.

The variations in the composition and structure of extracellular matrix in multi-species biofilms of *A. pleuropneumoniae*, with respect to the mono-specie biofilms, were tested again like with the NAD supplementation (Figures 30 - 37). With the polysaccharide PGA component were observed in most multi-species biofilms, as with the NAD supplementation, clusters or filament-like structures. However, the most important is that PGA component remains like the primary component responsible for the biofilm structure.
In the case of eDNA, were observed an increase in the two-species biofilms formed with *S. suis, B. bronchiseptica, P. multocida* and *S. aureus*; and once more in the three-species biofilms with *S. suis* and *B. bronchiseptica*. The increase in the eDNA appreciated in the biofilms of *A. pleuropneumoniae* - *B. bronchiseptica* and *A. pleuropneumoniae* - *P. multocida*, were highly visible (Figures 31 and 32).

Regarding to proteins, in this case, only was observed an increase in the biofilms of *A. pleuropneumoniae* - *P. multocida.* At last, like with the NAD supplementation, in all biofilms with *E. coli* (two and three-species) the proteins composition was lower.

Regarding with the stain FilmTracer FM 1-43, changes were observed in the two-species biofilms with *S. aureus* and *E. coli* without NAD, and again, in all three-species

biofilms in which was involving *E. coli*. The biofilms formed by *A. pleuropneumoniae - P. multocida* appeared to be composed for microcolonies. Moreover and like with the NAD supplementation, in the three-species biofilms with *S. suis* and *B. bronchiseptica*, there was observed an increase in the occurrence of clusters (Figures 30 - 37).

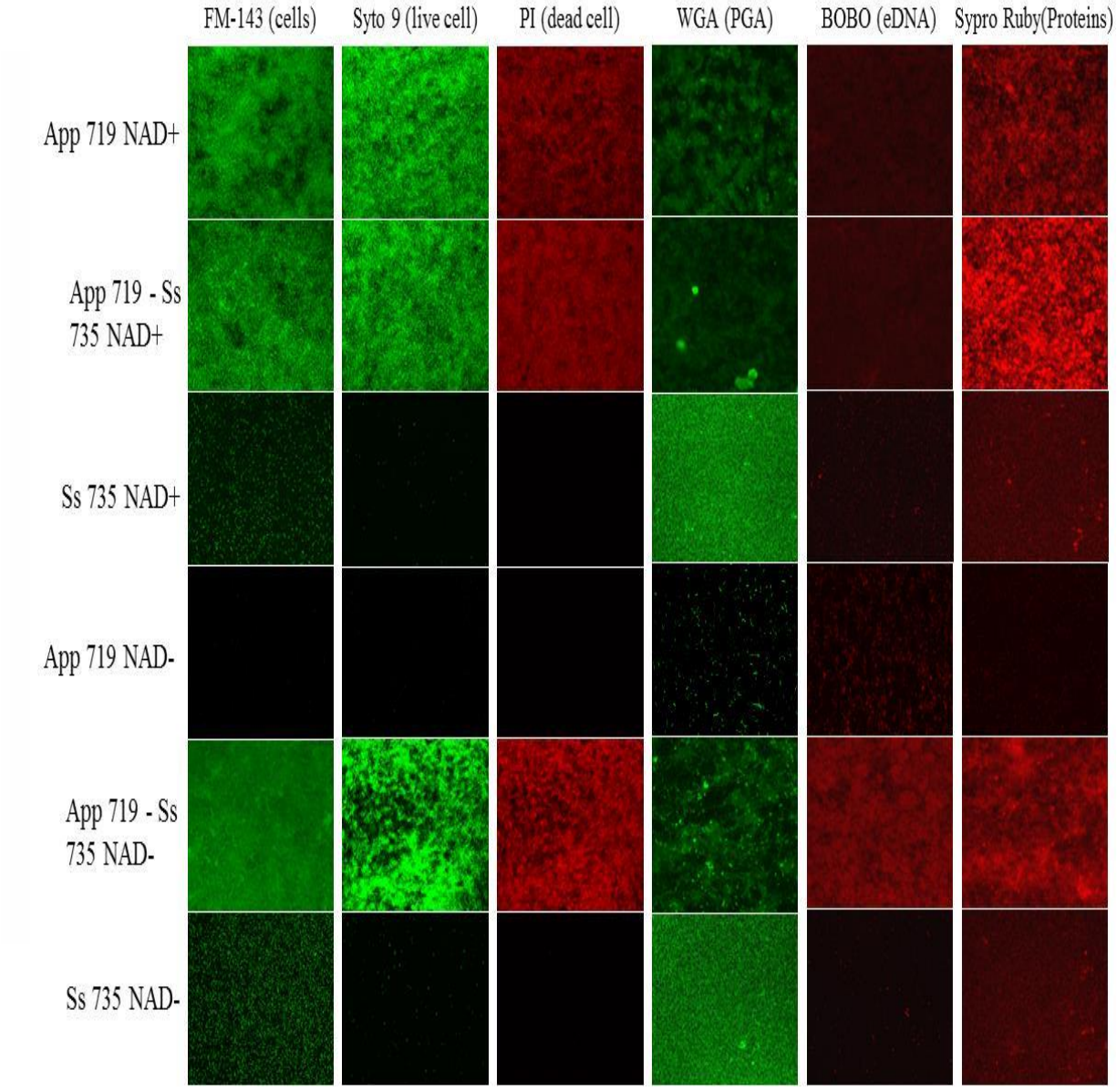


Figure 30: Extracellular matrix composition of the mono and di-specie biofilms of *A. pleuropneumoniae*, *S. suis* and *A. pleuropneumoniae - S. suis* in *A. pleuropneumoniae* conditions with and without the supplementation of NAD. App 719: *A. pleuropneumoniae* 719; Ss 735: *S. suis* 735.

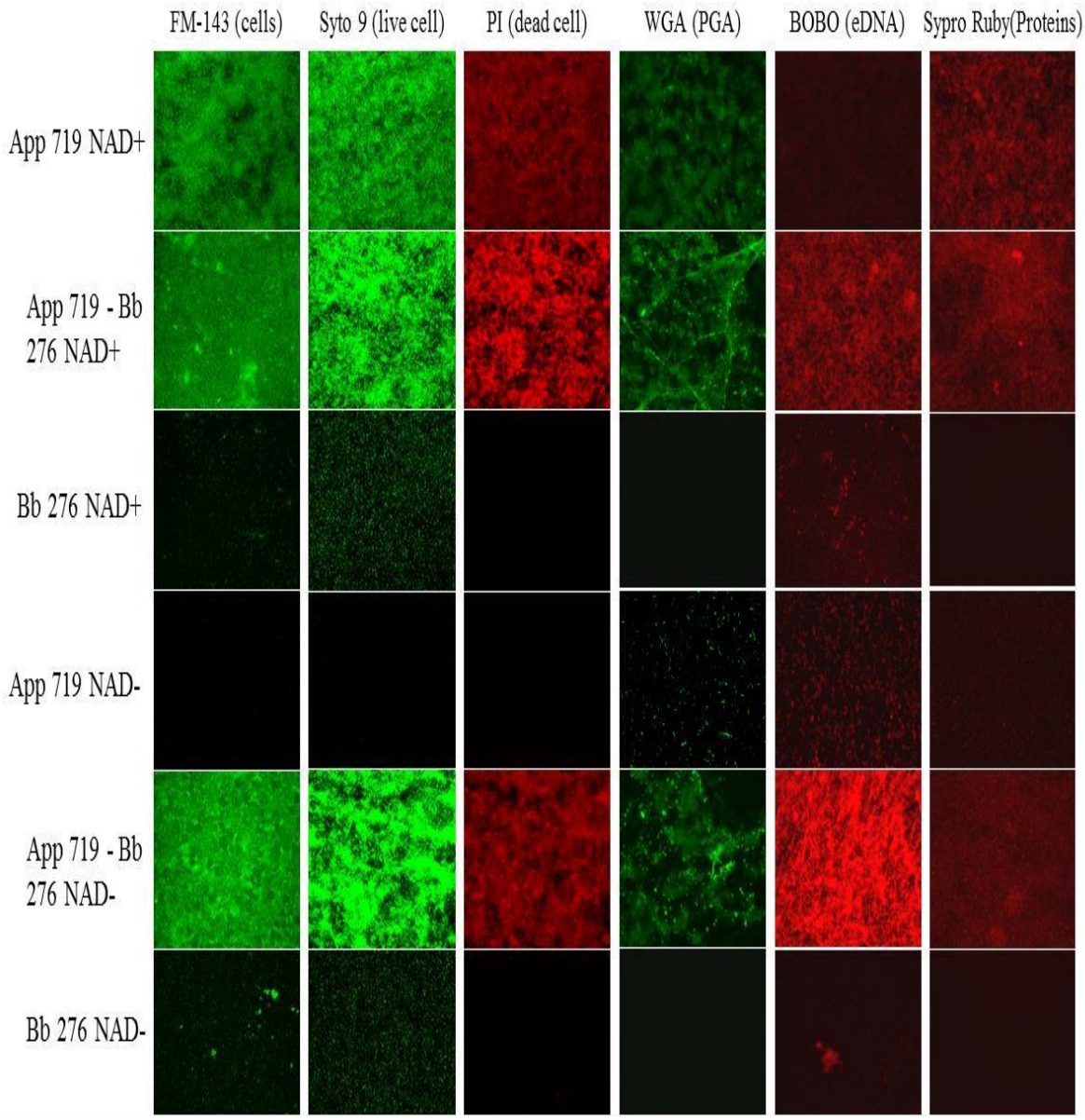


Figure 31: Extracellular matrix composition of the mono and di-specie biofilms of *A. pleuropneumoniae*, *B. bronchiseptica* and *A. pleuropneumoniae* - *B. bronchiseptica* in *A. pleuropneumoniae* conditions with and without the supplementation of NAD. App 719: *A. pleuropneumoniae* 719; Bb 276: *B. bronchiseptica* 276.

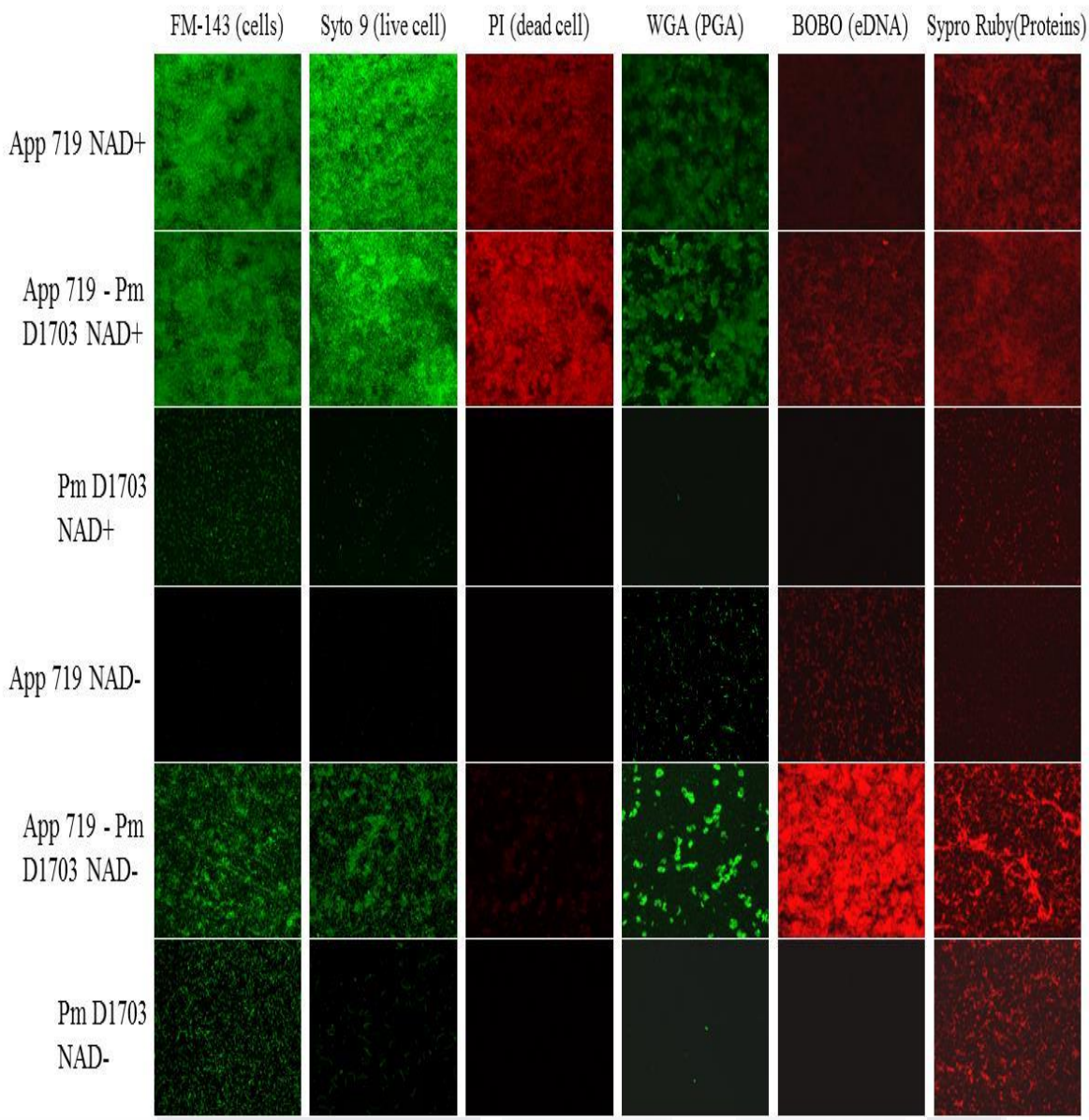


Figure 32: Extracellular matrix composition of the mono and di-specie biofilms of *A. pleuropneumoniae*, *P. multocida* and *A. pleuropneumoniae* - *P. multocida* in *A. pleuropneumoniae* conditions with and without the supplementation of NAD. App 719: *A. pleuropneumoniae* 719; Pm D1703: *P. multocida* D1703.

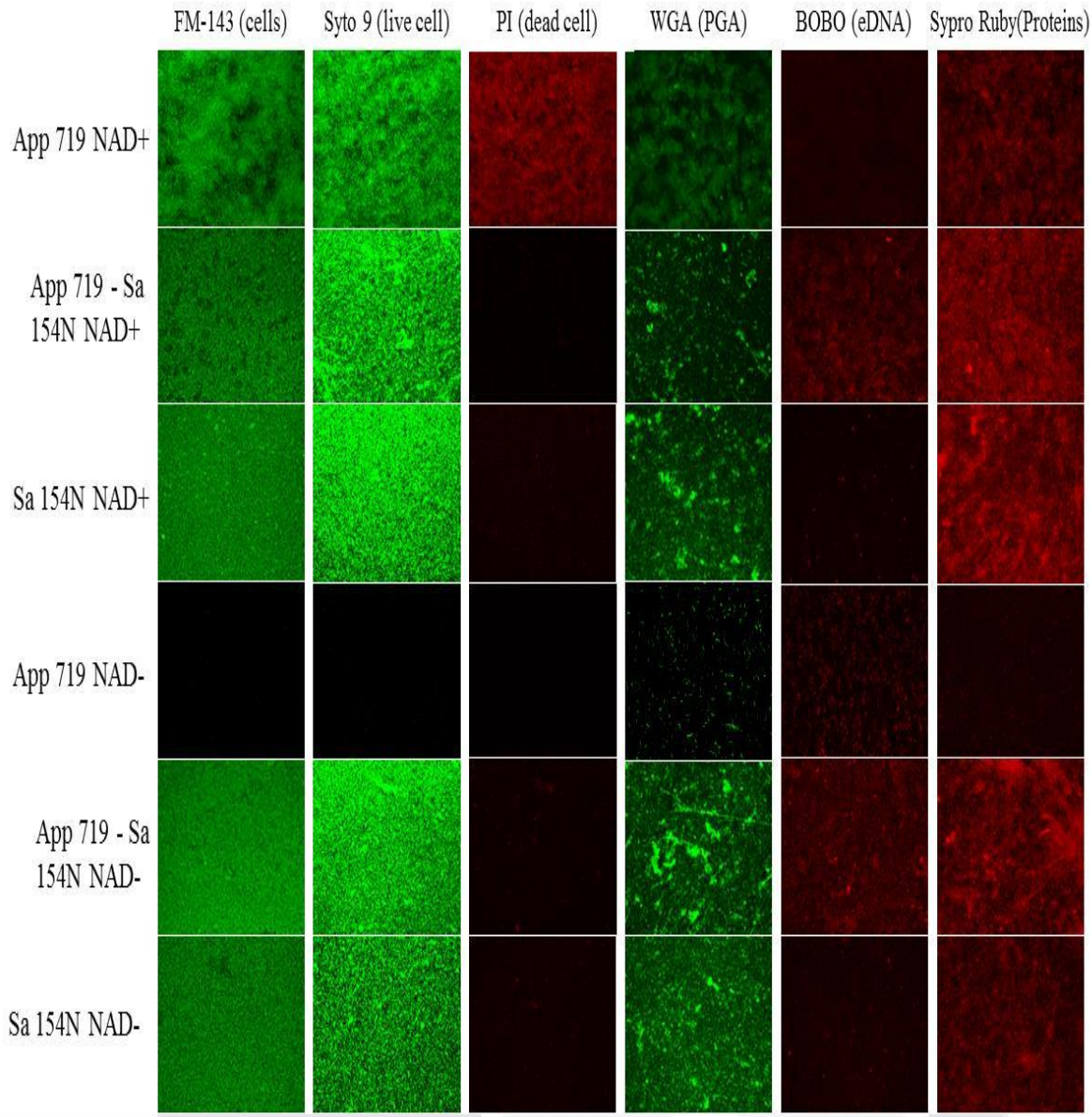


Figure 33: Extracellular matrix composition of the mono and di-specie biofilms of *A. pleuropneumoniae*, *S. aureus* and *A. pleuropneumoniae* - *S. aureus* in *A. pleuropneumoniae* conditions with and without the supplementation of NAD. App 719: *A. pleuropneumoniae* 719; Sa 154N: *S. aureus* 154N.

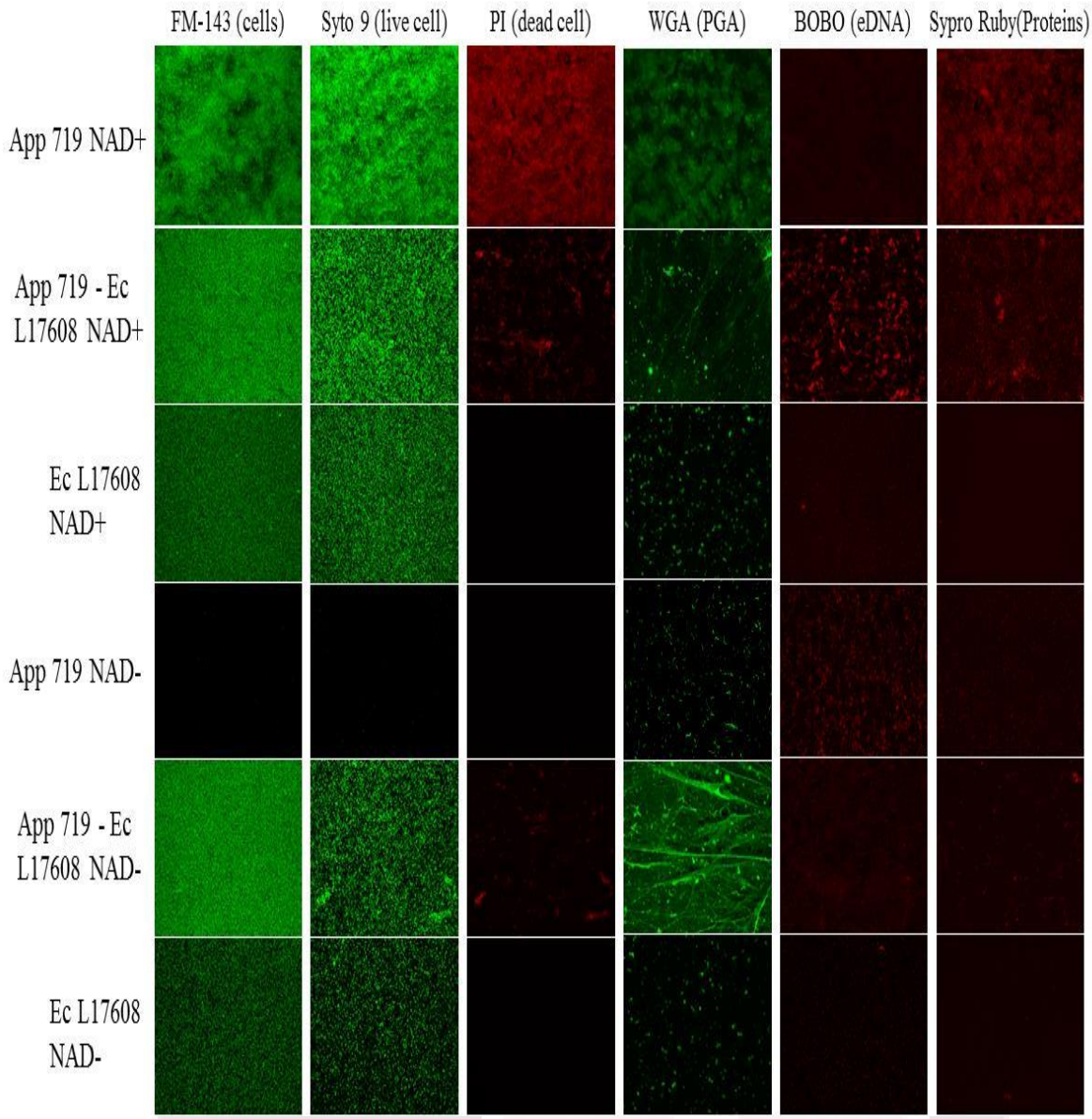


Figure 34: Extracellular matrix composition of the mono and di-specie biofilms of *A. pleuropneumoniae*, *E. coli* and *A. pleuropneumoniae* - *E. coli* in *A. pleuropneumoniae* conditions with and without the supplementation of NAD. App 719: *A. pleuropneumoniae* 719; Ec L17608: *E. coli* L17608.

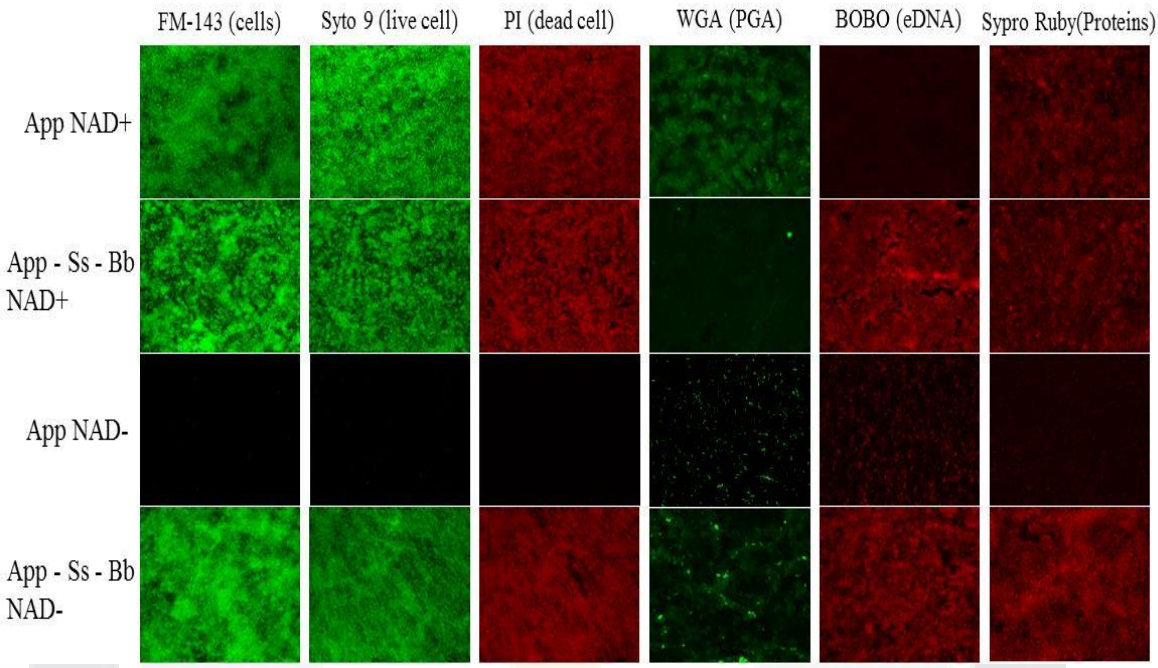


Figure 35: Extracellular matrix composition of the mono and tri-specie biofilms of *A. pleuropneumoniae* and *A. pleuropneumoniae - S. suis - B. bronchiseptica* in *A. pleuropneumoniae* conditions with and without the supplementation of NAD. App: *A. pleuropneumoniae* 719; Ss: *S. suis* 735; Bb: *B. bronchiseptica* 276.

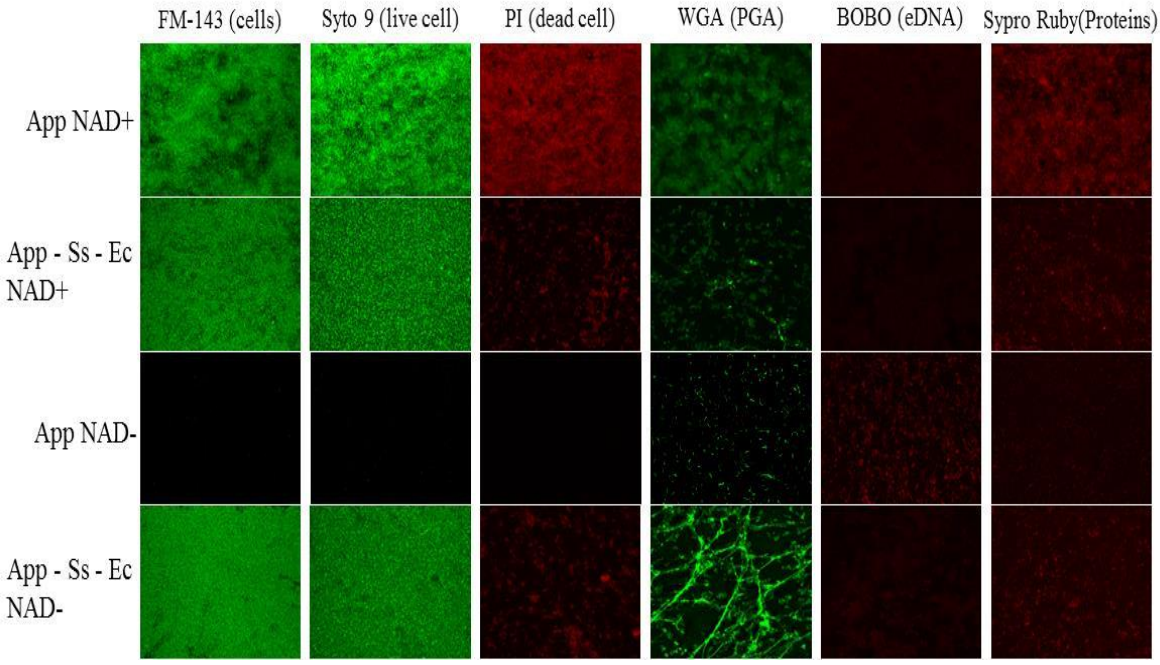


Figure 36: Extracellular matrix composition of the mono and tri-specie biofilms of *A. pleuropneumoniae* and *A. pleuropneumoniae - S. suis - E. coli* in *A. pleuropneumoniae* conditions with and without the supplementation of NAD. App: *A. pleuropneumoniae* 719; Ss: *S. suis* 735; Ec: *E. coli* L17608.

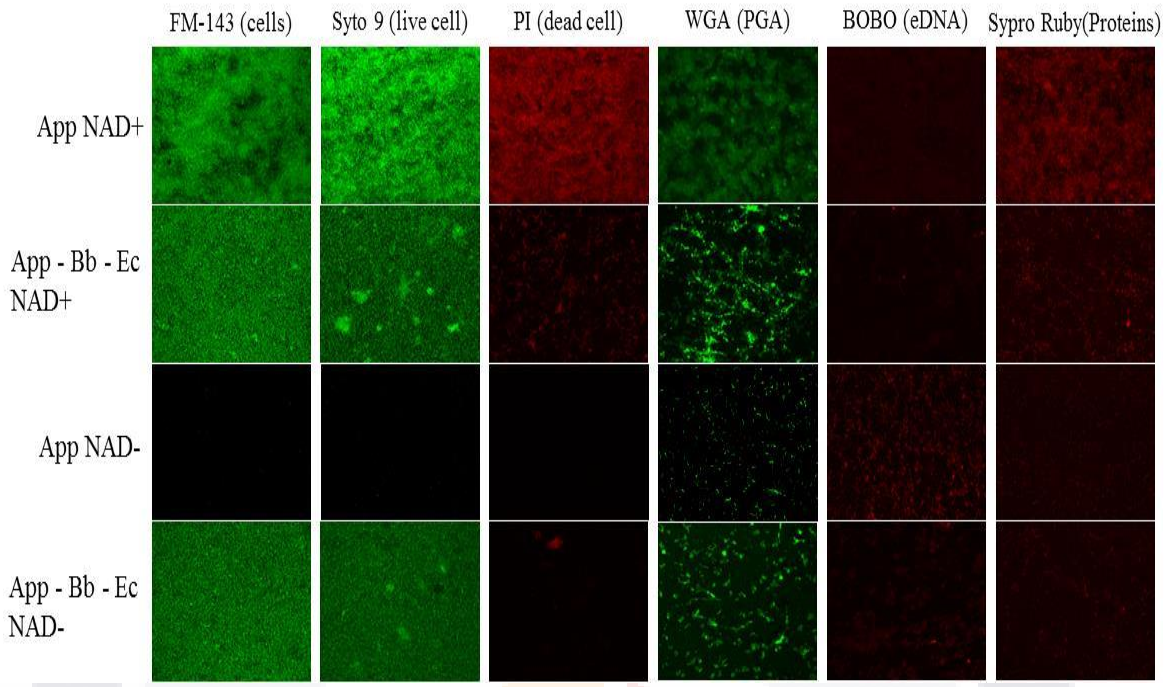


Figure 37: Extracellular matrix composition of the mono and tri-specie biofilms of *A. pleuropneumoniae* and *A. pleuropneumoniae - B. bronchiseptica - E. coli* in *A. pleuropneumoniae* conditions with and without the supplementation of NAD. App: *A. pleuropneumoniae* 719; Bb: *B. bronchiseptica* 276; Ec: *E. coli* L17608.

6.10.6 Effect of enzymatic treatment on multi-species biofilms without NAD supplementation.

Newly, enzymatic treatments of biofilms with proteinase K, DNase I and dispersin B were made for relate changes in the structural function (Figures 38 - 42). Again, in the case of proteinase K, there were no changes with respect to the control of mono-species biofilms formed by *A. pleuropneumoniae*, and also with the biofilms without enzymatic treatment. With respect to the DNase I, which helps to analyze if the eDNA has structural roles in the biofilms, only a change was observed in the two-species biofilms of *A. pleuropneumoniae - P. multocida* without NAD supplementation. This biofilms decreased approximately 50%, compared to untreated biofilms (Figure 40). Above indicates that in this case, eDNA take a structural importance not viewed on mono-species biofilms. Finally,

the tests with dispersin B, again showed significant increments in the resistance against the action of this enzyme. These increments were observed in the two-species biofilms with *P. multocida* (26%), and, like with the NAD supplementation, in the biofilms with *S. aureus* (59%), *E. coli* (30%), and also in all the three-species biofilms with *E. coli* (Figure 42). In all cases, these increases in resistance to the enzyme were higher in the absence of NAD.

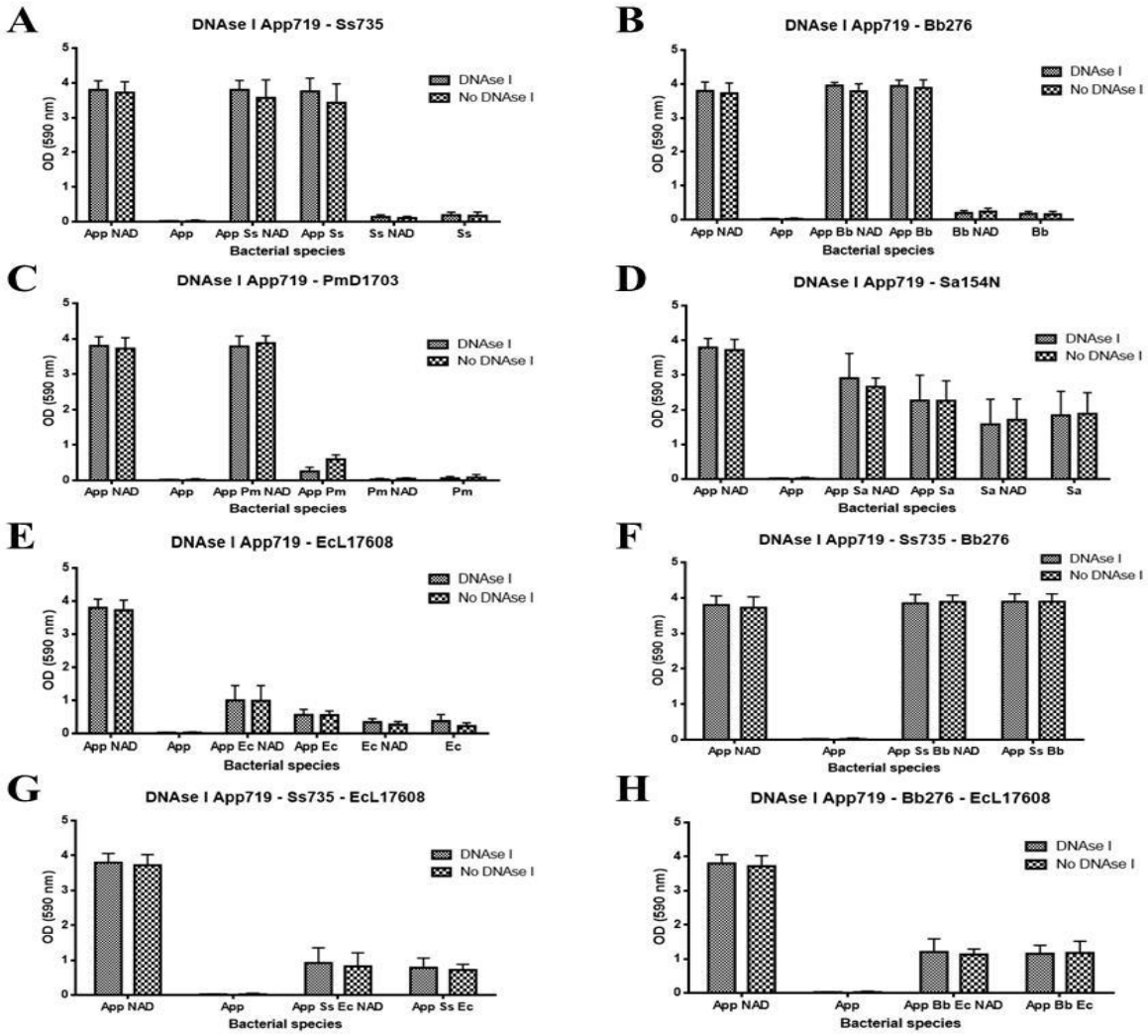


Figure 38: Dispersion of (A) *A. pleuropneumoniae - S. suis*, (B) *A. pleuropneumoniae - B. bronchiseptica*, (C) *A. pleuropneumoniae - P. multocida*, (D) *A. pleuropneumoniae - S. aureus*, (E) *A. pleuropneumoniae - E. coli*, (F) *A. pleuropneumoniae - S. suis - B. bronchiseptica*, (G) *A. pleuropneumoniae - S. suis - E. coli* and (G) *A. pleuropneumoniae - B. bronchiseptica - E. coli*, grown in multi-species biofilms in BHI media with or without NAD by DNAse I. App: *A. pleuropneumoniae* 719; Ss: *S. suis* 735; Bb: *B. bronchiseptica* 276; Pm: *P. multocida* D1703; Sa: *S. aureus*; Ec: *E. coli* L17608.

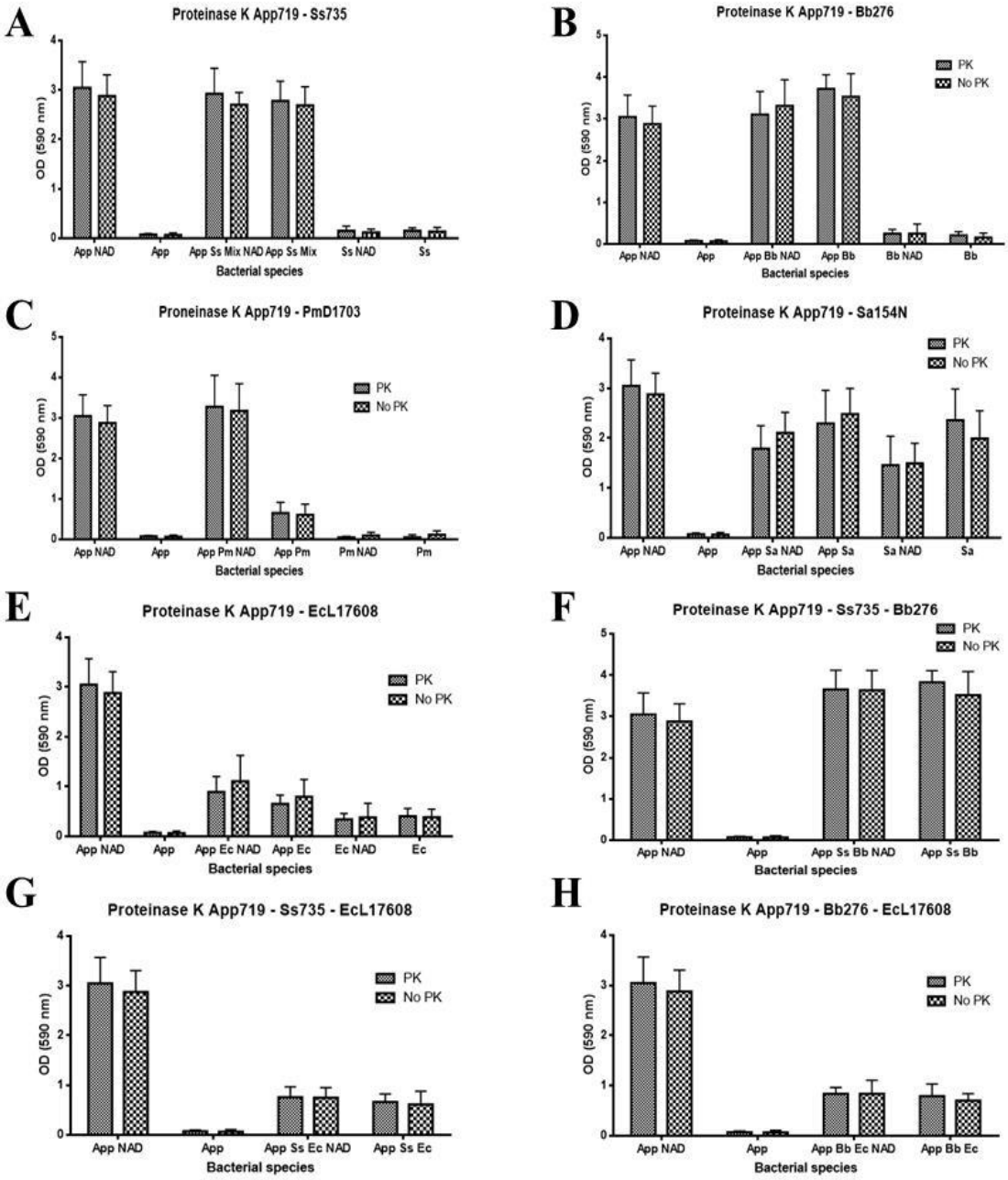


Figure 39: Dispersion of (A) *A. pleuropneumoniae - S. suis*, (B) *A. pleuropneumoniae - B. bronchiseptica*, (C) *A. pleuropneumoniae - P. multocida*, (D) *A. pleuropneumoniae - S. aureus*, (E) *A. pleuropneumoniae - E. coli*, (F) *A. pleuropneumoniae - S. suis - B. bronchiseptica*, (G) *A. pleuropneumoniae - S. suis - E. coli* and (G) *A. pleuropneumoniae - B. bronchiseptica - E. coli*, grown in multi-species biofilms in BHI media with or without NAD by Poteinase K. App: *A. pleuropneumoniae* 719; Ss: *S. suis* 735; Bb: *B. bronchiseptica* 276; Pm: *P. multocida* D1703; Sa: *S. aureus*; Ec: *E. coli* L17608.

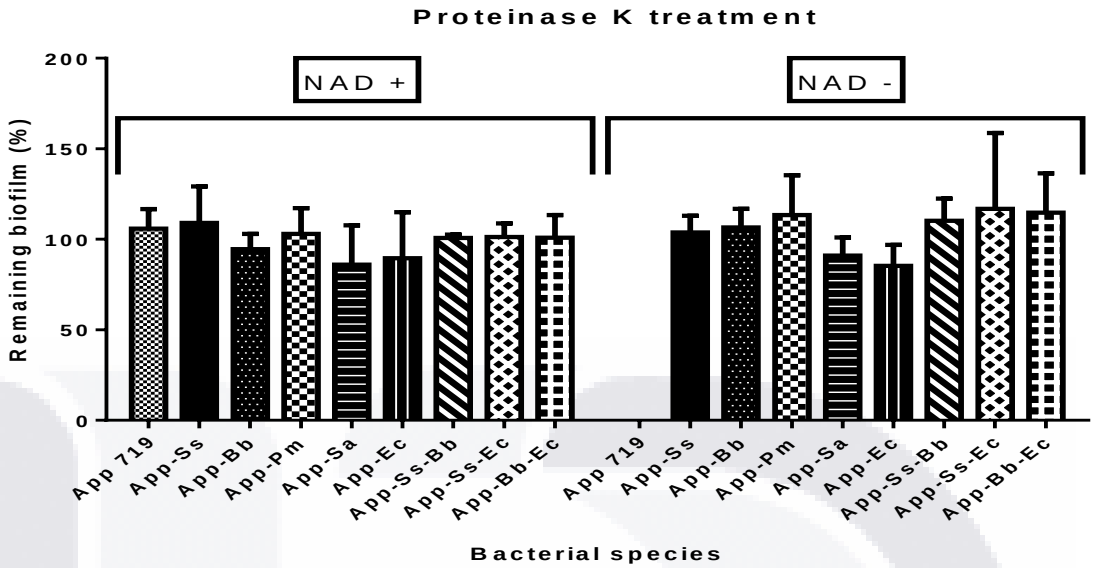


Figure 40: Remaining biofilms percentage of *A. pleuropneumoniae*, *S. suis*, *B. bronchiseptica*, *P. multocida*, *S. aureus*, and *E. coli*, grown in multi-species biofilms in BHI media with or without NAD by DNAse I. App: *A. pleuropneumoniae* 719; Ss: *S. suis* 735; Bb: *B. bronchiseptica* 276; Pm: *P. multocida* D1703; Sa: *S. aureus*; Ec: *E. coli* L17608.

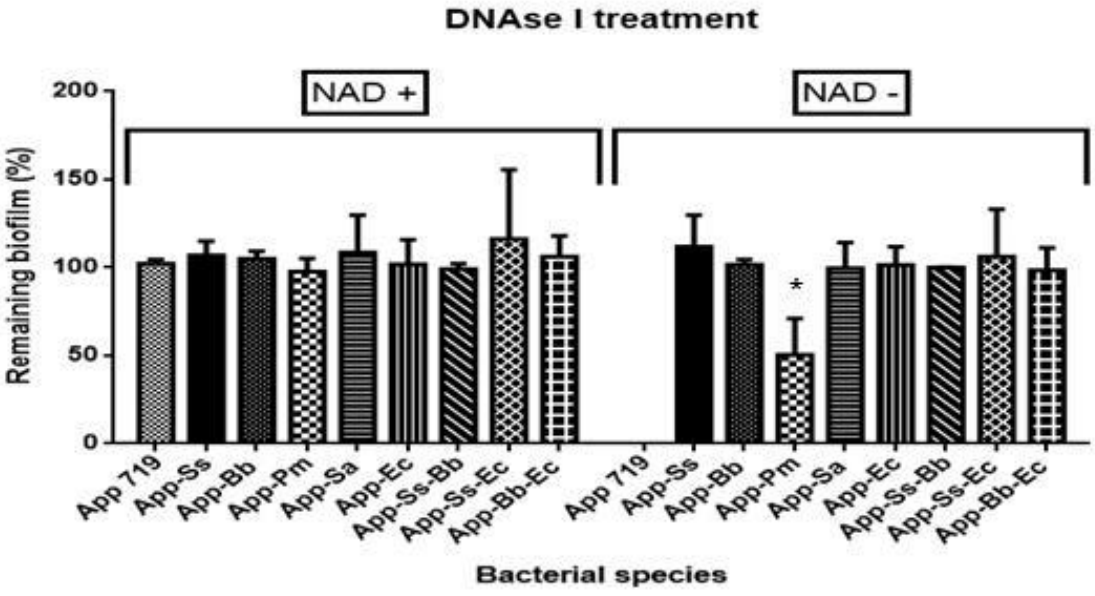


Figure 41: Remaining biofilms percentage of *A. pleuropneumoniae*, *S. suis*, *B. bronchiseptica*, *P. multocida*, *S. aureus*, and *E. coli*, grown in multi-species biofilms in BHI media with or without NAD by Proteinase K. App: *A. pleuropneumoniae* 719; Ss: *S. suis* 735; Bb: *B. bronchiseptica* 276; Pm: *P. multocida* D1703; Sa: *S. aureus*; Ec: *E. coli* L17608.

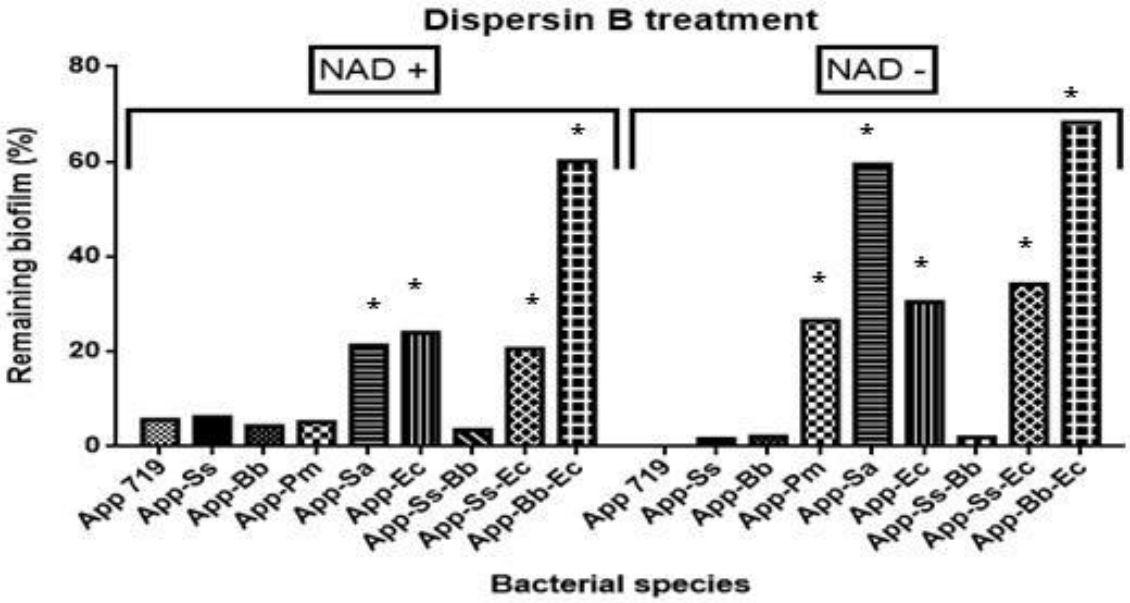


Figure 42: Remaining biofilms percentage of *A. pleuropneumoniae*, *S. suis*, *B. bronchiseptica*, *P. multocida*, *S. aureus*, and *E. coli*, grown in multi-species biofilms in BHI media with or without NAD by Dispersin B. App: *A. pleuropneumoniae* 719; Ss: *S. suis* 735; Bb: *B. bronchiseptica* 276; Pm: *P. multocida* D1703; Sa: *S. aureus*; Ec: *E. coli* L17608.

6.10.7 Confirmation of the presence of *A. pleuropneumoniae* in multi-species biofilms by FISH.

To confirm the presence of *A. pleuropneumoniae* in these multi-species biofilms, FISH assays were performed in the cases of dual biofilms of *A. pleuropneumoniae-S suis* and *A. pleuropneumoniae-B. bronchiseptica*. In both cases, the presence of *A. pleuropneumoniae* was confirmed in the biofilms. Above, takes a greater importance in the case of no supplementation with NAD (Figure 43). With respect to the distribution presented by these bacteria in the two-species biofilms, it should be noted that *A. pleuropneumoniae* and *S. suis* showed a highly homogeneous distribution in the biofilm; while *A. pleuropneumoniae* and *B. bronchiseptica* presented a heterogeneous distribution through the biofilm (Figure 44).

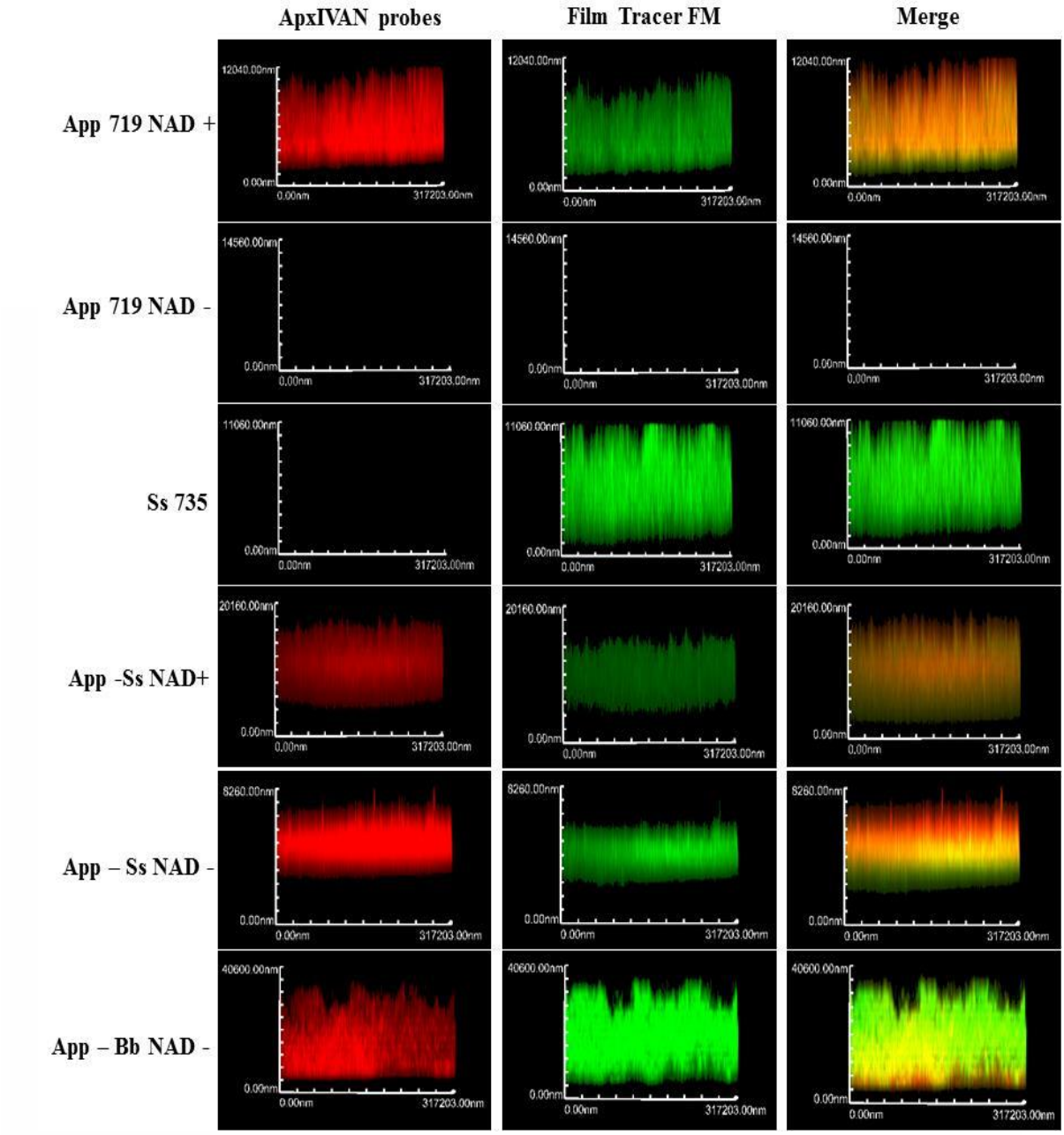


Figure 43: Detection of *A. pleuropneumoniae* in the di-species biofilm of *A. pleuropneumoniae* 719 and *S. suis* 735 or *B. bronchiseptica* 276 by FISH with an ApxIVAN-AlexaFluor 633 probe (red). Images of the X-Z plane of biofilm mono and dual-species biofilms grown in BHI with or without NAD. Bacterial cell in the biofilms were stained with FilmTracer ™ FM ® 1-43 (Molecular Probes) which are represented in green. Yellow represent the co-localization of both the ApxIVAN probe and the stain FM 1-43. App: *A. pleuropneumoniae*; Ss: *S. suis*; Bb: *B. bronchiseptica*.

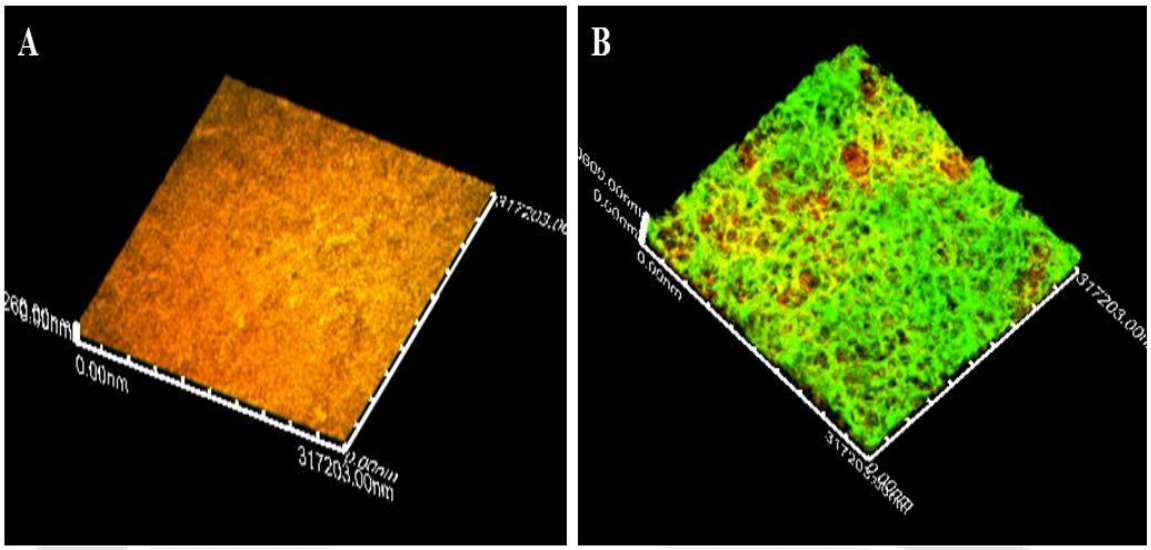


Figure 44: Detection of *A. pleuropneumoniae* in the di-species biofilm of *A. pleuropneumoniae* 719 and *S. suis* 276 (A) or *A. pleuropneumoniae* 719 and *B. bronchiseptica* 276 (B) by FISH with an ApxIVAN-AlexaFluor 633 probe (red). 3D images of biofilm dual-species biofilms grown in BHI without NAD. Bacterial cell in the biofilms were stained with FilmTracer ™ FM ® 1-43 (Molecular Probes) which are represented in green. Yellow represent the co-localization of both the ApxIVAN probe and the stain FM 1-43.

6.10.8 Scanning electron microscopy.

The two-species biofilms of *A. pleuropneumoniae* and *E. coli* was observed by EM (Figure 45). In this two-species biofilms formed by *A. pleuropneumoniae* and *E. coli* is possible to observed the existence of two populations in the biofilms, a population of larger bacteria which are the main bacteria in the multi-species biofilm, and a population of smaller bacteria that form a minority in the biofilm ($p < 0.01$, Figure 45d). That is agrees with the observed by CFU and extracellular matrix composition assays, where *E. coli* was the main bacteria in these biofilms, and where the extracellular matrix looks very similar to the matrix of a mono-species biofilms of *E. coli*. It is also interesting to observer fimbriae-like or curlis-like structures, and how these structures are promoting the interaction between all the bacteria presents in the biofilm. Moreover, these structures look in more quantities than in the *A. pleuropneumoniae* mono-species biofilm (Figure 45a) but in less number than in the mono-species biofilm of *E. coli* (Figure 45b). Another interesting

situation is that is unknown if the interaction between the two bacteria promote morphological changes in the two-species biofilm. However, it is difficult the exactly differentiation between both bacteria in the two-species biofilm by this technique.

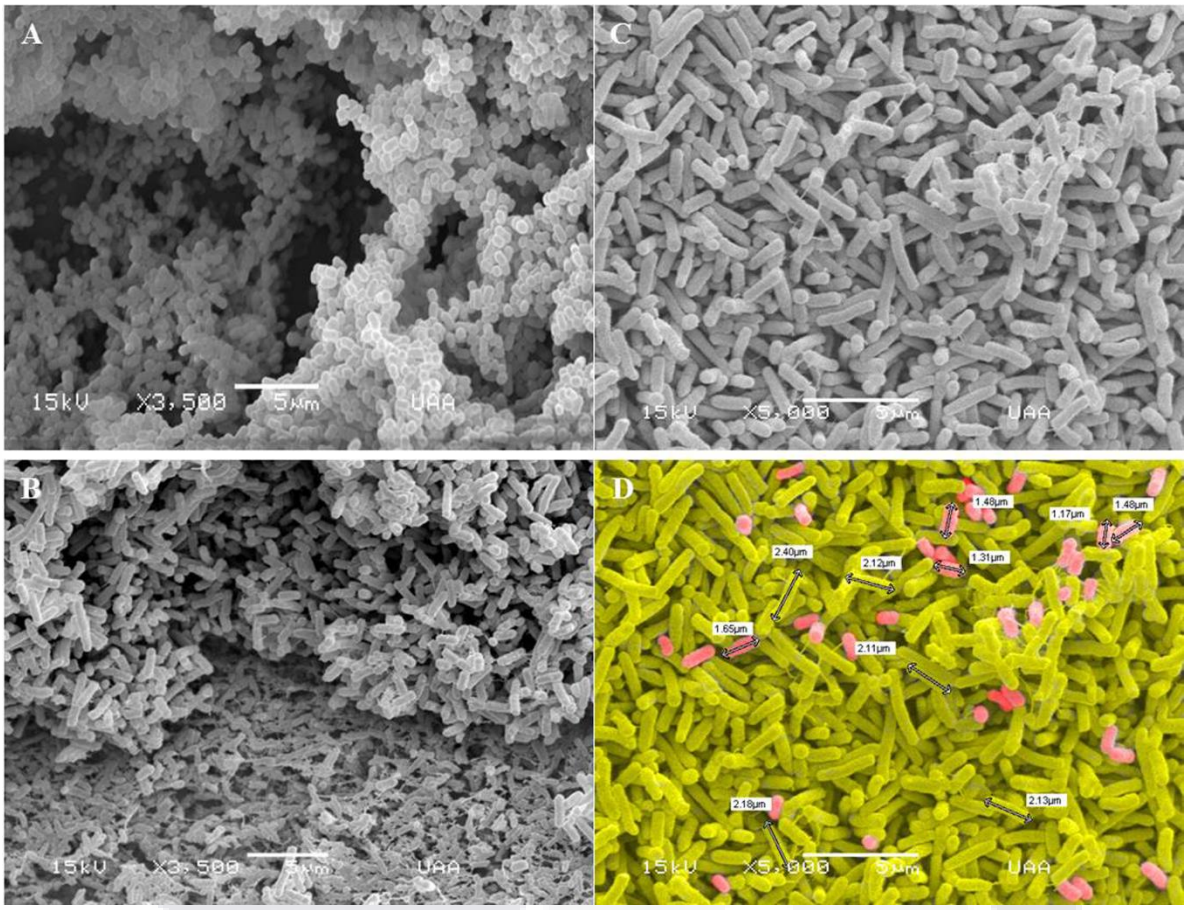


Figure 45: EM of two-species biofilm formed by *A. pleuropneumoniae* and *E. coli*. Mono-species and two-species biofilms formed by (A) *A. pleuropneumoniae*, (B) *E. coli* and (C-D) *A. pleuropneumoniae* and *E. coli*. In this two-species biofilms of *A. pleuropneumoniae* - *E. coli* is possible to observed the existence of two populations in the biofilm, a population of larger bacteria which are the main bacteria in the multi-species biofilm, and a population of smaller bacteria that form a minority in the biofilm. Likewise, is possible to observed fimbriae-like or curlis-like structures in this multi-species biofilm. However, it is difficult the exactly differentiation between both bacteria in the two-species biofilm.

6.10.9 Incorporation of *A. pleuropneumoniae* in pre-formed biofilms in conditions for *S. aureus*, *S. suis* and *E. coli*.

Finally, *A. pleuropneumoniae* was tested to form multi-species biofilms with *S. suis* or *E. coli* but this time in conditions for biofilms formation of *S. suis* or *E. coli*, and the capacity for incorporate in a pre-formed biofilms of *S. aureus*, *S. suis* and *E. coli*. In both cases, *A. pleuropneumoniae* was able to survive, grow and incorporated into previously biofilms formed by these two bacteria, even though the conditions, growth medium, temperature, absence of NAD, etc., were very different to *A. pleuropneumoniae* needed to growth and form biofilms (Figures 46 - 48). Likewise, *A. pleuropneumoniae* was able to grow and form biofilms when inoculated simultaneously with *S. suis* or *E. coli* (Figures 47 and 48). With this, we can say that *A. pleuropneumoniae* is capable to form and/or incorporated into other bacterial species biofilms, in this case *S aureus*, S. *suis* or *E. coli*, and the above is allowing that *A. pleuropneumoniae* can survive even in environments that would be hostile to its development when is part of a multi-species biofilm.

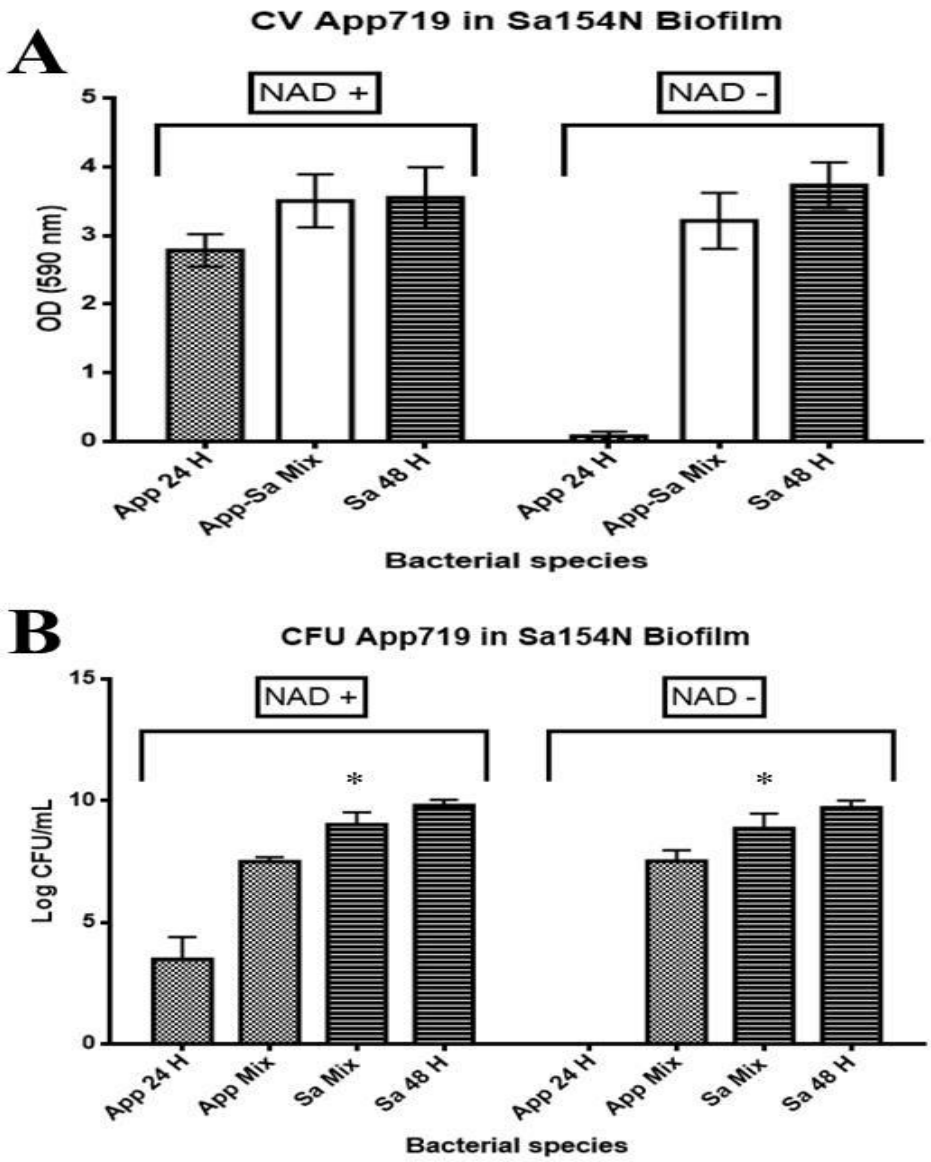


Figure 46: Di-specie biofilms between *S. aureus* and *A. pleuropneumoniae* in microplates in *S. aureus* conditions. A) Crystal Violet (CV) measuring of *S. aureus* - *A. pleuropneumoniae* biofilms and B) Colony Forming Units (CFU) of *S. aureus* - *A. pleuropneumoniae* biofilms. Biofilms to 48 h indicate that first was made the *S. aureus* biofilm for 24 h and then was added *A. pleuropneumoniae* for observe the incorporation in *S. aureus* biofilm for 24 h for a total of 48 h. Biofilms to 24 h indicate that *A. pleuropneumoniae* biofilm was formed in 24 h. App: *A. pleuropneumoniae* 719; Ss: *S. suis* 735.

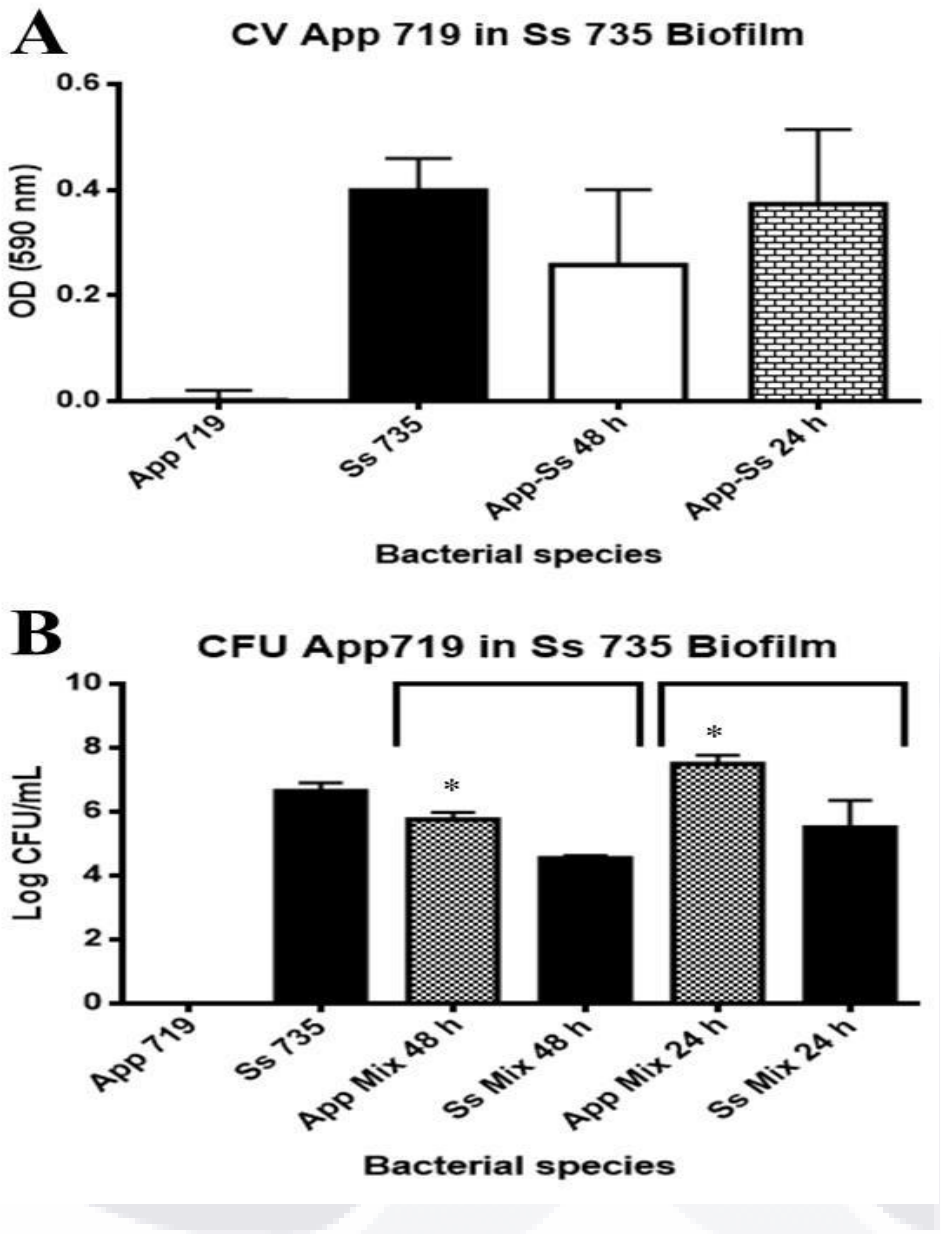


Figure 47: Di-specie biofilms between *S. suis* and *A. pleuropneumoniae* in microplates in *S. suis* conditions. A) Crystal Violet (CV) measuring of *S. suis* - *A. pleuropneumoniae* biofilms and B) Colony Forming Units (CFU) of *S. suis* - *A. pleuropneumoniae* biofilms. Biofilms to 48 h indicate that first was made the *S. suis* biofilm for 24 h and then was added *A. pleuropneumoniae* for observe the incorporation in *S. suis* biofilm for 24 h for a total of 48 h. Biofilms to 24 h indicate that both bacteria, *S. suis* and *A. pleuropneumoniae* were added at same time and the biofilm was formed in 24 h. App: *A. pleuropneumoniae* 719; Ss: *S. suis* 735.

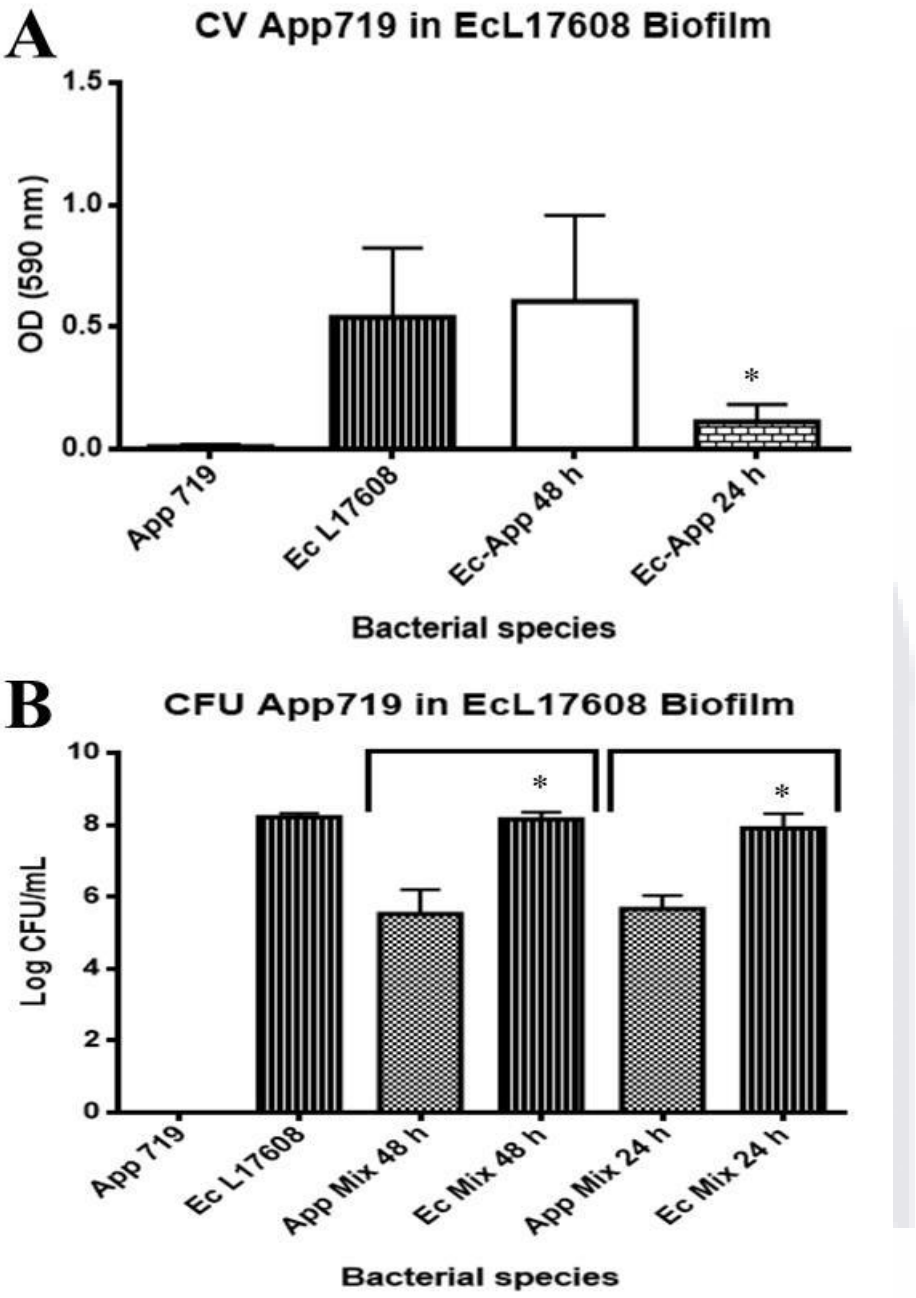


Figure 48: Di-specie biofilms between *E. coli* and *A. pleuropneumoniae* in microplates in *E. coli* conditions. A) Crystal Violet (CV) measuring of *E. coli* - *A. pleuropneumoniae* biofilms and B) Colony Forming Units (CFU) of *E. coli* - *A. pleuropneumoniae* biofilms. Biofilms to 48 h indicate that first was made the *E. coli* biofilm for 24 h and then was added *A. pleuropneumoniae* for observe the incorporation in *E. coli* biofilm for 24 h for a total of 48 h. Biofilms to 24 h indicate that both bacteria, *E. coli* and *A. pleuropneumoniae* were added at same time and the biofilm was formed in 24 h. App: *A. pleuropneumoniae* 719; Ec: *E. coli* L17608.

6.10.10 Measurement of NAD production by the mono and multi-species biofilms.

To verify if the other bacteria founded in the multi-species biofilm produced and gave directly NAD to *A. pleuropneumoniae* for growth and development, quantification of NAD production by the mono and multi-species biofilms was performed directly from the supernatant. For this, was used the NAD/NADH Extraction Kit (Sigma-Aldrich). In general, in the case of bacteria *B. bronchiseptica*, *P. multocida*, *S. aureus* and *E. coli*, no production of NAD was observed in the supernatant after incubation and mono-species biofilm formation. On the case of these four bacteria, *A. pleuropneumoniae* could be taking any of the NAD precursors to conduct their growth and development within the multi-species biofilm. However, only in the case of mono-species biofilm of *S. suis*, we observed an increase in NAD concentration, so, this pathogen was capable to produce NAD while in biofilm mono-species (Figure 49, $p = 0.0041$). Thus, in this case *A. pleuropneumoniae* could be taking directly NAD produced by *S. suis* when is in the mixed biofilm.

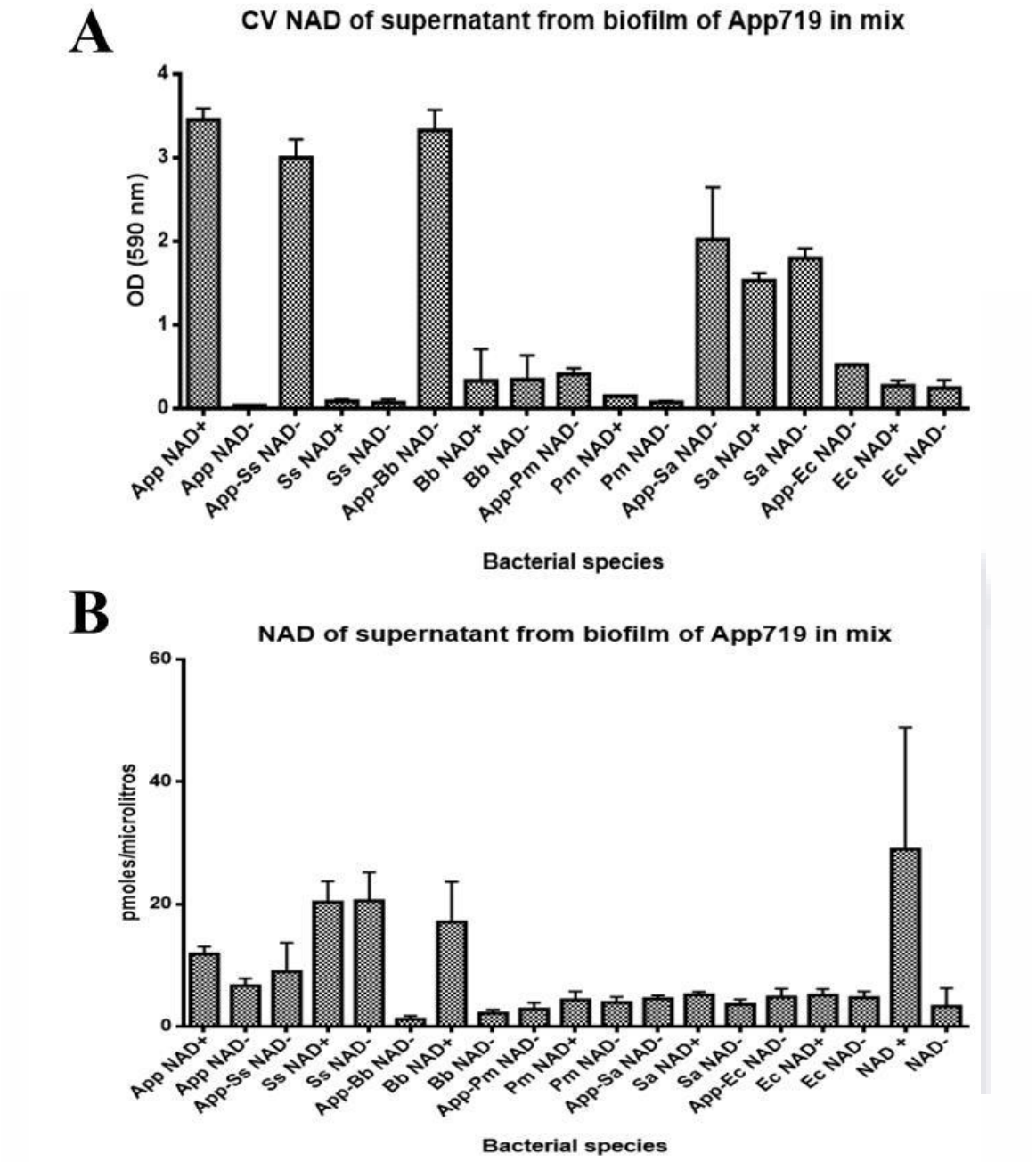


Figure 49: Quantification of NAD production by the mono and multi-species biofilms. A) CV staining was performed to the plate to verify that there were biofilms formations and B) The production of NAD in the supernatant by the mono and multi-species biofilms was quantified by the NAD/NADH Extraction Kit (Sigma-Aldrich).

6.10.11 *A. pleuropneumoniae* biofilm formation with crude cell-free supernatants.

In order to investigate the effects of crude cell-free supernatants of each bacterium for the *A. pleuropneumoniae* mono-species biofilm formation, the standard 96-well microtiter plate technique was used with CV. In general, four different patterns were observed. First, in the case of the supernatant from *B. bronchiseptica*, was observed a positive effect on the biofilm of *A. pleuropneumoniae* in all proportions tested. This allowed the development of a normal biofilm. The second pattern was observed in the case of the supernatant from *S. aureus*. In this case, a positive effect was observed when was treated at concentration of 100% of the supernatant, allowing the development of a strong biofilm *A. pleuropneumoniae*. However, the effect was decreased as decreased the concentrations of the supernatant, until it could not allow the biofilm development of *A. pleuropneumoniae*. The third case was seen with supernatant from *S. suis*. In this case, there was an inverse effect observed with supernatant of *S. aureus*, where concentrations of 100% did not permit the development of *A. pleuropneumoniae* biofilm. However, by lowering its concentrations, this allowed the development of a weak biofilm of *A. pleuropneumoniae* (25%, Figure 50). Finally, the fourth pattern observed was in the case of supernatants from *P. multocida* and *E. coli*. These supernatants in any concentration were unable to allow of biofilm development of *A. pleuropneumoniae*.

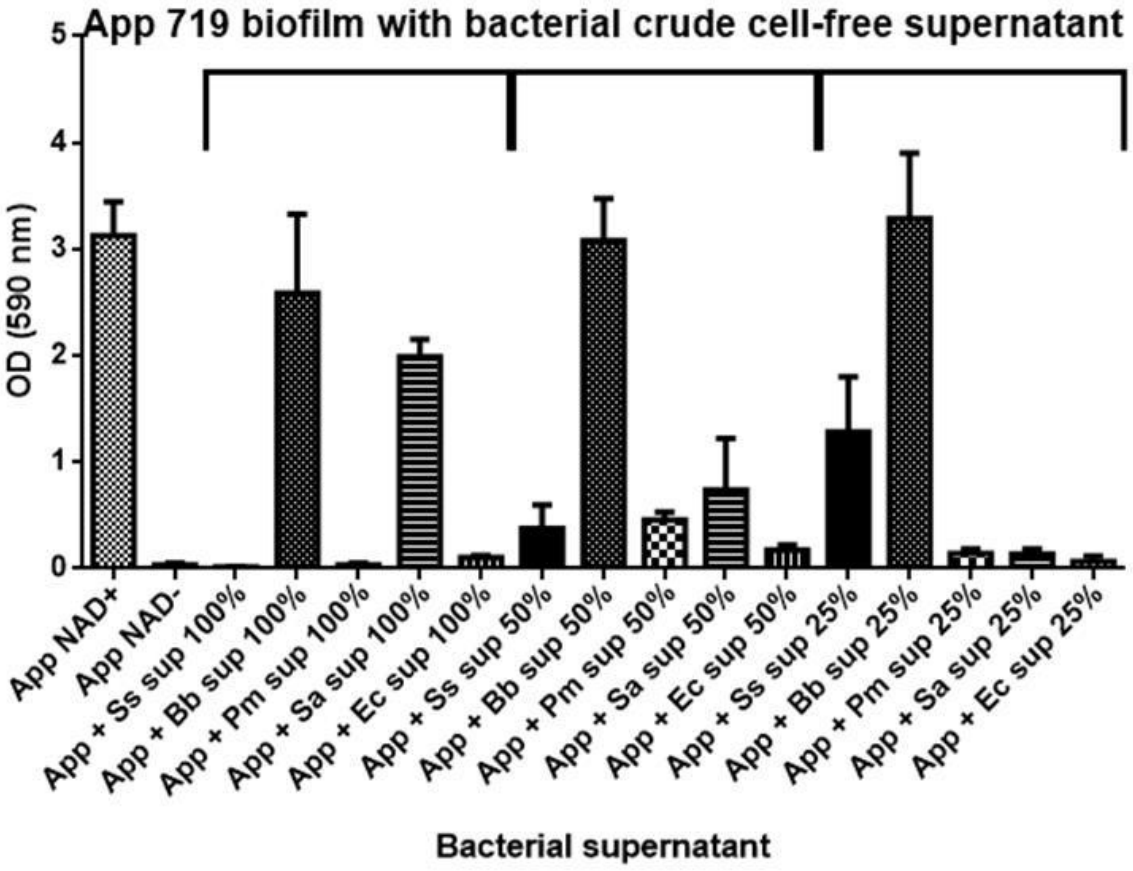


Figure 50: Standard 96-well microtiter plate technique with CV in a single species biofilms formation of *A. pleuropneumoniae* with the crude cell-free supernatants of the other five different bacteria in order to investigate the effects of crude cell-free supernatants of each bacterium for the *A. pleuropneumoniae* mono-species biofilm.

6.10.12 Multi-species biofilms formed by *A. pleuropneumoniae* with the *E. coli* isolated from drinking water of swine farm.

Due to the above results, where we observed that *A. pleuropneumoniae* was able to form multi-species biofilms with different porcine respiratory pathogens, with no respiratory pathogenic bacteria, but even more surprising, with bacteria that are not regular inhabitants of porcine respiratory tract, such as *E. coli*, we decided to test whether *A. pleuropneumoniae* was able to grow and form multi-species biofilms with the *E. coli* isolated from drinking water in swine farms. In all cases, the biofilm formed by the two

bacterial species increased with respect to the mono-species biofilms formed by the *E. coli*. Surprisingly, in 7 of the 12 cases (counting *E. coli* strains ATCC 25922 and L17608 used as controls) this increase was significant (Figure 51); but more importantly, in all cases were able to detect both bacterial species when the conditions were suitable for the development of *E. coli* biofilms and not to the growth and development of *A. pleuropneumoniae*. Remarkably, the proportions of these bacteria seen in two-species biofilm was irregular, and which depended upon the isolated with *A. pleuropneumoniae* biofilm formed (Figure 52), but in all cases, no significant differences between populations, although *E. coli* was slightly higher population in most biofilms. With this results, we can confirm that *A. pleuropneumoniae* has the ability to incorporate into biofilms produced by environmental bacteria, which gives support to *A. pleuropneumoniae* are using the multi-species biofilms as a survival strategy outside its host.

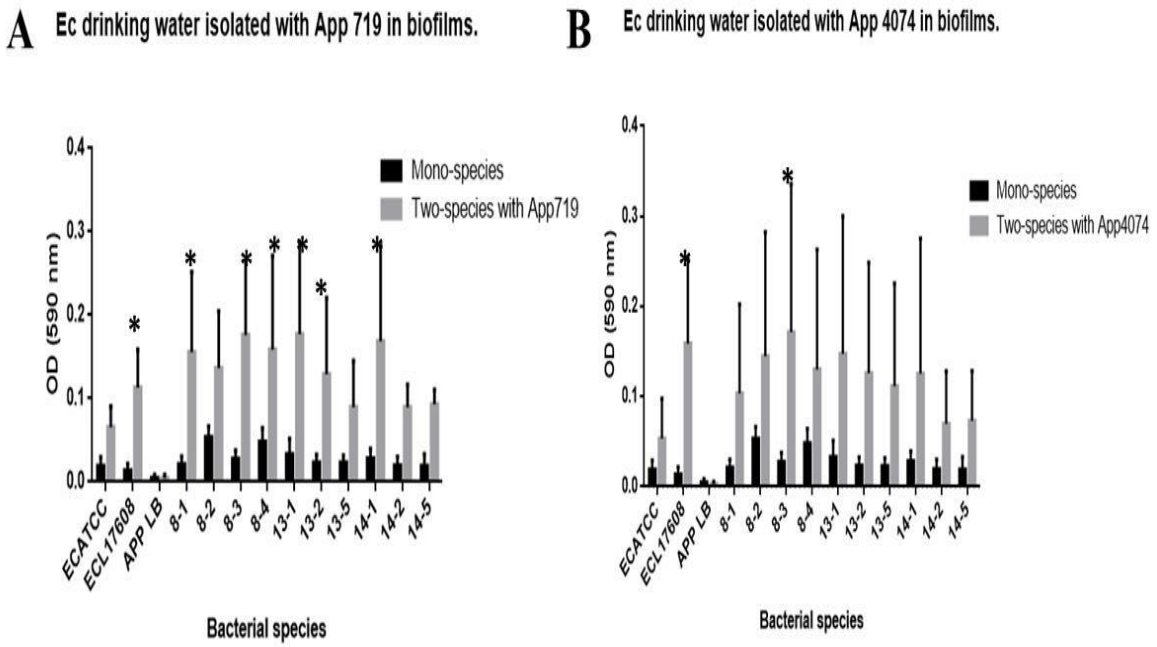


Figure 51: Multi-species biofilms formed by *A. pleuropneumoniae* with the *E. coli* isolated from drinking water of swine farm. A) Two-species biofilms formed by *A. pleuropneumoniae* 719 with all the *E. coli* isolated. B) Two-species biofilms formed by *A. pleuropneumoniae* 4074 with all the *E. coli* isolated. * significant different.

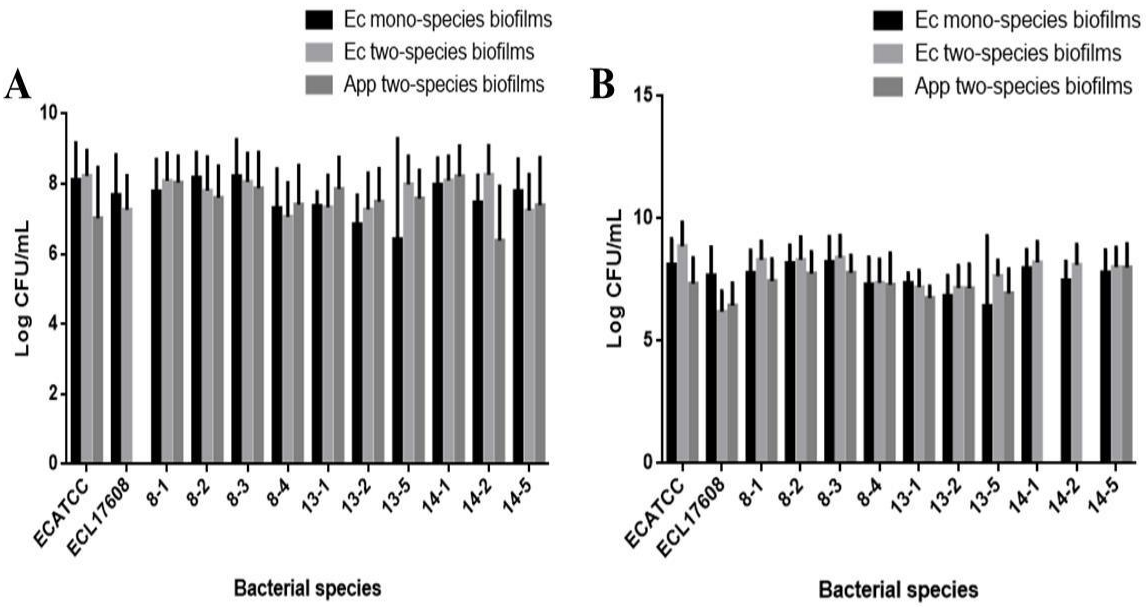


Figure 52: Mono and multi-species biofilms formed by *A. pleuropneumoniae* with the *E. coli* isolated from drinking water of swine farm. A) CFU of two-species biofilms formed by *A. pleuropneumoniae* 719 with all the *E. coli* isolated. B) CFU of two-species biofilms formed by *A. pleuropneumoniae* 4074 with all the *E. coli* isolated. * significant different.

6.11 Mobile device for obtaining biofilms directly from the environment.

Actually, there are no devices that will permit obtein biofilm from a natural environment and that provides protection to the biofilm to allow the study of the same with the least interference. Therefore, the "mobile device for obtaining biofilm directly from the environment" is created in order to enable the production of nature bacterial biofilm directly into a standard or non-standard means, on a flat surface, on the which can be applied different laboratory tests that allow the study of biofilms (Figure 51). The device consists of two parts with a system comprising a housing assembly which has small holes on five faces for the flow of water and /or air. With these two pieces, can be introduce the basis on which it is desired that this microbial community is formed to further study in the laboratory. It also has a couple of holes with standard rope attached to a base with a screw and a pair of small eyes on the sides of the device to tie ropes or cords.

Figure 53: Mobile device for obtaining biofilms directly from the environment. The device can be placed in a great number of places where you want to get the environmental biofilm.

7. DISCUSSION.

A total of 14 farms producing pigs in the State of Aguascalientes, Mexico, were selected for this study. This area is a semi-arid area with little rain in summer, with an average temperature of 17-18 °C, where much of year may be increased above 30 °C (INEGI, 2012). For this study a total of 162 pigs were sampled, with about 12 pigs per farm. Pigs were sampled at random, they had no apparent signs of illness and were in normal production process on farms. Most pigs sampled are 1-2 years, although, also sampled reproductive sows with different ages were sampled.

Of the 162 nasal swabs, 20.37% were positive to *A. pleuropneumoniae* and localized in 78.57% of the farms sampled. Eighteen point fifty-two percent of the samples were positive for *S. suis* with a distribution in 85.71% of farms, of these; corresponding to 3.09% *S. suis* serotype 2. This serotype is the most important because it is the most virulent serotype. In the case of *H. parasuis*, 30.86% of the samples tested positive and were distributed in 92.86%; of the farms. For *P. multocida*, 28.40% were positive with a distribution in the 78.57% of the farms. Nineteen point thirty-nine percent were positive for *M. hyopneumoniae* with a distribution of samples in 64.29% of the farms. Finally, no evidence of the presence of the pathogen *B. bronchiseptica* in nasal swab samples in any of the farms sampled in this study (Table 5, Figure 6 and 7). Moreover, of the 14 farms sampled in this study, 35.71% (5 farms) found the presence of 5 and 4 bacterial pathogens of PRDC, respectively, in 21.43% of cases were 3 pathogens on the same farm, in the 7.14% with 2 pathogens and found no farm that was being affected by 6 pathogens in this study and who were free of any of them (Table 6 and Figure 8). Likewise, the pathogens found a greater number of times to affect the same farm were *S. suis* and *H. parasuis* in 85.71% of cases (12 farms), and in the 71.43% of cases (10 farms): *A. pleuropneumoniae* and *H. parasuis*, *H. parasuis* and *P. multocida*, *P. multocida* and *S. suis* (Table 7 and Figure 9).

In Mexico, there are numerous studies of different pathogens that are part of the RPDC, mainly the pathogen *A. pleuropneumoniae* (Negrete-Abascal *et al.*, 1998; Negrete-Abascal *et al.*, 2003; García-González *et al.*, 2004; Hamer-Barrera *et al.*, 2004; Serrano *et al.*, 2008; Gutiérrez *et al.*, 2011; Loera-Muro *et al.*, 3013), but there are fewer studies in the field (Williams *et al.*, 2000, Álvarez *et al.*, 2004) on the same that make up this complex which is, the biggest health problem faced by pork producers today. One of these studies conducted by Álvarez *et al.* (2004), estimate the frequency of *A. pleuropneumoniae*, *M. hyopneumoniae* and swine influenza virus (SIV), and they found a frequency of 100% for *A. pleuropneumoniae* and *M. hyopneumoniae*. Although in our study, we found a lower frequency of both pathogens, probably due to different methodologies used, it is remarkable the high percentage of farms affected by these pathogens observed in both studies, which shows the health problems facing pig production in our country.

The swine industry is affected by sanitary problems, among them the PRDC is one of the main problems (Loera-Muro *et al*., 2013). The presence of this complex in farms has the effect of economic loss in the market. Within this complex, *A. pleuropneumoniae* is one of the main agents causing economic losses worldwide, being the causative agent of swine pleuropneumonia (Labrie *et al.*, 2010; Ohba *et al.*, 2010; Buettner *et al.*, 2011; Grasteau *et al.*, 2011; Li *et al.*, 2011; Li *et al.*, 2012; Sadilkova *et al.*, 2012). In Mexico, serotypes 1a, 3, 5a, 5b and 7 biotypes 1 are generally found (Ontiveros *et al.*, 1995; Williams *et al.*, 2000; Serrano *et al.*, 2008) and subtype 1a from serotype 1 has been associated with acute cases of disease. Álvarez *et al.* (2004) reported in a study conducted in the State of Yucatan, southern Mexico, all sampled farms had pigs infected with *A. pleuropneumoniae*, finding serotypes 1, 3 and 7. In previous studies had already reported the presence of serotypes 1, 3, 4, 5, 7 and 11. In Mexico, serotypes of biotypes 2 have not been studied (Serrano *et al.*, 2008) and no exist report of presence of *A. pleuropneumoniae* biotypes 2. However, biotypes 2 variants of servers 4, 7 and 11 have been reported (Nicolet, 1992; Kamp *et al.*, 1996; Nielsen *et al.* 1997; Gouré *et al.*, 2009; Maldonano *et al.*, 2009; Deslandes *et al.*, 2010; Perry *et al.* 2011), therefore, it is likely that our positive samples of *A. pleuropneumoniae* from drinking water in swine farms could belong to any of these variants of servers of biotype 2. However, further testing needed because was possible only

the *apxIV* gene detection in all samples and a few cases the *apxIB* and *apxII* genes, although this *apxIV* gene is specific of *A. pleuropneumoniae*, and with this information we could know only one serotype of *A. pleuropneumoniae* found in the water samples, which belongs to serotype 7 frequently reported in North America (Serrano *et al.*, 2008), but belong to biotype 1 (Schaller *et al*., 2000; Rayamajhi *et al*., 2005). This could be due to the limit of detection presenting in the different PCR used in this study (*apxIA*: 50 ng, *apxIB:* 5 ng, *apxII*: 50 ng, *apxIII*: 500 pg and *apxIV*: 400 pg [data not shown]) and probably, the low DNA concentration of *A. pleuropneumoniae* in the samples. However, further studies are necessary to confirm that. Moreover, as in a sample are other bacteria, like *S. maltophilia, A. schindleri* and *E. coli*, these can supply the nutrients needed to grow in media without NAD. Hansen *et al*. (2007) reported that in multi-species biofilms formed by *Acinetobacter* sp. and *Pseudomonas putida* (*P. putida*), the coexistence of the *P. putida* population is dependent on the benzoate excreted from *Acinetobacter* during the catabolism of benzyl alcohol, the sole carbon source. In preliminary results, our group found that *A. pleuropneumoniae* can get NAD from others bacteria like *E. coli* when grow into a mixed biofilm.

A most interesting point is that these samples were obtained from the environment around pigs, such as drinking water, and that could prove their viability and above all, biofilm formation *in vitro* and *in vivo*, demonstrating that *A. pleuropneumoniae*, is surviving in the environment of swine farms in form of biofilm, probably with other bacteria, like *S. maltophilia, A. schindleri* or *E. coli*. In the first case, it is important to mention that there are few studies on the survival of *A. pleuropneumoniae* in the environment, outside the pig. Assavacheep and Rycroft (2012), demonstrated that survival of *A. pleuropneumoniae* was shown to be limited to 3 – 4 days under controlled laboratory conditions, and survived better in cool temperatures and with NaCl. Also, previous work from our laboratory had already detected their presence in the environment in a viable and culturable form. Recently, our group has described the first report of *A. pleuropneumoniae* in drinking water from pig's farm in Mexico. Additionally, viable *A. pleuropneumoniae* was selected and isolated using the cAMP test and the identity of the isolated bacteria were confirmed by Gram staining, a specific polyclonal antibody and an *A. pleuropneumoniae*-

specific PCR and biofilms formation were observed by scanning electron microscopy in *A. pleuropneumoniae*-positive samples (Loera-Muro *et al*., 2013).

Moreover, the ability of *A. pleuropneumoniae* for form biofilms has been widely demonstrated *in vitro* (Kaplan *et al.*, 2004; Kaplan & Mulks, 2005; Izano *et al.*, 2007; Buettner *et al.*, 2008; Kerrigan *et al.*, 2008; Li *et al.*, 2008; Liu *et al.*, 2008; Auger *et al.*, 2009; Dalai *et al.*, 2009; Ganeshnaryan *et al.*, 2009; Tegetmeyer *et al.*, 2009; Bossé *et al.*, 2010; Labrie *et al.*, 2010), under specific growth conditions, when cultured under static (Kaplan & Mulks, 2005; Labrie *et al.*, 2010) and low shear conditions at the air–liquid interface (Jacques *et al.*, 2010); and external factors and genes are well known involved in the development of the same (Kaplan *et al.*, 2004; Izano *et al.*, 2007; Ganeshnarayan *et al.*, 2009; Bossé *et al.*, 2010; Chiers *et al.*, 2010**;** Jacques *et al.*, 2010; Labrie *et al.*, 2010; Grasteau *et al.*, 2011; Li *et al.*, 2011). Also, there are several studies of the function of these biofilms formed by *A. pleuropneumoniae* during development of infection (Bossé *et al.*, 2010; Li *et al.*, 2011) and to increase resistance to antibiotics (Izano *et al.*, 2007; Ganeshnarayan *et al.*, 2009; Archambault *et al.*, 2012). However, here are two interesting points to consider; the first is that *A. pleuropneumoniae* was detected and co-isolated with other bacteria, *S. maltophilia, A. schindleri* and *E. coli*. This might indicate that *A. pleuropneumoniae* may be interacting with other species of bacteria that form biofilms in water in some form of multi-species biofilm. In nature, multi-species biofilms represent the lifestyle preferred by bacteria (Yang *et al*.., 2011), where these structures allow them to survive even in extremely adverse for the development of planktonic life (Bordi & De Bentzmann, 2011), as would be the case for *A. pleuropneumoniae*. These multi-species biofilms are regulated by a variety of interactions inter and intra-specific very important for development, composition, structure and function (De Vriendt *et al.*, 2005; Parsek & Greenberg, 2005; Bordi y Bentzmann, 2011; Hoiby *et al.*, 2011). As already mentioned above, Hansen *et al*. (2007) reported that multi-species biofilms formed by *Acinetobacter* sp. and *Pseudomonas putida* (*P. putida*). Moreover, Ryan *et al*. (2008) reported that CF pathogen *S. maltophilia* can interact with *P. aeruginosa* in a multi-specie biofilms, where *S. maltophilia* diffusible signal factor affects biofilm formation and polymyxin tolerance in *P. aeruginosa* through a sensor kinase encoded by *P. aeruginosa* PA1396 gene. A recent

study of Bridier *et al.* (2012), was evaluated the biofilm resistance of a *Bacillus subtilis* strain (called hereafter $ND_{medical}$) recently isolated from endoscope washer-disinfectors to peracetic acid and its ability to protect the pathogen *Staphylococcus aureus* in mixed biofilms. When grown in mixed biofilm with *S. aureus*, the $ND_{medical}$ strain demonstrated the ability to protect the pathogen from PAA action, thus enabling its persistence in the environment. This work points out the ability of bacteria to adapt to an extremely hostile environment. The second point is that *A. pleuropneumoniae* biofilms was detected in pig's drinkers, and these may be a continual inoculum for animals. In this point, one example is the pathogen *Campylobacter jejuni*, this has the ability to form biofilms in the water supplies and plumbing systems of animal husbandry facilities and animal-processing plants. These biofilms may provide a continual inoculum for domesticated animals (Reeser *et al*., 2007; Jacques *et al*., 2010). Also, *Campylobacter* can form mono-species biofilms as wells as join pre-established multi-species biofilms (Sulaeman et al., 2010; Jacques et al., 2010).

In the other hand, the formation of the consortium can be correlated with increased resistance to antimicrobial agents in infections in animals and/or human (Jacques *et al.*, 2010; Pereira *et al.*, 2010; Almeida *et al.*, 2011). Archambault *et al.* (2012), reported for *A. pleuropneumoniae*, that biofilm formation increases from 100 to 30,000 times the level of resistance to different antibiotics compared to planktonic counterpart. Moreover, in *A. pleuropneumoniae* the formation of biofilm on polystyrene microtiter plates is dependent on the production of PGA (Kaplan *et al.*, 2004; Izano *et al.*, 2007). PGA biosynthesis is dependent on the proteins encoded within the *pgaABCD* operon (Kaplan *et al.*, 2004) and this compound is used by other bacteria to also form biofilms, such as *E. coli*, *S. aureus*, *Staphylococcus epidermidis*, *Yersinia pestis*, *Actinobacillus* spp., *Aggregatibacter actinomycetemcomitans* and *Bordetella* spp., among other (Izano *et al.*, 2007; Jacques *et al*., 2010).

With regard to resistance to 12 antibiotics investigated in this study, the samples shown resistance against 9 antibiotics: nitrofurantoin (40%), pefloxacin (20%), gentamicin (5%), cefotaxime (40%), amikacin (35%), ampicillin (40%), ceftriaxone (40%), chloramphenicol (40%), and cephalothin (40%). Intermediate resistance was observed to

nitrofurantoin (10%), pefloxacin (5%), gentamicin (15%), cefotaxime (10%), amikacin and cefotaxime (5%), and chloramphenicol (15%). Yang *et al.* (2010) reported a higher resistance from isolates of *A. pleuropneumoniae* in Taiwan to lincospectin, ampicillin and amoxicillin, and reports that had a higher susceptibility to ceftiofur, cephalothin and chloramphenicol. Archambault *et al.* (2012), reports on a study conducted antibiotic resistance to 43 isolates of *A. pleuropneumoniae* from Canada, that all isolates were susceptible to ceftiofur, florfenicol, enrofloxacin, erythromycin, clindamycin, trimethoprim/sulfamethoxazole and tilmicosin. A low level of resistance was observed toward tiamulin, penicillin and ampicillin as well as danofloxacin. And high level of resistance to chlortetracycline and oxytetracycline. The above might indicate, that although these isolates did not possess great resistance to multiple drugs, whether they might be developing it because of possible interactions of this pathogen with other pathogens or commensal bacteria, where the phenomenon could be causing horizontal gene transfer, where may be several genes for resistance to these antimicrobials. Also, as mentioned above, our samples have the ability to form biofilms *in vitro* and *in vivo*, which may provide greater resistance to different antibiotics, since this is an established fact in the case of *A. pleuropneumoniae* (Ganeshnarayan *et al.*, 2009; Jacques *et al.*, 2010; Archambault *et al.*, 2012), although further studies are needed to verify this fact.

In this research was demonstrated than *A. pleuropneumoniae* is able to form multi-species biofilms with other swine isolates; remarkably strong biofilms with other respiratory pathogen of swine belonging to PRDC: *S. suis*, *B. bronchiseptica* and *P. multocida*, and with non-pathogenic nasal isolated *S. aureus*; but even was also able to form weak multi-species biofilms with not respiratory pathogen like *E. coli*. Little is known about the ability of some swine respiratory pathogen, such as *A. pleuropneumoniae*, to form multi-species biofilms with other bacteria, and its importance in the process of infection, persistence in the host and in the environment (Jacques *et al*., 2010; Loera-Muro *et al*., 2013). Respiratory disease in pigs has a polymicrobial nature (Opriessnig *et al*., 2011), but it is not known yet if this disease could involve multi-species biofilms. However, multi-species biofilms represent the most important lifestyles of microorganisms in nature (Yang *et al*., 2011) and several cases of multi-species biofilms formation can be found in the

literature (Standar *et al*., 2010; Biyikoglu *et al*., 2012; Bridier *et al*., 2012; Chávez de Paz, 2012; Lopez *et al*., 2012; Stewart *et al*., 2012; Orell *et al*., 2013; Serra *et al*., 2013). Additionally, *A. pleuropneumoniae* serotype 1 was able to survive, grown and form multi-species biofilms even without supplementation of NAD with all the bacteria tested in this study; therefore, it was able to get pyridine compounds (NAD or some of its precursors [NMN and NR] that can be used to produce NAD) (O'Reilly *et al*., 1986; Martin *et al*., 2001) from the other bacteria present in these multi-species biofilms. Our results suggest that *A. pleuropneumoniae* could be using the strategy to form multi-species biofilms by persist in its host (Chiers *et al*., 2010; Labrie *et al*., 2010) and/or to survive in the environment (Assavacheep *et al*., 2012; Loera-Muro *et al*., 2013). In the literature we can find several human and animal pathogens, like *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Legionella pneumophila, Klebsiella pneumoniae, Fusobacterium nucleatum*, *Streptococcus mutans* and *E. coli*, among others, that are known to form multi-species biofilms, and how those associations help them to increase their pathogenicity, resistance and its persistence in the environment (Ryan *et al*., 2008; Almeida *et al*., 2011; Yang *et al*., 2011; Biyikoglu *et al*., 2012; Bridier *et al*., 2012; Chávez de Paz, 2012; Lopez *et al*., 2012; Schlafer *et al*., 2012; Stewart *et al*., 2012; Ali Mohammed *et al*., 2013). However, more studies are needed to know the exact role that these multi-species communities could be playing in the case of *A. pleuropneumoniae*.

In *A. pleuropneumoniae*, the formation of biofilms on polystyrene microtiter plates is dependent on the production of PGA (Izano *et al.,* 2007; Kaplan *et al*., 2004; Jacques *et al*., 2010; Wu *et al*., 2013;). Likewise, can also be found protein and eDNA (Wu *et al*., 2013). However, little is known about the variations that occur in the extracellular matrix composition on biofilms from mono to multi-species. In this study, significant changes in the extracellular matrix composition of *A. pleuropneumoniae* were observed, related to the formation of multi-species biofilms, as well as structural changes in the function that these components have in the biofilm. Important changes in the proportion of PGA polysaccharide were detected; moreover, structural variations, in the form of clusters or filament-like structures, were also observed in most multi-species biofilms. These conformation changes in the polysaccharide component could be related with structural

modifications on PGA, or with the capacity of other bacteria in the biofilm to produce different polysaccharides in the extracellular matrix; as occur with *E. coli* biofilms, where cellulose is also found in the extracellular matrix (Jacques *et al*., 2010), or with *S. aureus* that despite to have PGA in the extracellular matrix, enzymatic treatments with dispersin B, which attacks PGA, do not eliminate the biofilm (Tremblay *et al*., 2013). The significant increments observed in the resistance against the action of this enzyme in several multi-species biofilms, support this last explanation. The largest change was observed in the eDNA component in the two-species biofilms formed by *A. pleuropneumoniae* and *P. multocida* without NAD supplementation. An important increment in the eDNA levels was detected, and it has a role in biofilm structure, since DNase I treatment resulted in 50% disruption of the biofilms. More studies are needed in order to know if this increment in the eDNA could be also related with the interchange and acquisition of new genes (Dominiak *et al*., 2011; Ali Mohammed *et al*., 2013; Jakubovics *et al*., 2013; Tang *et al*., 2013). Likewise, there were changes in the proteins composition in the matrix in this two-species biofilm.

In conclusion, our data show that *A. pleuropneumoniae* has the ability to form multi-species biofilms with the respiratory porcine pathogen *S. suis*, *B. bronchiseptica*, and *P. multocida*, and with other swine no pathogen or pathogen bacteria like *S. aureus* and *E. coli*. Likewise, that *A. pleuropneumoniae* was able to acquire pyridine compounds (NAD or some of its precursors), from those bacteria, in order to enable growth and biofilm development. Significant variations in extracellular matrix composition were observed, as well as structural and functional changes. Morphological variations in the biofilms structure, and in live/dead bacterial composition were also detected. However, further studies are needed to understand if these changes observed in multi-species biofilms are involved in the onset and/or development of swine respiratory diseases; and likewise, if the interactions that occurring in these multi-species biofilms could be related to increase the *A. pleuropneumoniae* resistance and/or persistence in their host, as well as in the environment.

CONCLUSIONS.

With this study we observed that bacterial pathogens of porcine respiratory disease complex are present in pigs in most of the swine farms in the State of Aguascalientes, Mexico. We also observed the great problems existing in our swine farms, which is a reflection of the current situation of the Mexican field. This demonstrates that is necessary to implement a continuous sampling to assess the state of our swine farms, special to small producers.

This work is the first reported of the detection of NAD-independent *A. pleuropneumoniae* with other different bacteria, *S. maltophilia*, *A. schindleri* and *E. coli*, founded in environmental samples from drinking water and in drinkers in swine farms. In addition, was detected the biofilms formation in both, *in vitro* and *in vivo*, this proves that *A. pleuropneumoniae* could be surviving in the environment drinkers in multi-species biofilms. Finally, mentioned that the serotype of this *A. pleuropneumoniae* was serotype 7. However, more studies are needed to show that *A. pleuropneumoniae* found in the environmental biofilms is able to infect swines in a herd and this way is a means of transmission between swines. In addition, why was impossible to obtain a pure isolate these *A. pleuropneumoniae* despite the efforts and confirmatory tests for this pathogen is unknown.

In the other hand, our data show that *A. pleuropneumoniae* has the ability to form multi-species biofilms with *S. suis*, *B. bronchiseptica*, *P. multocida*, *S. aureus* and *E. coli*, in presence or absence of NAD in *A. pleuropneumoniae*, *S. suis* or *E. coli* conditions; suggesting that it is able to acquire NAD or some of its precursors of from those bacteria to enable growth and biofilm development. However, further studies are needed to understand the interactions that may be occurring in these multi-species biofilms formed with *A. pleuropneumoniae*, and if these could be related to increased resistance and/or persistence of both their host as environment.

The bacterial community of the drinking water in swine farms were composed of species such as *Escherichia coli, Provetella* spp., *Ideonella dechloratans, Novosphigobium* spp., *Propionivibrio* spp., *Erythrobacter, Burkholderia, Pseudomonas, Enterobacter,* among others. In this analysis of 16S rDNA did not detect the species *A. schindleri* and *S. maltophilia*.

In conclusion, our data suggest that *A. pleuropneumoniae* has the ability to survive in aquatic environments and may use multi-species biofilms as mean to survive outside its host. Finally, if this capacity for form multi-species biofilms is involved in the persistence and virulence in its host, should be investigated in more detail.

GLOSSARY.

EPS: Extracellular Polymeric Substances.

QS: Quorum Sensing.

AHL: Acyl-Homoserine-Lactone.

PGA: Poly-*N*-Acetylglucosamine.

eDNA: Extracellular DNA.

CLSM: Confocal Laser Scanner Microscopy.

REFERENCES.

Ali Mohammed, M.M., Nerland, A., Al-Haroni, M. & Bakken, V. 2013. Characterization of extracelular polymeric matrix, and treatment of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms with DNase I and proteinase K. *J Oral Microbiol* 5, doi:10.3402/jom.v5i0.20015.

Almeida, C., Azevedo, Santos, S., Keevil, C. & Vieira, M. 2011. Discriminating Multi-species Populations in Biofilms with Peptide Nucleic Acid Fluorescence In Situ Hybridization (PNA FISH). *PLoS ONE* 6, e14786.

Álvarez, M., J. Rodríguez, A. Ciprián, L. Rodríguez, Ayora, G. & Segura, J. 2004. Serological profile of porcine influenza virus, *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae*, in farms of Yucatán, México. *Vet Mex* 35, 296 - 305.

An, D., Danhorn, T., Fuqua, C. & Parsek, MR. 2006. Quorum sensing and motility mediate interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm cocultures. *Proceedings of the National Academy of Sciences of the United States of America* 103, 3828 - 3833.

Archambault, M., Harel, J., Gouré, J., Tremblay, Y.D. & Jacques, M. 2012. Antimicrobial Susceptibilities and Resistance Genes of Canadian Isolates of *Actinobacillus pleuropneumoniae*. *Microb Drug Resist* 18, 198 - 206.

Assavacheep, P. & Rycroft, A.N. 2012. Survival of *Actinobacillus pleuropneumoniae* outside the pig. *Res Vet Sci* 94, 22 - 26.

Auger, E., Deslandes, V., Ramjeet, M., Contreras, I., Nash, J., Harel, J., Gottschalk, M., Olivier, M. & Jacques, M. 2009. Host Pathogen Interactions of *Actinobacillus pleuropneumoniae* with Porcine Lung and Tracheal Epithelial Cells. *Infect Immun* 77, 1426 - 1441.

Bales, P.M., Renke, E.M., May, S., Shen, Y. & Nelson, D. 2013. Purification and Characterization of Biofilm-Associated EPS Exopolysaccharides from ESKAPE Organisms and Other Pathogens. *PLoS ONE* 8, e67950.

Baltes, N. & Gerlach, G.F. 2004. Identification of genes transcribed by *Actinobacillus pleuropneumoniae* in necrotic porcine lung tissue by using selective capture of transcribed sequences. *Infect Immun* 72, 6711 - 6716.

Berk, V., Fong, J., Dempsey, G., Develioglu, O., Zhuang, X., Liphardt, J., Yildiz, F. & Chu, S. 2012. Molecular Architecture and Assembly Principles of *Vibrio cholerae* Biofilms. *Science* 337, 236 - 239.

Biyikoglu, B., Ricker, A. & Diaz, P. 2012. Strain-specific colonization patterns and serum modulation of multi-species oral biofilm development. *Anaerobe* 18, 459 - 470.

Bjarnsholt, T., Alhede, M., Alhede, M., Eickhardt-Sørensen, S., Moser, C., Kühl, M., Jensen, P.O., & Høiby, N. 2013. The in vivo biofilm. *Trends Microbiol*, 1 - 9.

Blanco, I., Canals, A., Evans, G., Mellencamp, M., Cia, C., Deeb, N., Wang, L. & Galina-Pantoja, L. 2008. Differences in susceptibility to *Haemophilus parasuis* infection in pigs. *Can J Vet Res* 72, 228 - 235.

Blume, V., Luque, I., Vela, A., Borge, C., Maldonado, A., Domínguez, L., Tarradas, C. & Fernández-Garayzábal, J. 2009. Genetic and virulence-phenotype characterization of serotypes 2 and 9 of *Streptococcus suis* swine isolates. *Int Microbiol* 12, 161-166.

Bochev, I. 2007. Porcine Respiratory Disease Complex (PRDC): A Review. I. Etiology, Epidemiology, Clinical Forms and Pathoanatomical Features. *Bulgarian Journal of Veterinary Medicine* 10, 131 - 146.

Boekema, B.K., Van Putten, J.P., Stockhofe-Zurwieden, N. & Smith, H.E. 2004. Host cell contact-induced transcription of the type IV fimbria gene cluster of *Actinobacillus pleuropneumoniae*. *Infect Immun* 72, 691 - 700.

Bolland, J. & Dybvig, K. 2012. *Mycoplasma pulmonis* Vsa proteins and polysaccharide modulate adherence to pulmonary epithelial cells. *FEMS Microbiol Lett* 331, 25 - 30.

Bolland, J., Simmons, W., Daubenspeck, J. & Dybvig, K. 2012. Mycoplasma polysaccharide protects against complement. *Microbiology* 158, 1867 - 1873.

Bonifait, L., Grignon, L. & Grenier, D. 2008. Fibrinogen induces biofilm formation by *Streptococcus suis* and enhances its antibiotic resistance. *Appl Environ Microbiol* 74, 4969 - 4972.

Bonifait, L., Gottschalk, M. & Grenier, D. 2010. Cell surface characteristics of nontypeable isolates of *Streptococcus suis*. *FEMS Microbiol Lett* 311, 160 - 166.

Bossé, J.T., Janson, H., Sheehan, B.J., Beddek, A.J., Rycroft, A.N., Kroll, J.S. & Langford, P.R. 2002. *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. *Microbes Infect* 4, 225 - 235.

Bordi, C. & De Bentzmann, S. 2011. Hacking into bacterial biofilms: a new therapeutic challenge. *Ann Intensive Care* 1, 1-8.

Bowen, W. & Koo, H. 2011. Biology of *Streptococcus mutans*- Derived Glucosyltransferases: Role in Extracellular Matrix Formation of Cariogenic Biofilms. *Caries Res* 45, 69 - 86.

Brenci, A., McFarland, K.A., McManus, H.R., Castang, S., Mogno, I., Dove, S.L. & Lory, S. 2009. The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. *Mol Microbiol* 73, 434 - 445.

Bridier, A., Dubois-Brissonnet, F., Boubetra, A., Thomas, V. & Briandet, R. 2010. The biofilm architecture of sixty opportunistic pathogens deciphered using a high throughput CLSM method. *J Microbiol Methods* 82, 64 - 70.

Bridier, A., Sanchez, M., Le Coq, D., Aymerich, S., Meylheuc, T., Maillard, J., Thomas, V., Dubois-Brissonnet, F. & Briandet, R. 2012. Biofilms of a *Bacillus subtilis* Hospital Isolate Protect *Staphylococcus aureus* from Biocide Action. *PLoS ONE* 7, e44506.

Brogden, K. & Guthmiller, J. 2002. Polymicrobial Diseases. American Society for Microbiology. E.U.A. 427 pp.

Brothers, M.C., Geissler, B., Hisao, G.S., Satchell, K., Wilson, B. & Rienstra, C. 2013. Backbone and side-chain resonance assignments of the membrane localization domain from *Pasteurella multocida* toxin. *Biomol NMR Assign* 8, 221 - 224.

Buettner, F., Konze, S., Maas, A. & Gerlach., G. 2011. Proteomic and immunoproteomic characterization of a DIVA subunit vaccine against *Actinobacillus pleuropneumoniae*. *Proteome Sci* 9, 1 - 23.

Burmølle, M., Ren, D., Bjarnsholt, T. & Sørensen, S. 2014. Interactions in multispecies biofilms: do they actually matter?. *Trends Microbiol* 22, 84 - 91.

Cai, H., Dreumel, T., McEwen, B., Hornby, G., Bell-Rogers, P., McRaild, P., Josephson, G. & Maxie, G. 2007. Application and field validation of a PCR assay for the detection of *Mycoplasma hyopneumoniae* from swine lung tissue samples. *J Vet Diagn Invest* 19, 91 - 95.

Calzas, C., Goyette-Desjardins, G., Lemire, P., Lachance, C., Van Calsteren, M.R. & Segura, M. 2013. Group B *Streptococcus* and *Streptococcus suis* Capsular Polysaccharides Induce Chemokine Production by Dendritic Cells via Toll-Like Receptor Via TLR2- and MyD88-Dependent and -Independent Pathways. *Infect Immun* 81, 3106 - 3118.

Campuzano, V., González, A., Hernández, R., Suárez, F., Trigo, F. & Jaramillo, C. 2011. Caracterización fenotípica y molecular de cepas de *Pasteurella multocida* aisladas de exudado nasal de bovinos, en dos cuencas lecheras de México. *Vet Mex* 42, 1 - 10.

Cao, M., Feng, Y., Wang, C., Zheng, F., Li, M., Liao, H., Mao, Y., Pan, X., Wang, J., Hu, D., Hu, F. & Tang, J. 2011. Functional Definition of LuxS, an Autoinducer-2 (AI-2) Synthase and Its Role in Full Virulence of *Streptococcus suis* Serotype 2. *J. Microbiol* 49, 1000 - 1011.

Cezairliyan, B.O., & Sauer, R.T. 2009. Control of *Pseudomonas aeruginosa* AlgW protease cleavage of MucA by peptide signals and MucB. *Mol Microbiol*, 72: 368 - 379.

Ciofu, O. & Tolker-Nielsen, T. 2011. Antibiotic Tolerance and Resistance in Biofilms in Biofilms Infections. Springer, 215 - 219.

Chávez de Paz, L.E. 2012. Development of a Multispecies Biofilm Community by Four Root Canal Bacteria. *J Endod* 38, 318 - 323.

Chen, Z., Chien, M.S., Chang, N.Y., Chen, T.H., Wu, C.M., Huang, C., Lee, W.C. & Hsuan, S.L. 2011. Mechanisms underlying *Actinobacillus pleuropneumoniae* exotoxin ApxI induced expression of IL-1b, IL-8 and TNF-a in porcine alveolar macrophages. *Vet Res* 42, 2 - 10.

Chen, L., & Wen, Y. 2011. The role of bacterial biofilms in persistent infections and control strategies. *Int J Oral Sci* 3, 66 - 73.

Chen, H., Yu, S., Hu, M., Han, X., Chen, D., Qiu, X. & Ding, C. 2012. Identification of biofilm formation by *Mycoplasma gallisepticum*. *Vet Microbiol* 161, 96 - 103.

Chiers, K., De Waele, T., Pasmans, F., Ducatelle, R. & Haesebrouck, F. 2010. Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and induction of lesions in its porcine host. *Vet Res* 41, 65 - 70.

Chomnawang, M., Nabnuengsap, J., Kittiworakarn, J. & Pathanasophon, P. 2009. Expression and Immunoprotective Property of a 39-kDa PlpB Protein of *Pasteurella multocida*. *J Vet Med Sci* 71, 1479 - 1485.

Chu, Y., Gao, P., Zhao, P., He, Y., Zhang, N., Liu, Y., Liu, J. & Lu, Z. 2011. Genotyping of *Haemophilus parasuis* Isolated from Northwest China Using PCRRFLP Based on the ompA Gene. *J Vet Med Sci* 73, 337 - 343.

Clermont, O., Christenson, J.K., Denamur, E. & Gordon, D. 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ Microbiol Rep* 5, 58 - 65.

Clinical and Laboratory Standard Institute. 2011. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First. Informational Supplement: approved standard M100-S21 Vol. 31 No. 1. Clinical and Laboratory Standards Institute, Wayne, PA.

Conover, M., Mishra, M. & Deora, R. 2011. Extracellular DNA Is Essential for Maintaining *Bordetella* Biofilm Integrity on Abiotic Surfaces and in the Upper Respiratory Tract of Mice. *PLoS ONE* 6, e16861.

Conover, M., Redfern, C., Ganguly, T., Sukumar, N., Sloan, G., Mishra, M. & Deora, R. 2012. BpsR Modulates *Bordetella* Biofilm Formation by Negatively Regulating the Expression of the Bps Polysaccharide. *J Bacteriol* 194, 233 - 242.

Dalai, B., Zhou, R., Wan, Y., Kang, M., Li, L., Li, T., Zhang, S. & Chen, H. 2009. Histone-like protein H-NS regulates biofilm formation and virulence of *Actinobacillus pleuropneumoniae*. *Microb Pathog* 46, 128 - 134.

Davis, B., Rasmus Jensen, R., Williams, P. & O'Shea, P. 2010.The Interaction of N-Acylhomoserine Lactone Quorum Sensing Signaling Molecules with Biological Membranes: Implications for Inter-Kingdom Signaling. *PLoS ONE* 5, e13522.

De Vriendt, K., Theunissen, S., Carpentier, W., De Smet, L., Devreese, B. & Van Beeumen, J. 2005. Proteomics of *Shewanella oneidensis* MR-1 biofilm reveals differentially expressed proteins, including AggA and RibB. *Proteomics* 5, 1308 - 1316.

Deslandes, V., Denicourt, M., Girard, C., Harel, J., Nash, J. & Jacques, M. 2010. Transcriptional profiling of *Actinobacillus pleuropneumoniae* during the acute phase of a natural infection in pigs. *BMC Genomics* 11, 1 - 15.

Dominiak, D., Nielsen, J.N. & Nielsen, P.H. 2011. Extracellular DNA is abundant and important for microcolony strength in mixed microbial biofilms. *Environ Microbiol* 13, 710 - 21.

Donlan, R. 2002. Biofilms: Microbial life on surfaces. *Emerg Infect Dis* 8, 881 - 890.

Dugal, F., Girard, C. & Jacques, M. 1990. Adherence of *Bordetella bronchiseptica* 276 to Porcine Trachea Maintained in Organ Culture. *Appl Environ Microbiol* 56, 1523 - 1529.

Enríquez, I., Godinez, D., Martínez, R., Serrano, J., de la Garza, M., Hamer, R. & Guerrero, A., 2003. Adhesión de *Actinobacillus pleuropneumoniae* a componentes de matriz extracelular de cerdo. *Ciencia Veterinaria* 9, 269 - 293.

European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 1.3, January 5. (2011). http://www.eucast.org/eucast_disk_diffusion_test/breakpoints/.

Feng, Y., Zhang, H., Ma, Y. & Gao, J. 2010. Uncovering newly emerging variants of *Streptococcus suis*, an important zoonotic agent. *Trends Microbiol* 30, 1 - 8.

Fittipaldi, N., Takamatsu, D., Domínguez, M., Lecours, M., Montpetit, D., Osaka, M., Sekizaki, T. & Gottschalk, M. 2010. Mutations in the gene encoding the ancillary pilin subunit of the *Streptococcus suis* srtf cluster result in pili formed by the major subunit only. *PLoS ONE* 5, e8426.

Fröls, S. 2013. Archaeal biofilms: widespread and complex. *Biochem Soc Trans* 41, 393 - 398.

Ganeshnarayan, K., Shah, S.M., Libera, M.R., Santostefano, A. & Kaplan, J.B. 2009. Poly-N-acetylglucosamine matrix polysaccharide impedes fluid convection and transport of the cationic surfactant cetylpyridinium chloride through bacterial biofilms. *Appl Environ Microbiol* 75, 1308 - 1314.

Ganguly, S. & Mitchell, A. 2011. Mucosal biofilms of *Candida albicans*. *Curr Opin Microbiol* 14, 1 - 6.

García-González, O., García, R.M., de la Garza, M., Vaca-Pacheco, S., Paniagua, G.L., Mejía, R., *et al.* 2004. *Actinobacillus pleuropneumoniae* metalloprotease: cloning and in vivo expression. *FEMS Microbiol Lett* 234, 81 - 86.

Garnett, J.A. & Matthews, S. 2012. Interactions in Bacterial Biofilm Development: A Structural Perspective. *Curr Protein Pept Sci* 13, 739 - 755.

Grant, S.S. & Hung, D.T. 2013. Persistent bacterial infections, antibiotic tolerance, and the oxidative stress response. *Virulence* 4, 273 - 283.

Grasteau, A., Tremblay, Y., Labrie, J. & Jacques, M. 2011. Novel genes associated with biofilm formation of *Actinobacillus pleuropneumoniae*. *Vet Microbiol* 153, 134 - 143.

Giao, M., Azevedo, N.F., Wilks, S.A., Vieira, M. & Keevil, C. 2008. Persistence of *Helicobacter pylori* in Heterotrophic Drinking-Water Biofilms. *Appl Enviromen Microbiol* 74, 5898 - 5904.

Giao, M., Azevedo, NF., Wilks, SA., Vieira, M. & Keevil, C. 2010. Effect of Chlorine on Incorporation of *Helicobacter pylori* into Drinking Water Biofilms. *Appl Enviromen Microbiol* 76, 1669 - 1673.

Gorbushina, A. 2007. Life on the rocks. *Environ Microbiol* 9, 1613 - 1631.

Gottschalk, M., Lacouture, S., Bonifait, L., Roy, D., Fittipaldi, N. & Grenier, D. 2013. Characterization of *Streptococcus suis* isolates recovered between 2008 and 2011 from diseased pigs in Québec, Canada. *Vet Microbiol* 162, 819 - 825.

Gouré, J., Findlay, W., Deslandes, D., Bouevitch, A., Foote, S., MacInnes, J., Coulton, J., Nash, J. & Jacques, M. 2009. Microarray-based comparative genomic profiling of reference strains and selected Canadian field isolates of *Actinobacillus pleuropneumoniae. BMC Genomics* 10, 88.

Grasteau, A., Tremblay, Y., Labrie, J. & Jacques, M. 2011. Novel genes associated with biofilm formation of *Actinobacillus pleuropneumoniae*. *Vet Microbiol* 153, 134 - 143.

Gu, H., Zhu, H. & Lu, C. 2009. Use of in vivo-induced antigen technology (IVIAT) for the identification of *Streptococcus suis* serotype 2 in vivo-induced bacterial protein antigens. *BMC Microbiol* 9, 1 - 15.

Gutiérrez, L., Vargas, D., Ocampo, L., Sumano, H., Martínez, R. & Tapia, G. 2011. Plasma concentrations resulting from florfenicol preparations given to pigs in their drinking water. *J Anim Sci* 89, 2926 - 2931.

Hamer-Barrera, R., Godínez, D., Enríquez, V.I., Vaca-Pacheco, S., Martínez-Zúñiga, R., Talamás-Rohana, P., Suárez-Güemez, F. & de la Garza, M. 2004. Adherence of *Actinobacillus pleuropneumoniae* serotype 1 to swine buccal epithelial cells involves fibronectin. *Can J Vet Res* 68, 33 - 41.

Hannan, S., Ready, D., Jasni, A., Rogers, M., Pratten, J. & Roberts, A. 2010. Transferofantibiotic resistance by transformation with eDNA within oral bio¢lms. *FEMS Immunol Med Microbiol* 59, 345 - 349.

Hansen, S.K., Haagensen, J.A., Gjermansen, M., Jørgensen, T., Tolker-Nielsen, T. & Molin, S. 2007. Characterization of a *Pseudomonas putida* rough variant evolved in a mixed-species biofilm with *Acinetobacter* sp. strain C6. *J Bacteriol* 189, 4932 - 4943.

Hao, N., Nga, T. & Schultsza, C. 2011. Slaughterhouse Pigs Are a Major Reservoir of *Streptococcus suis* Serotype 2 Capable of Causing Human Infection in Southern Vietnam. *PLoS ONE* 6, e17943.

Harper, M., Boyce, J. & Adler, B. 2006. *Pasteurella multocida* pathogenesis: 125 years after Pasteur. *FEMS Microbiol Lett* 265, 1 - 10.

Harriott, M.M. & Noverr, M.C. 2010. Ability of *Candida albicans* mutants to induce *Staphylococcus aureus* vancomycin resistance during polymicrobial biofilm formation. *Antimicrob Agents Chemother* 54, 3746 - 3755.

Hay, I.D., Gatland, K., Campisano, A., Jordens, J.Z. & Rehm, B.H. 2009. Impact of alginate overproduction on attachment and biofilm architecture of a supermucoid *Pseudomonas aeruginosa* strain. *Appl Environ Microbiol* 75, 6022 - 6025.

Heegaard, P., Stockmarr, A., Piñeiro, M., Carpintero, R., Lampreave, F., Campbell, F., P Eckersall, D., Toussaint, M., Gruys, E. & Sorensen, N. 2011. Optimal combinations of acute phase proteins for detecting infectious disease in pigs. *Vet Res* 42, 1 - 13.

Henrich, B., Schmitt, M., Bergmann, N., Zanger, K., Kubitz, R., Haüssinger, D. & Pfeffer, K. 2010. *Mycoplasma salivarium* detected in a microbial community with Candida glabrata in the biofilm of an occluded biliary stent. *J Med Microbiol* 59, 239 - 241.

Hoa, N., Chieu, T. & Schultsz, C. 2011. The antimicrobial resistance patterns and associated determinants in *Streptococcus suis* isolated from humans in southern Vietnam, 1997-2008. *BMC Infect Dis* 11, 2 - 8.

Hotchkiss, E., Hodgson, C., Lainson, A. & Zadoks, R. 2011. Multilocus sequence typing of a global collection of *Pasteurella multocida* isolates from cattle and other host species demonstrates niche association. *BMC Microbiol* 11, 2 - 8.

Høiby, N., Ciofu, O., Johansen, H.K., Song, Z., Moser, C., Jensen, P.Ø., Molin, S., Givskov, M., Tolker-Nielsen, T. & Bjarnsholt, T. 2011. The clinical impact of the bacterial biofilms. *Int J Oral Sci* 3, 55 - 65.

Hozbor, D., Fouque, F., & Guiso, N. 1999. Detection of *Bordetella bronchiseptica* by the polymerase chain reaction. *Res Microbiol* 150, 333 - 341.

INEGI (Instituto Nacional de Estadística y Geografía). 2012. www.inegi.org.mx

Irie, Y., Preston, A. & Yuk, M.H. 2006. Expression of the Primary Carbohydrate Component of the *Bordetella bronchiseptica* Biofilm Matrix Is Dependent on Growth Phase but Independent of Bvg Regulation. *J Bacteriol* 188, 6680 - 6687.

Izano, E., Sadovskayab, I., Vinogradovc, E., Mulksd, M., Velliyagoundera, K., Ragunatha, C., Khera, W., Ramasubbua, N., Jabbourib, S., Perryc, M. & Kaplan, J. 2007. Poly-N-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*. *Microb Pathog* 43, 1 - 9.

Jacques, M. 2004. Surface polysaccharides and iron-uptake systems of *Actinobacillus pleuropneumoniae*. *Can J Vet Res* 68, 81 - 85.

Jacques, M., Aragon, V. & Tremblay, Y.D. 2010. Biofilm formation in bacterial pathogens of veterinary importance. *Anim Health Res Rev* 11, 97 - 121.

Jakubovics, N.S., Shields, R.C., Rajarajan, N. & Burgess, J.G. 2013. Life after Death: The Critical Role of Extracellular DNA in Microbial Biofilms. *Lett Appl Microbiol* 6, 467 - 475.

Jensen, H.E., Gyllensten, J., Hofman, C., Leifsson, P.S., Agerholm, J.S., Boye, M. & Aalbæk, B. 2010. Histologic and bacteriologic findings in valvular endocarditis of slaughter-age pigs. *J Vet Diagn Invest* 22, 921 - 927.

Jin, H., Zhou, R., Kang, M., Luo, R., Cai, X. & Chen, H. 2006. Biofilm formation by field isolates and references strains of *Haemophilus parasuis*. *Vet Microbiol* 118, 117 - 123.

Jolivet-Gougeon, A. & Bonnaure-Mallet, M. 2014. Biofilms as a mechanism of bacterial resistance. *Drug Discovery Today Technologies*, in press.

Jonas, K., Melefors, O. & RömLing, U. 2009. Regulation of c-di-GMP metabolism in biofilms. *Future Microbiol* 4, 341 - 358.

Kamp, E., Bokken, G., Vermeulen, T., Jong, M., Buys, H., Reek, F. & Smits, M. 1996. A specific and sensitive PCR assay suitable for large-scale detection of toxigenic *Pasteurella multocida* in nasal and tonsillar swabs specimens of pigs. *J Vet Diagn Invest* 8, 304 - 309.

Kaplan, J.B., Velliyagounder, K., Ragunath, C., Rohde, H., Mack, D., Knobloch, J.K. & Ramasubbu, N. 2004. Genes involved in the synthesis and degradation of matrix polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* biofilms. *J Bacteriol* 186, 8213 - 8220.

Kaplan, J. & Mulks, M. 2005. Biofilm formation is prevalent among field isolates of *Actinobacillus pleuropneumoniae*. *Vet Microbiol* 108, 89 - 94.

Kaut, C., Duncan, M., Kim, J., Maclaren, J., Cochran, K. & Julio, S. 2011. A Novel Sensor Kinase Is Required for *Bordetella bronchiseptica* To Colonize the Lower Respiratory Tract. *Infect Immun* 79, 3216 - 3228.

Kerrigan, J.E., Ragunath, C., Kandra, L., Gyémant, G., Liptak, A., Janossy, L., Kaplan, J.B. & Ramasubbu, N. 2008. Modeling and biochemical analysis of the activity of antibiofilm agent Dispersin B. *Acta Biol Hung* 59, 439 - 451.

Kim, H., Lee, S., Moon, H., Kim, Y., Lee, S., Hur, M. & Yun, Y. 2011. *Streptococcus suis* Causes Septic Arthritis and Bacteremia: Phenotypic Characterization and Molecular Confirmation. *Korean J Lab Med* 31, 115 - 117.

Kim, T., Son, C., Lee, J. & Kim, K. 2012. Vaccine potential of an attenuated *Pasteurella multocida* that expresses only the N-terminal truncated fragment of *P. multocida* toxin in pigs. *Can J Vet Res* 76, 69 - 71.

Klitgaard, K., Friis, C., Jensen, T., Angen, Ø. & Boye. M. 2012. Transcriptional Portrait of *Actinobacillus pleuropneumoniae* during Acute Disease – Potential Strategies for Survival and Persistence in the Host. *PLoS ONE* 7, e35549.

Kolenbrander, P.E., Palmer Jr., R.J., Periasamy, S. & Jakubovics, N.S. 2010. Oral multispecies biofilm development and the key role of cell–cell distance. *Nat Rev Microbiol* 8, 471 - 480.

Krasteva, P.V., Giglio, K.M. & Sondermann, H. 2012. Sensing the messenger: The diverse ways that bacteria signal through c-di-GMP. *Protein Sci* 21, 929 - 948.

Labrie, J., Pelletier-Jacques, G., Deslandes, V., Ramjjet, M., Nash, J. & Jacques, M. 2010. Effects of growth conditions on biofilm formation by *Actinobacillus pleuropneumoniae*. *Vet Res* 41, 3 - 10.

Lachance, C., Segura, M., Gerber, P.P., Xu, J. & Gottschalk, M. 2013. Toll-Like Receptor 2-Independent Host Innate Immune Response against an Epidemic Strain of *Streptococcus suis* That Causes a Toxic Shock-Like Syndrome in Humans. *PLoS ONE* 8, e65031.

Lasa, U. 2005. Biofilms bacterianos. *Actualidad SEM* 37, 14 - 18.

Lax, A. 2012. The *Pasteurella multocida* Toxin: A New Paradigm for the Link Between Bacterial Infection and Cancer. *Curr Top Microbiol Immunol* 361, 131 - 144.

Lee, J., Kang, H. & Wooa, H. 2012. Protective Immunity Conferred by the C-Terminal Fragment of Recombinant *Pasteurella multocida* Toxin. *Clin Vaccine Immunol* 19, 1526 - 1531.

Lehtola, M., Torvinen, E., Kusnetsov, J., Pitkanen, T., Maunula, L., Bondsdorff, C., Martinkainen, C., Wilks, S., Keevil, W. & Miettinen, E. 2007. Survival of *Mycobacterium avium, Legionella pneumophila, Escherichia coli*, and calciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. *Appl Enviromen Microbiol* 73, 2859 - 2859.

Li, L., Zhou, R., Li, T., Kang, M., Wan, Y., Xu, Z. & Chen, H. 2008. Enhanced biofilm formation and reduced virulence of *Actinobacillus pleuropneumoniae* luxS mutant. *Microb Pathog* 45, 192 - 200.

Li, L., Xu, Z., Zhou, Y., Li, T., Sun, L., Chen, H. & Zhou, R. 2011. Analysis on *Actinobacillus pleuropneumoniae* LuxS regulated genes reveals pleiotropic roles of

LuxS/AI-2 on biofilm formation, adhesion ability and iron metabolism. *Microb Pathog* 50, 293 - 302.

Li, L., Xu, Z., Zhou, Y., Sun, L., Liu, Z., Chen, H. & Zhou, R. 2012. Global Effects of Catecholamines on *Actinobacillus pleuropneumoniae* Gene Expression. *PLoS ONE* 7, e31121.

Li, L., Liao, X., Sun, J., Yang, Y.R., Liu, B., Yang, S.S., Zhao, D. & Liu, Y. 2012. Antimicrobial Resistance, Serotypes, and Virulence Factors of *Streptococcus suis* Isolates from Diseased Pigs. *Foodborne Pathog Dis* 9, 583 - 588.

Liu, J., Tan, C., Li, J., Chen, H., Xu, P., He, Q., Bei, W. & Chen, H. 2008. Characterization of ISApl1, an insertion element from *Actinobacillus pleuropneumoniae* field isolate in China. *Vet Microbiol* 132, 348 - 354.

Liu, W., Feng, Z., Fang, L., Zhou, Z., Li, Q., Li, S., Luo, R., Wang, L., Chen, H., Shao, G. & Xiao, S. 2011. Complete Genome Sequence of *Mycoplasma hyopneumoniae* Strain 168. *J Bacteriol* 193, 1016 - 1017.

Liu, W., Yang, M., Xu, Z., Zheng, H., Liang, W., Zhou, R., Wu, B. & Chena, H. 2012. Complete Genome Sequence of *Pasteurella multocida* HN06, a Toxigenic Strain of Serogroup D. *J Bacteriol* 194, 3292 - 3293.

Loera-Muro, V., Garcidueñas. C., Avelar, F., Ramírez, E. & Guerrero, A. 2008. Biopelículas: Estrategias de resistencia y ataque para la patogénesis bacteriana. *Scientiae Nature* 10, 5 - 16.

Loera-Muro, A., Ramírez, F., Avelar, F. & Guerrero, A. 2012. Biopelículas Multi-especie: asociarse para sobrevivir. *Investigación y Ciencia* 54, 49 - 56.

Loera-Muro, V., Jacques, M., Tremblay, Y., Avelar-González, F., Loera-Muro, A., Ramírez-López, E., Medina-Figueroa, A., González-Reynaga, H. & Guerrero-Barrera, A. 2013. Detection of *Actinobacillus pleuropneumoniae* in drinking water from pig farms. *Microbiology* 159, 536 - 544.

Lone, A., Deslandes, V., Nash, J., Jacques, M. & MacInnes. J. 2009. *malT* knockout mutation invokes a stringent type gene-expression profile in *Actinobacillus pleuropneumoniae* in bronchoalveolar fluid. *BMC Microbiol* 9, 195.

Lopes, S.P, Ceri, H., Azevedo, N.F. & Pereira, M.O. 2012. Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection. *Int J Antimicrob Agents* 40, 260 - 263.

MacArthur, I., Jones, J., Goodlett, D., Ernst, R. & Preston, A. 2011. Role of pagL and lpxO in *Bordetella bronchiseptica* Lipid A Biosynthesis. *J Bacterol* 193, 4726 - 4735.

MacInnes, J., Gottschalk, M., Lone, A., Metcalf, D., Ojha, S., Rosendal, T., Watson, S. & Friendship, R. 2008. Prevalence of *Actinobacillus pleuropneumoniae, Actinobacillus suis, Haemophilus parasuis, Pasteurella multocida*, and *Streptococcus suis* in representative Ontario swine herds. *Can J Vet Res* 72, 242 - 248.

Maddox, T., 2011. Biofilms and Antimicrobial Resistance in Companion Animals in Biofilms and Veterinary Medicine. Springer Series on Biofilms 6, 223- 252.

Maldonado, J., Valls, L., Martínez, E. & Riera, P. 2009. Isolation rates, serotypes, and toxin genotypes or nicotinamide adenine dinucleotide-independent *Actinobacillus pleuropneumoniae* among pigs suffering from pleuropneumoniae in Spain. *J Vet Diagn Invest* 21, 854 - 857.

Marcinkiewicz, J., Strus, M. & Pasich, E. 2013. Antibiotic resistance: a "dark side"ofbiofilm-associated chronic infections. *Pol Arch Med Wewn* 123, 309 - 313.

Martin, P.R., Shea, R.J. & Mulks, M.H. 2001. Identification of a Plasmid-Encoded Gene from *Haemophilus ducreyi* Which Confers NAD Independence. *J Bacteriol* 183, 1168 - 1174.

Martínez, P., Espinal, P., Bustos, A. & Mattar, S. 2005. Prevalencia de *Klebsiela pneumoniae* y *Escherichia coli* productoras de β-lactamasas de espectro extendido (BLEE), en el Hospital de San Jerónimo de Montería, Pedro Javier. *Med UNAB* 8, 15 - 22.

Marois, C., Bougeard, S. Gottschalk, M. & Kobisch, M. 2004. Multiplex PCR Assay for Detection of *Streptococcus suis* Species and Serotypes 2 and 1/2 in Tonsils of Live and Dead Pigs. *J Clin Microbiol* 42, 3169 - 3175.

Muckle, A., Giles, J., Lund, L., Stewart, T. & Gottschalk, M. 2010. Isolation of *Streptococcus suis* from the urine of a clinically ill dog. *CVJ* 51, 773 - 774.

Mullins, M., Register, K., Bayles, D., Dyer, D., Kuehn, J. & Phillips, G. 2011. Genome sequence of *Haemophilus parasuis* strain 29755. *Stand Genomic Sci* 5, 61 - 68.

Nadkarni, M., Martin, E., Jacques, N. & Hunter, N. 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 148, 257 - 266.

Nazar, J. 2007. Biofilms bacterianos. *Rev Otorrinolaringol* 67, 61 - 72.

Negrete-Abascal, E., Tenorio, V., Guerrero, A.L., García, R.M., Reyes, M. & de la Garza, M. 1998. Purification and characterization of a protease from *Actinobacillus pleuropneumoniae* serotype 1, an antigen common to all the serotypes. *Can J Vet Res* 62, 183 - 190.

Negrete-Abascal, E., Reyes, M., García, R., Vaca, S., Girón, J., García, O., Zenteno, E. & de la Garza, M. 2003. Flagella and Motility in *Actinobacillus pleuropneumoniae*. *J Bacteriol* 185, 664 - 668.

Nga, T., Nghia, H. & Schultsza, C. 2011. Real-time PCR for detection of *Streptococcus suis* serotype 2 in cerebrospinal fluid of human patients with meningitis. *Diagn Microbiol Infect Dis* 70, 461 - 467.

Nicholson, T.L., Conover, M. & Deora, R. 2013. Transcriptome Profiling Reveals Stage-Specific Production and Requirement of Flagella during Biofilm Development in *Bordetella bronchiseptica*. *PLoS ONE* 7, e49166.

Nicolet, J. 1992. *Actinobacillus pleuropneumoniae*. In: Leman A.D., Straw B.E., Mengeling W.L., D'Allaire D., Taylor D.J. Diseases of Swine. Iowa State University Press, Ames, Iowa, U.S.A.

Nielsen, R., Andresen, L.O., Plambeck, T., Nielsen, J.P., Krarup, L.T. & Jorsal, S.E. 1997. Serological characterization of *Actinobacillus pleuropneumoniae* biotype 2 strains isolated from pigs in two Danish herds. *Vet Microbiol* 54, 35 - 46.

Nijland, R., Hall, M., J. & Burgess, G. 2010. Dispersal of Biofilms by Secreted, Matrix Degrading, Bacterial DNase. *PLoS ONE* 5, e15668.

Ohba, T., Shibahara, T., Kobayashi, H., Takashima, A., Nagoshi, M. & Kubo, M. 2010. Granulomatous lymphadenitis associated with *Actinobacillus pleuropneumoniae* serotype 2 in slaughter barrows. *Can Vet J* 51, 733 - 737.

Oliveira, S., Galina, L. & Pijoan, C. 2001. Development of a PCR test to diagnose *Haemophilus parasuis* infections. *J Vet Diagn Invest* 13, 495 - 501.

Olson, M., Ceri, H., Morck, D., Buret, A. & Read, R. 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can J Vet Res* 66, 86 - 92.

Onishi, H., Sugawara, M., Okura, M., Osaki, M. & Takamatsu, D. 2012. Prevalence of *Streptococcus suis* Genotypes in Isolates from Porcine Endocarditis in East Japan. *J Vet Med Sci*, [Epub ahead of print].

Ontiveros, C.L., Camacho, M.J. & Álvarez, M.J. 1995. Correlación entre la serotipificación de *Actinobacillus pleuropneumoniae* y su aislamiento en cerdos de rastro. *Téc Pecu Méx* 33, 1 - 7.

Opriessnig, T., Giménez-Lirola, L.G. & Halbur, P.G. 2011. Polymicrobial respiratory disease in pigs. *Anim Health Res Revi* 12, 133 - 148.

O'Reilly, T. & Niven, D.F. 1986. Defining the Metabolic and Growth Responses of Porcine Haemophili to Exogenous Pyridine Nucleotides and Precursors. *J Gen Microbiol* 132, 807 - 818.

Orell, A., Fröls, S. & Albers, S.V. 2013. Archaeal Biofilms: The Great Unexplored. *Annu Rev Microbiol* 67, 337 - 354.

Pachirat, O., Taksinachanekit, S., Mootsikapun, P. & Kerdsin, A. 2012. Human *Streptococcus suis* Endocarditis: Echocardiographic Features and Clinical Outcome. *Clin Med Insights Cardiol* 6, 119 - 123.

Parise, G., Mishra, M., Itoh, Y., Romeo, T. & Deora, R. 2007. Role of a Putative Polysaccharide Locus in *Bordetella* Biofilm Development. *J Bacteriol* 189, 750 - 760.

Park, C., Ha, Y., Kim, S., Chae, C. & Ryu, D. 2009. Construction and Characterization of an *Actinobacillus pleuropneumoniae* Serotype 2 Mutant Lacking the Apx Toxin Secretion Protein Genes apxIIIB and apxIIID. *J Vet Med Sci* 71, 1317 - 1323.

Parsek, M.R. & Greenberg, E.P. 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* 13, 27 - 33.

Pereira, A., Silva, T., Gomes, A., Araújo, A. & Giugliano, L. 2010. Diarrhea-associated biofilm formed by enteroaggregative *Escherichia coli* and aggregative *Citrobacter freundii*: a consortium mediated by putative F pili. *BMC Microbiol* 10, 1 - 18.

Perry, M.B., Angen, O., Maclean, L.L., Lacouture, S., Kokotovic, B. & Gottschalk, M. 2011. An atypical biotype I *Actinobacillus pleuropneumoniae* serotype 13 is present in North America. *Vet Microbiol* 4, 403 - 410.

Pósa, R., Donkó, T., Bogner, P., Kovács, M., Repa, I. & Magyar, T. 2011. Interaction of *Bordetella bronchiseptica, Pasteurella multocida*, and fumonisin B1 in the porcine respiratory tract as studied by computed tomography. *Can J Vet Res* 75, 176 - 182.

Post, J., Stoodley, P., Hall-Stoodley, L. & Ehrlich, G. 2004. The role of biofilms in otolaryngologic infections. *Curr Opin Otolaryngol Head Neck Surg* 12, 185 - 190.

Rajagopal, R., Nair, G.K., Mini, M., Joseph, L., Saseendranath, M.R. & John, K. 2013. Biofilm formation of *Pasteurella multocida* on bentonite clay. *Iran J Microbiol* 5, 120 - 125.

Ramadam, H. 2006. Chronic rhinosinusitis and bacterial biofilms. *Curr Opin Otolaryngol Head Neck Surg* 14, 183 - 186.

Ramírez, F.Y., Avelar, F.J., Garneau, P., Márquez, F., Guerrero-Barrera, A.L. & Harel, J. 2013. Presence of multi-drug resistant pathogenic *Escherichia coli* in the San Pedro River located in the State of Aguascalientes, Mexico. *Front Immunol* 4, 147.

Ramjeet, M., Cox, A., Hancock, M., Mourez, M., Labrie, J., Gottschalk, M. & Jacques, M. 2008. Mutation in the LPS outer core biosynthesis gene, galU, affects LPS interaction with the RTX toxins ApxI and ApxII and cytolytic activity of *Actinobacillus pleuropneumoniae* serotype 1. *Mol Microbiol* 70, 221 - 235.

Rashid, R., Morohoshi, T., Someya, N. & Ikeda, T. 2011. Degradation of *N*-Acylhomoserine Lactone Quorum Sensing Signaling Molecules by Patato Root Surface-Associated *Chryseobacterium* strain. *Microbes Environ* 26, 1444 - 1448.

Rayamajhi, N., Shin, S.J., Kang, S.G., Lee, D.Y., Ahn, J.M. & Yoo, H.S. 2005. Development and use of a multiplex polymerase chain reaction assay based on Apx toxin genes for genotyping of *Actinobacillus pleuropneumoniae* isolates. *J Vet Diagn Invest* 17, 359 - 362.

Reeser, R.J., Medler, R.T., Billington, S.J., Jost, B.H. & Joens, L.A. 2007. Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. *Appl Environ Microbiol* 73, 1908 - 1913.

Register, K. & DeJong, K. 2006. Analytical verification of a multiplex PCR for identification of *Bordetella bronchiseptica* and *Pasteurella multocida* from swine. *Vet Microbiol* 117, 201 - 210.

Rendueles, O., Travier, L., Latour-Lambert, P., Fontaine, T., Margnus, J., Denamur, E. & Chingo, J.M. 2011. Screening of *Escherichia coli* Species Biodiversity Reveals New Biofilm- Associated Antiadhesion Polysaccharides. *MBio* 2, e00043-11.

Rickard, A.H., Gilbert, P., High, N.J., Kolenbrander, P.E. & Handley, PS. 2003. Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* 11, 94 - 100.

Rodriguez-Nuñez, J., Avelar, F., Marquez, F., Rivas-Santiago, B., Quiñones, C. & Guerrero-Barrera, A. 2012. *Mycobacterium tuberculosis* complex detected by modified fluorescent in situ hybridization in lymph nodes of clinical samples. *J Infect Dev Ctries* 6, 58 - 66.

Ryan, R.P., Fouhy, Y., Garcia, B.F., Watt, S., Niehaus, K., Yang, L., Tolker-Nielsen, T. & Dow, M. 2008. Interspecies signalling *via* the *Stenotrophomonas maltophilia* diffusible signal factor influences biofilm formation and polymyxin tolerance in *Pseudomonas aeruginosa*. *Mol Microbiol* 68, 75 - 86.

Sadilkova, L., Nepereny, J., Vrzal, V., Sebo, P. & Osicka, R. 2012. Type IV fimbrial subunit protein ApfA contributes to protection against porcine pleuropneumoniae. *Vet Res* 43, 1 - 12.

Sambrook, J. & Rossell, D. 2001. Molecular Cloning, a laboratory manual. 3a ed, CSHL PRESS. E.U.A.

Sanderson, A., Leid, J. & Hunsaker, D. 2006. Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. *Laryngoscope* 116, 1121 - 1126.

Schaller, A., Djordjevic, S. & Eamens, G. 2001. Identification and detection of *Actinobacillus pleuropneumoniae* by PCR based on the gene *apxIVA*. *Vet Microbiol* 79, 47 - 62.

Scherr, T., Heim, C., Morrison, J. & Kielian, T. 2014. Hiding in plain sight: interplay between staphylococcal biofilms and host immunity. *Front Immunol* 5, 37.

Schlafer, S., Raarup, M.K., Wejse, P.L., Nyvad, B., Städler, B.M., Sutherland, D.S., Birkedal, H. & Meyer, R.L. 2012. Osteopontin Reduces Biofilm Formation in a Multi-Species Model of Dental Biofilm. *PLoS ONE* 7, e41534.

Scraber, S., Helmi, K., Willame, R., Ferreol, M., Gantzer, C., Hoffmann, L. & Cauchie, H. 2007. Ocurrence and persistence and bacterial and viral fecal indicators in wastewater biofilms. *Water Sci Technol* 55, 377 - 387.

Serra, D.O., Richter, A.M., Klauck, G., Mika, F. & Hengge, R. 2013. Microanatomy at cellular resolution and spatial order of physiological differentiation in a bacterial biofilm. *mBio* 4, e00103-13.

Serrano, L., Tenorio-Gutiérrez, V., Suárez, F., Reyes-Cortés, R., Rodríguez-Mendiola, M., Arias-Castro, C., Godínez-Vargas, D. & de la Garza, M. 2008. Identification of *Actinobacillus pleuropneumoniae* biovars 1 and 2 in pigs using a PCR assay. *Mol Cell Probes* 22, 305 - 312.

Shivachandra, S.B., Kumar, A., Yogisharadhya, R., Ramakrishnan, M.A. & Viswas, K.N. 2013. Carboxyl terminus heterogeneity of type IV fimbrial subunit protein of *Pasteurella multocida* isolates. *Vet Res Commun* 37, 269 - 275.

Simmons, W.L. & Dybvig, K. 2007. Biofilms Protect *Mycoplasma pulmonis* Cells from Lytic Effects of Complement and Gramicidin. *Infect Immun* 75, 3696 - 3699.

Siqueira, F., Schrank, A. & Schrank, I. 2011. *Mycoplasma hyopneumoniae* Transcription Unit Organization: Genome Survey and Prediction. *DNA Research* 18, 413 - 422.

Sisti, F., Ha, D.G., O'Toole, G., Hozbor, D. & Fernández, J. 2013. Cyclic-di-GMP signalling regulates motility and biofilm formation in *Bordetella bronchiseptica*. *Microbiology* 159, 869 - 879.

Sloan, G.P., Love, C.F., Sukumar, N., Mishra, M. & Deora, R. 2007. The *Bordetella* Bps Polysaccharide Is Critical for Biofilm Development in the Mouse Respiratory Tract. *J Bacteriol* 189, 8270 - 8276.

Sokoli, A., Groebel, K., Hoelzle, K., Amselgruber, W., Mateos, J.M., Schneider, M., Ziegler, U., Felder, K.M. & Hoelzle, L. 2013. *Mycoplasma suis* infection results endothelial cell damage and activation: new insight into the cell tropism and pathogenicity of hemotrophic mycoplasma. *Vet Res* 44, doi: 10.1186/1297-9716-44-6.

Srivastava, D. & Waters, C.M. 2012. A Tangled Web: Regulatory Connections between Quorum Sensing and Cyclic Di-GMP. *J Bacteriol* 194, 4485 - 4493.

Stahel, A., Hoop, R., Kuhnert, P. & Korczak, B. 2009. Phenotypic and genetic characterization of *Pasteurella multocida* and related isolates from rabbits in Switzerland. *J Vet Diagn Invest* 21, 793 - 802.

Standar, K., Kreikemeyer, B., Redanz, S., Münter, W.L., Laue, M. & Podbielski, A. 2010. Setup of an In Vitro Test System for Basic Studies on Biofilm Behavior of Mixed-Species Cultures with Dental and Periodontal Pathogens. *PLoS ONE* 5, e13135.

Stevenson, A., Macdonald, J. & Roberts, M. 2003. Cloning and characterization of type 4 fimbrial genes from *Actinobacillus pleuropneumoniae*. *Vet Microbiol* 92, 121 - 134.

Stewart, C.R., Muthye, V. & Cianciotto, N.P. 2012. *Legionella pneumophila* Persists within Biofilms Formed by *Klebsiella pneumoniae*, *Flavobacterium* sp., and *Pseudomonas fluorescens* under Dynamic Flow Conditions. *PLoS ONE* 7, e50560.

Stock, A.M., Robinson, V.L. & Goudreau, P.N. 2000. Two-component signal transduction. *Annu Rev Biochem* 69, 183 - 215.

Stoodley, P., Sauer, K., Davies, D.G. & Costerton, J.W. 2002. Biofilms as complex differentiated communities. *Annu. Rev Microbiol* 56, 187 - 209.

Strait, E., Madsen, M., Minion, C., Christopher-Hennings, J., Dammen, M., Jones, K. & Thacker, E. 2008. Real-Time PCR Assays To Address Genetic Diversity among Strains of *Mycoplasma hyopneumoniae*. *J Clin Microbiol* 46, 2491 - 2498.

Sulaeman, S., Le Bihan, G., Rossero, A., Federighi, M., De´, E. & Tresse, O. 2010. Comparison between the biofilm initiation of *Campylobacter jejuni* and *Campylobacter coli* strains to an inert surface using BioFilm Ring Test. *J Appl Microbiol* 108, 1303 - 1312.

Susan, E. Manual Merck de Veterinaria. Quinta edición. Merck y Co., Inc. España. 2558.

Talavera, M., Gottschalk, M. & Velázquez, V. 2001. Evaluación de la virulencia y serotipos de *Streptococcus suis* aislados de trabajadores de rastros en el valle de Toluca, estado de México, México. *Vet Méx* 32, 201 - 205.

Tang, X., Zhao, Z., Hu, J., Wu, B., Cai, X., He, Q. & Chen, H. 2009. Isolation, Antimicrobial Resistance, and Virulence Genes of *Pasteurella multocida* Strains from Swine in China. *J Clin Microbiol* 47, 951 - 958.

Tang, J., Kang, M., Chen, H., Shi, X., Zhou, R., Chen, J. & Du, J. 2011. The staphylococcal nuclease prevents biofilm formation in Staphylococcus aureus a nd other biofilm-formingbacteria. *Sci China Life Sci* 54, 863 - 869.

Tang, L., Schramm, A., Neu, T., Revsbech, N.P. & Meyer, R.L. 2013. Extracellular DNA in adhesion and biofilm formation of four environmental isolates: a quantitative study. *FEMS Microbiol Ecol* 86, 394 - 403.

Tegetmeyer, H.E., Fricke, K. & Baltes, N. 2009. An isogenic *Actinobacillus pleuropneumoniae* AasP mutant exhibits altered biofilm formation but retains virulence. *Vet Microbiol* 137, 392 - 396.

Thomas, J. & Nakaishi, L. 2006. Managing the complexity of a dynamic biofilm. *J Am Dent Assoc* 137, 10S - 15S.

Trappetti, C., Ogunniyi, A., Oggioni, M. & Paton, J. 2011. Extracellular Matrix Formation Enhances the Ability of *Streptococcus pneumoniae* to Cause Invasive Disease. *PLoS ONE* 6, e19844.

Tremblay, Y.D., Lamarche, D., Chever, P., Haine, D., Messier, S. & Jacques, M. 2013. Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. *J Dairy Sci* 96, 234 - 246.

Vaerewijck, M., Huys, G., Palomino, J., Swings, J. & Portaels, F. 2005. Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiol Rev* 29, 911 -934.

Van Calsteren, M.R., Gagnon, F., Calzas, C., Goyette-Desjardins, G., Okura, M., Takamatsu, D., Gottschalk, M. & Segura, M. 2013. Structure determination of *Streptococcus suis* serotype 14 capsular polysaccharide. *Biochem Cell Biol* 91, 49 - 58.

Vaz-Moreira, I., Nunes, O. & Manaia, C. 2012. Diversity and antibiotic resistance in *Pseudomonas* spp. from drinking water. *Sci Total Environ* 426, 366 - 374.

Villarreal, I., Vranckx, K., Calus, D., Pasmans, F., Haesebrouck, F. & Maes, D. 2012. Effect of challenge of pigs previously immunized with inactivated vaccines containing homologous and heterologous *Mycoplasma hyopneumoniae* strains. *BMC Vet Res* 8, 2.

Vranckx, K., Maes, D., Marchioro, S., Villarreal, I., Chiers, K., Pasmans, F. & Haesebrouck, F. 2012. Vaccination reduces macrophage infiltration in bronchus-associated lymphoid tissue in pigs infected with a highly virulent *Mycoplasma hyopneumoniae* strain. *BMC Vet Res* 8, 24.

Wang, K., Sun, X. & Lu, C. 2011. Development of rapid serotype-specific PCR assays for the eight serotypes of *Streptococcus suis*. *J Clin Microbiol* 50, 3329 - 3334.

Wang, Y., Zhang, W., Wu, Z. & Lu, C. 2011. Reducedvirulence is an important characteristicof bio¢lminfection of *Streptococcus suis*. *FEMS Microbiol Lett* 316, 36 - 43.

Weia, B., Li, F., Yanga, H., Yua, L., Zhaoa, K., Zhoua, R. & Hua, Y. 2012. Magnetic beads-based enzymatic spectrofluorometric assay for rapid and sensitive detection of antibody against ApxIVA of *Actinobacillus pleuropneumoniae. Biosens Bioelectron* 35, 390 - 393.

Wertheim, H., Nghia, H., Taylor, W. & Schultsz, C. 2009. *Streptococcus suis*: An Emerging Human Pathogen. *Emerg Infect* 48, 617 - 625.

Williams, J., Torres-León, M., Echeverria-Coello, P. & Matos-Medina, M. 2000. Aislamiento e identificación de *Actinobacillus pleuropneumoniae* en pulmones de cerdos con pleuroneumonía crónica sacrificados en el rastro municipal de Mérida, Yucatán, México. *Rev Biomed (Méx)* 11, 175 - 181.

Wu, C., Labrie, J., Tremblay, Y.D., Haine, D., Mourez, M. & Jacques, M. 2013. Zinc as an agent for the prevention of biofilm formation by pathogenic bacteria. *J Appl Microbiol* 115, 30 - 40.

Xu, Z, Zhao, G. & Chen, H. 2008. Genome Biology of *Actinobacillus pleuropneumoniae* JL03, an Isolate of Serotype 3 Prevalent in China. *PLoS ONE* 1, e1450.

Yang, C., Lin, C., Lin, C.F., Chang, T. & Chiou, M. 2010. Serotypes, Antimicrobial Susceptibility, And Minimal Inhibitory Concentrations Of *Actinobacillus pleuropneumoniae* Isolated From Slaughter Pigs In Taiwan (2002–2007). *J Vet Med Sci* 73, 205 - 208.

Yang, L., Liu, Y., Wu, H., Høiby, N., Molin, S. & Song, Z. 2011. Current understanding of multi-species biofilms. Int. *J Oral Sci* 3, 74 - 81.

Zhang, N., Chu, Y., Gao, P., Zhao, P., He, Y. & Lu, Z. 2012. Immunological identification and characterization of extracellular serine protease-like protein encoded in a putative *espP2* gene of *Haemophilus parasuis. J Vet Med Sci* 74, 983 - 987.

Zhou, M., Zhang, Q., Zhao, J. & Jin, M. 2012 Haemophilus parasuis Encodes Two Functional Cytolethal Distending Toxins: CdtC Contains an Atypical Cholesterol Recognition/Interaction Region. *PLoS ONE* 7, e32580.

Zou, Y., Feng, S., Xu, C., Zhang, B., Zhou, S., Zhang, L., He, X., Li, J., Yang, Z. & Liao, M. 2013. The role of galU and galE of *Haemophilus parasuis* SC096 in serum resistance and biofilm formation. *Vet Microbiol* 162, 278 - 284.

APPENDIX.

APPENDIX.

Some of the results presented in this work have been published or have been presented during scientific meetings:

Publications:

- Presence of *Actinobacillus pleuropneumoniae, Streptococcus suis, Pasteurella multocida, Bordetella bronchiseptica, Haemophilus parasuis* and *Mycoplasma hyopneumoniae* in upper respiratory tract of swine in farms from Aguascalientes, Mexico. 2013. **Abraham Loera-Muro**, Francisco J. Avelar-González, Víctor M. Loera-Muro, Mario Jacques, Alma L. Guerrero-Barrera. *Open Journal of Animal Science* 3, 132 - 137.
- Biopelícula Multi-especie formada por *Actinobacillus pleuropneumoniae* y *Escherichia coli*. 2013. **Abraham Loera Muro.** Ciencia y Desarrollo, 19: 44.
- Biopelículas multiespecie: asociarse para sobrevivir. Multi-species biofilms: association to survive. 2012. **Abraham Loera Muro**, Flor Yazmín Ramírez Castillo, Francisco Javier Avelar González, Alma Lilián Guerrero Barrera. *Investigación y Ciencia* 54, 49 - 56.

Additional Publications:

- Detection of *Actinobacillus pleuropneumoniae* in drinking water from pig farms. 2013. Victor M. Loera-Muro, Mario Jacques, Yannick D. N. Tremblay, Francisco J. Avelar-González, **Abraham Loera-Muro**, Elsa M. Ramírez-López, Alejandra Medina-Figueroa, Higinio M. González-Reynaga and Alma L. Guerrero-Barrera. *Microbiology* 159, 536 - 544.
- Matriz extracelular: ¿es el andamio de los tejidos?. Extracellular matrix: Is it a tissue scaffolding? 2012. María del Carmen Díaz-Galindo; **Abraham Loera-Muro**;

Flor Yazmín Ramírez-Castillo; Roxanne Olvera-Farías y Alma Lilián Guerrero-Barrera. *Investigación y Ciencia* 56, 53 - 60.

Oral Presentations:

- *A. PLEUROPNEUMONIAE* EN BIOPELÍCULAS MULTIESPECIE. 2013. **Loera Muro Abraham**, Jacques Mario, Avelar González Francisco Javier, Labrie Josée, Tremblay Yannick D.N., Oropeza Navarro Ricardo, Guerrero Barrera Alma Lilián. Cuarto Congreso Internacional "La Investigación en el Posgrado". UAA. Aguascalientes, México.
- SUSCEPTIBILIDAD ANTIMICROBIAL DE AISLADOS AMBIENTALES DE *A. PLEUROPNEUMONIAE*. 2012. **Loera Muro Abraham**, Avelar González Francisco Javier, Jacques Mario, Guerrero Barrera Alma Lilián. Tercer Congreso Internacional "La Investigación en el Posgrado". UAA. Aguascalientes, México.

Poster Presentations:

- *Actinobacillus pleuropneumoniae* can acquire pyridine compounds from other swine pathogens to form multispecies biofilms. 2014. **Abraham Loera-Muro**, Mario Jacques, Francisco Javier Avelar-González, Josée Labrie, Yannick D.N. Tremblay and Alma L. Guerrero-Barrera. International Union of Microbiological Societies (IUMS 2014) XIVth International Congress of Bacteriology and Applied Microbiology, XIVth International Congress of Mycology and Eukaryotic Microbiology and XVIth International Congress of Virology. Montreal, Canada.
- *Actinobacillus pleuropneumoniae* can acquire pyridines compounds from other swine pathogens and form or incorporate into biofilms with other swine pathogens. 2014. **Abraham Loera-Muro**, Mario Jacques, Francisco Javier Avelar-González, Josée Labrie, Yannick D.N. Tremblay, Ricardo Oropeza-Navarro and Alma L. Guerrero-Barrera. Biofilms 6. Vienna, Austria.
- Las biopelículas ambientales como un reservorio de patógenos potenciales de interés medico y veterinario en ambientes contaminados del Estado de

Aguascalientes. 2014**. Loera Muro Abraham**, Ramírez Castillo Flor Yazmín, Avelar González Francisco Javier, Jacques Mario, Harel Josée, Moreno Flores Adriana, Guerrero Barrera Alma L. Feria de Posgrados, CONACYT.

- *Actinobacillus pleuropneumoniae* in biofilms in swine farms in Mexico. 2014. **Abraham Loera-Muro**, Alma L. Guerrero-Barrera, Mario Jacques, Josée Labrie, Yannick D.N. Tremblay, Flor Y. Ramírez-Castillo, Ricardo Oropeza Navarro and Francisco Javier Avelar-González. International Pasteurellacea Conference. Prato, Italy.
- *Actinobacillus pleuropneumoniae* can acquire pyridines compounds from other swine pathogens and form or incorporate into biofilms with other swine pathogens. 2014. **Abraham Loera-Muro**, Mario Jacques, Francisco Javier Avelar-González, Josée Labrie, Yannick D.N. Tremblay, Ricardo Oropeza Navarro and Alma L. Guerrero-Barrera. International Pasteurellacea Conference. Prato, Italy.
- Detection of NAD-independent *Actinobacillus pleuropneumoniae* in biofilms isolated from drinking water of farms located in the state of Aguascalientes, Mexico. 2013**. Abraham Loera Muro**, Mario Jacques, Francisco J. Avelar, Yannick Tremblay, Alma L. Guerrero Barrera. Feria de Posgrados, CONACYT.
- Multi-species Biofilm formation by *Actinobacillus pleuropneumoniae* with other swine respiratory pathogens. 2013. **Abraham Loera-Muro**, Mario Jacques, Francisco Javier Avelar-González, Josée Labrie, Yannick D.N. Tremblay and Alma L. Guerrero-Barrera. CSM-SCM Conference. Ottawa, Canada.
- Detection of NAD-independent *Actinobacillus pleuropneumoniae* in biofilms isolated from drinking water of farms located in the state of Aguascalientes, Mexico. 2012. **Abraham Loera-Muro**, Mario Jacques, Francisco Javier Avelar, Alma Lilián Guerrero Barrera. 6th ASM Conference on Biofilms. ASM Biofilms. Miami, E.U.A.

Participation in Contest:

- We participate in the National Scientific Photography Contest organized by CONACYT. We get fourth place with an electron microscopy photograph of a two-

species biofilm formed by *A. pleuropneumoniae* and *E. coli* in BHI medium + NAD.

PUBLICATIONS.

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Open Journal of Animal Sciences

Presence of *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Haemophilus parasuis* and *Mycoplasma hyopneumoniae* in upper respiratory tract of swine in farms from Aguascalientes, Mexico

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ABSTRACT

Respiratory diseases are one of the most important health problems in pig herds. The porcine respiratory disease complex (PRDC) is the term used to describe pneumonic diseases caused by multiple infectious agents that provoke weight loss in animals or death. In the PRDC multiple pathogens (bacteria and/or viruses) work in combination to induce this respiratory disease. Within this complex, *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Haemophilus parasuis* and *Mycoplasma hyopneumoniae* are the main bacterial pathogens involved in great economic losses to the swine industry. The aim of this work was to estimate the presence of *A. pleuropneumoniae*, *S. suis*, *P. multocida*, *B. bronchiseptica*, *H. parasuis* and *M. hyopneumoniae* in the upper respiratory tract of pigs in representative swine farms in Aguascalientes, Mexico, using PCR technique. The study was performed in 14 swine farms. We obtained a total of 212 nasal swabs. Near 20% of samples were positive for *A. pleuropneumoniae* (located in the 79% of farms); 17% were positive for *S. suis* (in 86% of farms), of these, 3% were *S. suis* serovar 2; 30% were positive for *H. parasuis* (93% of farms); 23% of the samples to *P. multocida* (in 79% of farms); and 19% to *M. hyopneumoniae* (in 64% of farms). *B. bronchiseptica* was not detected in this study. The results obtained show that bacterial pathogens of PRDC were present in the upper respiratory tract of pigs in all farms studied; therefore, these pathogens are widely disseminated in pig farms of Aguascalientes, Mexico.

Keywords: Porcine Respiratory Disease Complex; *Actinobacillus pleuropneumoniae*; *Streptococcus suis*; *Pasteurella multocida*; *Bordetella bronchiseptica*; *Haemophilus parasuis*; *Mycoplasma hyopneumoniae*

1. INTRODUCTION

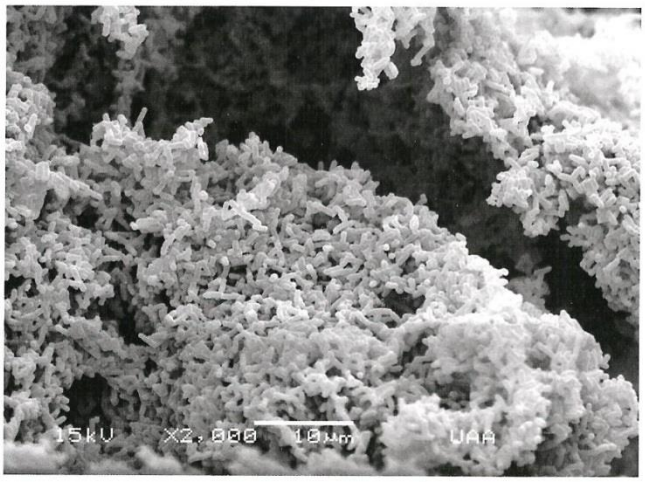
Respiratory diseases are one of the most important health problems in pig herds. Due to the multifactorial nature of these diseases, they are considered as a porcine respiratory disease complex (PRDC). The PRDC is the term used to describe pneumonic diseases is caused by multiple infectious agents, which produce weight loss in animals or death. The PRDC is a major health problem in the current production of pigs [1,2]. Within this complex, the bacteria *A. pleuropneumoniae*, *S. suis*, *P. multocida*,

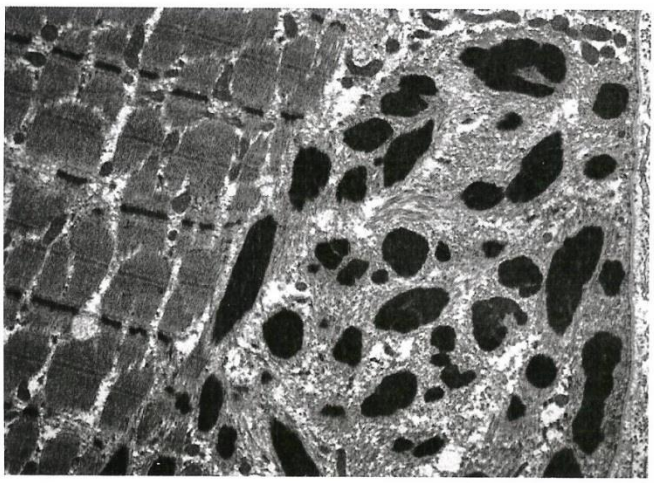
MENCIÓN HONORÍFICA

BIOPELÍCULA MULTI-ESPECIE FORMADA POR *ACTINOBACILLUS PLEUROPNEUMONIAE* Y *ESCHERICHIA COLI*

Abraham Loera Muro

Fotografía tomada con un microscopio electrónico a 2000X que muestra una biopelícula multi-especie formada por las bacterias *Actinobacillus pleuropneumoniae*, patógeno de vías respiratorias en cerdos, causante de la pleuropneumonia porcina, y *Escherichia coli*, bacteria comensal, habitante del tracto intestinal de mamíferos que, en ocasiones, se puede convertir en patógeno de éstos, y suele ser encontrada en cuerpos de agua y suelos.





MENCIÓN HONORÍFICA

CUERPOS NEMALÍNICOS

Alma Delia Hernández Pérez

Miopatía nemalínica es una patología que pertenece a un grupo de enfermedades musculares denominado miopatías estructurales congénitas.

El diagnóstico se da por Microscopía Electrónica de Transmisión, al identificar cambios morfológicos en una biopsia de tejido muscular. Como se observa en esta micrografía electrónica, ultraestructuralmente se identifican grupos de bastones o cuerpos nemalínicos muy electrodensos, constituidos por actina, que indican una alteración de las bandas Z y,por lo tanto, de la sarcomera, que es la unidad de contracción muscular.

INVESTIGACIÓN y CIENCIA
DE LA UNIVERSIDAD AUTÓNOMA DE AGUASCALIENTES
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Biopelículas multi-especie: asociarse para sobrevivir

Multi-species biofilms: association to survive

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Revisión Científica

Loera Muro, A.; Ramírez Castillo, F. Y.; Avelar González, F. J.; Guerrero Barrera, A. L., Biopelículas multi-especie: asociarse para sobrevivir. 54, 49-56, 2012.

RESUMEN

Las biopelículas son una estrategia de supervivencia para los microorganismos que les permite la colonización de ambientes hostiles, tejidos del hospedero o superficies inertes, aún en condiciones cambiantes y para las bacterias patógenas representan un mecanismo de dispersión de infecciones. Debido a lo anterior, el estudio de las biopelículas permite comprender nuevas formas de colonización, resistencia a antibióticos, transferencia horizontal de genes, entre otros mecanismos compartidos por los microorganismos que las conforman. Así, el propósito de la presente revisión es brindar un conocimiento general de estas comunidades, resaltando su importancia en el ambiente y las interacciones entre las especies que participan en su formación.

Palabras clave: biopelículas multi-especie, ciclo celular, matriz extracelular, resistencia antimicrobiana, quorum sensing, FISH.

Key words: multi-species biofilms, cell cycle, extracellular matrix, antimicrobial resistance, quorum sensing, FISH.

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ABSTRACT

Biofilms are an ancient survival microorganism strategy that allows the colonization of hostile environments, host tissues or inert surfaces, even under changing conditions. For pathogenic this represents, a dispersal mechanism of infections. Therefore, the study of biofilms is important to understand new colonization strategies, antibiotic resistance, and horizontal gene transfer, among other mechanisms shared by microorganisms at the consortia. The purpose of this review is to provide a general understanding of these communities, highlighting their importance in the environment and interactions among species that form them.

INTRODUCCIÓN

La formación de biopelículas es reconocida como una estrategia de supervivencia microbiana en diferentes ambientes que brinda resistencia a la desinfección, estrés ambiental y condiciones hostiles en microambientes adversos dentro de los tejidos del hospedero (Pereira *et al.*, 2010; Almeida *et al.*, 2011).

Las biopelículas son complejas comunidades tridimensionales de microorganismos embebidos en una matriz extracelular (MEC), en las cuales despliegan fenotipos únicos o característicos de adaptación especiales, comparados con la forma de vida libre de estos microorganismos, también conocida como planctónica (Ganguly y Mitchell, 2011; Trappetti *et al.*, 2011). En la naturaleza, las biopelículas multi-especie representan

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Detection of *Actinobacillus pleuropneumoniae* in drinking water from pig farms

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Actinobacillus pleuropneumoniae is the aetiological agent of porcine pleuropneumonia and is normally transmitted by aerosols and direct contact between animals. *A. pleuropneumoniae* has traditionally been considered an obligate pathogen of pigs and its presence in the environment has yet to be investigated. Here, the presence of *A. pleuropneumoniae* was detected in drinking water of pig farms in Mexico using a PCR specific for the RTX toxin gene, *apxIV*. The presence of *A. pleuropneumoniae* in farm drinking water was confirmed by indirect immunofluorescence using an *A. pleuropneumoniae*-specific polyclonal antibody and by fluorescent *in situ* hybridization. Viable bacteria from the farm drinking water were detected using the Live/Dead *Bac*Light stain. Additionally, viable *A. pleuropneumoniae* was selected and isolated using the cAMP test and the identity of the isolated bacteria were confirmed by Gram staining, a specific polyclonal antibody and an *A. pleuropneumoniae*-specific PCR. Furthermore, biofilms were observed by scanning electron microscopy in *A. pleuropneumoniae*-positive samples. In conclusion, our data suggest that viable *A. pleuropneumoniae* is present in the drinking water of swine farms and may use biofilm as a strategy to survive in the environment.

INTRODUCTION

Actinobacillus pleuropneumoniae is a member of the family *Pasteurellaceae* and is responsible for porcine pleuropneumonia (Dousse *et al.*, 2008; Reiner *et al.*, 2010). *A. pleuropneumoniae* can be divided in two biotypes based on their NAD requirement. Serotypes 1, 5 and 7 are the predominant serotypes found in North America while serotype 2 is the predominant serotype in many European countries (Chiers *et al.*, 2010; Kim *et al.*, 2010). The clinical signs of the disease can be hyperacute, acute or chronic (Xu *et al.*, 2008; Ohba *et al.*, 2010). Chronic porcine pleuropneumonia is usually very contagious because the bacteria persist in the lungs, the tonsils and, in rare cases, the nasal cavity. The persistence of *A. pleuropneumoniae* within chronically infected pigs helps the dispersion of the disease within a herd (Jacobsen *et al.*, 2005; Wagner & Mulks, 2007). The pathogenesis of *A. pleuropneumoniae* involves several virulence factors, including RTX exotoxins (Apx toxins), lipopolysaccharides, capsular polysaccharides, adhesins, proteases and outer-membrane proteins (Archambault *et al.*, 2003; Kim *et al.*, 2010). Among the virulence factors, the Apx toxins are the factors responsible for the lesions observed in the lungs (Rayamajhi *et al.*, 2005; Xie *et al.*, 2010; Reiner *et al.*, 2010). It has been reported that aerosols are responsible for the dispersion of *A. pleuropneumoniae* over short distances especially when animals are in close proximity. The presence of this pathogen in the environment surrounding pig farms has yet to be reported (Brockmeier *et al.*, 2003; Gião *et al.*, 2010; Reiner *et al.*, 2010). Assavacheep & Rycroft (2013) proposed that *A. pleuropneumoniae* may be transferred between farms if carried on inanimate surfaces in a wet or dried state. They observed that *A. pleuropneumoniae* survived in an aqueous suspension in the presence of NaCl and mucin. Additionally, survival of *A. pleuropneumoniae* was prolonged at lower temperature. In a dry state, *A. pleuropneumoniae* survival improved on hydrophobic

Abbreviations: FISH, fluorescent *in situ* hybridization; SEM, scanning electron microscopy; WGA, wheatgerm agglutinin.

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Matriz extracelular: ¿es el andamio de los tejidos?

Extracellular matrix: Is it the tissue scaffolding?

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Revisión Científica

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RESUMEN

La matriz extracelular se considera un componente extracelular multi-funcional fundamental que participa en la morfología, supervivencia, desarrollo, migración y en las relaciones inter- e intra-celulares de un tejido. El objetivo principal de esta revisión es dar a conocer una visión amplia de la importancia de la matriz extracelular, sus componentes principales, y las funciones e interacciones que estos componentes realizan para mantener la homeostasis de los tejidos.

Palabras clave: matriz extracelular (MEC), glicosaminoglucanos (GAG), colágena, elastina, fibronectina, vitronectina.

Key words: extracellular matrix (ECM), glycosaminoglycans (GAG), collagen, elastin, fibronectin, vitronectin.

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ABSTRACT

The extracellular matrix is considered a multifunctional extracellular component involved in morphology, survival, development, migration, and inter- and intra-cellular tissue relationships. The main objective of this review is to provide a broad view of the importance of the extracellular matrix, its main components, as well as the functions and interactions of these components performance in order to maintain tissue homeostasis.

INTRODUCCIÓN

Uno de los eventos más importantes en la evolución de la vida en la Tierra, es la presencia de la multicelularidad, la cual se considera como un grupo o equipo de células en donde cada una desarrolla funciones especializadas en respuesta a los problemas que confronten. Lo que hace que este equipo funcione, es la comunicación efectiva entre las células y la división de las labores. Sin embargo, los tejidos no están formados únicamente por células. Una buena parte de su volumen lo constituye el espacio extracelular, el cual está ocupado por una intrincada red macromolecular, que constituye la matriz extracelular (MEC) (Lewin *et al.*, 2007; Özbek *et al.*, 2010).

La MEC es una red tridimensional, no celular, presente en todos los tejidos y órganos que estabiliza su estructura física e inicia mecanismos bioquímicos y biomecánicos necesarios para la morfogénesis, diferenciación y homeostasis celular (Alvaro *et al.*, 2010).