UNIVERSIDAD POLITÉCNICA DE MADRID

**ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA,
ALIMENTARIA Y DE BIOSISTEMAS**

**VIRULENCE CHARACTERIZATION OF AVIAN
ENTEROCOCCUS FAECALIS FIELD ISOLATES AND
GENETIC APPROACH TO SELECT MORE
RESISTANT LAYING HENS**

TESIS DOCTORAL

Ana Estefanía Blanco García

Ingeniero Agrónomo

2017

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2017

*“The scientific man does not aim at an immediate result.
He does not expect that his advanced ideas will be readily taken up.
His work is like that of the planter - for the future.
His duty is to lay the foundation for those who are to come, and point the way”*

Nikola Tesla

A mi familia

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ABBREVIATION LIST

AA	Amyloid arthropathy	K_{dyn}	Dynamic stiffness
AC	Allantoic cavity	LSMEANS	Least squares means
AEF	Amyloid enhancing factor	MALDI-TOF	Matrix-assisted linear desorption/ionization time-of-flight
apoSAA	Serum Amyloid A apolipoprotein	MLST.	Multilocus sequence typing
BS	Breaking strength	MS	Mass spectrometry
BW	Body weight	PBS	Phosphate-buffered saline
CFU	Colony-forming units	PFGE	Pulsed-field gel electrophoresis
LD₅₀	Median lethal dose	PG's	Proteoglycans
ELA	Embryo lethality assay	p.i.	Post-inoculation
EMR	Embryo mortality rate	RED	Restriction endonuclease digestion
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction	RT-PCR	Real-time polymerase chain reaction
EW	Egg weight	SAA	Serum Amyloid A
GAGs	Glycosaminoglycans	SAP	Serum Amyloid P
h²	Heritability	SPF	Specific pathogen-free
HDL	High-density lipoprotein	ST	Sequence type
IAPP	Islet Amyloid Polypeptide	TTR	Transthyretin
IL	Interleukin		

SUMMARY

In the last twenty years, major progress in the understanding of systemic amyloid-A amyloidosis, also known as amyloid arthropathy (AA) in chickens, has been gained. However, several topics related to this disease remain unclear, such as the higher susceptibility of brown layers compared to white layers, the biochemical mechanism of the pathogenesis and the transmission route as well as a possible effective treatment to prevent or cure the disease.

AA is the only type of amyloidosis that has been associated with chronic infections induced by *Enterococcus (E.) faecalis* strains and, therefore, the treatment of this disease has focused to date on the prevention and/or treatment of this infection. Given the intrinsic and acquired resistance of *E. faecalis* to several antimicrobials and the new regulations to reduce the antibiotic use in animal production, its treatment becomes more complicated. Additionally, little is known about the risk from field avian *E. faecalis* strains and it is currently difficult to estimate its virulence degree. Consequently, control measures against *E. faecalis* infection such as biosecurity, hygiene, responsible use of antibiotics, development of effective vaccines or the breeding and genetic selection are essential. Therefore, in the present dissertation different avian *E. faecalis* field strains were characterized and the possibility to select more resistant laying hens against *E. faecalis* infection through the Embryo Lethality Assay (ELA) was investigated. The ELA is proposed as an alternative model to replace the adult avian challenge assays.

To date, adult avian challenge assay is the most used animal model in order to determine the degree of virulence of isolated fungi and bacteria, as well as in the selection for disease resistance. However, this model is currently limited by the exposure to pathogens on a large scale, its technical complexity, the cost associated and for ethical reasons. Therefore, the

aim of the **Experiment 1** was to develop a generic methodology of the ELA as a diagnostic model to establish the pathogenicity of *E. faecalis* strains in embryos of White Leghorn layers. Of particular interest was to determine the virulence of the avian *E. faecalis* strain K923/96 and assess its median lethal dose (**LD₅₀**) in order to use it as reference strain in further analyses. The ELA was repeated four times. 0.2 ml of different infectious doses of the cited strain (from 2.5 to 2500 colony-forming units (**CFU**) per ml) were inoculated into the allantoic cavity (**AC**) of 10-day-old embryos. The embryo mortality rate (**EMR**) was determined by daily candling of the eggs over a period of seven days and based on this information the LD₅₀ was calculated. The infectious dose showed a significant effect on the EMR. The EMR with the doses of 2.5, 5, 25, 250 and 2500 CFU/ml was 43%, 45%, 63%, 90% and 93%, respectively. The estimated dose at LD₅₀ was 6.6 CFU/ml. The highest EMR was recorded after three and four days post-inoculation (**p.i.**) in all doses. In summary, the methodology proposed in this experiment can be used as reference in further researches on *E. faecalis* virulence taking into account that the EMR produced in the ELA depends on the dose; the higher the infectious dose, the greater the EMR and the lower the embryo survival time.

In order to characterize the virulence of different avian *E. faecalis* field strains and thus corroborate the ELA capacity for distinguishing *E. faecalis* strains, 68 avian *E. faecalis* strains through the ELA were characterized in the **Experiment 2**. Of particular interest was to characterize the genetic diversity of these strains by Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (**ERIC-PCR**) under the hypothesis that strains with similar DNA banding patterns might be similar also in its infective ability. The ELA was performed ten times with subsets of 7-8 *E. faecalis* strains on a sample of 9987 eggs, including two control groups (positive and negative). An estimated 3-24 CFU were inoculated into the AC of 10-day-old embryos of White Leghorn layer hens. The EMR was determined

by means of candling the eggs over a period of seven days. The ELA was able to distinguish the virulence of the *E. faecalis* strains. Twenty-six strains were considered as avirulent strains with an EMR of below 40%. Five strains were highly virulent with an EMR above 80%, and the remaining 37 strains were classified as strains of moderate virulence, causing an EMR between 40% and 80%. The highest EMR occurred three and four days p.i. From the fourth day p.i., almost no embryonic mortality was observed. Therefore, the ELA could be optimized by reducing experiment duration to four days p.i. ERIC-PCR did not cluster the strains according to its virulence, although ERIC banding patterns revealed a considerable genetic diversity. In conclusion, the ELA can be considered a reliable and useful tool to predict the virulence of avian *E. faecalis* strains. The results obtained in this experiment can be used as a basis for future researches on the *E. faecalis* virulence, and high pathogenic isolates tested might be good candidates for the production of vaccines.

The aim of the **Experiment 3** was to investigate the possibility to select more resistant laying hens against *E. faecalis* infection through the ELA, which is proposed as an alternative model to replace the adult avian challenge assays. A preliminary study was first carried out under the hypothesis that chicken embryos of brown layers would be more susceptible compared with embryos of white layers when they are infected with the same *E. faecalis* strain and infectious dose since white layers are less susceptible to AA than brown layers. To this end, the ELA was performed six times in the preliminary study. 0.2 ml of two infectious doses of the avian *E. faecalis* strain K923/96 (2.5 and 5 CFU/per ml) were inoculated into the AC of 10-day-old embryos of brown layers. The EMR was determined by daily candling of the eggs over a period of four days. The infectious dose showed a significant effect on the EMR. The average EMR estimated for the doses of 2.5 and 5 CFU/ml was 69% and 83%, respectively. They differed significantly from each other. On the basis of these results, 7563 embryos of 10-day-old from 500 families of brown layers (from one to eight embryos per

family) were inoculated into the AC with only one infectious dose of 2.5 CFU/ml of the avian *E. faecalis* strain K923/96 in three more ELAs. The EMR was determined by daily candling of the eggs over a period of four days. Genetic parameters of embryonic survivability to *E. faecalis* infection were estimated to judge the possibility to apply the value of genetic selection. Furthermore, the genetic correlation between embryonic survivability and some traits of economic interest such as laying performance, egg weight (**EW**), body weight (**BW**), breaking strength (**BS**) and dynamic stiffness (**K_{dyn}**) was calculated. The average EMR estimated of these three ELAs was 50% and the highest EMR occurred three and four days p.i. as expected. The estimated heritability for embryonic survivability to the infection by *E. faecalis* was $h^2 = 0.17 - 0.20$ indicating that the selection of more resistant laying hens to *E. faecalis* infection is feasible. However, a high genetic negative correlation was estimated between embryonic survivability and laying performance at the peak of lay ($r_g = -0.69$) and at the end of the production ($r_g = -0.55$), as well as with BS ($r_g = -0.48$ to -0.30), which is undesirable from a breeding point of view. A positive correlation was found between EW and the embryonic survivability ($r_g = +0.16$) and no genetic correlation with BW and **K_{dyn}** was found. Therefore, although it was demonstrated that it is possible to breed for *E. faecalis* resistance, the improvement of other important traits will be penalized.

On the basis of the results obtained in the present dissertation it can be concluded that the ELA is a reliable and useful tool to characterize the virulence of avian *E. faecalis* strains as well as to select more resistant laying hens against *E. faecalis* infection, which can be use as alternative model to replace the adult avian challenge assays. Virulence characterization of avian *E. faecalis* field isolates allows us to know the risk that the strains represent and provide valuable information, which can be important for the selection of isolates for the production of vaccines. Regarding to the possibility to select more resistant laying hens against *E. faecalis* infection, the estimated heritability for embryonic survivability to the infection of *E.*

faecalis indicates that it is feasible. However, the undesirable correlations between the embryonic survivability and some analysed traits show the need to take into account all traits and their genetic correlations to achieve a balanced genetic progress.

A new perspective for the implementation of genetic selection for *E. faecalis* susceptibility in laying hens is offered in the present dissertation. Practical implementation of selection against susceptibility to *E. faecalis* infection will require that breeding company is convinced that AA is of significant economic importance, which depends on disease incidence, production costs and losses associated with sick and/or dead birds, in addition to welfare and food safety concerns of consumers.

RESUMEN (Spanish summary)

En los últimos 20 años ha habido un importante avance en la comprensión de la amiloidosis sistémica A-amiloide, también conocida en las aves como artropatía amiloide (AA). Sin embargo, algunas cuestiones relacionadas con esta enfermedad permanecen sin resolver, tales como la mayor susceptibilidad de las estirpes semipesadas (marrones) en comparación con las estirpes ligeras (rubias), el mecanismo bioquímico de la patogénesis y las vías de transmisión, así como un posible tratamiento eficaz para prevenir o curar la enfermedad.

La AA es el único tipo de amiloidosis asociado con infecciones crónicas inducidas por cepas de *Enterococcus (E.) faecalis* y por lo tanto el tratamiento de esta enfermedad se ha enfocado hasta la fecha en la prevención y/o tratamiento de dicha infección. Dada la resistencia intrínseca y adquirida de *E. faecalis* hacia los antimicrobianos y las nuevas regulaciones para la reducción del uso de antibióticos en la producción animal, su tratamiento es cada vez más limitado. Además, se desconoce el riesgo que suponen las cepas de campo y actualmente es difícil estimar el grado de virulencia de éstas. En consecuencia, las medidas de control de la infección por *E. faecalis*, tales como la bioseguridad, la higiene, el uso responsable de antibióticos, el desarrollo de vacunas efectivas o la selección genética para aumentar la resistencia a la infección por *E. faecalis*, son imprescindibles. Por lo tanto, en la presente tesis se caracterizaron diferentes cepas aviares de *E. faecalis* y se evaluó la posibilidad de seleccionar gallinas ponedoras más resistentes a la infección de *E. faecalis* mediante el Ensayo de Letalidad Embrionaria (ELA), el cual se propone como un modelo alternativo para reemplazar los ensayos de desafío con aves adultas.

La prueba de desafío en aves adultas es el modelo animal más utilizado hasta la fecha para determinar el grado de virulencia de hongos y bacterias, así como para la selección

genética de aves más resistentes a enfermedades. Sin embargo, este modelo está actualmente limitado por la exposición a gran escala de las aves a patógenos, su complejidad, los costes que supone y por razones éticas. Por lo tanto, el objetivo del **Experimento 1** fue desarrollar una metodología genérica del ELA como modelo diagnóstico para caracterizar la virulencia de cepas de *E. faecalis* en embriones de gallinas ponedoras ligeras Leghorn. De particular interés fue determinar la virulencia de la cepa aviar de *E. faecalis* K923/96 y estimar su dosis letal media (**LD₅₀**) para utilizarla como cepa de referencia en estudios posteriores. Se realizaron un total de cuatro ELAs. Se inocularon 0,2 ml de cinco dosis infecciosas (de 2,5 a 2500 unidades formadoras de colonias (**CFU**)/ml) de la citada cepa en la cavidad alantoidea (**AC**) de embriones de 10 días de edad. Durante siete días se registró diariamente la tasa de mortalidad embrionaria (**EMR**) mediante ovoscopia y, en base a esta información, se calculó la LD₅₀. La dosis infecciosa mostró un efecto significativo sobre la EMR. La EMR con las dosis 2,5, 5, 25, 250 y 2500 CFU/ml fue 43%, 45%, 63%, 90% y 93% respectivamente. Se estimó una LD₅₀ de 6,6 CFU/ml. La mayor EMR se produjo tres y cuatro días post-inoculación (**p.i.**) con todas las dosis. En resumen, la metodología propuesta en este estudio puede utilizarse como referencia en estudios futuros sobre la virulencia de cepas de *E. faecalis*, aunque se debe tener en cuenta que la EMR producida en un ELA depende de la dosis; cuanto mayor es la dosis de infección, mayor es la EMR y menor el tiempo de supervivencia de los embriones.

Con el objetivo de caracterizar la virulencia de diferentes cepas aviares de campo de *E. faecalis*, y así poder corroborar la capacidad del ELA para distinguir diferentes cepas, en el **Experimento 2** se caracterizaron 68 cepas aviares de *E. faecalis* mediante el ELA. De particular interés fue caracterizar la diversidad genética de estas cepas mediante la Amplificación de Secuencias Intergénicas de Consenso Repetitivas de Enterobacterias (**ERIC-PCR**) bajo la hipótesis de que las cepas con patrones de bandas de ADN similares

pueden ser similares también en su capacidad de producir infección. Se llevaron a cabo un total de 10 ELAs con subgrupos de 7-8 cepas y dos grupos control (positivo y negativo). Se inocularon de 3 a 24 CFU en la AC de embriones de 10 días de edad de gallinas ponedoras ligeras Leghorn. Durante siete días se registró diariamente la EMR mediante ovoscopia. El ELA fue capaz de diferenciar las cepas de *E. faecalis* con respecto a la EMR que produjeron. Se clasificaron 26 cepas de *E. faecalis* como avirulentas con una EMR inferior al 40%. Cinco cepas fueron altamente virulentas con una EMR por encima del 80%, y las 37 cepas restantes presentaron moderada virulencia con una EMR entre 40% y 80%. La mayor EMR se registró tres y cuatro días p.i. A partir del cuarto día apenas se observó mortalidad embrionaria, por lo que el ELA podría ser optimizado reduciendo el tiempo de observación a cuatro días. La ERIC-PCR no agrupó las cepas de acuerdo a su virulencia, aunque los patrones de bandas revelaron una considerable diversidad genética. En base a los resultados obtenidos, se puede concluir que el ELA es una herramienta fiable y útil para predecir la virulencia de cepas aviares de *E. faecalis*. Los resultados obtenidos en este experimento pueden utilizarse como base para futuras investigaciones sobre la virulencia de *E. faecalis*, y las cepas de alta patogenicidad halladas podrían ser buenas candidatas para la producción de vacunas.

El objetivo del **Experimento 3** fue investigar la posibilidad de seleccionar aves más resistentes a la infección por *E. faecalis* mediante el ELA, el cual se propone como un modelo alternativo para reemplazar el ensayo con aves adultas. Se llevó a cabo un estudio preliminar bajo la hipótesis de que los embriones de gallinas ponedoras semipesadas podrían ser más susceptibles que los embriones de gallinas ponedoras ligeras cuando se infectan con la misma cepa de *E. faecalis* y la misma dosis, ya que las gallinas ponedoras ligeras son menos susceptibles a la AA que las gallinas ponedoras semipesadas. Durante el estudio preliminar se llevaron a cabo seis ELAs. Se inocularon 0,2 ml de dos dosis infecciosas (2,5 y 5 CFU/ml) de la cepa de *E. faecalis* K923/96 en la AC de embriones de 10 días de edad de gallinas

ponedoras semipesadas. Durante cuatro días se registró diariamente la EMR mediante ovoscopia. La dosis infecciosa mostró un efecto significativo sobre la EMR. La EMR media con las dosis de 2,5 UCF/ml y 5 CFU/ml fue 69% y 83% respectivamente, y fueron significativamente diferentes. En base a estos resultados, se infectaron 7563 embriones de 10 días de edad de 500 familias de gallinas ponedoras semipesadas (de uno a ocho embriones por familia) con una única dosis infecciosa de 2,5 CFU/ml de la cepa de *E. faecalis* K923/96 en tres ELAs adicionales. Durante cuatro días se registró diariamente la EMR mediante ovoscopia. Se estimaron los parámetros genéticos de la supervivencia embrionaria a la infección por *E. faecalis* para juzgar el valor de la selección genética, y se investigó la relación entre la supervivencia embrionaria y algunos parámetros de interés económico tales como el rendimiento de producción, el peso del huevo (**EW**), el peso corporal (**BW**), la resistencia a la rotura (**BS**) y la rigidez dinámica (**K_{dyn}**). La EMR media de estos tres ELAs fue 50% y la mayor mortalidad se produjo los días tres y cuatro p.i., como era de esperar. La heredabilidad estimada para la supervivencia embrionaria a la infección por *E. faecalis* fue $h^2 = 0,17 - 0,20$ indicando que la selección de gallinas ponedoras más resistentes a la infección por *E. faecalis* es factible. Sin embargo, se estimó una alta correlación genética entre la supervivencia embrionaria y el rendimiento de producción en el pico de puesta ($r_g = -0,69$) y al final de la producción ($r_g = -0,55$), así como con BS (de $r_g = -0,48$ a $-0,30$), lo cual es indeseable desde el punto de vista de la selección genética. Se encontró una correlación positiva entre EW y la supervivencia embrionaria ($r_g = +0,16$) y no se observó correlación genética con BW y **K_{dyn}**. Por lo tanto, aunque se ha demostrado que es posible seleccionar aves más resistentes a la infección por *E. faecalis*, esto podría penalizar la mejora de otros parámetros importantes.

En base a los resultados obtenidos en la presente tesis se puede concluir que el ELA es una herramienta útil y fiable para la caracterización de la virulencia de cepas aviarias de *E.*

faecalis así como para la selección de gallinas ponedoras más resistentes a la infección por *E. faecalis*, el cual puede utilizarse como modelo alternativo para sustituir los ensayos de desafío con aves adultas. La caracterización de la virulencia de las cepas aviares de *E. faecalis* aisladas en brotes de campo nos permite conocer el riesgo que éstas suponen y proporciona información valiosa para futuras investigaciones epidemiológicas. Además, esta información puede ser importante en la selección de aislados para la producción de vacunas. En cuanto a la posibilidad de seleccionar gallinas ponedoras más resistentes a la infección por *E. faecalis*, la heredabilidad estimada para la supervivencia embrionaria a la infección por *E. faecalis* indica que es factible. No obstante, las correlaciones indeseables entre la capacidad de supervivencia embrionaria y algunos de los parámetros analizados muestran la necesidad de tener en cuenta todos los parámetros y sus correlaciones genéticas para lograr un progreso genético equilibrado.

La presente tesis ofrece una nueva perspectiva para la implementación de la selección genética para la susceptibilidad de *E. faecalis* en gallinas ponedoras. La implementación práctica de la selección contra la susceptibilidad a la infección por *E. faecalis* requerirá que la empresa de genética se convenza de que la AA tiene una importancia económica significativa, la cual depende de la incidencia de la enfermedad, los costes de producción y las pérdidas asociadas a los animales enfermos además de las preocupaciones sobre el bienestar de los animales y la seguridad alimentaria de los consumidores.

CHAPTER 1.

GENERAL INTRODUCTION AND OBJECTIVES.

1.1. LITERATURE REVIEW

1.1.1. Amyloidosis

Amyloidosis is a general term that refers to diseases associated with the deposition of amyloid fibril protein, mainly in the extracellular spaces of organs and tissues of animals and humans. These amyloid fibril proteins are the main component of the amyloid and are deposited as insoluble fibrils due to the sequential changes in protein folding under certain conditions.

Amyloidosis can be classified based on the tissue distribution of amyloid deposits (local or systemic amyloidosis), the absence or presence of pre-existing disease (primary or secondary amyloidosis) and the chemical type of amyloid fibril protein. To date, there are 31 amyloid fibril proteins characterised in humans (Sipe *et al.* 2014), which differ in primary amino acid sequence (Landman *et al.*, 1998a). Ten of these 31 fibrils proteins have also been described in domestic and wild cage animals (Sipe *et al.* 2014) ([Table 1.1.](#)).

Due to the great number of recognised amyloid types, the pathogenesis of amyloidosis is extremely variable and, therefore, research has focused on fibrillogenesis (Landman *et al.*, 1998a). Amyloid fibril formation begins with abnormal folding of an amyloidogenic

precursor protein. Depending on the precursor protein, the misfolding can result from an inadequate proteolytic cleavage (e.g., Islet Amyloid Polypeptide (IAPP)), an amino acid substitution (e.g., transthyretin (TTR)) or an excessive production of serum concentrations in response to acute phase (e.g., Serum Amyloid A (SAA)) (Dember, 2005). According to Woldemeskel (2012), all resulting fibrils are arranged in a characteristic β -pleated sheet fibrillar structure and they are morphologically indistinguishable, despite the great diversity in structure and function of amyloidogenic protein precursors.

Table 1.1. Amyloid fibril proteins and their precursors in animals.¹

Fibril protein	Precursor protein	Occurrence
AA	(Apo) Serum Amyloid A (S)	Chicken, duck, turkey, cat, etc.
AL	Inmunoglobulin Light Chain (S, L)	Cat, horse
AApoAI	Apolipoprotein AI (S)	Dog
AApoAII	Apolipoprotein AII (S)	Mouse
ATTR	Transthyretin (S)	Monkey
AFib	Fibrinogen Aa (S)	Stone martens
A β	A β precursor protein (L)	Dog, sheep, bear, ape
AIAPP	Islet Amyloid Polypeptide (L)	Apes, cat, racoon, cougar
AIns	Insulin (L)	Degu
ACas	A-S2C Casein (L)	Cow

¹ Modified from (Sipe *et al.*, 2014). S: systemic; L: local.

Besides the fibril protein that defines the type of amyloidosis, all types of amyloid deposits contain several common components such as, proteoglycans (PG's), serum amyloid P (SAP) and glycosaminoglycans (GAGs). The latter is a promoter of fibrillogenesis (Dember, 2006).

1.1.2. Amyloid-A amyloidosis

Amyloid-A amyloidosis (also referred to as systemic amyloid according to the distribution of amyloid deposition and secondary amyloidosis by the presence of pre-existing disease) occurs as a consequence of chronic inflammatory or infectious conditions. This leads to a dramatic increase in the levels of serum amyloid A (SAA) to fight the inflammation (Westermarck, 2005). It should be noted that SAA is an acute phase protein synthesised by the liver (Gruys *et al.*, 2005).

According to Landman *et al.* (1998a) amyloid fibrils are formed from SAA prior to C-terminal proteolytic cleavage and transformation into mature amyloid-A fibrils, which are produced at a different site from the sites of deposition. Among the chronic infections and inflammatory diseases that lead to the development of amyloid-A amyloidosis in humans are tuberculosis, rheumatoid arthritis and leprosy (Dember, 2005).

In chickens, amyloid-A amyloidosis is known as amyloid arthropathy (AA), which was first described by Landman *et al.* (1994) as a complication of bacteraemia derived chronic (poly) arthritis. AA is characterised by amyloid-A depositions in the joints (mainly in knee and hock joints and with a higher incidence in the left leg) (Steentjes *et al.*, 2002 and Bautista *et al.*, 2009). Landman *et al.* (1994) observed that the first signs of AA in chickens occur mainly during rearing period from five to six weeks of age onwards. Lamé birds have difficulties accessing food and water, and consequently can become dehydrated and die. Therefore, AA compromises the welfare of chickens and has a significant economic impact since the disease affects about 20-30% of all European flocks (Landman *et al.*, 1998a). Interestingly, it occurs mainly associated with chronic infections induced by arthropathic and amyloidogenic *Enterococcus (E.) faecalis* strains (Landman *et al.*, 1997), which has only been described in association with this disease in chickens and not in humans or other species so

far (Landman *et al.*, 1999a). This form of amyloidosis is the only amyloid type found in chickens so far (Landman *et al.*, 1998a).

1.1.3. Aetiology of amyloid arthropathy

There are various bacteria, which may induce AA. Landman *et al.* (1998b) observed that brown layers inoculated intravenously with doses between 10^2 and 10^8 CFU of *E. faecalis*, *Staphylococcus* (*S.*) *aureus*, *Escherichia* (*E.*) *coli* and *Salmonella* (*S.*) *Enteritidis* showed joint amyloidosis. However, the most severe joint amyloidosis was observed in layers infected with *E. faecalis*. Landman & Feberwee (2001) observed that brown layers developed joint amyloidosis after intra-articular inoculation of *Mycoplasma synoviae*. However, *E. faecalis* is the bacterium which has been isolated mostly in amyloidogenic joints of field cases of AA in brown layers (Landman *et al.*, 1994) and which has been showed to produce a large amount of amyloid deposits when inoculated (Landman, 1999).

According to the results of the analysis of routine post-mortem conducted by Landman *et al.* (1998b) in field cases, 12.9% (249/1930) of the necropsied brown layers during 1996 and 1997 suffered from AA. *E. faecalis* were isolated in 76.6% of the affected birds. *S. aureus* and *E. coli* were only isolated in 8.3% and 5% of the affected brown layers respectively. In the case of the broiler breeders, only 2.9% (53/1815) suffered from AA in the same period of time. *S. aureus*, *E. faecalis* and *E. coli* were isolated in 73.4%, 25% and 1.5% of the affected birds, respectively. Steentjes *et al.* (2002) examined 30 birds from five affected broiler breeder flocks by unilateral (left side) lameness. They found 83% (25/30) amyloid-positive hock joint of broiler parent stock. *E. faecalis* was isolated in 77% (23/30) from the hock joints of the affected birds, and *S. aureus* was isolated from the joint in only one affected bird (1/30).

Additionally, *E. faecalis* is a trigger of the acute phase response in chickens and, therefore, is considered to be a causative agent of AA (Landman *et al.*, 1994). For this reason, it has been deemed important to underline the main features of *E. faecalis* and its role in AA, as well as its possible routes of transmission in the following sections.

1.1.3.1. *Enterococcus faecalis* bacterium

1.1.3.1.1. Features

Enterococci are Gram-positive bacteria, which can appear as single cocci, arranged in pairs or form short-chains. They are mainly catalase negative, facultative anaerobic, non-spore forming and non-motile. They are able to grow in 6.5% NaCl, can hydrolyse esculin and tolerate 40% bile salts (Fisher & Philips, 2009).

E. faecalis is able to survive a wide range of conditions in nature. The bacterium can grow at a temperature range of 10 to 45°C and in environments with pH of 4.5 - 9.9. *E. faecalis* colonies are characterised by its round form, smooth and grey colour. They have a diameter of approximately 2 mm eventually surrounded by an alpha-haemolysis on Columbia sheep blood agar. *E. faecalis* constitutes a part of the endogenous intestinal microflora of birds and humans. The bacterium is considered the third leading cause of nosocomial bacteraemia in patients with underlying conditions (Gregersen *et al.*, 2010). Fisher & Philips (2009) and Gregersen *et al.* (2010) reported that *E. faecalis* and *Enterococcus* (*E.*) *faecium* are the species most frequently isolated from the microbial intestinal flora of one-day-old chicks.

1.1.3.1.2. Diseases in chickens

E. faecalis has been associated with high numbers of poultry diseases. However, very little is known about the epidemiology and pathogenesis of *E. faecalis* infections in animal species (Gregersen *et al.*, 2010). The bacterium has been isolated from pulmonary

hypertension syndrome in broilers (Tankson *et al.*, 2001), septicaemia and endocarditis in a broiler parent flock (Chadfield *et al.*, 2004). According to Gregersen *et al.* (2010), *E. faecalis* were isolated in 5.4% (167/3100) of the necropsied broiler breeders affected by different lesion types such as arthritis, amyloidosis, valvular endocarditis, salpingitis, peritonitis and septicaemia. Landman *et al.* (1998b) and Steentjes *et al.* (2002) observed by Pulsed-Field Gel Electrophoresis (PFGE) that several *E. faecalis* strains associated with AA from different origins showed similar Restriction Endonuclease Digestion (RED) patterns. Therefore, Landman *et al.* (1998b) suggested a wide distribution of a single virulent clone of *E. faecalis* causing amyloid-A amyloidosis in chickens. Petersen *et al.* (2009) observed the same tendency through Multilocus Sequence Typing (MLST). With this technology, they showed the specific association between amyloid-A amyloidosis in poultry and the Sequence Type (ST) 82 of *E. faecalis*, which has been related with this disease worldwide (Petersen *et al.*, 2007). Later, Petersen *et al.* (2010) developed a Real-Time Polymerase Chain Reaction (RT-PCR) method for the rapid and specific detection of the MLST 82 of *E. faecalis*, based on a so-called kinetic PCR approach.

According to the database from the Multi Locus Sequence Typing website (<http://www.mlst.net>), ST36, ST59, ST82, ST170, ST171, ST172 and ST174 have been associated with amyloid-A amyloidosis, and ST34, ST82, ST174 and ST177 have been associated with first week mortality in layers (Olsen *et al.*, 2012b). Additionally, Gregersen *et al.* (2010) observed that ST32, ST176, ST177 and ST249 have the capacity of inducing AA in broiler breeders, and ST16, ST82, ST174 and ST249 have the capacity of inducing septicaemia and/or salpingitis. Of all these STs, ST82, ST174 and ST177 constitute more than 80% of the mortality related with broiler breeders (Gregersen *et al.*, 2010). It should be noted that of all the STs cited above, only ST16, ST34, ST36, ST59 and ST82 have, until now, been identified in both poultry and humans (<http://www.mlst.net>). Therefore, Freitas *et al.* (2009)

suggested that isolated strains in animals and humans might have the same origin which could be spread through the food chain. Furthermore, Hayes *et al.* (2003) argued that poultry meat in particular, might be a route of transmission of *E. faecalis* to humans. In regards to its zoonotic potential, *E. faecalis* is a serious challenge due to its antimicrobial resistance and is therefore gaining great attention.

E. faecalis possess the ability to acquire antimicrobial resistance and virulence factors which are not exclusively associated with a disease (Olsen *et al.*, 2012a). *E. faecalis* have a natural intrinsic resistance towards several antimicrobials such as chloramphenicol, clindamycin, erythromycin, tetracycline, high-level aminoglycosides, beta-lactam antibiotics and vancomycin (McBride *et al.*, 2007). Many virulence genes related with enterococcal infections have been identified: aggregation substance (*asaI* or *agg*), endocarditis antigen (*efaA*), cytolisine (*cylA*), gelatinase (*gelE*), collagen-binding protein (*ace*), hyaluronidase (*hyl*) and extracellular surface proteins (*esp*). The research done by Ciftci & Diker (2009) showed that the virulence genes *gelE*, *cylA* and *asaI* (or *agg*) could have an effect on the pathogenesis of AA. The chickens inoculated (intraarticular and intravenous inoculations) with *gelE* negative strains did not show any pathological signs, whereas 65.2% of *gelE* positive strains caused AA. Additionally, the chickens inoculated with *asaI* (or *agg*) or *cylA* strains (both negative and positive strains) showed AA. In a study done by Olsen *et al.* (2012a), the virulence genes *ace*, *efaA* and *gelE* were present in all isolates obtained from poultry and humans. However, these virulence genes cannot be the only cause for the pathogenicity of *E. faecalis* in poultry since these genes were also present in all commensal isolates. The *esp* gene was the only gene not detected among any poultry isolates. Additionally, Olsen *et al.* (2012a) did not observe any difference on the virulence profile among isolates from clinical birds and cloacal isolates from healthy birds. Furthermore, these authors did not find major differences

in the virulence gene profile from *E. faecalis* isolates of human and poultry which would confirm the possible zoonotic potential of this bacterium.

1.1.3.1.3. Transmission of *E. faecalis*

Several experimental investigations to demonstrate the possible routes of transmission of *E. faecalis* in chickens have been reported in the literature (Landman *et al.*, 1999a; 1999b; 2000; 2001 and 2003; Fertner *et al.*, 2011 and Olsen *et al.*, 2012c).

Landman *et al.* (1999b) found that the same *E. faecalis* strain which infected the parent stock was also present in an analysed offspring population. But the reproductive organs from the infected birds were not affected. Therefore, they concluded that vertical transmission is possible. Landman *et al.* (1999a and 1999b) discarded the theory that *E. faecalis* is spread by the transovarial route because yolk sac infections of chicken embryos with *E. faecalis* resulted in a massive mortality within a few days of incubation. However, after inoculation of egg albumen with low doses of *E. faecalis*, some chicks were successfully hatched. This suggests that an infection via the oviduct is a possible way of vertical transmission (Landman *et al.*, 1999a and 1999b).

Landman *et al.* (2000) observed that some hatchery air samples (up to 10^3 CFU/l air), Marek's disease vaccine suspensions (up to 10^6 CFU/ml vaccine suspension) and injection needles (9500 to 61000 CFU/needle) of two hatcheries were contaminated with an arthropathic and amyloidogenic *E. faecalis* strain during processing and vaccination of the newly hatched layer chickens. Besides, three isolates showed a similar RED pattern to *E. faecalis* 6085.94 (amyloidogenic *E. faecalis* strain used as control) and the other 17 isolates were close or unrelated to control *E. faecalis* strain. Although the authors could not explain

the high concentration of *E. faecalis* found in Marek vaccine suspensions, they suggested that this could be a possible iatrogenic infection route in chickens.

Fertner *et al.* (2011) carried out studies on the transmission of *E. faecalis* among chickens during a hatch in two hatcheries. They sampled a cloacal swab from each single chicken and assumed that an immediate massive growth of *E. faecalis* in pure culture (at 0 h), would be an indicator for vertical transmission. Not immediate but massive growth within the next 24 h indicated either a horizontal or vertical transmission due to the interaction of the chicks in the hatchery, as well as from infected or contaminated eggs. Fertner *et al.* (2011) observed the same tendency for ST82 and ST249. A massive increase of the bacterial growth from 0 to 24 h in brown layers suggests horizontal transmission - orally and cloacally by means of the 'drinking' route between chicks. In white layers, only the ST177 was isolated at 0 h and no later than 24 h. However, other STs like ST82 and ST249 were isolated only after 24 h suggesting the horizontal transmission of these STs.

In accordance with the results of Fertner *et al.* (2011), Olsen *et al.* (2012c) observed an increment of the growth of *E. faecalis* in broiler chickens during the hatch. *E. faecalis* colonised 1% of all the chickens at the beginning of the hatch and after 24 h was detected in 70% of them. This suggests a vertical and horizontal transmission. Besides, all of the STs of *E. faecalis* that were isolated at the time of hatch were also present after 24 h. The authors therefore suggest that the intestine of broiler chickens is a possible route for the horizontal transmission of *E. faecalis* from the hatchery to the farm.

On the basis of the information obtained, both vertical and horizontal transmissions of *E. faecalis* seem possible through different transmission routes. Moreover, Landman *et al.* (2000) suggested that AA could be an iatrogenic infection in chickens after intramuscular

inoculation of Marek vaccine suspensions contaminated with arthropathic and amyloidogenic *E. faecalis* strains.

1.1.4. Pathogenesis of amyloid arthropathy

AA is associated with chronic inflammation and infection, which appears to be induced by *E. faecalis* strains in chickens (Westermarck, 2005). The acute phase response is a complex systemic early-defence mechanism activated to fight the inflammation or infection. Pro-inflammatory cytokines play an important role in initiating the acute phase response (Sevimli *et al.* 2008). The increase in the levels of interleukin (IL) -1 and IL-6 during an infection is induced by the synoviocytes (type B cells) and macrophages (type A cells) as a local response, as shown in [Figure 1.1](#). Although there is very little literature on the role of cytokines in AA, Sevimli *et al.* (2008) reported that IL-1 appears to have an important role in the amyloid pathogenesis due to its positive association with high SAA levels in blood and the severity of AA. However, the role of IL-6 is still unclear.

SAA is mainly synthesised by the liver during the acute phase response. However, the extrahepatic production of SAA has been reported in different human tissues such as ovary, brain or kidney (Upragarin *et al.*, 2005). Ovelgönne *et al.* (2001) reported that SAA is synthesised in the synoviocytes and in the vessel walls of the synovium in chickens affected by AA while the healthy chickens did not express SAA mRNA in the synoviocytes. According to Kisilevsky & Manley (2012), SAA is gaining more attention not only because it is the precursor protein of amyloid-A amyloidosis, but also as its plasma concentration may increase 1000-fold during the acute phase response, its influence on high-density lipoprotein (HDL) structure and function, and on HDL's role in reverse cholesterol transport. However, the physiological role for SAA still remains contradictory among the different proposals of the current literature.

Figure 1.1. Speculative illustration of pathogenesis of amyloid arthropathy.

According to Upragarin *et al.* (2005) and Abbas (2005), SAA can be degraded by intra- and extracellular pathways (Figure 1.1.). Through the intracellular pathway, SAA degradation products may act as a source of the amyloid-A fibril precursor (Upragarin *et al.*, 2005), that is to say, like an amyloid enhancing factor (AEF). According to Landman *et al.* (1998a), AEF is considered to be a material with high content of β -pleated sheets that acts as nest or template for the generation and growth of amyloid-A fibrils. Through the extracellular pathway, SAA is degraded by proteinases (Upragarin *et al.*, 2005) and a possible defect in the

cells of monocyte origin that degrade SAA could generate insoluble amyloid-A molecules rather than soluble amyloid-A molecules (Abbas, 2005).

According to Upragarin *et al.* (2005), a persistent acute phase response will provide an elevated precursor protein SAA necessary for SAA and amyloid-A fibril formation. However, an elevated production of SAA by itself is not sufficient for the deposition of amyloid since it is common to inflammatory states that do not necessarily lead to amyloid-A amyloidosis (Abbas, 2005). Furthermore, other factors are involved in the development of amyloid-A amyloidosis since the amyloid formation does not occur in all individuals in the same way (Upragarin *et al.*, 2005). Thus, factors such as the isoform or proteolytic cleavage site of SAA (amyloid fibrils are transformed from SAA prior to C-terminal proteolytic cleavage), the SAP and GAGs are thought to contribute to amyloid pathogenesis (Landman, 1999).

1.1.5. Pathology of amyloid arthropathy

The published literature of Landman *et al.* (1994 and 1998b), Peperkamp *et al.* (1997), Ovelgönne *et al.* (2001), Petersen *et al.* (2008) and Bautista *et al.* (2009) describes field cases of avian amyloidosis and agrees that the gross features of AA are characterised by deposits of orange-coloured amyloid materials in the joints (mainly in knee joints and with a higher incidence in the left knees) and a turbid orange-yellow amyloid material in the synovial fluid, as shown in [Figure 1.2](#).

Landman *et al.* (1994) observed the first signs of AA in field cases in brown layer breeds between 5 and 6 weeks of age. Affected chickens were smaller than healthy ones. The chickens had ruffled feathers and chirped like younger chickens, showing reduced weight gain, uni- and bilateral swelling of tibio-metatarsal and femorotibial joints, mainly on the left side. Petersen *et al.* (2008) observed erosion of the articular cartilage as well as in bone tissue

in commercial layers between 24 and 37 weeks of age. Bautista *et al.* (2009) reported that 5% of the commercial layers showed lameness and inflammation of the knee joints after 17 weeks of age, though no significant mortality was observed.

Figure 1.2. Inner view of a femoral-tibial joint of a spontaneous case of AA. (Image courtesy of Ibertec, 2012).

Additionally, AA is often accompanied by hepatomegaly and renomegaly, and sometimes by hydropericardium and ascites. The most affected organs are the liver, spleen and kidneys. At necropsy, deposits of amyloid can be seen in the liver, located in Disse's space, in the kidney, located in the interstitium of cortex and medulla, and in the spleen, located in the white pulp. The liver usually shows a pale brownish hue and the spleen appears pale (Bautista *et al.*, 2009). Amyloid deposits occasionally appear in the heart, and can be found in the digestive tract, in the proventriculus, and the small intestine, located in the duodenal or jejunal section, the pancreas, being the least affected organ (Peperkamp *et al.*, 1997). Rarely brain, lungs and skin are affected (Landman *et al.*, 1998a).

Regarding the reproductive system, Landman *et al.* (1999b) observed that four out of six 27-week-old brown layer breeds with arthritis, which were inoculated intravenously with

10^9 CFU of *E. faecalis*, had productive ovaries and remained in production (from 50 to 100%) although with oscillations. Landman *et al.* (2001) examined 18 brown layer breeds that displayed lameness. At necropsy, it was observed that ten of them suffered from AA, of which seven had active reproductive organs (70%) and only one bird stopped laying eggs (10%). This means a diminished number of eggs in affected birds and, therefore, an important financial loss for the poultry industry.

Field cases of AA in broiler parent stocks have also been reported by Landman *et al.* (1998b), Ovelgönne *et al.* (2001) and Steentjes *et al.* (2002), and the signs were very similar to those previously described in layers.

Peperkamp *et al.* (1997) observed that amyloid deposition is more severe in field amyloid cases than in experimentally infected birds. There are several reports about experimentally infected birds with *E. faecalis* and the many possible ways of infection. However, pathogenesis and clinical manifestations of induced avian amyloidosis follow a similar pattern to field cases of AA. Landman *et al.* (1999b) studied the induction of AA in embryonated eggs; neither the dipped embryonated eggs nor the inoculation of the air chamber with *E. faecalis* resulted in AA. However, yolk sac inoculation produced a high EMR within two days while egg albumen inoculation hardly produced any embryonic deaths. In one-day-old chick the intramuscular inoculation of 10^6 CFU of *E. faecalis* caused arthritis (60%) and articular amyloidosis (40%) whereas oral inoculation was ineffective (Landman, 1999). Additionally, according to Landman *et al.* (1997; 1998b; 1999a and 1999b), six-week-old brown layer chickens developed AA after intravenous, intra-articular and intraperitoneal inoculations of 10^8 or 10^9 CFU of *E. faecalis*. Landman *et al.* (1999a) found that intra-tracheal, oral or intramuscular inoculations were ineffective. Landman *et al.* (2003) reported in a later study that both intra-tracheal and intramuscular inoculations (in *musculus*

gastrocnemius and *musculus pectoralis*) induce lameness and arthritis. The joint lesions with the inoculations in the *m. gastrocnemius* were more severe than in the *m. pectoralis*. Besides, they reported that the exposure to aerosolized *E. faecalis* produced bacteraemia.

In general, inoculations with high doses of *E. faecalis* could develop AA and show the typical clinical and pathological lesions according to Landman (1999), and according to Ciftci & Diker (2009) *E. faecalis* has a predilection for joints of chickens regardless of the inoculation route. Nevertheless, it is to be noted that although AA is characterised by the deposition of amyloid, there are experimentally inoculated chicken cases where these depositions have not been observed. Ovelgönne *et al.* (2001) observed amyloid deposits in the joints of all infected brown layers at six weeks of age. However, at necropsy (i.e. at 13 weeks of age), the amyloid deposits were not observed in all infected white layers although their joints were bacteriologically positive. According to the study of Landman *et al.* (2003), the experimentally inoculated chickens at one day of age did not show any amyloid deposits at necropsy (at ten weeks of age). These authors reported that outbred brown layer chickens were used. These have an immune system with a genetically determined diversity (Hawken *et al.*, 1998) which might serve as a possible explanation for this fact. Consequently, the acute phase reaction necessary to amyloid deposition might not occur in the same way in all birds.

An extensive diagnosis is therefore necessary to confirm amyloid deposits and rule out similar extracellular deposits such as collagen and fibrin (Woldemeskel, 2012). Congo red stain is the most commonly used tool for the identification of amyloid (Picken, 2007). Green birefringence under polarised light is used with amyloid orange deposits (Snyder, 2007), as well as immunohistochemistry (antibody against apolipoprotein AI) and biochemical characterisation which helps to identify the specific constituents composing the amyloid deposits (Picken, 2007). Histopathologic identification of amyloid deposits is required to

assess the amyloid type (Woldemeskel, 2012). Serology and bacteriology of blood samples and/or of affected joints are complementary proofs to amyloid diagnosis which have been used in research conducted, for instance, by Landman *et al.* (2003) and Sevimli *et al.* (2005), to name a few.

1.1.5.1. Breed susceptibility

The incidence of AA has been reported in field cases in brown layer breeds (Peperkamp *et al.*, 1997 and Bautista *et al.*, 2009) and occasionally in broiler breeder flocks (Gregersen *et al.*, 2010). AA has never been reported in broiler chicks in field cases probably because they are slaughtered at an early age of seven weeks. Interestingly, AA has never been reported in field cases in white layer breeds and they are more resistant to induced AA than brown layers (Ovelgönne *et al.*, 2001). Therefore, differences in susceptibility to AA between brown and white layers have been investigated in many cases (Zekarias *et al.*, 2000 and Ovelgönne *et al.*, 2001). However, it still remains puzzling.

Zekarias *et al.* (2000) observed that the defence reaction that recognises an invading bacterium and produces antibodies specific against that antigen appears to be different between breeds, i.e. brown and white layers had a different immune reaction when they were infected with an amyloidogenic *E. faecalis* strain. Therefore, the greater susceptibility to the disease of brown layers as compared to white layers may be associated with the immunological response pattern.

Zekarias *et al.* (2000) detected more CD8⁺ T cells present in the synovial membrane and peripheral blood of affected joints from white layer breeds resistant to AA induced by *E. faecalis* than on the susceptible brown layer breeds. Likewise, chickens resistant to *Salmonella* infection showed more CD8⁺ T cell according to Berndt & Methner (2001).

However, chicken strains susceptible to coccidiosis showed more CD8⁺ T cells than resistant lines (Lillehoj, 1994). Therefore, the amount of T cells appears unrelated to the resistance to disease (Zekarias *et al.*, 2002).

Zekarias *et al.* (2000) observed that white layers showed a cell-mediated response characterised by T cell proliferation and macrophage activity whilst brown layers showed a high humoral antibody response due to the large number of plasma cells present in its arthritic synovial membrane. Besides, Sevimli *et al.* (2005) reported that the most affected brown layers by AA presented the highest numbers of macrophages and heterophil. Therefore, they concluded that the rate of macrophages and heterophil migration into the tissue may be associated with the severity of AA.

Additionally, Ovelgönne *et al.* (2001) reported that the differences in susceptibility to AA among individuals could have a genetic basis but it cannot be explained by the SAA genes. These authors reported that although there are minor differences located in the untranslated regions of both mRNAs, the amino acid sequences of SAA of both chicken breeds are identical with 4 exons and 3 introns. Therefore, they concluded that the promoters of the brown and white layers must house the functional differences between the SAA genes. It should be noted that only one SAA gene has been identified in the chicken genome while SAA is encoded by four different genes in humans and mice (Upragarin *et al.*, 2005).

Furthermore, Ovelgönne *et al.* (2001) reported that the fact that the brown layers might have higher blood SAA levels than white layers cannot explain the differences in susceptibility to AA. When the brown and white layers were inoculated intra-articularly with *E. faecalis*, the amyloid deposits were only observed in the injected joints, whereas the other joints remained free of amyloid. However, local production of SAA in the affected joints might play a role in amyloid depositions. Therefore, they suggested that the synoviocytes of

brown layers might proliferate more strongly than the synoviocytes of white layers and, therefore, produce larger amounts of extrahepatic SAA that may act as nidus of amyloid fibrils. Quantitative studies of the extrahepatic production of SAA are therefore required to investigate this possibility.

1.1.6. Approaches for a possible treatment

AA is an irreversible disease (Landman *et al.*, 1998a), probably due to the fact that amyloid fibrils have low solubility and a relative resistance to proteolytic digestion under physiological conditions. Therefore, the best option would be to treat the underlying inflammatory disease and prevent the occurrence of fibrillogenesis. Substances which facilitate or inhibit the amyloid depositions have been reported by Sevimli *et al.* (2005, 2008 and 2013). These authors observed that high doses of vitamin A increased the severity of AA induced by *E. faecalis* and Freund's adjuvant, while methylprednisolone and pentoxifylline inhibit amyloidosis. Additionally, Shtrasburg *et al.* (2012) observed the possible role of alkylating agents in the prevention of amyloidogenesis in mice where cyclophosphamide and chlorambucil were the most potent inhibitors.

Therefore, the treatment of AA has focused to date on the prevention of chronic inflammation. However, the recent study conducted by Kanata *et al.* (2015) concluded that chickens previously exposed to AA may improve with age or even the amyloid deposits may tend to regress when chronic infection disappears.

In regard to chronic infection induced by *E. faecalis*, measures to prevent *E. faecalis* colonisation could have a direct impact on animal welfare by reducing the burden of infection thereby lowering environmental stress and, therefore, to reduce the incidence rate of AA. It is therefore necessary to learn more about the different avian *E. faecalis* field strains. Until

today, the characterisation of *E. faecalis* strains has enabled us to relate its STs with the disease, as well as to identify the possible virulence genes involved. Worldwide, it is well known that the ST82 of *E. faecalis* is associated with AA in poultry. However, it is still not known whether ST82 as well as other STs which were found both in poultry and human, are normal inhabitants of the gastro-intestinal tract or represent a genuine zoonosis. Therefore, it would be interesting to conduct further studies on the different strain of *E. faecalis* affecting brown layer chickens with emphasis on the specific STs that are associated with AA.

In addition, *E. faecalis* can be transmitted among birds vertically and horizontally. Investigations to prevent and reduce enterococci contamination in birds and to figure out if the AA actually represents a zoonosis should be developed on a parallel basis. It should be taken into account that *E. faecalis* have a natural resistance towards antimicrobials and, therefore, the enterococcal infection treatments are increasingly limited. Consequently, efficient control measures against *E. faecalis* infection are required. Different general strategies could be applied in the fight against *E. faecalis* infection, such as the development of effective vaccines made from proven high pathogenic isolates or breeding for more resistant laying hens against *E. faecalis* infection.

1.1.7. Breeding for disease resistance

Estimations from FAO (2003) suggest that the world eggs' production will reach 89.9 million metric tons in 2030, at a growth rate of 1.6 percent per year from 2015 until 2030. It is therefore expected that the egg poultry industry progress steadily. However, infectious diseases are still responsible for high losses in the poultry industry due to mortality, veterinary costs, product contamination and subclinical symptoms. The major tools to combat avian infectious diseases are antibiotics and vaccines. Changes in regulations about the use of antibiotics in animal production and the fact that some vaccines are only partly effective

underline the need for other approaches to disease control (Warner *et al.*, 1987). Selection for disease resistance could be useful for this purpose.

The development of a disease in an animal occurs after the interaction between the environment and the genotype of the individual. The environment can be controlled under strong hygienic measures and biosecurity. However, the genetic control of the disease resistance is highly complex and is generally controlled by many genes. Estimates of heritability do not indicate which or how many genes are involved in the control of a particular trait (Warner *et al.*, 1987). However, the estimation of the heritability of resistance to a disease shows the additive genetic component and allows predicting the selection response. According to Arthur & Albers (2003), heritability of resistance to a disease is generally under 10%. Berthelot *et al.* (1998) estimated heritabilities for resistance to the cecal carrier state of *S. Enteritidis* from $h^2=0.08$ to $h^2=0.09$. Janss & Bolder (2000) reported heritability of $h^2=0.12$ for mortality to *S. Enteritidis*. Cavero *et al.* (2009) reported heritability for resistance to *E. coli* of $h^2=0.17$, and different studies estimated direct heritabilities for ascites syndrome up to $h^2=0.44$ (Ahmadpanah *et al.*, 2017).

Additionally, the estimation of the genetic correlation between disease resistance and other traits of economic significance is very important in order to carry out an indirect selection through a correlated response. Thus, antagonistic correlations might make simultaneous improvement of these both traits difficult. Unfortunately, information on genetic correlations among disease resistance and production traits is scarce. Han & Smyth (1972) reported that chickens selected for higher growth rate were more susceptible to develop of Marek's disease than chickens selected for slower growth rate. Gavora *et al.* (1974) observed that more resistant chickens against Marek's disease had lower body weight and their eggs were smaller than susceptible chickens. Chickens selected by Spencer *et al.* (1979) for high

egg production showed more resistance to lymphoid leukosis virus. More recently, Greef *et al.* (1998) and Moghadam *et al.* (2001) reported the genetic correlation between ascites syndrome and body weight, which is not clear. Greef *et al.* (1998) reported a negative correlation and, on the contrary, Moghadam *et al.* (2001) reported a positive correlation between these traits. Caverio *et al.* (2009) reported positive correlations between resistance to colibacillosis and important production traits. These authors observed that laying hens which are more resistant against *E. coli* infection have a better start of lay and lower feed intake while susceptible laying hens to colibacillosis showed better breaking strength and egg weight, although these differences between resistant and susceptible laying hens were not large. Therefore, they concluded that the improvement of the analysed traits will not be penalized if the selection for *E. coli* resistance is added into a commercial selection program.

Direct selection has made remarkable progress, but it still has some limitations (Jie & Liu, 2011). Breeding for disease resistance requires that the birds are exposed to pathogens, which cannot be applied to high number of hens because of animal welfare constraints in addition to the associated costs. Usually, mortality is the criterion for disease resistance. However, it can be an obstacle when the infection does not cause death and the infected birds do not present clinical symptoms. Besides, the possible interaction among pathogens should be considered because the economic importance of infectious diseases as a whole is much higher than that of one disease alone. Consequently, breeding for resistance is usually carried out whenever losses were increasing and other means of control seemed ineffective.

1.2. OBJECTIVES

The overall aims of this doctoral thesis were i) to characterize the virulence of different avian *E. faecalis* field isolates through the Embryo Lethality Assay (ELA) and ii) to investigate the possibility to select more resistant laying hens against *E. faecalis* infection through the ELA, which is proposed as an alternative model to replace the adult avian challenge assay.

To achieve the main objective, the following specific objectives are addressed:

- Develop a generic methodology to evaluate the pathogenicity of avian *E. faecalis* strains through the ELA in chicken embryos.
- Assess the median lethal dose (LD₅₀) and the virulence of a well-known pathogenic avian *E. faecalis* strain (K923/96), which can be used as reference strain in further analyses.
- Corroborate the capacity of the ELA for distinguishing *E. faecalis* strains in chicken embryos.
- Characterize the genetic diversity of different avian *E. faecalis* strains by ERIC-PCR under the hypothesis that strains with similar DNA banding patterns may be similar also in its infective ability.
- Evaluate the ELA in chicken embryos of brown layers under the hypothesis that these embryos are more susceptible compared with embryos of white layers when they are infected with the same *E. faecalis* strain and infectious dose.
- Estimate the heritability for embryonic survivability to *E. faecalis* infection and its relationship with traits of economic interest.

CHAPTER 2.

GENERIC METHODOLOGY TO EVALUATE THE PATHOGENICITY OF AVIAN *ENTEROCOCCUS FAECALIS* STRAINS (EXPERIMENT 1).

2.1. INTRODUCTION

E. faecalis is gaining more attention, not only because it has been associated with several poultry diseases (Gregersen *et al.*, 2010), but also because of its ability to acquire antimicrobial resistance and its capacity to transfer pathogenicity islands, which encode virulence genes (Coburn *et al.*, 2007). Consequently, *E. faecalis* could acquire the ability to survive in an environment in which antimicrobial agents are heavily used and, therefore, the enterococcal infection treatments are increasingly limited (Hayes *et al.*, 2003). Hence, a better understanding of the virulence mechanisms of the *E. faecalis* strains is needed to develop preventive measures.

In order to study the role of *E. faecalis* in the pathogenesis of AA, adult avian challenge assay is the most used animal model. AA has been experimentally induced with *E. faecalis* in one-day-old chickens (Landman, 1999) and in six-week-old brown layer chickens (Landman *et al.*, 1997; 1998b; 1999a and 1999). However, relatively high costs, complexity and ethical considerations limit the application of such challenge tests. Therefore, it seems appropriate to use the chicken ELA, which can provide an alternative tool to study the

virulence of different strains in a faster, more sensitive, less expensive, more specific and relatively simple form (Wooley *et al.*, 2000; Gibbs *et al.*, 2003; Gibbs & Wooley, 2003; Oh *et al.*, 2012 and Seo *et al.*, 2013).

Chicken ELA has been proposed to determine the degree of virulence of isolated fungi (Jacobsen *et al.*, 2010), and bacteria such as *E. coli* (Wooley *et al.*, 2000; Gibbs *et al.*, 2003; Gibbs & Wooley, 2003; Montgomery *et al.*, 2005 and Oh *et al.*, 2012), *Francisella spp.* (Nix *et al.*, 2006), *Yersinia enterocolitica* (Townsend *et al.*, 2008), *Campylobacter jejuni* (Stewart-Tull *et al.*, 2009), *S. aureus* (Polakowska *et al.*, 2012), *Riemerella anatipestifer* (Seo *et al.*, 2013) and *Listeria (L.) monocytogenes* (Andersson *et al.*, 2015). In the case of *Enterococcus spp.*, Borst *et al.* (2014) reported that the ELA is able to differentiate between virulent and avirulent *Enterococcus (E.) cecorum* strains, whose genotype has been closely related to each other through PFGE. Rudolph (2004) reported that different *E. faecalis* strains produced different degrees of mortality when the embryos were infected in the allantoic cavity (AC) with a single infectious dose (2500 CFU/ml). Furthermore, they were able to compare and confirmed the results obtained by the ELA with the results obtained by the adult avian challenge assay in a following experiment, where six-week-old chicks were infected with an infectious dose of 10^{10} CFU of two *E. faecalis* strains tested previously in embryos.

According to the revised literature cited above, it seems necessary to develop a methodology in accordance with the isolate types and the purpose of the inoculation, taking into account that the embryo mortality rate (EMR) produced during an ELA is influenced by several factors such as:

- The source of fertilized eggs and good embryo quality, which are necessary to ensure that the inoculation is the only cause of mortality (Wooley *et al.*, 2000; Gibbs *et al.*, 2003 and Gibbs & Wooley, 2003).

- The age of the embryos at the inoculation since strains' virulence appears to differ based on embryonic development (Seo *et al.*, 2013).
- The route of infection, which depends largely on the isolate type and the purpose of the inoculation (Wooley *et al.*, 2000).
- The infectious dose, which appears to be related to the embryo survival time and rate (Nix *et al.*, 2006).

Therefore, the main goal of this research was the evaluation of the ELA as a diagnostic model that could be used to establish the pathogenicity of *E. faecalis* strains cultured from field samples of AA. Of particular interest was to assess the median lethal dose (LD₅₀), which is defined as the dose required to kill half of the embryos at a defined time for a tested population, and the virulence of a well-known pathogenic avian *E. faecalis* strain (K923/96), which has been previously related with avian amyloidosis (Rudolph, 2004 and Petersen *et al.*, 2009) and which will be used as the reference strain in further analyses.

2.2. MATERIAL AND METHODS

2.2.1. Sample

A total of 3443 eggs of White Leghorn Layers at an age of 47 - 51 weeks were used for the different ELAs (see [Table 2.1.](#) for further details). The tested flocks were free from diseases like *Salmonella* and *Mycoplasma*. Only first quality hatching eggs were used, which means soiled eggs as well as eggs with hairline cracks were sorted out. Eggs were stored at 15 °C for a maximum of four days prior to incubation and incubated in an auto-rotating egg incubator at 37.8 °C and 52 - 56% relative humidity.

Table 2.1. Characteristics of the ELAs I-IV carried out in order to evaluate the *E. faecalis* strain K923/96.

ELA	I	II	III	IV	Viable count (CFU/ml) ¹
No. embryos/control ²	60	57	108	148	-
No. embryos/dose	100	290	300	300	-
Infectious dose (CFU/ml) ³	2500	-	-	-	> 1000
	250	-	-	-	> 250
	25	25	25	25	85 - 258
	-	5	5	5	15 - 60
	2.5	2.5	2.5	2.5	10 - 35

¹ Verification of the number of CFU/ml contained in each used infectious dose.

² Embryos of the control group were inoculated in the AC with 0.2 ml of sterile PBS.

³ By an inoculation volume of 0.2 ml/embryo, 500, 50, 5, 1 and 0.5 CFU/embryo were inoculated respectively.

2.2.2. Experimental design

The overall scope of this research included four ELAs (I to IV) that are illustrated in [Table 2.1](#). White fertilised eggs of a commercial layer programme of Lohmann Tierzucht GmbH were inoculated via the AC after 10 days of incubation. A single known and well-characterized pathogenic avian *E. faecalis* strain K923/96 (Rudolph, 2004 and Petersen *et al.*, 2009) was used in all these ELAs. Different doses of the *E. faecalis* strain mentioned above were used in the present research in order to determine the LD₅₀. As shown in [Table 2.2.](#), the doses used in the ELA I were reduced in the following ELAs due to the high mortality recorded with the higher doses. In addition to the infected embryos, there was always one control group in each ELA, which was inoculated in the AC with 0.2 ml of sterile phosphate-buffered saline (PBS).

2.2.3. Inoculum

The *E. faecalis* strain K923/96 isolated from the amyloidogenic joint of a brown layer, which was already used by Rudolph (2004) and Petersen *et al.* (2009), was used in all ELAs. The preparation of the inoculum was applied in accordance with the following protocol (Rudolph, 2004).

The bacteria, which were preserved in a CRYOBANK™ tube, were recovered by removing the cryobank tube from the freezer. A sterile forceps was used to roll one bead on a Columbia sheep blood agar plate. The agar plate was incubated aerobically for 24 h at 37 °C. After incubation, colonies were scraped off and a suspension of 10 ml was prepared in PBS. This suspension was set to the photometer, of a wavelength at 650 nm. To obtain a suspension around 5×10^7 CFU/ml (culture I), it was adjusted with an absorbance to approximately 0.164 ± 0.01 . To adjust the suspension, 100 µl of the culture I were given into 10 ml of PBS (culture II). For the culture III another 50 µl of the culture II were given into 10 ml of PBS (dilution 1:1 with an infectious dose of 2500 CFU/ml). From this dose, different dilutions were obtained: 1/10, 1/100, 1/500 and 1/1000 with infectious doses of 250, 25, 5 and 2.5 CFU/ml, respectively (Table 2.1.). By an inoculation volume of 0.2 ml/embryo, 500, 50, 5, 1 and 0.5 CFU/embryo were inoculated respectively. However, Rudolph (2004) reported that the number of counted colonies in the experiments they did may vary between 500 and 5000 CFU/ml. Therefore, in order to guarantee that the infectious dose was correctly prepared, the number of CFUs contained in each dose was verified by viable count on a Columbia sheep blood agar plate. For this purpose, 0.1 ml of inoculum per dose was incubated aerobically. Viable counts were determined two times per dose. After 24 h at 37°C the exact number of grown bacteria was counted with a traditional click-counter. The real number of CFUs contained in each infectious dose is shown in Table 2.1.

2.2.4. Embryo lethality assay

According to the previously described methods for AC inoculation of chicken embryos, the ELAs were assayed as follows.

Prior to inoculation, the unfertilized eggs and eggs with dead embryos were removed by candling. Hatching eggs, which had been incubated for 10 days and were in good condition, were inoculated after disinfection of the egg surface with Bacillol AF (Bode Chemie GmbH). Each egg was opened carefully on the blunt end of the egg with a punch and inoculated with a disposable and sterile syringe in the AC either with 0.2 ml of bacterial suspension or with 0.2 ml of PBS in the case of the control group. All holes were sealed with wood glue and the embryos returned to incubation at 37.8°C for another seven days. The incubated eggs were candled every 24 h for seven days and embryonic mortalities were daily recorded per group. The number of dead embryos was used to classify the pathogenicity of *E. faecalis* according to the infectious dose.

At the end of each ELA, at 17 days of incubation, all remained embryos were sacrificed by hypothermia. They were stored for 48 h at 4°C.

2.2.5. Macroscopic lesions and re-isolation

Random selections of the embryos dead by the infection, as well as some surviving embryos at the end of the ELAs, were used for further examinations. After disinfection of the egg surface with Bacillol AF, the eggshell was opened with sterile material. The embryo was carefully separated from its surrounding membranes in order to evaluate macroscopic tissue changes in embryos caused by *E. faecalis*. In addition, smear samples from the allantoic fluid of some dead and surviving embryos were cultivated in a blood plate for 24 h at 37°C in order to re-isolate the *E. faecalis* strain.

2.2.6. Statistical analysis

Statistical analyses of mortality data were carried out by applying a linear logistic model for repeated measurements with a binary response variable, which was modelled as a binomial random variable (y_i). The dependent variable (y_i) can take the value 1 with a probability for embryonic mortality π_i or the value 0 with a probability to survive of $1 - \pi_i$. The logistic model uses a link function $g(\mu_i)$, linking the expected value to the linear predictors η_i . The logit link function is defined by $\log [\pi_i / (1 - \pi_i)] = \eta_i$, where π_i is the probability of mortality until the end of the research at 17 days of incubation. The data were then analysed with the GLIMMIX procedure (SAS Institute Inc., 2011) using the following generalized linear model (Littell *et al.*, 1999):

$$\text{Logit}(\pi_{ij}) = \eta_{ij} = \log [\pi_{ij} / (1 - \pi_{ij})] = \mu + \alpha_i + \beta_j,$$

where η_{ij} denotes the linear predictor, π_{ij} is a binary outcome (i.e., probability of embryonic mortality), μ is the overall mean effect, α_i is the fixed effect of the infectious dose (from 2.5 to 2500 CFU/ml) and β_j is the random effect of repeated measurement of ELA. Least squares means (LSMEANS) were estimated on the logit scale and then back-transformed using the inverse link function $\pi = \exp(x\beta) / [1 + \exp(x\beta)]$ to the original scale (probability) applying the LSMEANS statement. Significant differences between LSMEANS were tested using a *t*-test procedure by inclusion of the DIFF option in the LSMEANS statement. Differences were considered significant when $P < 0.05$.

Different statistical models using different probability distributions, normal, logistic and extreme value distribution, were tested in order to compare its fit statistics for the infectious dose. Underlying normal distribution using the coefficient of determination was found to be the best fit and therefore was applied to estimate the LD₅₀.

The LD₅₀ was estimated by the SAS PROBIT procedure (SAS Institute Inc., 2011) using the maximum likelihood method of regression parameters. Since the response Y is binary, the probit equation is:

$$p = \Pr(Y = 1) = C + (1 - C) F(x' \beta)$$

where p is the probability of a response, C is the natural response rate, F is the cumulative distribution function, x' is the vector of explanatory variables and β is the vector of parameter estimates. The response rate was calculated using the INVERSECL statement option by applying the following formula:

$$x' = 1/b_1 (F^{-1}(p) - x^* b^*)$$

The course of embryonic survivability over the seven post-inoculation (p.i.) days within each treatment group was illustrated according to the Kaplan-Meier method (survival analysis) by applying the LIFETEST procedure of SAS System 9.3 (SAS Institute Inc., 2011) and using the following model:

$$\hat{S}(t) = \prod_{j: t_j \leq t} \left[1 - \frac{d_j}{n_j} \right]; \text{ for } t_1 \leq t \leq t_k$$

where $\hat{S}(t)$ is the survivor function and t is the lifetime of a randomly selected experimental unit. For each j: $t_j \geq t$, let $t_1 < t_2 < \dots < t_k$ represent the different event times. n_j is the number of individuals at risk just prior to t_j , and d_j is the number of individuals that die at time t_j .

2.3. RESULTS

2.3.1. Embryo lethality assay

The ELAs showed a different EMR according to the infectious dose. The infectious dose showed a significant effect ($P < 0.0001$) on the EMR. As shown in [Table 2.2.](#), the infectious doses of 250 CFU/ml and 2500 CFU/ml used in the ELA I resulted in a high EMR of more than 90% lethality after seven days p.i. and did not differ significantly from each other. Therefore, neither of these doses were used in the following ELAs in order to avoid a massive embryonic mortality that would not allow the differentiation between the *E. faecalis* strains. The dose of 25 CFU/ml produced an average EMR of 63%, which was significantly different than all other used doses. The infectious dose of 5 CFU/ml was added into ELAs II, III and IV ([Table 2.1.](#) and [2.2.](#)) in order to estimate the lethal effect of the low infectious dose and calculate the proper LD₅₀. The average EMR estimated for the doses of 5 CFU/ml and 2.5 CFU/ml was 45% and 43%, respectively, and did not differ significantly from each other. Moreover, in ELA III a higher EMR was reported with the infectious dose of 2.5 CFU /ml (64%) as compared to the infectious dose of 5 CFU/ml (51%).

The average EMR of the control group was 2.5%, with variation from 0% to 7%. Thus, these low mortalities in the control group confirm the appropriate environment in the incubator during the research, as well as the low negative impact of the injection. Therefore, the mortalities obtained in the different groups can be unequivocally associated to the effect of the infectious doses. The control group differed significantly from all other infectious doses.

Table 2.2. Percentage of chicken embryo mortality with increasing infectious dose of the *E. faecalis* strain K923/96 following inoculation of 0.2 ml into the AC.

Infectious dose (CFU/ml)	ELA ¹				LS-means ²
	I ₍₄₆₀₎	II ₍₉₂₇₎	III ₍₁₀₀₈₎	IV ₍₁₀₄₈₎	
2500	92	-	-	-	93 ^a
250	89	-	-	-	90 ^a
25	71	48	83	56	63 ^b
5	-	50	51	38	45 ^c
2.5	25	45	64	30	43 ^c
Control ³	7	0	4	1	2.5 ^d

¹ Overall number of infected embryos in each ELA is indicated in brackets.

² The effects were estimated using a generalized linear model with binomial error distribution and logit link function. Different letters indicate statistically significant differences between groups.

³ Embryos of the control group were inoculated in the AC with 0.2 ml of sterile PBS.

2.3.2. Median lethal dose

Figure 2.1. shows probit analysis on infectious dose of *E. faecalis* strain K923/96. The results of the dose level effect on the embryonic mortality (Figure 2.1., left) indicate a mean tolerance for the embryos inoculated with the *E. faecalis* strain K923/96 of 6.6 CFU/ml, i.e., the dose corresponding to an embryonic mortality probability of 0.5 (LD₅₀) was 6.6 CFU/ml, with a 95% confidence interval of 3.5 and 11.4 CFU/ml. It can be observed that infectious doses of this strain above 100 CFU/ml (with a 95% confidence interval of 45 and 500 CFU/ml) can produce 75% or greater embryonic mortality.

Figure 2.1. on the right shows the relationship between infectious doses, observed embryonic mortality values, and estimated probability values of the *E. faecalis* strain K923/96. Embryonic mortality produced with the infectious doses of 2.5 and 250 CFU/ml was higher than the estimated probability. However, the embryonic mortality observed with the infectious doses of 5 and 2500 CFU/ml was lower than the estimated probability. The observed and estimated embryonic mortality with the infectious dose of 25 CFU/ml was the same.

Figure 2.1. Probit analysis on infectious dose of *E. faecalis* strain K923/96. On the left, the dose level effect on the embryonic mortality and, on the right, the distribution dependent on the dose.

2.3.3. Embryo survival

The survival curve for embryos infected with the *E. faecalis* strain K923/96 is presented in Figure 2.2. Higher infectious doses of this strain were significantly more virulent than the lower doses ($P < 0.0001$). The highest embryonic mortality occurred three and four days p.i. for all doses. As expected, the higher the infectious dose, the lower the embryonic survival time, i.e., the embryos infected with the infectious doses of 250 and 2500 CFU/ml had less survival probability at day 3 p.i. than the embryos infected with doses of 25, 5 and 2.5 CFU/ml. As shown in Figure 2.2., after four day p.i. the embryonic mortality declined substantially. Around 98% of the embryos in the control group survived until the end of the study duration.

Figure 2.2. Survival curves of 10-day-old chicken embryos inoculated into the AC with different infectious doses of the *E. faecalis* strain K923/96.

2.3.4. Macroscopic lesions

No lesions were observed in the embryos of the control group. However, the embryos infected with *E. faecalis* succumbed to bacterial infection. Malformed and underdeveloped embryos were observed in all treated groups. Bodies of the analysed infected embryos showed profound subcutaneous oedema, cranial and skin haemorrhages and loss of the plumage regardless of the infectious dose, as shown in [Figure 2.3](#). Therefore, a clear relationship between the embryo lesions and the respective infectious dose was not observed. It should be noted that some dead and surviving infected embryos at the end of the ELAs did not show any visible signs.

Figure 2.3. Macroscopic lesions of white laying hen embryos infected with different infectious doses of the *E. faecalis* strain K923/96.

2.3.5. Re-isolation.

Smear samples from the allantoic fluid of random analysed dead embryos as well as from surviving embryos were taken in order to re-isolate the *E. faecalis* strain. The bacterial counts of the infected embryos were always positive and their allantoic fluid contained more CFU/ml compared to the infectious doses administered to the embryos (> 1000 CFU/ml), although some of them did not show any signs and any abnormalities. In the case of the control group, the bacterial count was negative for all analysis.

2.4. DISCUSSION

Based on the revised literature (Landman *et al.*, 1999b; Wooley *et al.*, 2000; Gibbs *et al.*, 2003; Gibbs & Wooley, 2003; Rudolph, 2004; Montgomery *et al.*, 2005; Townsend *et al.*, 2008; Stewart-Tull *et al.*, 2009; Jacobsen *et al.*, 2010; Oh *et al.*, 2012; Polakowska *et al.*, 2012; Seo *et al.*, 2013 and Borst *et al.*, 2014) an ELA was carried out as a diagnostic model to establish the pathogenicity of a single *E. faecalis* strain.

The results on the use of specific pathogen-free (SPF) embryos in the ELA obtained by Borst *et al.* (2014) with *E. cecorum* showed that the SPF embryos are as susceptible as normal embryos, suggesting that SPF embryos have no innate resistance to bacterial infection. Therefore, in the present research no SPF embryos were used. Additionally, only embryos of the best quality were used in order to avoid biased results, i.e., to ensure that the inoculation is the only cause of mortality (Wooley *et al.*, 2000).

According to Jacobsen *et al.* (2010) and Seo *et al.* (2013), the embryo age at inoculation has an influence on the embryonic lethality rate. The younger the embryos (seven days old), the more susceptible they were to the virulent strains compared to embryos at later stage of embryonic development (13 and 15 days old) when they are inoculated with *R. anatipestifer* (Seo *et al.*, 2013) and *Aspergillus fumigatus* (Jacobsen *et al.*, 2010). However, the greatest differences in EMR between the virulent and avirulent *R. anatipestifer* strains were most evident in 10-day-old chicken embryos (Seo *et al.*, 2013). Therefore, for the present research 10-day-old embryos were used.

The number of inoculated embryos per strain varies among the revised literature. Nix *et al.* (2006), Townsend *et al.* (2008) and Stewart-Tull *et al.* (2009) used less than ten embryos per strain. Wooley *et al.* (2000), Gibbs *et al.* (2003), Montgomery *et al.* (2005),

Rudolph (2009), Jacobsen *et al.* (2010), Oh *et al.* (2012), Polakowska *et al.* (2012), Seo *et al.* (2013), Borst *et al.* (2014) and Jung *et al.* (2017) inoculated from 10 to 20 embryos per strain. Specifically, Wooley *et al.* (2000) infected 100 embryos (20 embryos per strain and ELA with five repetitions) and they estimated that at least 11 embryos are necessary to obtain significant differences among the strains of *E. coli*. However, Gibbs & Wooley (2003) observed that the overall ELA results seem to be more highly correlated with the results of the adult avian challenge assay when more embryos per ELA are used (100 vs. 44 embryos per strain). Therefore, the largest possible number of embryos was used in the present research, although a minimum of 11 embryos per strain is sufficient (Wooley *et al.*, 2000).

The AC route was chosen as the method of inoculation because other administration routes like yolk sac route and on the egg albumen with *E. faecalis* (Landman *et al.*, 1999b), as well as the infection on the chorioallantoic membrane with *C. jejuni* (Stewart-Tull *et al.*, 2009) produced a massive embryonic mortality, which would not allow a clear differentiation between the *E. faecalis* strains or the estimate of the LD₅₀. Landman *et al.* (1999b) studied the induction of AA and its possible routes of infection through chicken embryo infections with *E. faecalis*. These authors observed that the dipped fertilised eggs and the inoculation of the air chamber with *E. faecalis* were ineffective, the inoculation of egg albumen hardly produced any embryonic deaths; on the other hand, yolk sac inoculation resulted in a massive mortality within two days. Additionally, according to the results of Wooley *et al.* (2000) and Seo *et al.* (2013), the differentiation among avirulent and virulent strains of avian *E. coli* and *R. anatipestifer* respectively is possible and more effective through the AC route than into the yolk sac or on the chorioallantoic membrane respectively.

According to Landman (1999) and Ciftci & Diker (2009), regardless of the route of administration, the infectious dose of *E. faecalis* in an adult avian challenge assay also plays

an important role in the development of avian amyloidosis. Landman (1999) reported that the pathological and clinical lesions of AA can be reproduced through inoculation with high doses of *E. faecalis*. Ciftci & Diker (2009) observed that arthritis formation in chickens inoculated with *E. faecalis* depend on the infectious dose independent of the inoculation route. Besides, Nix *et al.* (2006) reported that inoculated embryos with higher infectious doses of *Francisella (F.) tularensis* strains (around 2.3×10^4 CFU) showed lower survival time and rate than inoculated embryos with lower infectious doses (around 1.3×10^1 CFU). Hence, different infectious doses of the *E. faecalis* strain K923/96 were used in the present research in order to estimate the LD₅₀ of this strain in chicken embryos.

As shown in [Table 2.2.](#), the results obtained showed a clear relationship between infectious dose and EMR in accordance with the results obtained by Seo *et al.* (2013) and Borst *et al.* (2014), who observed higher EMR with the higher infectious doses than the lower infectious doses. The EMR produced with the infectious dose of 2500 CFU/ml used in the present research (93%) is consistent with the values reported by Rudolph (2004), who observed an EMR between 90% and 100% with the same strain and infectious dose. This indicates that the ELA results of this research can be considered reliable.

The estimated doses at LD₅₀ for the inoculation of chicken embryos with the *E. faecalis* strain K923/96 was 6.6 CFU/ml ([Figure 2.1.](#)). This result is the first to report the appropriate infectious dose of an *E. faecalis* strain to inoculate these chicken embryos lines. Borst *et al.* (2014) reported that the lowest dose of *E. cecorum* strains able to produce around 50% embryonic mortality was 10^3 CFU/ml, and Nix *et al.* (2006) observed that infectious doses of *F. tularensis* from 30 to 200 CFU produced 100% embryonic mortality. This observation suggests the importance to determine an appropriate infectious dose according to the type of isolate and probably the strain.

In addition, Nix *et al.* (2006) reported that the embryo survival time is usually correlated with the infectious dose; the lower the infectious dose, the greater the survival time of the embryos, which was confirmed in this research ([Figure 2.2.](#)).

In accordance with the results obtained by Wooley *et al.* (2000) and Gibbs *et al.* (2003), the results obtained showed a fast decrease in survival rate from two to four days p.i., whereas the survival rate decreased more slowly afterwards, as shown in [Figure 2.2](#). The lowest embryonic survival probability was observed at three and four days p.i. in all doses, which confirms the results of Wooley *et al.* (2000), Gibbs *et al.* (2003), Gibbs & Wooley (2003), Stewart-Tull *et al.* (2009), Seo *et al.* (2013) and Borst *et al.* (2014) with other bacterial strains.

Rudolph (2004) confirmed the ELA results with the chicken infection challenge model. Both models have the ability to distinguish among the virulence of different strains, although there are more deaths in the ELA model compared to the chicken challenge study. This author reported that 2500 CFU/ml of the *E. faecalis* strain K923/96 (the same strain used in the present research) produced between 90% and 100% embryonic mortality in the ELA while no deaths in the chicken model (10^{10} CFU/chicken) were reported, although 66% of the chickens showed a growth decline and swelling of the joints. These mortality differences may be due to the fact that the embryos succumb to serious bacterial and in part do not withstand the infection while the chickens are able to survive (Gibbs *et al.*, 2003; Gibbs & Wooley, 2003 and Borst *et al.*, 2014).

The embryos in this research also succumbed to *E. faecalis* infection. They suffered sepsis, cutaneous haemorrhages and subcutaneous oedema like the infected embryos in the study of the authors mentioned above. Besides, Wooley *et al.* (2000) observed that avirulent *E. coli* strains produced fewer lesions than virulent strains, which could not be confirmed in

this experiment due to the use of only one single strain. All dead infected embryos in the present research showed the same signs regardless of the infectious dose and, therefore, no relationship between the embryo lesions and the infectious doses was observed.

No lesions and no re-isolates were observed in the embryos of the control group, thus indicating that the research was reliable and the embryonic mortality was due to the infection and not due to external effects. On the contrary, the re-isolation of *E. faecalis* in all analysed infected embryos was always positive, although some of these embryos did not show any visible signs. Besides, an increase in the viable count of the allantoic fluid of dead embryos was observed compared to the initial number of CFU/ml contained in each infectious dose, confirming the results obtained by Wooley *et al.* (2000), Rudolph (2004), Townsend *et al.* (2008) and Stewart-Tull *et al.* (2009). Even an increase in the viable count was observed in surviving embryos, which is in accordance with the results obtained by Rudolph (2004) and Townsend *et al.* (2008). Therefore, the differences in bacterial growth after the inoculation cannot explain the differences in EMR because the number of CFU can fluctuate and no produce clear differences in the lethality, which is consistent with the results of Wooley *et al.* (2000) with *E. coli*.

It should be taken into account that the EMR produced in the ELA depends on the dose. It is therefore important to determine an appropriate infectious dose which should not be too high to avoid a massive embryonic mortality that would not allow discrimination between the *E. faecalis* strains. In this regard, the LD₅₀ can be considered appropriate in order to establish an infection limit of 50% lethality. In conclusion, the methodology used in the present research, as well as the obtained LD₅₀ of the *E. faecalis* strain K923/96, can be used as reference in further ELAs on *E. faecalis* virulence.

CHAPTER 3.

VIRULENCE CHARACTERIZATION OF AVIAN

***ENTEROCOCCUS FAECALIS* FIELD ISOLATES BY CHICKEN**

EMBRYO LETHALITY ASSAY AND ERIC-PCR

(EXPERIMENT 2).

3.1. INTRODUCTION

Treatment of AA has focused to date on the prevention and treatment of chronic inflammation produced by *E. faecalis* strains. However, the enterococcal infection treatments are increasingly limited. This, on the one hand, is due to the restrictions on antimicrobial use in animal production emerged since the global health crisis of antimicrobial resistance (World Health Organization, 2012). On the other hand, it is due to the zoonotic potential of the *E. faecalis* strains, its ability to acquire antimicrobial resistance and its capacity to acquire virulence genes by transfer of pathogenicity islands (Coburn *et al.*, 2007). Additionally, little is known about the risk from field avian *E. faecalis* strains and it is currently difficult to estimate its virulence degree. Therefore, a thorough understanding of the risk from field *E. faecalis* strains is needed in order to develop effective control strategies against bacterial infections to reduce the incidence rate of AA and, consequently, to guarantee the welfare of the birds and improve their economic performance.

Therefore, the main goal of this research was to characterize different avian *E. faecalis* field strains through the ELA taking the *E. faecalis* strain K923/96 and the infectious dose of 5 CFU/ml reported in the previous Experiment 1 (Chapter 2 of the present dissertation) as reference, and thus corroborates the ELA capacity for distinguishing avian *E. faecalis* strains. Of particular interest was to characterize the genetic diversity of these strains by ERIC-PCR under the hypothesis that strains with similar DNA banding patterns may be similar also in its infective ability.

3.2. MATERIAL AND METHODS

3.2.1. Bacterial strains

A total of 69 avian strains of *Enterococcus* spp. (1 *Enterococcus* (*E.*) *hirae* and 68 *E. faecalis* strains) were characterized in the present research through 10 ELAs (I to X). As shown in [Figure 3.2.](#), most of the known *E. faecalis* strains were obtained from clinical cases of AA at layers farms in Germany (n=31), France (n=2) and Switzerland (n=1). Only three *E. faecalis* strains (282-4, 513-5 and 515-3) were isolated from apparently healthy birds. Unfortunately, the information on the isolation from 34 strains is unknown. All strains were obtained during 2011 and 2012, except the *E. faecalis* strain K923/96, which was isolated in 1996 and it has been well-characterized and used in different studies by Rudolph (2004) and Petersen *et al.* (2009), and the strains 1082-01 and 607-01 isolated in 2004 and characterized by Petersen *et al.* (2009). Additionally, 49 of the 67 *E. faecalis* used in the present study were tested before by the laboratory of Lohmann Tierzucht GmbH (Germany) as ST82 by using the Real Time (RT-) PCR method described by Petersen *et al.* (2010). Besides, the genes *ace*,

efaA, *cyl*, *asaI* (or *agg*) and *gelE* were present in all these strains although the genes *esp* and *hyl* were not determined.

3.2.2. ERIC-PCR and phylogenetic analysis

All *Enterococcus* spp., except the *E. faecalis* strain 2323-96, were used to develop the ERIC-PCR method described by Jurkovič *et al.* (2007). The bacteria, which were preserved in a CRYOBANK™ tube, were recovered removing the cryobank tube from the freezer. Bacteria were cultured on a Columbia sheep blood agar plate. The agar plate was incubated aerobically for 24 h at 37 °C. After incubation, colonies were scraped off and a suspension of 400 µl was prepared in PBS. DNA was purified using the NucleoSpin Tissue Kit (MachereyNagel, Düren). Afterwards 3 µl of the suspension was used for PCR amplification. Isolates were genotyped by ERIC-PCR. Reactions were carried out in a total volume of 50 µl containing 18 µl of RNase free water, 3 µl of MgCl₂, 25 µl of dNTPs mix (10 mM of each), 1 µl of ERIC1-R primer (5'-ATGTAAGCTCCTGGGGATTAC-3') and 3 µl of template DNA. The ERIC-PCR was carried out with only one single ERIC 1 primer, which uses the total DNA and, therefore, provides results with good reproducibility. Besides, its discriminatory power is higher than when using both ERIC 1 and ERIC 2 primers (Jurkovič *et al.*, 2007). Amplifications were performed in an Eppendorf thermocycler with a cycling program consisting of an initial denaturing step at 94 °C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 48 °C for 1 min and elongation at 72 °C for 2 min and a final extension of 72 °C for 7 min.

ERIC-PCR results show banding patterns, which can easily be visualized by agarose gel electrophoresis. The genetic diversity of the chromosomal DNA may be explained by differences in the banding patterns among the strains (Odinot *et al.*, 1995). Therefore, all products were analysed by electrophoresis through 1.5% agarose gels at 70 V for 70 min in a

1x TAE buffer (40mM Tris-acetate, pH 8.3) and revelled in ethidium bromide (10 mg/ml). The gels were visualized and photographed under UV trans illuminator.

The phylogenetic analysis was performed based on the banding pattern of the different strains of *Enterococcus spp.* by using the Phylogenetic Inference Package, PHYLIP-3.68 (Felsenstein, 2005), after ERIC-PCR. The data were calculated by using parsimony. The tree was visualized using the program Archaeopteryx 0.9901 beta (Han & Zmasek, 2009).

3.2.3. Embryo lethality assay

The ELAs were performed on a sample of 9987 eggs of White Leghorn Layers at an age of 29 - 62 weeks. The tested flocks were free from diseases such as *Mycoplasma* or *Salmonella*. Only first-quality hatching eggs were used, i.e. soiled eggs as well as eggs with hairline cracks were sorted out. Fertilized eggs were stored at 15°C for a maximum of four days prior to incubation and incubated at 37.8 °C and humidity from 52% to 56% with an automatic turning.

Each individual ELA was composed of a subset of eight strains, with the exception of the ELA IX, in which were used seven strains. The known and well-characterized pathogenic avian *E. faecalis* strain K923/96 (Rudolph, 2004 and Petersen *et al.*, 2009) was included in all ELAs as the positive control (reference strain). Fertilized eggs were inoculated via the AC after 10 days of incubation. In addition to the infected embryos, there was always one negative control group in each ELA, which was inoculated in the AC with 0.2 ml of sterile PBS. In each ELA, 100 eggs per strain and control groups were used, with the exception of two negative control groups (ELA I and II), in which were used 177 and 110 eggs, respectively.

The inoculum was prepared as described in the Experiment 1 (Chapter 2, section '2.2.3. Inoculum' of the present dissertation) up to an infectious dose of 5 CFU/ml, which corresponds approximately with the LD₅₀ of the reference strain K923/96. By an inoculation volume of 0.2 ml/egg, the number of bacteria used for inoculation was one CFU/egg. The number of CFU contained in each dose was verified by viable count on a Columbia sheep blood agar plate in order to guarantee that the infectious dose was correctly prepared, as shown in [Figure 3.1](#). The number of bacteria inoculated into the embryos ranged from 15 to 120 CFU/ml (i.e. from 3 to 24 CFU/egg), depending on the strain. In the case of the *E. hirae* strain, the embryos were inoculated with 275 CFU/ml (55 CFU/egg). The real number of CFU of each strain inoculated into the embryos is shown in [Figure 3.3](#).

Figure 3.1. Inoculum verification through the incubation of 0.1 ml of inoculum per dose of the *E. faecalis* strain 2140-5 on Columbia sheep blood agar plates. From left to right, the viable counts for the theoretical doses of 2500, 25 und 5 CFU/ml.

The incubated eggs were candled every 24 h for seven days after infection, and embryonic mortalities were daily recorded per strain and control groups. The number of dead embryos was used to classify the virulence of the *E. faecalis* strains. As previously reported by Rudolph (2004), the strains with an EMR below 40% were considered avirulent strains;

above 80% virulent strains and between 40% and 80% were classified as strains of moderate virulence. It should be noted that the embryos that died within 24 h p.i. were removed from the research, since its deaths could be attributed to a lethal trauma during the manipulation (Wooley *et al.*, 2000).

At the end of each ELA, at 17 days of incubation, all surviving embryos were sacrificed by hypothermia. They were stored for 48 h at 4°C.

Random selections of the embryos dead by the infection, as well as some surviving embryos at the end of the ELAs, were used to evaluate macroscopic lesions and to re-isolate the strains from the allantoic fluid of the embryos, as described in the Experiment 1 (Chapter 2, section ‘2.2.5. *Macroscopic lesions and re-isolation*’) of the present dissertation.

3.2.4. Statistical analysis

Statistical analyses of mortality data were carried out by applying a linear logistic mixed model with a binary response variable, which was modelled as a binomial random variable (y_i). The dependent variable (y_i) can take the value 1 with a probability for embryonic mortality π_i or the value 0 with a probability to survive of $1 - \pi_i$. The data were then analysed with the GLIMMIX procedure (SAS Institute Inc., 2011) using the following generalized linear model (Littell *et al.*, 1999):

$$\text{Logit}(\pi_{ij}) = \eta_{ij} = \log [\pi_{ij} / (1 - \pi_{ij})] = \phi + b(x_i) + \alpha_i,$$

where η_{ij} denotes the linear predictor, the logit link function is defined by $\log [\pi_i / (1 - \pi_i)] = \eta_i$, π_{ij} is the probability of mortality until the end of the research at 17 days of incubation, ϕ is the overall mean effect, b is the linear regression coefficient of mortality values of the positive control group used in each individual ELA as reference for adjusting the effect of trial (x_i) on embryonic mortality ability and α_i is the random effect of the strain.

The mortality produced by each strain was estimated on the logit scale and then back-transformed using the inverse link function $\pi = \exp(x\beta) / [1 + \exp(x\beta)]$ to the original scale (probability).

The development of the embryonic survivability over the seven days p.i. for the different groups of strains classified by its virulence degree quantified by impairment on embryonic mortality ability was illustrated according to the Kaplan-Meier method (survival analysis) by applying the LIFETEST procedure of SAS System 9.3 (SAS Institute Inc., 2011) and using the following model:

$$\hat{S}(t) = \prod_{j: t_j \leq t} \left[1 - \frac{d_j}{n_j} \right]; \text{ for } t_1 \leq t \leq t_k$$

where $\hat{S}(t)$ is the survivor function and t is the lifetime of a randomly selected experimental unit. For each $j: t_j \geq t$, let $t_1 < t_2 < \dots < t_k$ representing the different event times. n_j is the number of individuals at risk just prior to t_i , and d_j is the number of individuals that die at time t_j .

3.3. RESULTS

3.3.1. ERIC-PCR method and phylogenetic analysis

Phylogenetic tree of 1 *E. hirae* and 67 *E. faecalis* strains based on the banding patterns after ERIC-PCR is shown in [Figure 3.2](#). A visual comparison of the banding patterns revealed DNA fragments ranging in sizes from 236 to 3380 base pairs. ERIC patterns consisted of four to nine DNA fragments. The phylogenetic analysis of banding pattern showed the most distant relationship to the *E. hirae* strain, which was implemented as an outgroup. This

indicates that the ERIC-PCR can be considered reliable. *E. faecalis* strains were clustered into five major groups, and only one strain (*E. faecalis* 1246-15) was unclustered. All clusters showed a remarkable diversity among the *E. faecalis* strains since differences among its banding patterns were observed, except the Cluster 2, whose strains showed identical banding patterns although they were classified into different pathogenicity degrees according to the ELA results. All clusters were composed of avirulent, virulent and moderate virulence strains except five avirulent *E. faecalis* strains clustered in the Cluster 4.

3.3.2. Embryo lethality assay

The ELAs showed different EMRs according to the strain. As shown in [Figure 3.3.](#), the overall EMR of the infected embryos ranged from 3% to 89% depending on the strain. On the basis of virulence classification of Rudolph (2004), 27 strains, including the *E. hirae* strain, were classified as avirulent with an EMR below 40%, and five strains were classified as highly virulent with an EMR above 80%. The remaining 37 strains were classified as strains of moderate virulence, whose EMR varied from 40% to 80%. The average EMR of the negative control group was 1%, and the positive control group (*E. faecalis* strain K923/96) showed an average of 50%. The *E. hirae* strain (strain 580) showed low virulence (31%) although this strain registered the greatest number of bacteria count (55 CFU/egg) during the verification of the CFU number, as shown in [Figure 3.3.](#)

	Strains	Virulence ^a	ST82 ^b	Origin ^c	Source ^d	Clinical signs ^f
Cluster 1	1246-7	A	No	Germany	- ^e	-
	1246-17	A	No	Germany	-	-
	1246-14	A	No	Germany	-	-
	1246-11	A	No	Germany	-	-
	1246-10	A	No	Germany	-	-
	1246-9	A	No	Germany	-	-
Cluster 2	1246-8	M	No	Germany	-	-
	542-3a	A	Yes	Germany	Joint	Fluid in right hock
	1246-12	A	No	Germany	-	-
	5158	A	Yes	Switzerland	Joint	Yellow fluid in hock
	282-8A	A	Yes	Germany	Thigh	Abscesses in left thigh musculature
	5223-3	A	Yes	Germany	Joint	Dwarfism. Yellow fluid all joints.
	282-5a	A	Yes	Germany	Joint	Yellow fluid in right knee
	542-4b	A	Yes	Germany	Joint	Yellow fluid in left knee
	371-2	A	Yes	Germany	Joint	Yellow fluid in left hock
	5223-2	M	Yes	Germany	Joint	Yellow fluid in left hock
	1607-2	M	Yes	UK	-	-
	542-1b	M	Yes	Germany	Joint	Yellow fluid in left hock
	542-1c	M	Yes	Germany	Joint	Yellow fluid in right knee
	386-01	M	Yes	UK	-	-
	282-8S	M	Yes	Germany	Joint	Yellow fluid in left hock
	282-5b	M	Yes	Germany	Joint	Yellow fluid in left hock
	371-1	M	Yes	Germany	Joint	Yellow fluid in right hock
	K923/96	M	Yes	Germany	Joint	Amyloid depositions in joints
	371-2G	M	Yes	Germany	Thigh	Abscesses in thigh musculature
	111-9	M	Yes	Greece	-	-
	262-01	M	Yes	UK	-	-
	679-4a	M	Yes	Germany	Thigh	Abscesses under right knee
	5223-1	M	Yes	Germany	Joint	Yellow fluid in hock
	417-01	M	Yes	Germany	-	-
	137-01	M	Yes	UK	-	-
	1246-2	M	No	Germany	-	-
	542-3b	M	Yes	Germany	Joint	Yellow fluid in left hock
	923-01	M	Yes	Germany	-	-
	503-01	M	Yes	Germany	-	-
	833-4a	M	Yes	Germany	Joint	Yellow fluid in left hock
	5104	M	Yes	Germany	Joint	Dwarfism. Yellow fluid in hock
	833-2b	M	Yes	Germany	Thigh	Abscess under left thigh musculature
	542-1a	M	Yes	Germany	Joint	Yellow fluid in right hock
	542-5a	M	Yes	Germany	Joint	Yellow fluid in left hock
	542-4a	M	Yes	Germany	Joint	Yellow fluid in left hock
	1246-1	V	Yes	Germany	-	-
	1246-18	V	Yes	Germany	-	-
	1246-21	V	Yes	Germany	-	-
	679-1a	V	Yes	France	-	-
	1201-2	V	Yes	Germany	Joint	Yellow fluid in hock
Cluster 3	554-01	M	Yes	UK	-	-
	590-01	M	Yes	UK	-	-
	554-02	M	Yes	UK	-	-
	554-03	M	Yes	UK	-	-
	1426-01	A	Yes	Germany	-	-
Cluster 4	607-01	A	Yes	France	Joint	Amyloid depositions in joints
	1082-01	A	No	France	Joint	Amyloid depositions in joints
	2300	A	No	Germany	Joint	Soft bones. Yellow fluid in hock
	2027-2	A	No	Germany	Joint	Amyloid depositions. No egg laying
	515-3	A	No	Germany	Carcass	Apparently healthy
Cluster 5	513-5	A	No	Germany	Carcass	Apparently healthy
	2140-5	A	No	Russia	-	-
	4694-CH ₁	A	Yes	UK	-	-
	282-7	M	Yes	Germany	Joint	Yellow fluid in left hock
	282-1	M	Yes	Germany	Thigh	Abscess in left thigh musculature
	282-4	M	Yes	Germany	Carcass	Apparently healthy
	1611-1	A	No	Russia	-	-
	1245-2	M	No	Germany	-	-
Unclustered	4694-CH ₂	A	Yes	UK	-	-
	4694-PO	M	Yes	UK	-	-
	1246-15	M	No	Germany	-	-
	580 ^g	A	-	Germany	-	-

Figure 3.2. Phylogenetic tree (drawn in the rectangular cladogram format) of 1 *E. hirae* and 67 *E. faecalis* strains based on the aligned nucleotide sequences after ERIC-PCR.

^a Virulence classification of the strains through the ELAs (A=Avirulent, M=Moderate and V=Virulent). ^b ST82 refers to the sequence type 82 reported by the laboratory of Lohmann Tierzucht GmbH (Germany) by using the RT-PCR. ^c Country of origin of the strain. ^d Source of isolation. ^e This information is unknown. ^f Clinical signs observed in the birds. ^g *E. hirae* strain implemented as an outgroup.

The real number of CFU of each strain inoculated into the embryos did not show any clear relationship with the EMR produced. As can be seen in [Figure 3.3.](#), some of the strains with the lowest CFU number were able to produce high EMR and, on the contrary, some strains with higher CFU number produced low EMR. For example, 3 CFU/egg of the *E. faecalis* strains 1246-8 and 1246-18 produced an EMR of 78 and 82%, respectively. However, 20 and 23 CFU/ml of the *E. faecalis* strains 1611-1 and 2323-96 produced an EMR of 14 and 18.4%, respectively.

The lack of relationship among the CFU administered to the embryos and the embryonic mortality produced by each strain was also observed in the positive control group used in all ELAs, as shown in [Figure 3.4](#). The number of bacteria inoculated into the embryos of the positive control group varied from 4 to 19 CFU/egg among each individual ELAs and did not shown any relationship with the produced embryonic mortality, which ranged from 24 to 65%.

Figure 3.3. Virulence classification of 68 *E. faecalis* strains, one *E. hirae* strain (strain 580) and a negative control group (Neg. Co) sorted by the EMR that produce in 10-day-old chicken embryos inoculated into the AC with the represented number of CFU(*). Strains with an EMR below 40% (n=27) were considered avirulent; from 40% up to 80% were classified as strains of moderate virulence (n=37) and above 80% virulent strains (n=5).

Figure 3.4. Chicken EMR produced by the *E. faecalis* strain K923/96 used as positive control in 10 individual ELAs when 10-day-old embryos were inoculated into the AC with the represented number of CFU (*).

3.3.3. Embryo survival

The survival curve for the embryos infected with three groups of strains classified by its virulence degree is presented in [Figure 3.5](#). Obviously, embryos infected with avirulent strains had more survival probability than the embryos infected with more virulent strains. However, regardless of the virulence degree of the strains, the highest embryonic mortality occurred at three and four days p.i., and after four day p.i. the embryonic mortality declined substantially.

Figure 3.5. Survival curves of 10-day-old chicken embryos inoculated into the AC with 5 CFU/ml of three groups of different *Enterococcus* spp. strains classified by its virulence degree.

3.3.4. Macroscopic lesions

No lesions were observed in the embryos of the negative control group, which received 0.2 ml of PBS. However, the infected embryos succumbed to the bacterial infection. The most frequent detrimental effects of artificial infection on embryonic organ and tissue were malformations, growth failure, cranial and skin haemorrhages, subcutaneous oedema and loss of the plumage. The detrimental effects resulting by the *E. faecalis* infection on the chicken embryos showed the same pattern regardless of the degree of virulence of the strains, as shows in [Figure 3.6](#). In addition, most severe lesions were observed at three and four days p.i., and less serious lesions were observed from the fourth day p.i. onwards. Therefore, the most severe lesions were observed when the higher EMR was registered. It should be noted

that some dead and surviving infected embryos at the end of the ELAs did not show any visible signs.

Figure 3.6. Macroscopic lesions of white laying hen embryos infected with different *E. faecalis* strain with different virulence degree.

3.3.5. Re-isolation

Smear samples from the allantoic fluid of random analysed dead embryos as well as from surviving embryos were taken in order to re-isolate the *E. faecalis* strains. The bacterial counts of the infected embryos were always positive and their allantoic fluid contained more CFU/ml compared to the infectious dose administered to the embryos (> 1000 CFU/ml),

although some of them did not show any visible lesions and any abnormalities. The bacterial count of the negative control group was for all analysis negative, confirming that there was no cross-contamination.

3.4. DISCUSSION

In order to characterize the genetic diversity of the avian *E. faecalis* strains and under the hypothesis that strains with similar banding patterns might show also similar infective ability, the ERIC-PCR was carried out.

ERIC-PCR has been successfully used in previous studies for genotyping of different bacteria such as *E. coli* (Leung *et al.*, 2004), *Vibrio parahaemolyticus* (Zulkifli, *et al.*, 2009) and *L. monocytogenes* (Moreno *et al.*, 2013). In the case of *Enterococcus spp.*, Jurkovič *et al.* (2007) reported that the ERIC-PCR produce a high discriminatory power among *E. faecium* strains isolated from Bryndza cheese in comparison with PFGE and (GTG)₅-PCR. Besides, Bachtiar *et al.* (2015) observed a high genetic diversity among *E. faecalis* strains from saliva and infected root canal samples by using the ERIC-PCR.

As shown in [Figure 3.2.](#), ERIC-PCR in the present research was able to distinguish *Enterococcus spp.*, specifically *E. faecalis* and *E. hirae* strains, which is important to ensure the specificity of the test for *E. faecalis*. ERIC-PCR revealed a considerable genetic diversity among avian *E. faecalis* strains, which was previously demonstrated in *Enterococcus spp.* (Jurkovič *et al.*, 2007 and Bachtiar *et al.*, 2015). However, since *E. faecalis* strains with similar banding patterns did not produce the same EMR through the ELA, ERIC-PCR results did not show a clear relationship to the infection ability of the strains. Similar results were reported by Borst *et al.* (2014), who observed that an *E. cecorum* strain with virulent genotype

by using the PFGE did not have a virulent phenotype through the ELA, suggesting that the ELA may be useful in distinguishing strains with the same genotype but different phenotype. Therefore, the molecular taxonomy of *Enterococcus spp.* might not be able to distinguish between avirulent and virulent strains, as previously reported by Eaton & Gasson (2001). Nevertheless, it is possible that the sequencing of the whole genome may lead to different results.

The typing of *E. faecalis* strains has revealed that several STs are related with different human and avian diseases. Specifically, the ST82 of *E. faecalis* has been related with AA worldwide (Petersen *et al.*, 2007). In this study, from the 49 *E. faecalis* strains that contained the ST82 through the RT-PCR, 10% were classified as virulent strains, 67% as moderate virulence and 22% as avirulent strains through the ELA, as shown in Figure 3.2. On the contrary, 78% from the 18 *E. faecalis* strains with absence of the ST82 were classified as avirulent strains and 22% as strains of moderate virulence. The fact that the *E. faecalis* strain 1082-1, which is not related to the ST82, was isolated from a field case of AA can be explained by the ST172 reported by Petersen *et al.* (2009), which has been also associated with avian amyloidosis (<http://www.mlst.net>). In addition, since all used strains presented the same profile of virulence genes and produced however different virulence degree through the ELA, no relationship between the virulence degree of the strains and their virulence genes was observed.

The ELA in the present research has demonstrated its ability to differentiate *E. faecalis* strains with respect to its virulence (Figure 3.3.), in accordance with the ELA results of several authors with different bacterial strains (Wooley *et al.*, 2000; Gibbs *et al.*, 2003; Gibbs & Wooley, 2003; Rudolph, 2004; Montgomery *et al.*, 2005; Townsend *et al.*, 2008; Stewart-

Tull *et al.*, 2009; Jacobsen *et al.*, 2010; Oh *et al.*, 2012; Polakowska *et al.*, 2012; Seo *et al.*, 2013 and Borst *et al.*, 2014).

The average EMR of the negative control group was 1%, which confirms the appropriate environment in the incubator during the ELAs. Therefore, the obtained embryonic mortalities with each strain can be unequivocally associated to its virulence. The average EMR of the embryos inoculated with 5 CFU/ml of the *E. faecalis* strain K923/96 used as positive control in each ELA was 50%, which is in accordance with the results obtained in the previous Experiment 1 (Chapter 2) of the present dissertation; an EMR of 45% with the same strain and infectious dose. Therefore, although the *E. faecalis* strain K923/96 showed variability between the individual ELAs, as shown in [Figure 3.4.](#), its relative virulence provides accurate results, which is in accordance with the results reported by Gibbs *et al.* (2003) and Montgomery *et al.* (2005) with *E. coli* strains. Hence, these authors suggest the need to be careful when determining the absolute virulence of a strain based on a single ELA.

The EMR which was produced by each strain cannot be explained by the number of CFU administered to the embryos, as shown in [Figure 3.3](#). This observation is consistent with the results obtained by Gibbs *et al.* (2003) with *E. coli* strains, who reported that the ability of a strain to colonize the embryo was not related to its capacity to invade the embryo and cause disease or death. This explains the fact that the number of CFU/egg of the *E. faecalis* strain K923/96 used as positive control did not show a clear relationship with the EMR in each individual ELA ([Figure 3.4.](#)). However, Seo *et al.* (2013) reported that virulent and avirulent *R. anatipestifer* strains showed different capacity to replicate *in ovo* after the inoculation. They observed that the maximum number of CFU/ml of a virulent strain was about 1000 times higher than those of an avirulent strain for four days, suggesting that the ability of replication of the strains into embryonic tissues is related to systemic infection of the embryo

and death. The *E. hirae* strain 580, which was inoculated into the embryos with the same infectious dose like the *E. faecalis* strains, showed the greatest number of bacterial count administered to the embryos (55 CFU/egg); however, it produced low embryonic mortality (31%). Therefore, the number of CFU able to produce embryonic mortality seems to depend not only on the strain but also on the specie. More researches with different species of *Enterococcus* are required in order to corroborate this theory.

In accordance with the results reported by Wooley *et al.* (2000), Gibbs *et al.*, (2003), Gibbs & Wooley (2003), Stewart-Tull *et al.* (2009), Seo *et al.* (2013) and Borst *et al.* (2014), regardless of the strain virulence, the survival rate of the embryos decreased faster from two to four days p.i., whereas the survival rate decreased slower afterwards, as shown in [Figure 3.5](#). Therefore, the ELA with *E. faecalis* strains might be optimized by reducing the time of study until four days p.i. as Wooley *et al.* (2000) and Montgomery *et al.* (2005) have previously reported with *E. coli* strains. Additionally, Wooley *et al.* (2000) reported that the survival rate and lesions of the embryos infected with *E. coli* strains showed a relationship with the strain virulence degree. The results obtained in the present research also showed a relationship between the embryonic survival rate and the strain virulence degree since more virulent strains produced a faster decrease in embryonic survival rate from two to four days p.i. than avirulent strains ([Figure 3.5](#)). However, all infected embryos showed the same lesions regardless of the virulence of the strains, in contrast with the results of Wooley *et al.* (2000) and Borst *et al.* (2014). The observed macroscopic lesions were similar than those in the broiler embryos infected with *E. cecorum* by Borst *et al.* (2014) ([Figure 3.6](#)).

No lesions and no re-isolations were observed in the embryos of the negative control group, thus indicating that the embryonic mortality was due to the infection and not due to external effects and, therefore, the research was reliable. On the contrary, the recovered

bacterial counts from the allantoic fluid of all analysed infected embryos with *E. faecalis* strains were always positive, although some of these embryos did not show any visible signs. Besides, an increase in the viable count of the allantoic fluid of dead embryos was observed compared to the initial number of CFU/ml contained in the infectious dose. This ability of the bacteria to replicate and invade the embryos after the inoculation, even without killing them, has been previously reported by Wooley *et al.* (2000), Rudolph (2004), Montgomery *et al.* (2005), Townsend *et al.* (2008), Stewart-Tull *et al.* (2009) and Seo *et al.* (2013).

In conclusion, the results obtained in this research suggest that the ELA can be considered a reliable and useful tool to evaluate the virulence of avian *E. faecalis* strains by using the EMR. Although the clustering based on the ERIC-PCR did not show a clear relationship with the virulence assessment obtained by the ELA in this research, it can be applied successfully and be useful to obtain the genetic distribution and epidemiology of these bacteria. The results obtained in the present research can be used as a basis for future researches on the *E. faecalis* virulence, and high pathogenic isolates tested in the present experiment might be good candidates for autogenous vaccines.

CHAPTER 4.

GENETIC APPROACH TO SELECT MORE RESISTANT LAYING HENS FOR *ENTEROCOCCUS FAECALIS* INFECTION (EXPERIMENT 3).

4.1. INTRODUCTION

It is estimated that over 25,000 people die each year in Europe from infections caused by antibiotic-resistant bacteria according to the World Health Organization (2011). Therefore, it is expected that the use of antibiotics substantially decrease in the future, specifically in animal production, in order to satisfy the regulations and consumer demands. However, avian infectious diseases cause important production losses in the poultry industry, which is not only an economic problem but also it compromises the welfare of the birds and raise humane concerns worldwide. Consequently, there is high requirement for control strategies against bacterial infections. Different general strategies could be applied in the fight against bacterial infections such as the development of vaccines and breeding for more resistant birds, in addition to hygienic measures and biosecurity.

The development of AA in chickens is associated with chronic inflammation and infection, which appears to be induced by arthropathic and amyloidogenic *E. faecalis* strains. AA is considered an irreversible disease (Landman *et al.*, 1998a) since the amyloid fibrils have low solubility and a relative resistance to proteolytic digestion under physiological

conditions. Therefore, the treatment of AA has focused to date on treating the underlying inflammatory disease caused by *E. faecalis* and thus prevent the occurrence of fibrillogenesis.

Antibiotics such as penicillin, erythromycin, novobiocin, nitrofurans, oxytetracycline, chlortetracycline and tetracycline are used in poultry to treat enterococcal infections (Stephan *et al.*, 2008). However, *E. faecalis* have a natural intrinsic resistance towards erythromycin and tetracycline (McBride *et al.*, 2007), which difficult its treatment and it can lead to resistant infections, issue that is becoming increasingly important. Consequently, control strategies against *E. faecalis* infections are required.

It has been demonstrated that selection for increased resistance of Marek's disease, avian leukosis viruses, salmonellosis, colibacillosis among other diseases can be effective (Kuhnlein *et al.*, 2003). Genetic selection to reduce susceptibility to diseases requires first the presence of genetic variation in susceptibility among individuals. The selection response can then be predicted from estimating heritability.

Genetic variation in susceptibility to AA between brown and white breed layers have been reported both in field and induced cases of AA, as previously mentioned in the introduction of the present dissertation (Chapter 1, section '1.1.5.1. Breed susceptibility'). The greater susceptibility to AA of chicken breeds may be associated with immunological response pattern since brown and white layers show a different immune reaction when they are infected with amyloidogenic *E. faecalis* strains (Zekarias *et al.* 2000). According to Ovelgönne *et al.* (2001), although the differences in susceptibility to AA among individuals could have a genetic basis, it cannot be explained by the SAA genes because its amino acid sequences in both chicken breeds are identical.

Therefore, the aim of the present study was to investigate the possibility to breed for higher resistance against *E. faecalis* infection through the ELA, which is proposed as an alternative model to replace the adult avian challenge assays.

4.2. MATERIAL AND METHODS

4.2.1. Embryo lethality assay

All ELAs carried out in the present research were developed as the ELAs previously described in the Experiment 1 and 2 (Chapter 2 and 3, respectively) of the present dissertation, but the experiment duration of these ELAs was four days. The embryos were inoculated via the AC after 10 days of incubation with the reference *E. faecalis* strain K923/96 and 0.2 ml of different infectious doses (5 and/or 2.5 CFU/ml) prepared as previously described in the Experiment 1 (Chapter 2, section '2.2.3. Inoculum') of the present dissertation.

The incubated eggs were candled every 24 h for four days and embryonic mortalities were daily recorded per group. It should be noted that the embryos that died within 24 h p.i. were removed from the experiment, since its deaths could be attributed to a lethal trauma during the manipulation rather than due to the infection (Wooley *et al.*, 2000). At the end of each ELA, at 14 days of incubation, all surviving embryos were sacrificed by hypothermia. They were stored for 48 h at 4°C.

Random selections of the embryos dead by the infection, as well as some surviving embryos at the end of the ELAs, were used to evaluate of macroscopic lesions and to recover the bacteria from the allantoic fluid of the embryos p.i., as reported in the Experiment 1 (Chapter 2, section '2.2.5. Macroscopic lesions and re-isolation') of the present dissertation.

4.2.2. Preliminary study

Since AA has never been reported in field cases in white layers and they are less susceptible to induced AA than brown layers (Ovelgönne *et al.*, 2001) and, therefore, the genetic selection to reduce susceptibility to *E. faecalis* infection would make sense for brown layer chickens, it could be expected that chicken embryos of brown layers would be less resistant to *E. faecalis* infection compared with embryos of white layers when they are infected with the same *E. faecalis* strain and infectious dose.

A total of 2711 embryos of Lohmann Brown Layers at an age of 35 - 55 weeks were inoculated in six individual ELAs (I to VI), as shown in Table 4.1. The embryos were inoculated with a single avian *E. faecalis* strain K923/96 (reference strain) and 0.2 ml of two infectious doses of 2.5 and 5 CFU/ml. Both infectious doses were used in all these ELAs, with the exception of two ELAs (IV and V), in which was only used the infectious dose of 5 CFU/ml. In addition to the infected embryos, there was always one control group in each ELA, which was inoculated in the AC with 0.2 ml of sterile PBS.

Table 4.1. Accumulated embryo mortality in six individual ELAs during the preliminary study.

Infectious Dose	ELAs						LS-means ³
	I	II	III	IV	V	VI	
Control ¹	39/93 ²	2/95	3/95	7/132	4/125	3/187	3 ^a
5 CFU/ml	81/84	85/94	70/93	352/424	356/425	148/194	83 ^b
2.5 CFU/ml	87/93	75/89	69/92	-	-	92/191	69 ^c

¹ Embryos of the control group were inoculated in the AC with 0.2 ml of sterile PBS.

² These embryos were accidentally contaminated with *E. faecalis* and the ELA I was therefore considered invalid.

³ The effects were estimated using a generalized linear model with binomial error distribution and logit link function. Different letters indicate statistically significant differences between groups.

As shown in Table 4.1., the EMR of the control group in the ELA I was surprisingly high (42%). We realized that the embryos were accidentally contaminated with *E. faecalis* after cultivating smear samples from the allantoic fluid of some of them and, therefore, this ELA was considered invalid and remove.

4.2.3. Main study

Once the appropriate dose for brown embryos was determined, three more ELAs (VII-IX) were carried out. These three ELAs were performed on a sample of 7563 embryos of 500 Brown Layers families at an age of 54 - 56 weeks from a commercial breeding program of Lohmann Tierzucht GmbH. As shown in Table 4.2., the number of embryos and families used were 2656 and 463 in the ELA VII, 2478 and 454 in the ELA VIII, and 2429 and 452 in the ELA IX, respectively. For each family and trial, an average of 5.5 embryos (ranging from one to eight) was inoculated with 0.2 ml of 2.5 CFU/ml of the *E. faecalis* strain K923/96.

Table 4.2. Characteristics of the ELAs VII-IX carried out in the main study.

ELA	VII	VIII	IX
N° Embryos	2656	2478	2429
N° Families	463	454	452
EMR (%)	54	30	74

4.2.4. Traits of economic importance

In order to investigate the possibility to breed for higher resistance against *E. faecalis* infection, the influence of this selection for other traits of economic importance in the poultry industry was assessed. Laying performance was recorded and estimated based on the average of measurement in three periods; period 1 (start of lay) between 20 and 23 weeks of age, period 2 (peak of lay) between 24 and 51 weeks of age, and period 3 (end of lay) between 56

and 71 weeks of age. The egg weight (EW) was estimated based on the mean of the three measurements at 28, 35 and 45 weeks of age. The body weight (BW) was recorded at 32 weeks of age. Additionally, two traits related with the shell stability were recorded. The egg shell breaking strength (BS) captures the maximum load that can be withstood by an egg and provides information about the strength required to crack the eggshell (Bain, 2005). This trait was recorded and estimated based on the average of measurement at 28, 35 and 45 weeks of age (Period 1) and at 60 and 65 weeks of age (Period 2). Dynamic stiffness (K_{dyn}) is based on acoustic resonance test and offers information about the uniformity and eggshell strength as well as the probability of cracking (Bain *et al.*, 2006), which was recorded at 40 weeks of age.

4.2.5. Statistical analysis

4.2.5.1. Preliminary study

Statistical analyses of mortality data were carried out by applying a linear logistic model for repeated measurements with a binary response variable, which was modelled as a binomial random variable (y_i). The dependent variable (y_i) can take the value 1 with a probability for embryonic mortality π_i or the value 0 with a probability to survive of $1 - \pi_i$. The logistic model uses a link function $g(\mu_i)$, linking the expected value to the linear predictors η_i . The logit link function is defined by $\log [\pi_i / (1 - \pi_i)] = \eta_i$, where π_i is the probability of mortality until the end of the experiment at 17 days of incubation. The data were then analysed with the GLIMMIX procedure (SAS Institute Inc., 2011) using the following generalized linear model (Littell *et al.* 1999):

$$\text{Logit}(\pi_{ij}) = \eta_{ij} = \log [\pi_{ij} / (1 - \pi_{ij})] = \mu + \alpha_i + \beta_j,$$

where η_{ij} denotes the linear predictor, π_{ij} is a binary outcome (i.e., probability of embryonic mortality), μ is the overall mean effect, α_i is the fixed effect of the infectious dose

and β_j is the fixed effect of repeated measurement of the ELA. LSMEANS were estimated on the logit scale and then back-transformed using the inverse link function $\pi = \exp(x\beta) / [1 + \exp(x\beta)]$ to the original scale (probability) applying the LSMEANS statement. Significant differences between LSMEANS were tested using a *t*-test procedure by inclusion of the DIFF option in the LSMEANS statement. Differences were considered significant when $P < 0.05$.

4.2.5.2. Main study

For the estimation of genetic parameters, the egg traits and the full pedigree information of all tested animals from four generations were used. For the estimation of the heritability of embryonic survivability to *E. faecalis* infection, the mean value of the three ELAs was calculated for each family. Heritability and repeatability were calculated using univariate animal model with the variance of the logit and probit link functions by applying the package ASREML (Gilmour *et al.*, 1998). The variance of the logit link function implies a correction of the residual variance by factor $\pi^2/3$ presented as follows:

$$h^2 = \frac{\sigma_A^2}{\sigma_A^2 + \sigma_{EP}^2 + \pi^2/3}$$

where σ_A^2 is the additive genetic variance and σ_{EP}^2 is the variance of permanent environment. Estimates of random effects of animal were calculated in a univariate model on the logit scale and then back-transformed to the original scale using the inverse link function $\pi = \exp(x\beta) / [1 + \exp(x\beta)]$.

Genetic correlations between embryonic survivability and egg traits were estimated using bivariate animal model analysis. Fixed effects for the bivariate model were the same as the univariate analysis. Genetic correlations between binomially distributed traits were also estimated via ASREML (Gilmour *et al.*, 1998) using the probit link function.

The course of embryonic survivability over the four post-inoculation days within each treatment group was illustrated according to the life table method (survival analysis) by applying the LIFETEST procedure of SAS System 9.3 (SAS Institute Inc., 2011) and using the following model:

$$\hat{S}(t) = \prod_{j: t_j \leq t} \left[1 - \frac{d_j}{n_j} \right]; \text{ for } t_1 \leq t \leq t_k$$

where $\hat{S}(t)$ is the survivor function and t is the lifetime of a randomly selected experimental unit. For each j : $t_j \geq t$, let $t_1 < t_2 < \dots < t_k$ represent the different event times. n_j is the number of individuals at risk just prior to t_j , and d_j is the number of individuals that die at time t_j .

4.3. RESULTS

4.3.1. Preliminary study

The ELAs I to VI showed different EMR according to the infectious dose. The infectious dose showed a significant effect ($P < 0.0001$) on the EMR. As shown in [Table 4.1](#), both infectious doses resulted in more than 60% lethality after four days p.i. The average EMR for the infectious dose of 5 CFU/ml was 83% and for the infectious dose of 2.5 CFU/ml was 69%. They differed significantly from each other. The highest EMR was recorded after three days p.i. with both doses. Malformed and underdeveloped embryos were observed in both treated groups regardless of the infectious dose. Additionally, the bacterial counts from the allantoic fluid of random analysed dead embryos, as well as from surviving embryos, were always positive.

In the case of the control group, with the exception of the control group of the ELA I, the bacterial count was always negative and no lesion were observed indicating that there was no cross-contamination and, therefore, the contamination of the control group of the ELA I had to be only caused by the accidental contamination of the bottle of sterile PBS. The average EMR of the control group was 3%, indicating the appropriated environment in the incubator during the study and the low negative impact of the injection. Besides, the control group differed significantly from each other infectious doses.

On the basis of these results, the embryos in the main study were infected with a single infectious dose of 2.5 CFU/ml in order to avoid a massive embryonic mortality that would not allow the differentiation between the susceptibility of the families.

4.3.2. Main study

4.3.2.1. Embryo lethality assay

The average EMR was 54% in the ELA VII, 30% in the ELA VIII and 74% in the ELA IX, as shown in [Table 4.2](#). Thirty four from 500 families were discarded because they did not present embryos in any ELA. The average EMR in the three ELAs varied among the families from 0% to 100%, with a standard deviation of 23%, as shown in [Figure 4.1](#). All embryos from 14 families survived the infection and 12 families showed an average EMR lower than 10% during the three ELAs. On the contrary, all embryos from seven families died during the three ELAs and two families suffered an average EMR of more than 95%. The embryos from 84 families suffered an average EMR between 50% and 60%.

Figure 4.1. Average EMR of 10-day-old embryos from 466 families during three ELAs when they were inoculated into the AC with 2.5 CFU/ml of the *E. faecalis* strain K923/96.

Figure 4.2. Macroscopic lesions of brown embryos infected with the *E. faecalis* strain K923/96.

The infected embryos succumbed to bacterial infection and their bodies showed malformations, underdevelopment, cranial and skin haemorrhages, loss of plumage and subcutaneous oedema, as shown in [Figure 4.2](#).

The survival curve for the embryos infected during the three ELAs is presented in [Figure 4.3](#). Obviously, the infected embryos in ELA VIII, whose EMR was lower, had more survival probability than the embryos infected in the ELAs VII and IX, respectively. Regardless of the ELA, the highest embryonic mortality occurred three and four days p.i..

Figure 4.3. Survival curves of 10-days old chicken embryos inoculated into the AC with the *E. faecalis* strain K923/96 in three ELAs.

4.3.2.2. Descriptive statistics

The Table 4.3. shows the descriptive statistics for the embryonic survival and each analysed traits including mean values and standard deviations. The average embryonic survivability estimated during the three ELAs was 50%. The average laying performance varied from 74% to 94% among the periods. The average EW estimated based on the mean of three measurements at 28, 35 and 45 weeks of age was 63.6 g. BW measured at 32 week of age ranged from 1.4 to 2.5 kg among the birds, with an average of 1.9 kg. Regarding to the traits related with shell stability, the average BS in the period 1 was 4.5 N higher than in the period 2, and the average K_{dyn} at 40 weeks of age was 163.

Table 4.3. Means, SD, as well as minimum (Min) and maximum (Max) values for the embryonic survivability and traits of economic importance.

Trait ¹		n	Mean	SD	Min	Max
Embryonic survivability		454	0.50	0.34	0	1
Laying performance (%) ²	Period 1	453	74.0	16.2	11.0	100
	Period 2	454	94.0	6.3	44.0	100
	Period 3	383	86.0	12.1	33.0	100
EW (g)		454	63.6	4.6	52.1	81.8
BW (kg)		454	1.9	0.2	1.4	2.5
BS (N) ³	Period 1	454	42.7	6.6	24.1	62.0
	Period 2	369	38.2	9.0	15.7	66.0
K_{dyn}		425	163	18.7	115	216

¹ Trait: EW = egg weight; BS = breaking strength; K_{dyn} = dynamic stiffness; BW= body weight.

² Period 1 (start of lay): average of measurement at 20 and 23 weeks of age, period 2 (peak of lay): at 24 and 51 weeks of age, and period 3 (end of production): at 56 and 71 week of age.

³ Period 1: average measurement at 28, 35 and 45 weeks of age and period 2: at 60 and 65 weeks of age.

4.3.2.2. Heritabilities and genetic correlations

Estimated genetic parameters for embryonic survivability are presented in [Table 4.4](#). The estimated heritability for embryonic survivability was on a moderate level in general. The estimated heritability was $h^2 = 0.17$ calculated with the logistic link function and $h^2 = 0.20$ calculated with the probit link function.

Table 4.4. Estimation of heritability and repeatability for embryonic survivability using univariate repeatability animal model with logit and probit link functions.

	Var (a)	Var (pe)	Heritability	Repeatability
Logistic	0.74±0.23	0.29±0.18	0.17±0.05	0.07±0.04
Probit	0.27±0.08	0.10±0.06	0.20±0.05	0.07±0.05

The estimated genetic correlations between the embryonic survivability and the analysed traits are shown in Table 4.5. The correlations were negative and higher in the two last periods ($r_g = -0.69$ in the period 2 and $r_g = -0.55$ in the period 3) compared with the first period of production, where the correlation was positive ($r_g = +0.31$). A positive correlation was also found between EW and the embryonic survivability ($r_g = +0.16$). No genetic correlation between BW and the embryonic survivability ($r_g = -0.04$) was found. The genetic correlation between BS and the embryonic survivability was $r_g = -0.30$ in the period 1 and $r_g = -0.48$ in the period 2. However, there was no correlation between K_{dyn} and the embryonic survivability ($r_g = -0.05$).

Table 4.5. Genetic correlations between the embryonic survivability and the analysed traits.

Trait ¹		r_g
Laying performance (%) ²	Period 1	+0.31±0.12
	Period 2	-0.69±0.73
	Period 3	-0.55±0.24
EW(g)		+0.16±0.13
BW (kg)		-0.04±0.14
BS (N) ³	Period 1	-0.30±0.16
	Period 2	-0.48±0.22
K_{dyn}		-0.05±0.15

¹ Trait: EW = egg weight; BS = breaking strength; K_{dyn} = dynamic stiffness; BW= body weight.

² Period 1 (start of lay): average of measurement at 20 and 23 weeks of age, period 2 (peak of lay): at 24 and 51 weeks of age, and period 3 (end of production): at 56 and 71 week of age.

³ Period 1: average measurement at 28, 35 and 45 weeks of age and period 2: at 60 and 65 weeks of age.

The relationships between embryonic survivability and analysed traits are plotted in [Figure 4.4](#). The graphs A, B and C show the relationship between embryonic survivability and laying performance at the beginning of the lay (period 1), at peak of lay (period 2) and at the end of the lay (period 3), respectively. A clear lineal negative effect on the laying performance in the periods 2 and 3 can be distinguished. The negative relationship between embryonic survivability and the traits related with shell stability are shown in graphs D and E for BS in the periods 1 and 2, respectively and in graph F for K_{dyn} . The positive relationship between EW and embryonic survivability is plotted in the graph G. The graph H shows the weak relationship between BW and embryonic survivability.

Figure 4.4. Breeding values for embryonic survivability plotted against breeding values for laying performance (A, B, C)^a, BS (D, E)^b, K_{dyn} (F), EW (G) and BW (H).

^a A for period 1 (start of lay), B for period 2 (peak of lay) and C for period 3 (end of lay).

^b D for period 1 and E for period 2.

4.4. DISCUSSION

Breeding for increased disease resistance have been one of the challenges of many breeders and geneticists. However, breeding for resistance to diseases is difficult because the estimated heritabilities for resistance against a particular pathogen are generally low, although the heritabilities for the trait resistance to one specific disease is normally higher than for mortality in general. It should be taken into account that, although the genetic improvements are perhaps small for each generation, they are permanent and cumulative (Lamont, 1998). Additionally, breeding for resistance involves exposure of the animals to pathogenic agents, which raises ethical questions from an animal welfare standpoint. Consequently, in the present study the ELA was proposed as an alternative to adult avian challenge assays.

Heritability for resistance to diseases reported in the literature by applying different statistical methods is usually low. Berthelot *et al.* (1998) reported heritabilities for resistance to the cecal carrier state of *S. Enteritidis* from $h^2=0.08$ to $h^2=0.09$. Janss & Bolder (2000) estimated heritability for mortality to *S. Enteritidis* of $h^2=0.12$. Different studies have reported direct heritabilities estimates for ascites syndrome from $h^2=0.12$ to $h^2=0.44$ (Ahmadpanah *et al.*, 2017) and Cavero *et al.* (2009) reported heritability for resistance to *E. coli* of $h^2=0.17$. According to the usual range of the heritabilities for resistance to diseases, the heritability for resistance to *E. faecalis* infection estimated in the present dissertation ranged from $h^2 = 0.17$, calculated with the logistic link function, to $h^2 = 0.20$, calculated with the probit link function, i.e., that 17-20% of the total variability of the resistance to the *E. faeacalis* infection is explained by additive genetic reasons. Traits with similar or even lower heritability are successfully included in the breeding programs. Therefore, it might be feasible to include this trait in the breeding programs taking into account all traits and their genetic correlations to achieve a balanced genetic progress.

Genetic correlation among traits allows carrying out an indirect selection through a correlated response, i.e., a favourable correlation allows making simultaneous improvement in the traits. On the contrary, an unfavourable correlation results in selection for one trait worsening another. Genetic correlations in the present study are in accordance with the results obtained by Cavero *et al.* (2009), who observed that laying hens more resistant against *E. coli* infection have a better initial laying performance and lower feed intake while susceptible laying hens to colibacillosis showed better BS and EW, although these differences between resistant and susceptible laying hens were not large and, therefore, the selection for *E. coli* resistance could be added into a commercial selection program without penalizing the improvement of the analysed traits.

As shown in Table 4.5., the estimated correlation between EW and embryonic survivability in this research was positive ($r_g = +0.16$), which imply a favourable genetic relation. However, the genetic correlation between embryonic survivability and the traits related with shell stability indicate that BS and K_{dyn} are not favoured by a lower mortality, which it is not desired from a breeding point of view. Due to the fact that the negative genetic correlation between BS and embryonic survivability ($r_g = -0.30$ to -0.48) was much higher than for embryonic survivability and K_{dyn} ($r_g = -0.05$), it would be advantageous to use K_{dyn} as a selection trait measurement to achieve an optimal combination of embryonic survivability and adequate shell strength.

On the other side, from the results obtained it can be concluded that the use of the ELA offers great benefits. Only 4 days are required to provide proper outcomes and it is an easy assay to carry out. Besides, the ELA does not require that the hens are exposed to pathogens saving labour and cost. This fact allows the selection on the pedigree population without the need of a challenge test with a large number of birds. Therefore, the ELA might

be feasible for implementation in selection programs. However, it would be important to establish if selection against *E. faecalis* infection alters susceptibility against other pathogens because the economic importance of bacterial diseases as a whole is much higher than that of one bacterium alone. Besides, the present study was carried out with a single pathogenic *E. faecalis* strain and, therefore, a very specific disease selection program might protect the current birds and however provide little protection for years to come.

In summary, the heritabilities estimated in the present study show that the selection for increased resistance to *E. faecalis* infection is feasible. However, negative correlations between embryonic survivability and some economically important traits were observed. Special care should therefore be taken to monitor and to take all traits and their correlations into account to achieve a balanced genetic progress. Thanks to the ELA, the labour and cost associated to the challenge is reduced and the ethical and animal welfare reasons are minimised. Whether selection against *E. faecalis* alters susceptibility against other pathogens is unknown, which is essential in the treatment of bacterial diseases as a whole that of *E. faecalis* alone. This research shows a new perspective for the implementation of genetic selection for *E. faecalis* susceptibility in laying hens.

CHAPTER 5.

GENERAL DISCUSSION AND FINAL CONCLUSIONS.

5.1. GENERAL DISCUSSION

In this final Chapter, the main findings of this dissertation will be evaluated and discussed. Finally, the conclusion and suggestions for further researches will be presented.

5.1.1. Amyloid arthropathy

The first Chapter of the present dissertation summarises the knowledge collected in the literature about AA in chickens during the last twenty years. Highlight concerning the different hypotheses and theories about pathogenesis and transmission of this disease, as well as the differences in susceptibility to AA between brown and white layers.

Since *E. faecalis* is the most frequently isolated bacterium in field outbreaks of AA and its specific association with this disease has been extensively confirmed, it has been deemed important to underline in this first Chapter the main features of *E. faecalis* and its role in AA pathogenesis as well as its possible routes of transmission.

The Chapter 1 of the present dissertation highlights the need of expanding and deepening more the knowledge about this disease. The pathology of AA in chickens is widely recognised and it is characterized by deposits of orange-coloured amyloid materials in the joints. Nevertheless, there are also cases in which amyloid depositions have not been reported and hence the real question is whether all field cases reported in poultry are AA or if there are

other hidden problems. Although there are substances and anti-inflammatory agents such as methylprednisolone and pentoxifylline which inhibit the amyloid deposition in birds, more investigations are required to determine the effects of different agents on the treatment of AA and clarify if the amyloid deposits are really irreversible.

Although there are various investigations about the pathogenesis of AA in layers, it still cannot be fully explained. The inflammatory cytokines play an important role in the pathogenesis of amyloidosis. Besides, some pro-inflammatory cytokines are produced by peripheral blood monocytes and tissue macrophages, which are likely to start an acute phase response after a stimulus. Furthermore, the differences in susceptibility to AA among individuals could be due to the differences in the immune system. Therefore, the role of these cytokines in the pathogenesis and in the susceptibility of AA could be studied together.

In most cases of AA, *E. faecalis* is the bacterium which has been isolated. To date, it is still not known whether the STs, which were found both in *E. faecalis* isolates from poultry and human isolates, are normal inhabitants of the gastro-intestinal tract or represent a genuine zoonosis. This fact shows the lack of knowledge about this bacterium. In addition, traditional management strategies such as preventive use of, or therapeutic treatment with, antibiotics are insufficient in preventing *E. faecalis* infections because of its ability to acquire antimicrobial resistance and its capacity to transfer virulence genes. In addition, antibiotic use in animal production is expected to substantially decrease in the future, which shows the necessity of a thorough understanding of the risk from field *E. faecalis* strains in order to develop effective control strategies against AA.

5.1.2. Embryo lethality assay

According to the Seventh Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States of the European Commission (2013), rodents and rabbits represented more than 80% of the total number of animal used in experiments in the European Union in 2011. The third largest group of animals used was birds with a 6% of the total use. The birds were mainly used for studies on animal diseases on avian influenza, Gumboro disease and bronchitis including studies on quality and safety of vaccines.

The adult avian challenge assay is the most commonly used method for determining the virulence of different microbial pathogens to date, as well as to study the pathogenesis of different avian infections and the selection for disease resistance. However, this method requires exposure of the birds to disease agents in a controlled manner, usually by chicken inoculation, which leads two main difficulties. On the one hand, such assay is complicate to carry out in diagnostic laboratories in order to characterize the avian strains or to study the pathogenesis of infections. On the other hand, it cannot normally be done in the pedigreed population under selection, due to the risk of killing excessive numbers of breeders and reducing effective selection for other traits. Additionally, the adult avian challenge assay is limited by relatively high costs, labour intensive and ethical animal welfare considerations. Consequently, the ELA has emerged as an alternative method to characterize the virulence of different fungi (Jacobsen *et al.*, 2010) and bacteria isolated (Wooley *et al.*, 2000; Gibbs *et al.*, 2003; Gibbs & Wooley, 2003; Rudolph 2004; Montgomery *et al.*, 2005; Oh *et al.*, 2012; Nix *et al.*, 2006; Townsend *et al.*, 2008; Stewart-Tull *et al.*, 2009; Polakowska *et al.*, 2012; Seo *et al.*, 2013; Borst *et al.*, 2014, Andersson *et al.*, 2015 and Jung *et al.*, 2017). Comparison of adult avian challenge assay and ELA has been reported by some authors. Gibbs & Wooley

(2003) compared the EMR produced by *E. coli* strains through the adult avian challenge assay and the ELA. A high positive correlation ($r=0.823$, $P<0.0001$) was reported and the correlation was even higher between the ELA and the combined mortality/morbidity of the adult avian challenge assay ($r=0.861$, $P<0.0001$) since the ELA results in more deaths compared to the chicken challenge. These results were confirmed by Rudolph (2004) with *E. faecalis* strains and by Seo *et al.* (2013) with *R. anatipestifer*, who reported that both assays have therefore the ability to distinguish among the virulence of different strains. These authors agree that the ELA is faster, more sensitive, less expensive, more specific and less labor intensive than the adult avian challenge assay. In addition, Gibbs & Wooley (2003) reported that the correlation between the ELA and the adult avian challenge assay is higher when more embryos per challenge are used, although Wolley *et al.* (2000) reported that 11 embryos per strain are sufficient to observe significant differences between strains versus the 30 chickens per strain required for the adult avian challenge assay.

Specifically, an ELA with two avian *E. faecalis* strains was already published by Rudolph (2004). However, this author used a single infectious dose (2500 CFU/ml) of the avian *E. faecalis* strain K923/96, which produced a high EMR from 90% to 100% that would not allow a clear differentiation among the *E. faecalis* strains. Additionally, since the EMR produced during an ELA is influenced by several factors as previously mentioned in Chapter 2, the first step was to establish a generic methodology of the ELA for avian *E. faecalis* field strains and thus identify any details needed to be addressed before the main data collection goes ahead.

The virulence and the LD₅₀ of the well-known pathogenic avian *E. faecalis* strain K923/96 were estimated in the present dissertation in order to use it as reference strain in further analyses. The results of the Experiment 1 (Chapter 2) showed that the EMR depends

on the infectious dose; the higher the infectious dose, the greater the EMR, thus indicating the importance to determine an appropriate infectious dose which should not be too high to avoid a massive embryonic mortality. In this regard, the LD₅₀ can be considered appropriate in order to establish an infection limit of 50% lethality. The estimated doses at LD₅₀ for the inoculation of these chicken embryos lines with the *E. faecalis* strain K923/96 was 6.6 CFU/ml (Figure 2.1.). To my knowledge, this is the first time that the LD₅₀ of an *E. faecalis* strain to inoculate chicken embryos is estimated. Noteworthy is that the LD₅₀ estimated is relatively low compared to the infectious dose of *E. cecorum* (10³ CFU/ml) strains able to produce around 50% embryonic mortality (Borst *et al.*, 2014). This might be explained by the differences in virulence among the bacterial species. However, this estimation was reliable since similar results were obtained after develop several ELAs with the same strain and infection dose in the present dissertation. In accordance with Gibbs *et al.* (2003) and Montgomery *et al.* (2005), it is important to interpret the results of an ELA with caution because it is possible to observe variability between individual ELAs, although the relative virulence provides accurate results. As it has been shown in the different experiments even with the same infectious doses, there is still a variation among the ELAs. Therefore, it is a good practice to do some repetitions of the experiments to obtain more reliable results.

In regard to the embryo survival, regardless of the infection dose and the strain virulence, all ELAs conducted in this dissertation showed a fast decrease in survival rate from two to four days p.i., whereas the survival rate decreased more slowly afterwards in accordance with the results of several authors (Wooley *et al.*, 2000; Gibbs *et al.*, 2003; Gibbs & Wooley, 2003; Stewart-Tull *et al.*, 2009; Seo *et al.*, 2013 and Borst *et al.*, 2014). Therefore, the experiment duration can be reduced to four days p.i. as was done in the Experiment 3 (Chapter 4) of the present dissertation in order to optimise the ELAs.

The results obtained showed that the embryo survival depends on the infectious dose; the higher the infectious dose, the lower the embryo survival time (Figure 2.2.), which confirms the results obtained by Nix *et al.* (2006). As shown in Figure 3.5., the embryonic survival probability also decrease significantly when the embryos are infected with more virulent than avirulent *E. faecalis* strains in accordance with the results of Wooley *et al.* (2000) with *E. coli* strains. These authors observed a relationship between the virulence degree of the strains and the lesions of the embryos infected in contrast with the results obtained in the present dissertation. As shown in Figure 3.6., all infected embryos in the Experiment 2 (Chapter 3) showed similar lesions regardless of the virulence degree of the strains, which were also similar than the embryos infected with *E. cecorum* strains by Borst *et al.* (2014). Most severe lesions were observed at three and four days p.i. when the higher EMR was registered, which could be explained by the capacity of the strains to replicate *in ovo* after the inoculation as previously reported by several authors (Wooley *et al.*, 2000; Rudolph, 2004; Montgomery *et al.*, 2005; Townsend *et al.*, 2008; Stewart-Tull *et al.*, 2009 and Seo *et al.*, 2013) with different bacteria. Unfortunately, the capacity of the strains to replicate *in ovo* after the inoculation could not be confirmed in this study due to the fast growth rate of the *E. faecalis* strains in the allantoic fluid. The viable count of the allantoic fluid of the infected dead and surviving embryos was usually much higher (> 1000 CFU) than the initial number of CFU/ml contained in the infectious dose. Additionally, as shown in Figures 3.3. and 3.4., the real number of CFU administered to the embryos cannot explain the EMR produced by each strain since the number of CFU for a same infectious dose varied among the strains and no showed relationship with the EMR in accordance with the results of Gibbs *et al.* (2003). Therefore, the results obtained suggest that for a same infectious dose, the ability of the strain to produce embryonic disease or death is not related with the number of CFU administered to the embryos, but with their capacity to replicate *in ovo* after the

inoculation. Researches on the growth rate of different species of *Enterococcus* during the ELAs are required in order to corroborate this theory.

In summary, the ELA can be considered a good method to achieve the principle of the “Three Rs” (3Rs) for more ethical use of animals in research:

- Replacement: the ELA replaces adult avian challenge assays with embryos.
- Reduction: the ELA enables researchers to obtain comparable information from 11 embryos (Wooley *et al.*, 2000) versus the 30 chickens required by the adult avian challenge assay (Gibbs & Wolley, 2003). Besides, the use of the chicks in further research concerning epidemiology and pathogenesis of *E. faecalis* associated diseases after the hatch might be possible.
- Refinement: the ELA minimizes potential pain, suffering or distress until 4 days p.i.

5.1.3. Virulence characterization of avian *E. faecalis* field strains

As described in Experiment 2 (Chapter 3) of the present dissertation, the ELAs showed different EMRs according to the strains. The virulence classification of the *E. faecalis* strains used in the present dissertation was the classification previously reported by Rudolph (2004), i.e., the strains with an EMR below 40% were considered avirulent, above 80% virulent strains and between 40% and 80% were classified as strains of moderate virulence (Figure 3.3.). However, Wooley *et al.* (2000) classified the *E. coli* strains with an EMR below 10% as avirulent, above 29% as virulent strains and between 10% and 29% as strains of moderate virulence according to the clinical cases of colibacillosis, and reported that commensal *E. coli* strains produced lower EMR. Seo *et al.* (2013) used this same classification to sort *R. anatipestifer* strains. However, these authors observed that some strains isolated from clinically normal ducks were classified through the ELA as moderate

virulence strains and, therefore, suggest the evaluation of these strains by the duck lethality assay. Similar findings were obtained in the present dissertation. As shown in [Figure 3.2.](#), no clear relationship was observed among the clinical signs observed in the chickens and the virulence obtained through the ELA, which could be partially explained by the infectious dose used. The LD₅₀ of the *E. faecalis* strain K923/96 was used to evaluate the virulence degree of all other strains, which does not mean that this infectious dose is able to kill half of the embryos with another *E. faecalis* strain. Therefore, although the LD₅₀ of each strain was not assayed, it might be different for each one. The LD₅₀ estimated in the present dissertation should be considered as an infectious dose of reference.

As shown in [Figure 3.5.](#), the embryos infected with avirulent strains had less probability of dying than the embryos infected with more virulent strains. Jung *et al.* (2017) also observed that virulent *E. cecorum* strains of different animal species isolated from clinical signs and/or pathological changes produced significantly higher EMR than avirulent strains. However, these authors did not observe differences among the avirulent and virulent strains identified through the Matrix-Assisted Linear Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS), neither by using 16S-rRNA-gene sequencing and either by the detection of the fatty acid composition by gas chromatography. As shown in [Figure 3.2.](#), *E. faecalis* strains with different pathogenic degree estimated in the present dissertation through the ELA were also not clearly clustered by the ERIC-PCR in accordance with Eaton & Gasson (2001), who reported that the molecular taxonomy of *Enterococcus spp.* might not lead to a differentiation between avirulent and virulent strains. Nevertheless, ERIC-PCR revealed a considerable genetic diversity among avian *E. faecalis* strains in accordance with the results of Jurkovič *et al.* (2007) and Bachtiar *et al.* (2015), and, therefore, it can be applied successfully and be useful to obtain the genetic distribution and epidemiology of *E. faecalis* strains.

Virulence of the Enterococci still cannot be fully explained because they are considered commensals pathogen of the endogenous intestinal microflora of humans and birds and, as such, have subtle virulence traits that are not easily identified. As revised by Eaton & Gasson (2001), this bacterium possesses virulence traits such as adherence and invasion of tissues and abscess formation, modulation of host inflammatory responses, and secretion of toxic products. The molecular typing methods have identified several STs within the *E. faecalis* population associated with different human and avian diseases. Although different STs have been related with AA in poultry, the ST82 has been specifically related with this disease worldwide (Petersen *et al.*, 2007). As shown in [Figure 3.2](#), the results obtained did not show a clear relationship between the ST82 and the virulence degree since 22% and 67% of the *E. faecalis* strain classified as avirulent and moderate virulence, respectively, were ST82. Although all virulent strains were ST82, they only represent the 10% of all strains that contained the ST82. On the contrary, 78% from the 18 *E. faecalis* strains with absence of ST82 were classified as avirulent and 22% as strains of moderate virulence. However, it seems that the ERIC-PCR tended to cluster the strains regarding the ST82, as shown in [Figure 3.2](#).

Additionally, Eaton & Gasson (2001) reported that there is a risk that an avirulent strain of *Enterococci* that lacks of virulence genes could acquire them by conjugation since some virulence genes are associated with highly transmissible plasmids. Skyberg *et al.* (2003) reported that there seems to be a relationship between the virulence of *E. coli* strains for embryos and the number of genes present in the strains, which is in contrast with the results obtained by Jung *et al.* (2017), who did not find significantly more virulence genes in the virulent *E. cecorum* strains than avirulent strains. The results obtained in the present dissertation neither show a clear relationship between the pathogenicity of the strains and their virulence genes since all strains used in the present dissertation showed the same profile of

virulence genes and produced however different virulence degree through the ELA. These findings suggest that the virulence genes are not the only cause of virulence and more investigations are therefore required to determine the role of the virulence genes and STs in the virulence of the *E. faecalis* strains. It is possible that a thorough analysis of a representative number of avirulent and virulent strains using the whole genome sequencing shows significant differences and maybe also new putative virulence factors.

5.1.4. Genetic variation in susceptibility to *E. faecalis* infection

The ELA with avian *E. faecalis* field strains was first carried out on white fertilized eggs because the candle is pretty easier and requires less labour intensive to see the embryos in white eggs than in brown eggs. However, AA has never been reported in field cases in white layer breeds and they are more resistant to induced AA than brown layers according to Ovelgönne *et al.* (2001). Therefore, the genetic selection to reduce susceptibility to *E. faecalis* would make sense for brown layer chickens. In this regard, a preliminary study with brown fertilized embryos was carried out under the hypothesis that chicken embryos of brown layers would be less resistant to *E. faecalis* infection compared with embryos of white layers when they are infected with the same strain and infectious dose.

The results obtained in the preliminary study of the Experiment 3 (Chapter 4) support this hypothesis. In accordance with the results of the Experiment 1 (Chapter 2) (Table 2.2.), the infectious dose in brown embryos used in the preliminary study of the Experiment 3 (Chapter 4) also showed a significant effect on the EMR (Table 4.1.). However, the infectious doses of 5 and 2.5 CFU/ml differed significantly from each other in contrast with the results of the Experiment 1 (Chapter 2) with white layers. The EMR of the brown embryos was higher than of the white embryos, even when the experiment duration was shorter. The EMR of the brown embryos was 83% with the infectious dose of 5 CFU/ml and 69% with 2.5

CFU/ml while the EMR of the white embryos was 45% with the infectious dose of 5 CFU/ml and 43% with 2.5 CFU/ml. These results prove genetic variation in susceptibility to *E. faecalis* infection between brown and white breed layers.

5.1.5. Genetic selection against susceptibility to *E. faecalis* infection

Breeding for disease resistance in poultry such as Marek's disease, avian leukosis viruses, salmonellosis and colibacillosis has been researched. However, to my knowledge, this is the first time that breeding for resistance to *E. faecalis* infection has been assessed. In accordance with the estimated heritabilities for resistance to different diseases, the estimated heritability for embryonic survivability in the present dissertation ($h^2 = 0.17 - 0.20$, depending on the model used) shows that selection for more resistant laying hens against *E. faecalis* infection is feasible (Table 4.4.). Besides, the average EMR obtained per family during the three ELAs showed a standard deviation of 23%, as shown in Figure 4.1. Genetic selection for resistance to *E. faecalis* infection will contribute to the reduction of the incidence of AA, the disease-related costs, decrease the consumption of antibiotics, and will improve the welfare of the future layers produced. However, it should be taken into account the estimated correlations between embryonic survivability and other economical important traits because it allows prediction of the extent to which selection for increased resistance will reduce the gain in the other criteria.

As shown in Table 4.5., favourable genetic correlations were found between embryonic survivability and EW ($r_g = +0.16$), as well as with the egg production at the beginning of the lay ($r_g = +0.31$), but not with BS ($r_g = -0.48$ to -0.30), the egg production at peak of lay ($r_g = -0.69$) and at the end of the egg production ($r_g = -0.55$). Besides, no genetic correlation with BW ($r_g = -0.04$) and K_{dyn} ($r_g = -0.05$) was found.

Selection for disease resistance based on adult avian challenge assays may not be feasible by the need of test an excessive number of breeders on the pedigree population. Therefore, marker gene(s) of resistance or gene itself has been proposed as the most promising research area, which could be assessed from a blood sample by molecular analysis. Cavero *et al.* (2009) reported that information on genetic markers will avoid the inconvenience of experimental challenges and it could be done before production traits are measured, even in pure-line pedigree stocks. However, very little is known to date about the effects of the genes involved in resistance to poultry diseases and genetic resistance to the majority of poultry diseases is usually controlled by many genes as well as influenced by environmental conditions (Beaumont *et al.*, 2003), which makes it difficult for practical implementation. In this regard, the results obtained from the ELA could be used to try to find markers related to resistance to *E. faecalis*. Besides, embryos of pure-line pedigree stocks could be tested and selected without subjecting breeding stock to disease conditions.

It should be noted that the Experiment 3 (Chapter 4) was done with a single virulent *E. faecalis* strain. However, many other different strains of *E. faecalis* have been isolated from AA field outbreaks, as shown in [Figure 3.2](#). Therefore, the lower susceptibility to one strain does not automatically mean a general improvement in immune response against all strains, but rather to a limited range of them. Besides, the economic importance of bacterial diseases as whole is much higher than of *E. faecalis* infection alone and, therefore, it would be important to know whether selection against *E. faecalis* infection alters susceptibility against other pathogens.

In summary, it has been demonstrated that it is possible to breed for *E. faecalis* infection resistance through the ELA although the relationship between *E. faecalis* resistance and some economically important traits should be take into account to achieve a balanced

genetic progress. The implementation of selection against susceptibility to *E. faecalis* infection in practice will require that breeding company is convinced that AA is of significant economic importance, which depends on disease incidence, production costs and losses associated with sick and/or dead chicks, in addition to welfare and food safety concerns of consumers.

5.2. FINAL CONCLUSIONS

The general conclusions of the present dissertation are summarized as follow:

- Virulence characterization of avian *E. faecalis* field isolates allows us to know the risk that the strains represent and provide valuable information, which can be important for the selection of isolated for the production of vaccines. The ERIC-PCR can be applied successfully to obtain the genetic distribution and epidemiology of *E. faecalis* strains.
- The estimated heritability for susceptibility to the infection by *E. faecalis* was $h^2 = 0.17-0.20$, depending on the model used, indicating that the selection for increased resistance to *E. faecalis* infection is feasible.
- The ELA can be considered a reliable and useful tool to evaluate the virulence of avian *E. faecalis* strains by using the EMR as well as to select more resistant laying hens against *E. faecalis* infection, which can be used as alternative model to replace the adult avian challenge assays. It is important to determine an appropriate infectious dose which should not be too high to avoid a massive embryonic mortality. The obtained LD₅₀ of the *E. faecalis* strain K923/96 can be used as reference in further ELAs on *E. faecalis* virulence and the ELA can be optimized by reducing the time of study until four days p.i.

5.3. SUGGESTIONS FOR FURTHER RESEARCHES

Although major progress in the understanding of AA has been gained in the last twenty years, the present dissertation revealed the lack of knowledge about it and the pathogenesis of *E. faecalis* associated disease. Besides, based on the results of the current dissertation and given the economic importance of AA, as well the animal welfare concerns, I would like to suggest some ideas for further research:

- Evaluate the effects of different agents on the treatment of AA and clarify if the amyloid deposits are really irreversible.
- Investigate the role of different agents involved in the pathogenesis of AA, such as the cytokines or ILs.
- Quantitative studies of the extrahepatic production of SAA, which may act as nidus of amyloid fibrils, to investigate the possibility that the synoviocytes of brown layers might proliferate more strongly than the synoviocytes of white layers.
- Study the grow rate of different species of *Enterococcus* during the ELAs in order to know if the ability of the strain to produce embryonic disease or death is related with their capacity to replicate *in ovo* after the inoculation.
- Determine the role of the virulence genes and STs in the virulence of the avian *E. faecalis* field strains as well as to clarify whether the STs are normal inhabitants of the gastrointestinal tract or represent a genuine zoonosis.
- Develop autogenous vaccines from proven high pathogenic avian *E. faecalis* strains.
- Investigate whether selection against *E. faecalis* infection alters susceptibility against other pathogens.

ETHICAL STATEMENT

Avian embryos are currently not regulated by legislation as animal experiments in Germany (<https://www.gesetze-im-internet.de/tierschg/BJNR012770972.html>) and, therefore, an approval from an ethics committee was not required for this thesis. Besides, all ELAs developed in the present dissertation were finished four and seven days prior to hatching, on developmental day 17 and 14, respectively.

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