Universitat Autònoma de Barcelona

MÁSTER UNIVERSITARIO EN ZOONOSIS Y UNA SOLA SALUD

Study of alternative strategies to antimicrobial use in poultry farming: the role of the intestinal microbiota

Official Master in Zoonosis and One Health

Curso 2018-2019

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ABSTRACT

Nowadays, antibiotic resistance is one of the main public health problems. Finding alternative strategies to the use of antibiotics in veterinary medicine is one of the great challenges. In poultry production (and in other food animals) some of these possible alternatives would be: a) to take advantage of bacterial cell to cell communication (quorum sensing) and the disruption of that communication (quorum quenching) to control the proliferation pathogens; b) the use of additives in animal feed which modulate the intestinal microbiota, promoting bacterial populations with antibacterial activity. In this study we aimed to: a) to investigate the production of acylhomoserine lactone (AHL) by *Yersinia enterocolitica* 057 under different conditions and its presence in the gut contents of chickens; b) to investigate the potential antimicrobial activity of the intestinal microbiota against different pathogens in three groups of chickens fed with different feed. These different diets (conventional diet, diet with xylanase, diet with xylanase and xylooligosaccharides) may promote different intestinal microbiota, and thus different antimicrobial activity, if any. The results suggest that the intestinal microbiota may interfere in the growth of *Y. enterocolitica* and/or its production of AHL. On the other hand, no antimicrobial activity against the pathogens tested could be detected in any of the 3 groups of chickens. The different growth conditions may have influenced the different bacterial populations able to grow on the agar plates and therefore the bacteria with potential antimicrobial activity.

1. INTRODUCCION

Antibiotic resistance is one of the main public health problems. Currently, finding strategies to find alternatives to the use of antimicrobials in livestock production is a key issue, to reduce the presence and spread of antimicrobial resistant bacteria. One of these alternatives could be to take advantage of bacterial cell to cell communication, the so called quorum sensing (QS), which is a key regulatory system in the pathogenesis of various bacterial infections (Boyen and al., 2000). The disruption of QS, called quorum quenching, would reduce the pathogenicity of the strains. Another tool of great utility in the veterinary field could be the use of feeding strategies, such as using feed additives (e.g., enzymes or prebiotic compounds) with antimicrobial activity or that modulates the intestinal microbiota, favouring bacterial populations with antimicrobial activity against pathogens of interest.

1.1 Quorum sensing

Several studies have evidenced that bacterial cells communicate with each other, allowing the control of a wide range of activities. The bacterial communication between bacteria belonging to the same species or different species is mediated by chemical signals synthesized and secreted by various types of bacteria (Jayaraman and Wood, 2008). These signals allow to regulate numerous physiological functions, such as symbiosis, virulence, motility, sporulation, antimicrobial peptide synthesis or the formation of biofilm (Lindum and al.,1998)

The bacterial communication system that modulates gene expression depending on cell density is called quorum sensing. In this communication system, cells produce and release diffusible signal molecules called autoinducers (Miller and Bassler, 2001). The signal molecules are produced and secreted at a basal level during bacterial growth. Their concentration in the environmental medium or matrix increases as the bacterial population expands, and when it reaches a threshold level (quorum level), it induces phenotypic effects (Czajkowski and Jafra 2009). This phenomenon occurs without any external intervention and is also referred to as autoinduction (Nealson and al., 1970). QS systems were originally described in the marine bacterium *Vibrio fischeri* (Nealson and al., 1970) as a being involved in the control of light production. This bacterium only emits light when it reaches a cell density or quorum.

1.2.1. Quorum sensing in gram-negative bacteria: autoinducing polypeptides

Gram-negative bacteria generally produce acylated homoserine lactones (AHL), which are also called auto-inducer 1 (AI-1). These consist of a lactone ring (homoserin lactone, HSL) linked by an amide bond to an acid that forms a side chain whose length can be between 4 and 18 carbons, saturated or unsaturated and with or without oxo- or hydroxy-substitutions in the third carbon (Whitehead and al., 2001) (Figure 1.1).

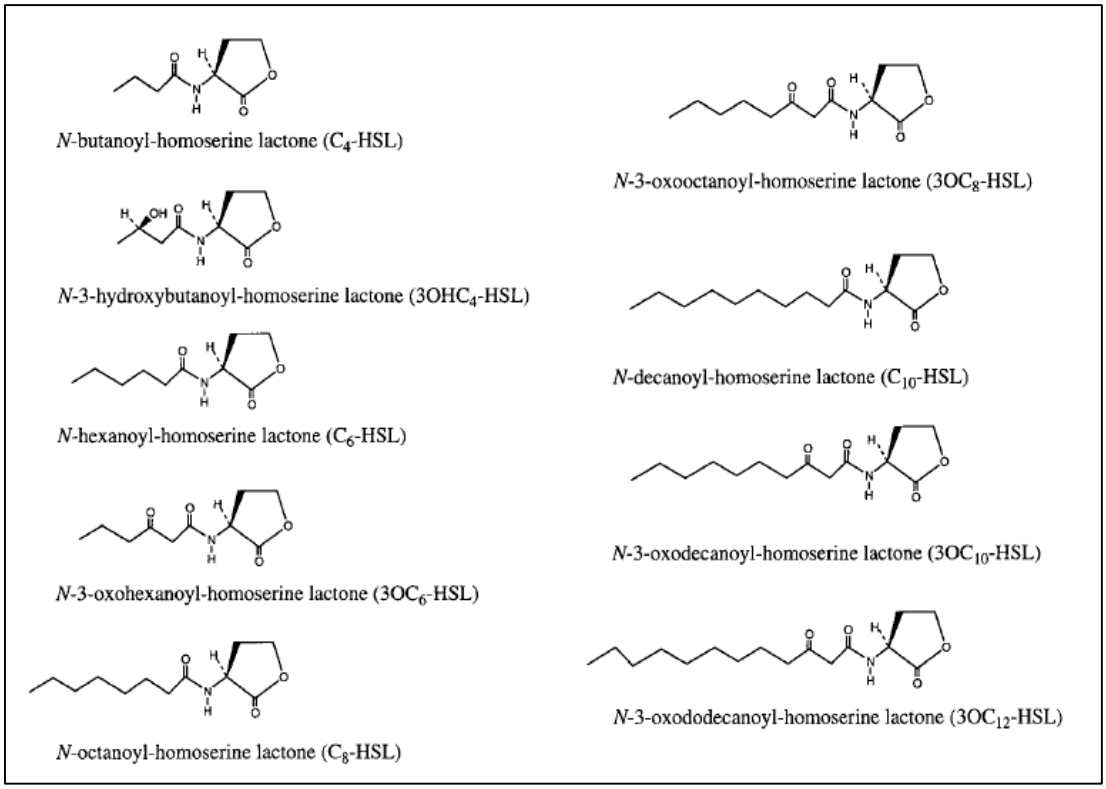


Figure 1.1. Chemical structures of N-acyl HSL autoinducers (Guan and al., 2000)

The quorum sensing mechanism of most gram-negative bacteria resembles that of the marine bacterium *Vibrio fischeri*. The *LuxI*-like proteins are responsible for the biosynthesis of a specific AHLs, which increase in concentration as the cell density also increases. When a threshold of concentration of AHL is reached, they bind to its receptor protein *LuxR*, which induces the expression of different target genes (Steidle and al. 2002) (Figure 1.2). There is a considerable diversity in the way in which the *LuxI* and *LuxR* homologues operate in the different bacterial species. Since *LuxR* type proteins only detect specific AHL (Walters and Sperandio 2006), this QS system is mainly used for intraspecific communication. For example, *V. fischeri* produces 3 chemically distinct autoinducers: N-hexanoyl-L-homoserin lactone (C6-HLS) and oxo-N-hexanoyl-L-

homoserin lactone (OC6-HSL) synthesized via *LuxI*. The third autoinducer, N-octanoyl-L-homoserin lactone (C8-HSL) dependent on a new gene called ainS, belongs to a second family of AHL synthetases that do not show homology with *LuxI* type enzymes (Gilson and al., 1995).

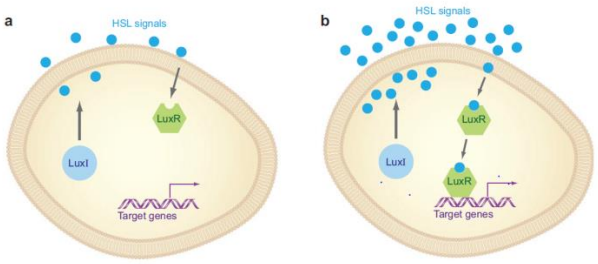


Figure 1.2. Quorum sensing in gram negative bacteria. Acyl-homoserine lactone (acyl-HSL) signals (blue circles) are produced by the LuxI enzyme homologues that bind to LuxR homologues to activate expression of target genes. a) at low cell densities, concentration of the signal is low both inside and outside the cell, with minimal activation of LuxR. b) at high cell densities, acyl-HSL activates LuxR through binding and leads to expression of downstream target genes (Waters and Bassler, 2005).

1.2.2. Quorum sensing in gram-positive bacteria: autoinducing polypeptides

The autoinducer molecules in gram-positive bacteria are modified oligopeptides. These molecules, unlike the acyl-homoserine lactones, are very specific and give the strain that possesses it the ability to communicate intraspecifically. Oligopeptides do not diffuse through the plasma membrane, and they need a specific transporter, which generally modifies the structure of the autoinducer. It also needs two receptors: a membrane histidine kinase and a protein that interacts with DNA and activates transcription. The signal produced is transmitted by a cascade of phosphorylation / dephosphorylation (Figure 1.3). Therefore, this mechanism is much more complex than that described in gram-negative bacteria (Sturme and al., 2002).

The simplest example of these QS systems is that described in *Staphylococcus aureus*. The Agr (accessory gene regulator) system of *S. aureus* consists of four agr genes that code for the expression of four proteins: AgrD that synthesizes the peptide autoinducer, AgrC which synthesizes the histidine kinase responsible for transmitting the signal from the plasma membrane to the regulatory molecule of the transcriptional response, AgrA responsible for synthesizing the regulatory molecule of the transcriptional response and finally AgrB excretory protein that modifies the thiolactone ring of the peptide autoinducer (Tenover and al., 2000).

When the concentration of *S. aureus* is very low, the Agr system is functioning constitutively, producing basal levels of the proteins mentioned above. In this way the autoinducer is present in the extracellular medium, and the adhesion and colonization factors to surfaces, which are QS-dependent, are produced. When *S. aureus* levels increase, the peptide autoinducer binds to the membrane histidine kinase produced by a phosphorylation signal of the transcriptional regulator which binds to DNA with two effects: one is to induce the transcription of a regulatory RNA (RNA III) that allows the expression of virulence factors and represses the expression of colonization factors and adhesion to surfaces. The second effect is the induction of the expression of the Agr operon (D, C, A, B), which reactivates the system (Waters and Bassler, 2005).

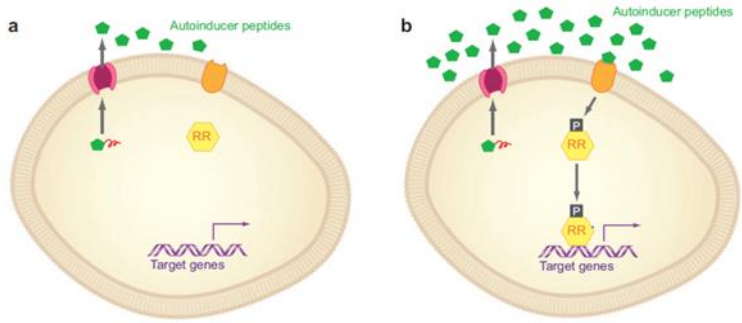


Figure 1.3. Quorum sensing with autoinducer peptides (AIPs) (green pentagon). AIPs are produced as precursor peptides and exported out of the cell (A). At low cell densities, concentration of the AIP signal is low outside the cell and there is no activation of the response regulator (RR). (B) At high cell densities, binding of the AIP to a histidine kinase receptor leads to phosphorylation of the RR and expression of downstream target genes (Waters and Bassler, 2005)

1.2.3. Autoinducer-2: QS of gram-positive and gram-negative bacteria

A third type of autoinducer is the so-called AI-2, whose structure is a diester furanosyl borate. This autoinducer was identified for the first time in another bioluminescent marine *Vibrio* species, *V. harveyi*. While AHLs and peptide autoinducers are highly specific and are used for intercellular communication within the same species, AI-2 is found in both gram-positive and gram-negative bacteria, indicating that it could act as the most universal interspecific chemical language, since in many cases both types of detection systems coexist (Miller and Bassler, 2001; Federle and Bassler, 2003).

1.3. Detection AHL

Several methods and sensor systems have been developed for the detection, characterization and quantification of AHL molecules.

One of them are biosensor bacteria. Biosensor bacteria do not have AHLs synthesis proteins but contain the related AHL receptor proteins and functional genes. Under exogenous AHLs stimulation, the expression of reporter genes can be initiated, which are then perceptible by the changes in colony colour, luminescence or enzyme activities. An example of this is strain CV026 of *Chromobacterium violaceum*. In this bacterium, the synthesis of purple violacein pigment, insoluble in water, is regulated by a QS system, based on the production of AHL, allowing its detection (McClean and al, 1997). Another example would be a strain of *E. coli* transformed with the *luxR* gene of *V. fischeri* coupled to the promoter and *lux* operon, but from which the *luxI* gene is eliminated and therefore there is no production of AHL. This strain does not produce light unless it is provided with an exogenous AHL (Romerno, 2010). There are other biosensor *E. coli* strains, such as *E. coli* 578 which produce a green fluorescent protein (gfp) in the presence of AHL (Andersen and al. 2001).

Thin Layer Chromatography (TLC) is a type of Liquid-Solid Absorption Chromatography (LSAC) commonly used in conjunction with biosensors. Usually, AHL standards and test samples are loaded in the TLC plate and immersed in the developing solution, causing samples to migrate with different speed. After the plate is dried, the culture media that contain biosensor is then added on the plate for culture. Signals used for determining the types of AHL via comparison with the AHL standards are colour changes or luminescence in the colonization sites of the biosensor (Huang and al., 2012).

High-Performance Liquid Chromatography tandem Mass Spectrometry (HPLC-MS) is a physicochemical detection method based on the different retention times of AHLs due to their molecular weights (Gui and al. 2018). The AHLs successively enter the mass spectrometer, and their molecular structures are determined based on ion charge-to-mass ratio. In recent years, Ultra-High-Performance Liquid Chromatography (UHPLC) has been used increasingly as the faster and more sensitive chromatography in the detection of AHLs for its great application potential. Other physicochemical and photochemical methods, such as Gas Chromatograph tandem MS (GCMS) and

Electrospray Ionization tandem MS (ESI-MS), are also widely applied in the detection of AHLs (Liu and al. 2018).

1.4. AHL producers, the case of *Yersinia* spp.

Y. enterocolitica is a gram-negative bacterium, belonging to the group of enterobacteria that is frequently colonizing the digestive system of animals. The species *Y. enterocolitica* is highly heterogeneous and can be subdivided in several bioserotypes, with only a few of them associated with human disease (Drummond and al. 2012). Most *Y. enterocolitica* strains associated with human yersiniosis belong to bioserotypes 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3, and 4/O:3. to 6 biotypes (Bottone, 1997).

Animals are asymptomatic carriers of the bacteria and they shed the enteropathogen in the environment with their stools. The pathogen is of interest in food safety for its zoonotic potential (Gnanasekaran and al. 2017). *Y. enterocolitica* is transmitted through the fecal-oral route and represents the third cause of bacterial diarrhea in Europe, after campylobacteriosis and salmonellosis (EFSA). Several studies have detected the presence of quorum sensing molecules in bacteria of the genus *Yersinia*. For example, Atkinson et al. (2006) showed that *Y. pseudotuberculosis* produces at least 8 different types of AHLs. Throup et al. (1995) reported the production of C6-HSL and 3-oxo-C6-HSL from *Y. enterocolitica*. But in most of these studies, the detection of lactones produced by *Yersinia* has been studied in laboratory culture media. There is no data available on the potential production of AHL by *Y. enterocolitica* in the digestive tract of animals, and the possible effects that other bacteria of the microbiota could have on the production of them.

1.5. Feeding strategies: the use of enzymes in animal feed.

The addition of additives in the feed is a widespread practice in poultry since they modify the conditions of the gastrointestinal tract, improving the digestive process. The improvement of the digestive process has numerous advantages such as increased production rates, better nutritional use, reduction of polluting waste, etc (Selle et Ravindran, 2007; Bedford, 2018). Feed additives include a wide variety of compounds, such as probiotics, prebiotics, organic acids, enzymes. There are two large groups of enzymes used as feed additives: a) phytases, which increase the release of phosphorus

from ingredients of plant origin, decreasing the incorporation of inorganic sources in the formulation and increasing the digestibility of amino acids; b) xylanases, acting on non-starchy polysaccharides, allowing the release of energy of the nutrients (González-Ortiz and al., 2019; Roofchaei and al, 2019)

Barley, wheat and rye are common ingredients in poultry feed. Among the components of these cereals there are macromolecules such as celluloses, hemicelluloses and pectins. These are difficult or almost impossible to digest by monogastric animals and are classified as non-starch polysaccharides (NSP). The arabinoxylans represent 50-70% of the NSP.

Poultry do not have enzymes of microbial origin in the stomach or small intestine that promote the digestion of NSP (De Maesschalck and al., 2015). These enzymes are only elaborated by the microbiota of the large intestine when the absorption of nutrients has already taken place.

The mechanisms of action of xylanases are (Bedford and Schulze, 1998):

1. Degradation of the cell wall of the plant cells, which retain easily digested nutrients in its interior, which, without the enzymatic contribution, cannot be used, resulting in lower growth and a lower conversion rate (Stone, 2004).

2. Decrease intestinal viscosity. The NSP form gels in the digestive tract, which produce an increase in the viscosity of the intestinal content. Viscosity retards intestinal transit and facilitates passage of pathogenic microorganisms from the large intestine to the thin one. Viscosity also reduces the efficiency of enzymes, since it does not allow access to the substrate (Danicke and al., 2000; Bedford and Schulze, 1998). Thus, the xylanases hydrolyze the NSP, contributing to the reduction of the viscosity and therefore, improving the intestinal transit.

3. The rupture of cell walls, and reduction of the length of the chains of viscous polymers of difficult digestion, results in compounds such as arabino oligosaccharides or xylo-oligosaccharides (XOS) that have prebiotic action. These promote lactate and butyrate producing bacteria, favouring the production of short chain fatty acids (SCFA) (Graham and al., 2003; De Maesschalck and al., 2015; González Ortiz and al. 2019). SCFA are easily absorbed contributing to a

better use of the feed, added to numerous beneficial effects as an influence on the immune system, specifically the butyrate that has an anti-inflammatory effect (Ros Berruezo and al., 2011)

Addition of enzymes or compounds with prebiotic potential such as XOS in animal feed has a direct impact on the intestinal microbiota, favouring the proliferation of beneficial bacteria such as *Bifidobacteria* or *Lactobacillus*, which have antagonistic activity against pathogenic bacteria (e.g. by inhibiting their growth) such as *Salmonella*, *Campylobacter*, *Clostridium* or *E. coli* that are frequently found colonizing the intestinal tract of poultry (Fooks and Gibson, 2002; Woodmansey, 2007; Boyen and al., 2009).

2. OBJECTIVES

- ✓ To investigate the production of acylhomoserine lactone (AHL) by *Yersinia enterocolitica* 057 under different conditions. This includes:

    a) To determine if the chicken intestinal microbiota influences the production of AHL by *Y. enterocolitica* 057.

    b) To investigate the presence of AHL in the caecal content of three groups of chickens fed with different feed and determine the potential differences among them.

- ✓ To assess the antimicrobial activity of the intestinal microbiota against different pathogens in three groups of chickens fed with different feed.

3. MATERIALS AND METHODS

3.1. Acylhomoserine lactone (AHL) detection

3.1.1. Production of AHL by *Y. enterocolitica* 057 in culture medium under different conditions

A colony of the AHL producer *Y. enterocolitica* 057 (Medina-Martínez et al., 2007), which had been grown in Luria-Bertani (LB) agar for 24h at 30ºC was inoculated in a series of 10 ml of LB broth. Each of the inoculated tubes were subjected to different incubation conditions (Table 3.1).

Table 3.1. Incubation conditions of the *Y. enterocolitica* cultures

	12 h		24 h		48 h	
30ºC	Aerobic (170 rpm)	Anaerobic	Aerobic (170 rpm)	Anaerobic	Aerobic (170 rpm)	Anaerobic
37ºC	Aerobic (170 rpm)	Anaerobic	Aerobic (170 rpm)	Anaerobic	Aerobic (170 rpm)	Anaerobic

After the incubation time, the resulting cultures were centrifuged (14000 rpm, 4ºC, 10 min) and the supernatants were filter sterilized (Whatman. FP 30/0.2 CA-S, pore size 0.22 μm). The filter sterilized supernatants were screened for the presence of AHL by the indirect fluorescence method using the biosensor strain *Escherichia coli* 578 (Andersen et al., 2001). All samples were tested in triplicate. An overnight culture of *E. coli* 578 in LB with 2 μg/ml tetracycline, grown at 30ºC with shaking (170 rpm) was prepared. The culture was diluted in LB to obtain a bacterial suspension of DO600≈ 0,40-0,5. A 100 μl of this *E. coli* 578 suspension was mixed with 50 μl of each of the sterile supernatants in a microtiter plate. Fluorescence detection was performed in a fluorescence reader FLx800 (Bio-Tek Instruments Inc., USA) with an excitation wavelength of 475 nm and emission detection at 515 nm. Positive (natural AHL in LB medium) and negative (LB medium) controls were run in parallel. The AHL detection was performed after 3 h, 5 h and 6.50 h of incubation with the biosensor *E. coli* at 30ºC.

3.1.2. Detection of AHL produced by *Y. enterocolitica* 057 after seeding samples of caecal content homogenates from hens

Ten caecum samples from 35-week-old hens of an ongoing trial were used. For convenience, these 10 caeca were divided into two groups (Group C, Group V), corresponding to the same groups of the trial. Caeca were stored at -80ºC until use.

Initially, to adjust the technique, 2 caeca from group C and 2 caeca from group V were used. The caecal content from each group were pooled and homogenized (5 g of caecal content in 2 ml LB). A 1 g aliquot for each group of samples was prepared in triplicate.

The AHL producer *Y. enterocolitica* 057 was grown in LB broth and incubated overnight at 30°C with shaking (170 rpm). After incubation, the culture was adjusted to a DO600 ≈ 0,5 and a volume of 500 µl, 100 µl or 0 µl was added to each of the tubes containing the 1g of caecal content, and vortexed (Figure 3.1). Those mixtures were centrifuged (14000 rpm, 4ºC, 10 min). The resulting supernatant was filter sterilized as above. All this process was carried out in parallel for both groups of samples (C and V).

The resulting filtered supernatants were frozen at -20°C until the detection of lactones was performed (≤ 3 days).

After the fine-tuning of the technique, the definitive lactone detection was carried out with the rest of the samples (C and V). The procedure was essentially the same, with some modifications: the pooled caecal contents (pools of 3 samples) were diluted 1/2 and 1/100 in LB.

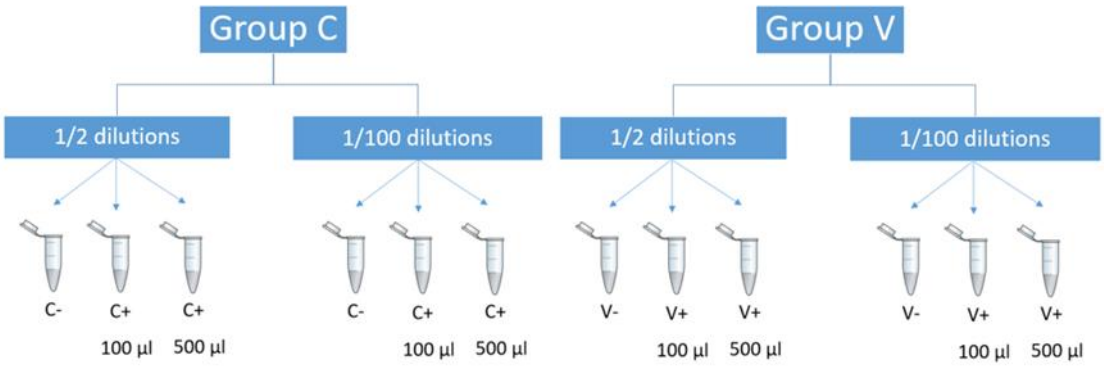


Figure 3.1. Diagram of the dilutions performed. Legend: C: samples group C; V: samples group V; -: uninoculated; +: 100 µl or 500 µl of *Y. enterocolitica* added .

The filter sterilized supernatants were screened for the presence of AHL by the indirect fluorescence method using the biosensor strain of *E. coli* 578 as described in section 3.1.1.

3.1.3. Detection of AHL production by *Y. enterocolitica* after seeding homogenates of caecal content of hens and subsequent incubation

The same caeca samples were used as in section 3.1.2. A 1/2 and 1/100 dilutions in LB of the caecum contents was performed and 4 aliquots of each of these two diluted caecal samples was prepared. Two aliquots of each subset of samples were inoculated with 500 µl of an overnight culture of *Y. entercolitica* 057 adjusted to DO600≈ 0,5 (as described in section 3.1.2), and the remaining two were uninoculated controls (Figure 3.2). Subsequently, each pair of inoculated and inoculated test tubes were subjected to different incubation conditions:

a) 24 h, 30 ºC, aerobiosis and shaking (170 rpm).

b) 24 h, 30ºC, anaerobiosis.

c) 48 h, 30ºC, aerobiosis and shaking (170 rpm)

d) 48 h, 30ºC, anaerobiosis.

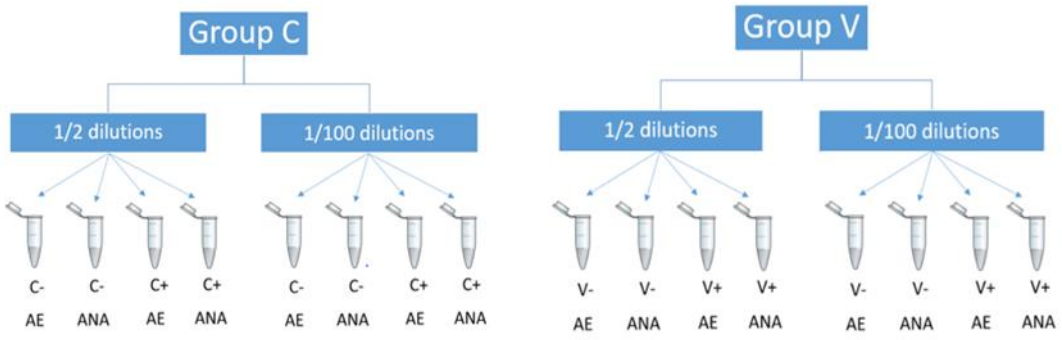


Figure 3.2. Diagram of the dilutions performed and incubation conditions. Legend: C: samples group C; V: samples group V; -: uninoculated +: 500 µl of *Y. enterocolitica* added. AE: aerobic conditions; ANA: anaerobic conditions

After incubation, centrifugation was carried out (14000 rpm, 4ºC, 10 min) and the supernatant was filtersterilized. The filtersterilized supernatants were screened for the presence of AHL by the indirect fluorescence method using the biosensor strain of *E. coli* 578 described in section 3.1.1.

3.1.4. Detection of AHL by *Y. enterocolitica* in caecal content of chickens fed with different animal diets

The dilution 10^{-1} of the samples of caecal content of chickens fed with different diets (see section 3.2.1) was used for the AHL detection. The 10^{-1} dilutions of each of the samples were centrifuged (14000 rpm, 4ºC, 10 min) and filter-sterilized. Those sterilized supernatants were screened for the presence of AHL by the indirect fluorescence method using the biosensor strain of *E. coli* 578 as described above (section 3.1.1).

3.2. Detection of the *in vitro* antimicrobial activity of the intestinal contents of chickens fed with different animal diets

3.2.1. Antimicrobial activity testing set up

To set up the method to detect the inhibitory capacity of intestinal contents of chickens from a trial where two different treatments were tested, samples of fresh chicken caecal discharges from a different study were used. Those were samples from an ongoing study at the farms of the Autonomous University of Barcelona.

Seven strains of four enteric pathogens of interest in poultry production were selected to be tested:

Table 3.2. Bacterial strains tested

Bacteria	**Strain**
Clostridium perfringens	GP435
C. jejuni	ES84
C. jejuni	ES115
***Salmonella* Enteritidis**	CS35
***Salmonella* Typhimurium**	CS34
E. coli	EDL933
E. coli	GN 709

The assessment of the antimicrobial activity was carried out by the agar diffusion method (Bauer and al., 1966). For this, a fresh culture of the selected pathogens was prepared on agar plates from stock cultures stored at -80 ºC in Brain- Heart Infusion (BHI) broth and supplemented with 20% glycerol. Each bacterial strain was and supplemented with 20% glycerol. Each bacterial strain was streaked and incubated under appropriate conditions (Table 3.3).

Table 3.3. Incubation conditions and culture media for the bacterial strains tested

Bacterial strains	**Culture media**	**Incubation conditions**
Clostridium GP435	BHI agar	24h, 37ºC, anaerobic
E. coli EDL933	BHI agar	24h, 37ºC, aerobic
E. coli GN709	BHI agar	24h, 37ºC, aerobic
Salmonella Enteritidis *CS35*	BHI agar	24h, 37ºC, aerobic
Salmonella Typhimurium CS34	BHI agar	24h, 37ºC, aerobic
C. jejuni ES84-3-21d-l12-C1	Blood agar	48h, 37ºC, microaerobic
C. jejuni ES84-3-21d-l12-C1	Blood agar	48h, 37ºC, microaerobic

Next, a suspension of each bacteria was prepared from the fresh cultures and adjusted to a turbidity equivalent to MacFarland 1 in saline for the strains of *C. jejuni* and 0.5 for the remaining strains. Within 15 minutes of having adjusted the inoculum, a sterile swab was soaked into the bacterial suspension and the agar plates (agar BHI agar or blood agar depending on the pathogen) were inoculated with the respective microorganisms by even streaking of the swab over the entire surface of the plate three times and rotating the agar plates 60º approximately after each application.

Homogenates of the caecal contents were prepared by diluting them 1/2 in PBS and serial ten-fold dilutions (10^{-1}, 10^{-2}, 10^{-3}) were made. A volume of 10 µl of those was deposited directly onto the agar plates previously streaked with each of the tested bacteria and allowed to dry for 10 min. The 10 µl volume was decided according to previous reports (Pérez andt al., 2011; Sanchez García and al., 2016). Plates were incubated in the appropriate conditions (Table 3.3)

After the incubation time, the plates were read. Positive samples were those in which an inhibition zone was observed around the mixed growth of the 10 µl drops. Negative samples were those in which no inhibition zone was observed. If there was a positive result, the mixed culture of one of the 10 µl drops was picked with a sterile loop and streaked onto the same agar medium and incubation conditions in which the inhibition was observed. After incubation, the bacterial growth (a mixed culture) was collected with

a sterile loop and a frozen stock culture at -80ºC in BHI with 20% glycerol was prepared for further analysis.

The mixed cultures showing antimicrobial activity (inhibition zone on the agar plate) and stored at -80ºC, where re-tested by making dilutions of a fresh culture and repeating the procedure. That is, inoculating 10 µl of the mixed bacterial suspension onto a previously streaked agar plate with the corresponding strain that was inhibited in the first test. Plates were checked for the presence/absence of an inhibition zone.

After having set up the technique, the study samples were analysed (Section 3.2.2).

3.2.2. Detection of the *in vitro* antimicrobial activity of the caecal content of chickens fed with different animal diets

Overall, 24 caecum samples (frozen at -80ºC) from 35 days-old Ross 308 chickens fed different diets were tested. These chickens were part of a trial with an overall 300 birds, which were allocated in three groups (n= 100 each group), corresponding to the three tested diets: control group (standard diet), treatment group 1 (diet supplemented with xylanase) and treatment group 2 (diet supplemented with xylo-oligosaccharides + xylanase). For each treatment there were ten replicates, and ten birds per pen. The caecum samples received corresponded to 8 chickens from each treatment group, from four different pens and two birds per pen (Annex, Table 1).

Thus, the caecum of 8 chickens from each group was analyzed for the presence of intestinal microbiota showing antimicrobial activity.

The agar diffusion test described in section 3.2.1 was followed to determine if there was antimicrobial activity in the caecum samples. However, after having analysed half of the samples, it was decided to remove the 10^{-3} dilution, and replace it by the initial caecal homogenate (cecal content diluted 1/2).

3.2.3. Detection of *in vitro* antimicrobial activity of the ileum content of chickens fed with different animal diets

Prospectively, three ileum samples per group (overall 9 samples) from the same birds from section 3.2.2 were tested. The same procedure as in section 3.2.2 was followed.

4. RESULTS AND DISCUSSION

4.1. Acylhomoserine lactone (AHL) detection

4.1.1. Production of AHL by *Y. enterocolitica* 057 in culture medium under different conditions

An assay was conducted to assess if there were substantial differences in AHL production by *Y. enterocolitica* 057 when grown under different conditions (aerobic vs anaerobic; different incubation times). No relevant differences were found among the different incubation conditions, with a similar fluorescence among the different groups of samples (Figure 4.1). However, a slight reduction of fluorescence was found in those cultures grown up to 24 h or 48 h, compared with those grown for 12h, thus indicating a lower amount of AHL present in the cultures. This might be due to an autoregulatory process of the bacterium, that when a threshold of AHL is achieved, the bacterium itself has the enzymatic machinery to degrade the QS molecules. This phenomenon has been described by numerous authors in different organisms, such as *Agrobacterium* spp. or *Pseudomonas putida* (Barbosa and al., 2010; Uroz and al., 2009).

Overall, the fluorescence signal was slightly higher in those samples incubated at 30ºC and aerobic conditions. AHL production by *Y. enterocolitica* at 30ºC and 37ºC and under aerobic conditions has been reported (Atkinson and al., 2006; Ng and al., 2018). However, there are no reports assessing the AHL production of *Y. enterocolitica* under anaerobic conditions. The production of AHL in the anaerobic environment suggests that the bacterium would be able to produce the QS signal in the intestine of animals. Such production however depends on many factors including metabolites derived from feed (Adachi and al., 2018; Ng and and., 2018). As expected, the longer the supernatants were incubated with the biosensor strain, the strongest the fluorescence signal.

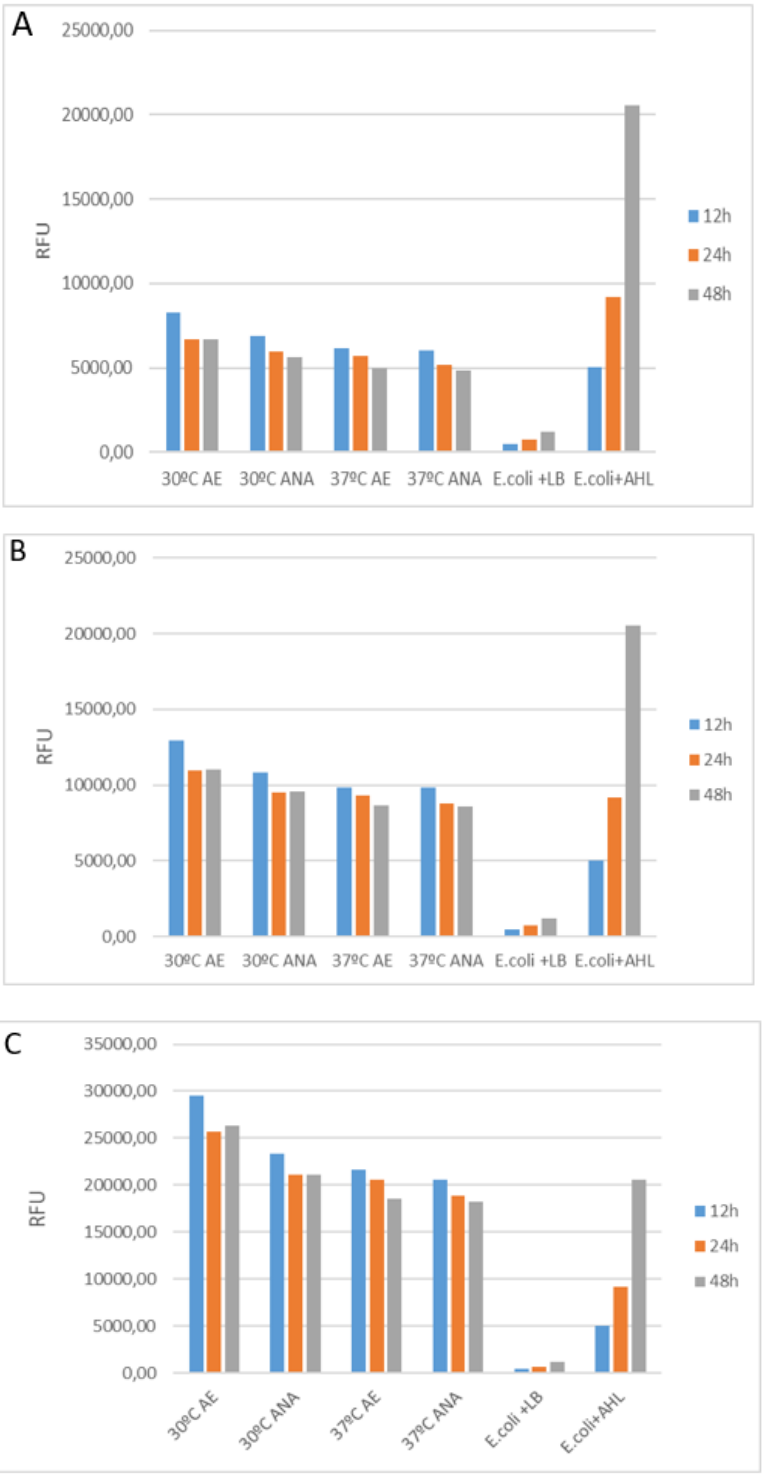


Figure 4.1_AHL detection in the sterile supernatant of *Y. enterocolitica* 057 after 12 h, 24 h and 48 h of incubation under different conditions. Detection of AHL was performed after incubation for: A) 3 h, B) 5h, C) 9h of the supernatants with the biosensor strain. The results are expressed as Relative Fluorescence Units (RFU). Legend: C (group C), V (group V), - (uninoculated), + (inoculated with Y. enterocolitica), E. coli + LB (negative control), E. coli + AHL (positive control).

4.1.2. Detection of AHL produced by *Y. enterocolitica* 057 after seeding samples of caecal content homogenates from hens

In order to assess if using the above method (section 4.1.1.) it was possible to detect AHLs present in the intestinal contents of caecal contents of poultry, and to adjust the methodology, aliquots of caecal contents homogenates from hens from an ongoing study were seeded with different volumes of an overnight culture of *Y. enterocolitica* 057. All sterile supernatants from caecal samples seeded with *Y. enterocolitica* 057 showed fluorescence, thus indicating the presence of AHL (Figure 4.2). Higher fluorescence was observed in those samples seeded with a higher volume of the AHL- producing bacterium. However, in those samples not seeded with the bacterium, no fluorescence was observed, which indicates that there was no AHLs or in two low concentrations to detect them.

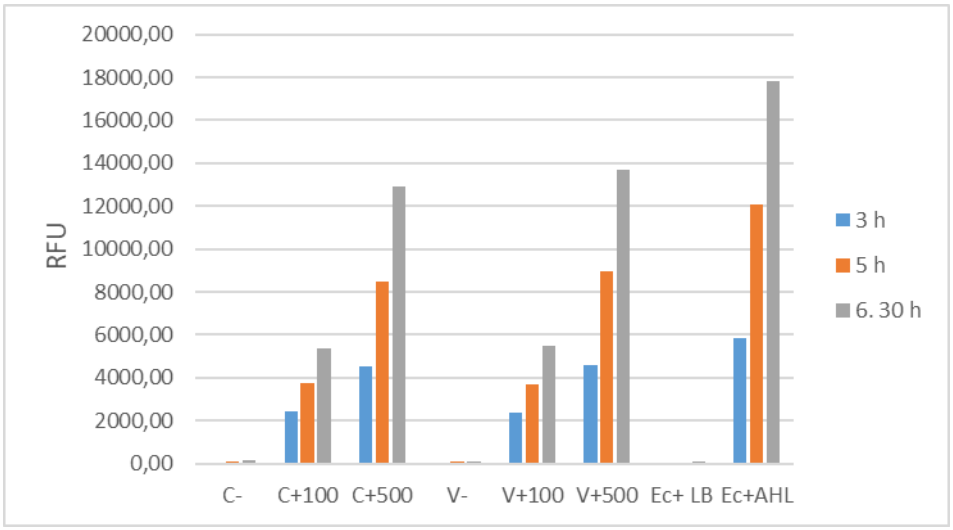
Caecal samples were diluted up to 1/100 to overcome potential inhibition of AHL production by compounds of bacteria present in the samples. No relevant differences were found in fluorescence between samples diluted 1/100 and samples diluted 1/2 seeded with *Y. enterocolitica* 057 (Figure 4.2).

As the incubation time of the supernatants with the biosensor strain *E. coli* 578 increases, the fluorescence signal increases, but after only 3 h of incubation it is already possible to detect fluorescence.

4.1.3. Detection of AHL production by *Y. enterocolitica* 057 after seeding homogenates of caecal content of hens and subsequent incubation

As observed in the previous assay, when adding the AHL-producer strain in caecal samples, lactones are detected in the sterile supernatants of the mixtures. In this assay we wanted to assess if the *Yersinia* strain is able to produce AHL when cultured with the microbiota present in the caeca of hens, since this accompanying bacterial population may inhibit *Yersinia* growth or its AHL production. Thus, caecal contents were inoculated with *Y. enterocolitica* 057 and incubated under different conditions. In the supernatants resulting from the caecal content diluted 1/2 and seeded with *Y. enterocolitica* 057 no fluorescence was detected in any of the samples (Figure 4.4), unlike the samples diluted 1/100 (Figures 4.3).

A



B

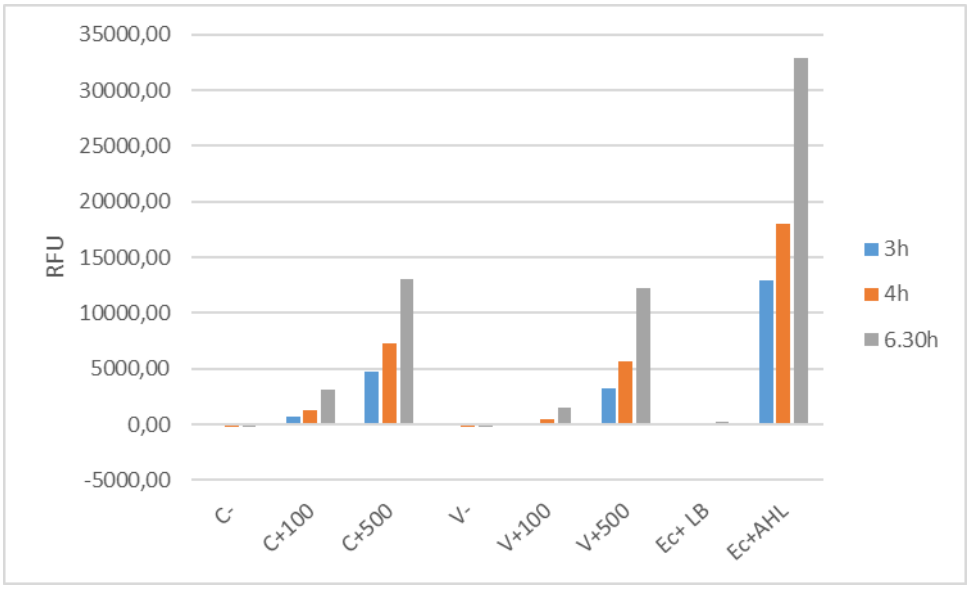
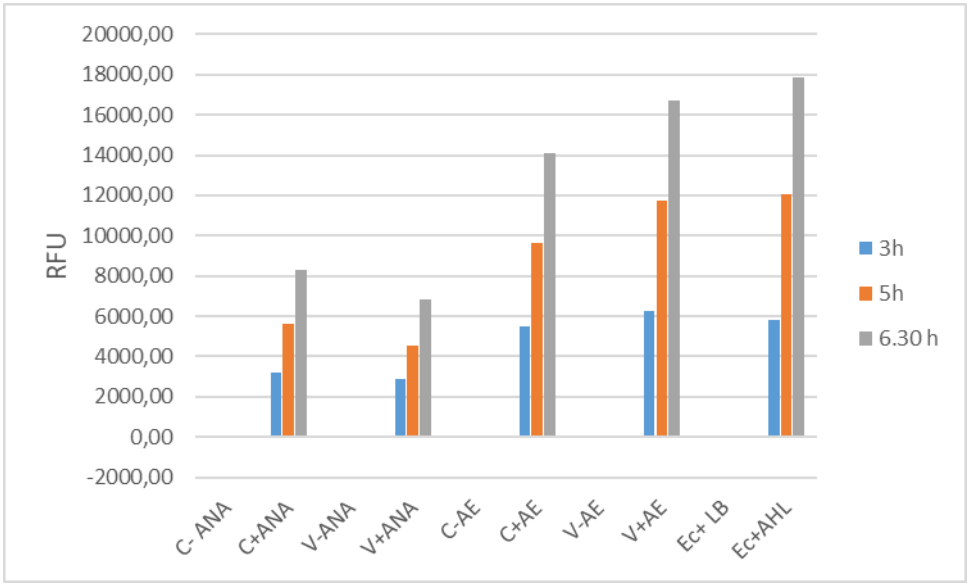


Figure 4.2_AHL detection in sterile supernatants after 3 h, 5 h and 6.30 h of incubation with *E. coli 578* biosensor strain. Samples tested are supernatants derived from the mixture of *Y. enterocolitica* 057 with caecal contents diluted A)1 /100 or B) 1/2. The results are expressed as Relative Fluorescence Units (RFU). Legend: C (group C), V (group V), - (uninoculated), + 100 (inoculated with 100 µl of *Y. enterocolitica*), + 500 (inoculated with 500 µl of *Y. enterocolitica*), *Ec* + LB (*E. coli* without AHL, negative control), *Ec* + AHL (*E. coli* with AHL, positive control).

A



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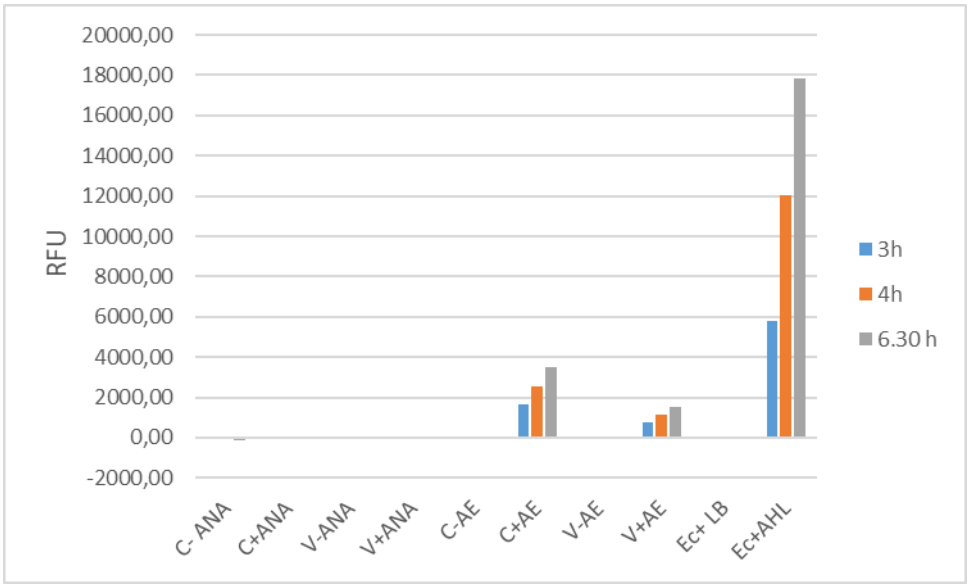
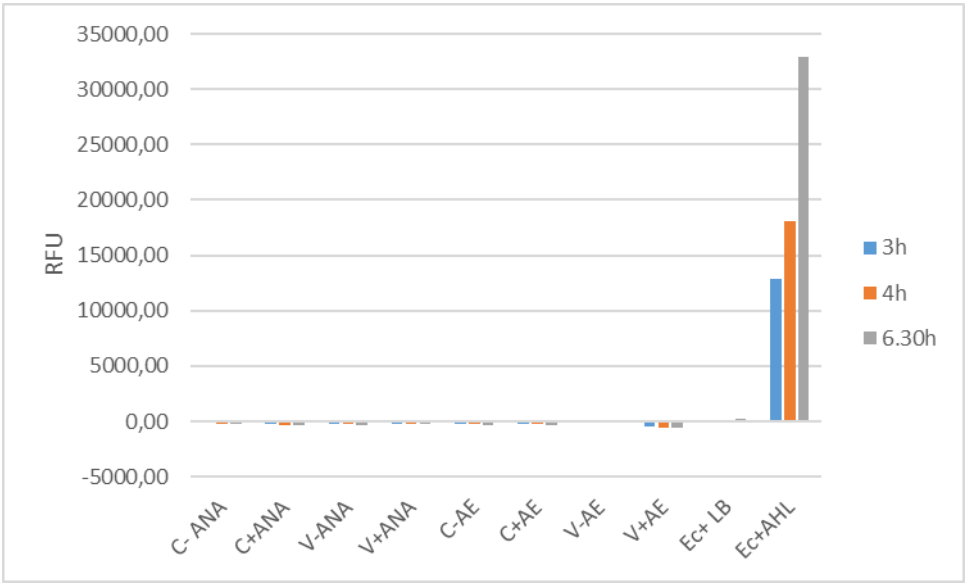


Figure 4.3_ AHL detection from cocultures of *Y. enterocolitica 057* with caecal content diluted 1/100 after A) 24 h B) 48 h of incubation under different conditions. Supernatants incubated with the biosensor strain *E.coli 578* during 3 h, 5 h and 6.30 h. Results are expressed as Relative Fluorescence Units (RFU). Legend: C (group C), V (group V), - (uninoculated), + 100 (inoculated with 100 µl of *Y. enterocolitica*), + 500 (inoculated with 500 µl of *Y. enterocolitica*), Ec + LB (negative control), Ec + AHL (positive control).

A



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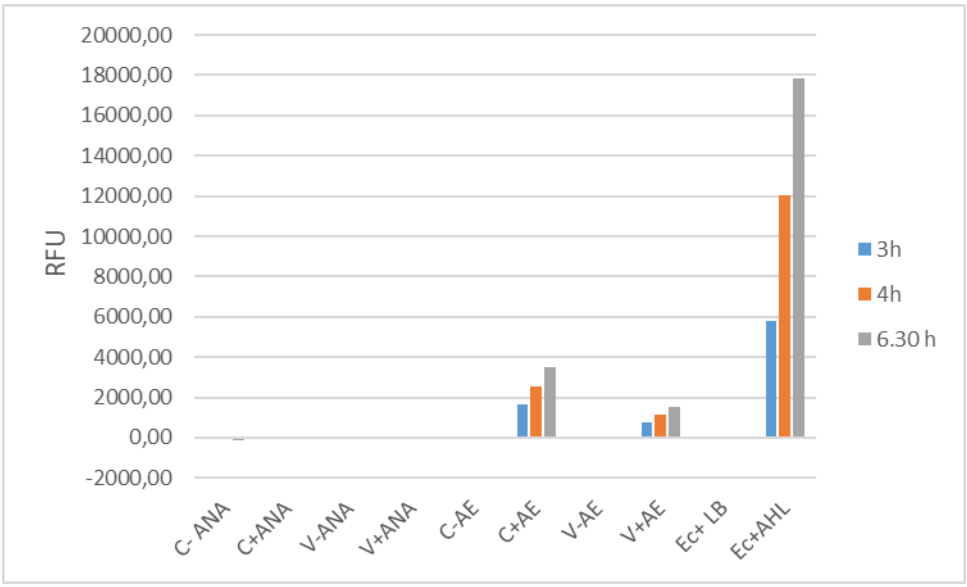


Figure 4.4_ AHL detection from cocultures of *Y. enterocolitica 057* with caecal content diluted 1/2 after A) 24 h B) 48 h of incubation under different conditions. Supernatants incubated with the biosensor strain *E.coli 578* during 3 h, 5 h and 6.30 h. Results are expressed as Relative Fluorescence Units (RFU). Legend: C (group C), V (group V), - (uninoculated), + 100 (inoculated with 100 µl of *Y. enterocolitica*), + 500 (inoculated with 500 µl of *Y. enterocolitica*), Ec + LB (negative control), Ec + AHL (positive control).

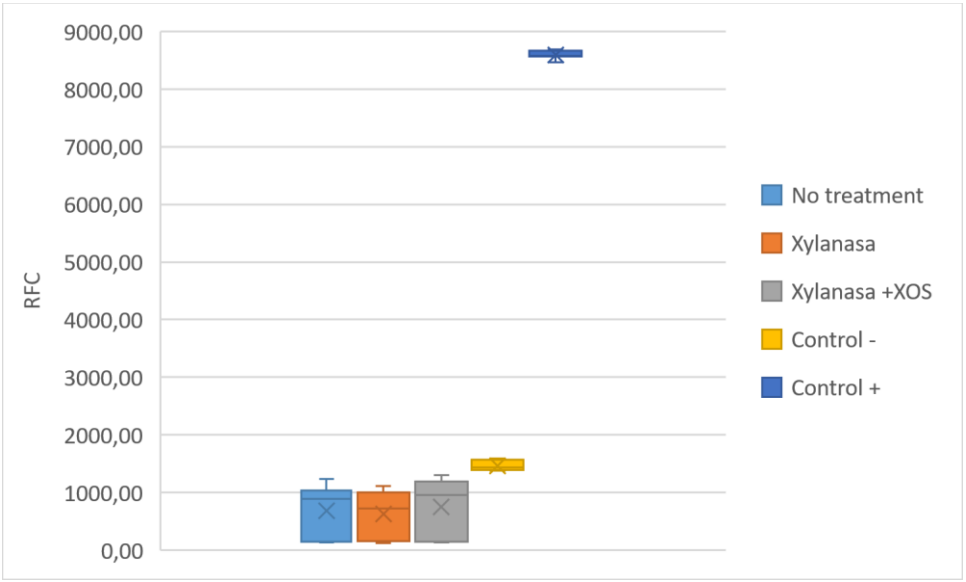
On the other hand, in caecal samples diluted 1/100, where AHLs are detected, different production of AHL occurs in cultures incubated in aerobic conditions vs anaerobic conditions (Figure 4.3 and 4.4). Under aerobiosis conditions a greater amount of AHL is produced, and therefore, detected. These differences may be due to the different bacteria that proliferates under this different growth conditions. In anaerobiosis certain microorganisms that have proliferated may have inhibited *Y. enterocolitica* growth (Kim and al., 2018), since in pure culture (section 4.1.1.) there are no relevant differences in AHL production by this bacteria when grown in aerobic or anaerobic conditions. Probably aerobic conditions favour the growth of different bacteria which may not interfere with the growth and production of AHL by *Y. enterocolitica*.

On the other hand, as noted in section 4.1.1, the levels of fluorescence detected at 48 h had decreased with respect to those recorded at 24 h. However, those differences between 24 h to 48 h incubation are clearly marked in this assay, compared with the assay performed in pure cultures of *Y. enterocolitica* (section 4.1.1) (Figures 4.3 and 4.4).

4.1.4. Detection of AHL in caecal content of chickens fed with different animal diets

The 1/10 dilution of the caecal contents of the 24 chickens investigated for its antimicrobial activity (see below, section 4.2.) were also investigated for the presence of AHL. Those chickens were distributed in three groups which were fed with 3 different diets. No AHL was detected in any of the samples (Figure 4.5). This, apparently, absence of AHLs in the intestinal contents might be because there has been no production of lactones by the caecal bacteria or, despite its presence in the gut, its concentration was in very low levels, below the limit of detection of the method used. With the biosensor *E. coli* 578 used in this study, it is possible to detect a minimum of 1 nM to 10 nM of OC6-HSL and C6-HSL, respectively (Andersen and al., 2001).

A



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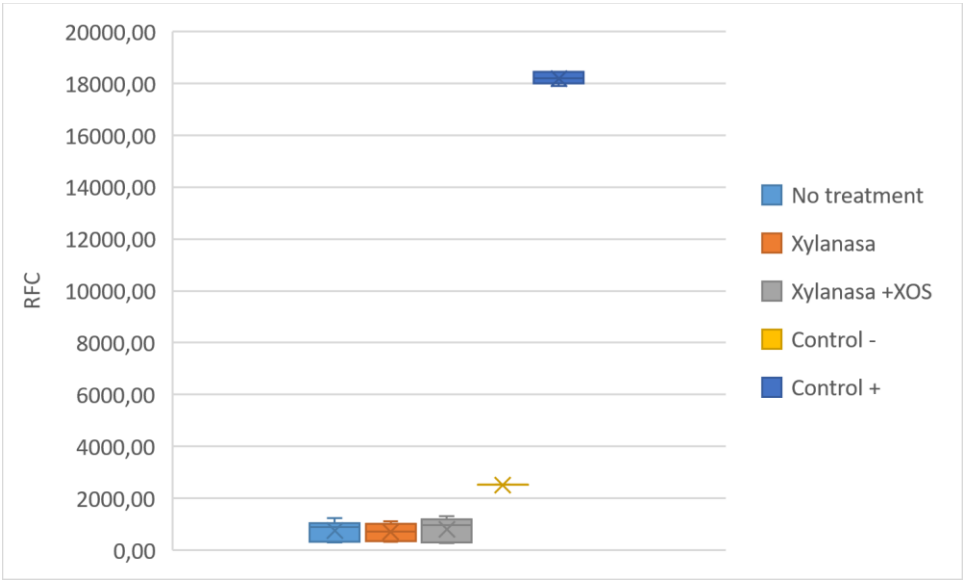

Figure 4.5_AHL detection of caecal content of chickens fed with different animal diets. Supernatants incubated with the biosensor strain *E. coli 578* during A) 3 h B) 4 h

4.2. Detection of the *in vitro* antimicrobial activity of the intestinal contents of chickens fed with different animal diets

4.2.1. Antimicrobial activity testing set up

Six samples of caecal discharges of chickens were used to set up the agar diffusion method to determine the antimicrobial activity of intestinal contents against enteric pathogens in poultry. Ten µl of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of each of the faecal discharges were deposited in previously inoculated plates with the tested pathogenic strains. The culture media used were BHI or blood agar, depending on the pathogenic strain (Table 3.3). After 24 h incubation of the plates, zones of inhibition against *E. coli* GN 709 and *E. coli* EDL 933 were observed in all the samples when the plates were incubated in aerobic conditions (Figure 4.6). The zone of inhibition observed was higher for *E. coli* EDL 933. Smaller inhibition zones were observed at dilutions 10^{-3} with respect to 10^{-1} and 10^{-2} of the caecal discharges.

In those samples where an inhibition zone was observed, the growth of the mixed culture from the 10 µl spotted onto the agar plate of one of the dilutions of the caecal discharges was picked and re-streaked to obtain a fresh growth. This newly obtained mixed culture was recovered and preserved in BHI broth with 20% glycerol for later re-test of the antimicrobial activity. When the diffusion agar test was repeated with this mixed culture an increase of the inhibition zone was observed.

No inhibition zone against *Salmonella*, *Clostridium* or *Campylobacter* was observed for any of the samples (Figure 4.7). Two of the *Campylobacter* plates were contaminated with *Proteus* and therefore it was not possible to determine if there was any inhibition zone (Figure 4.8).

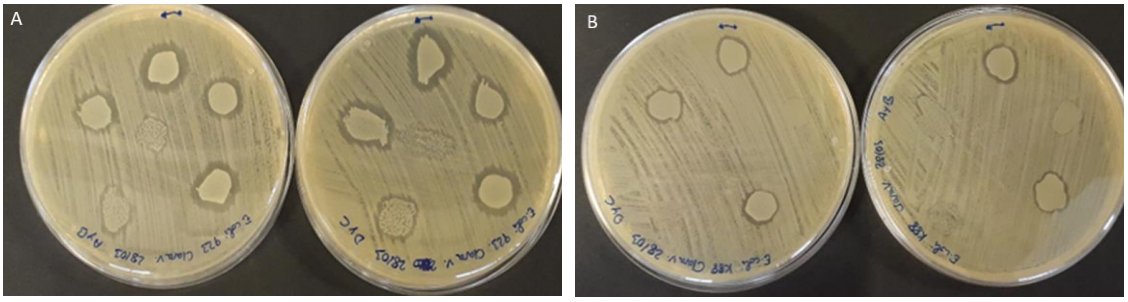

Figure 4.6_ Inhibition zone observed for A) *E.coli* EDL 933; B) *E.coli* GN 709 using the diffusion agar test.

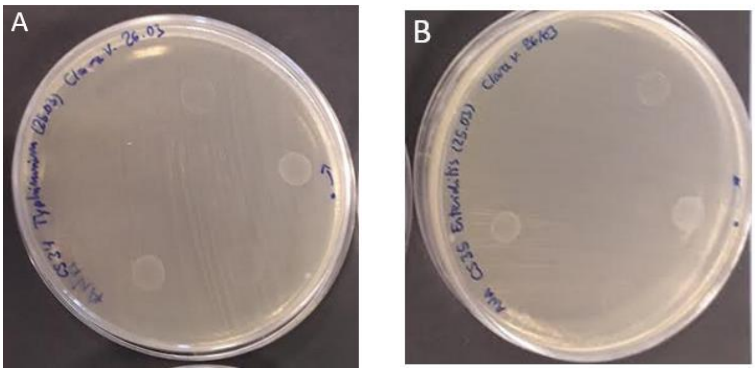

Figure 4.7_Absence of inhibition zone for *S. Tyfimurium* and *S. enteritidis* using diffusion agar test.

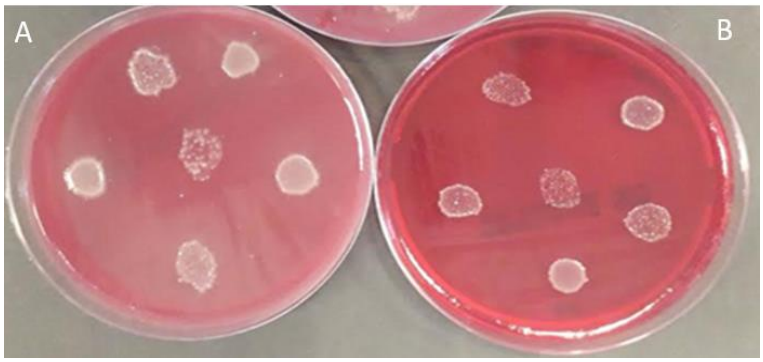

Figure 4.8_No zones of inhibition observed for A) *C. jejuni* ES84 and B) ES115 using diffusion agar test

The observation of inhibition zones suggests the presence of antimicrobial activity of the bacteria present in the gut microbiota. The antimicrobial activity can be due to several reasons such as the decrease of pH, the production of lactic acid, production of low molecular weight compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2) or diacetyls (2,3-butanedione) and high molecular weight compounds as bacteriocin (Piard and Desmazeaud, 1991; Ouwehand, 1998). Gut microbiota is mostly anaerobic (strict or facultatively) or microaerophilic. However, in this assay the antimicrobial activity was found only in *E. coli* when cutured in aerobiosis not in the plates incubated in anaerobic condions. This suggests that the bacterial population responsible for such inhibition belongs to a population of facultative anaerobic bacteria, which might be more abundant when plates are incubated in aerobic conditions compared when the incubation is in anaerobiosis. When plates are incubated in anaerobic conditions, the proportion of the different bacterial populations may vary, diminishing substantially those bacteria with antimicrobial activity, which results in the absence or not detectable of such activity. On the other hand, it is possible that a bacterium which

shows antibacterial activity when grown in aerobic conditions, does not have such activity when grown under different conditions, e.g., anaerobiosis.

Similarly, as explained above, the absence of antimicrobial activity against the other pathogens tested might be indicative of no bacteria in the caecal samples able to inhibit their growth, at least in the tested conditions.

The observation of an inhibition zone with some of the pathogens tested is an indication that the method of agar diffusion is a suitable method to perform a qualitatively screening of the possible antimicrobial activity of the samples we wanted to test. Thus, this method was chosen to screen for antimicrobial activity of samples from a trial (see section 4.2.2).

4.2.2. Detection of the *in vitro* antimicrobial activity of the caecal content of chickens fed with different animal diets

The 24 blind caecal samples belonging to 3 groups of animals subjected to 3 different treatments were tested for antimicrobial activity. No zone of inhibition was observed for *E. coli*, *Salmonella* (neither in the plates incubated in aerobiosis nor anaerobiosis), nor *Clostridium* (Annex, Table 2, 3 and 4).

For *C. jejuni*, in some of the samples from the 3 types of treatments, areas with different degree of transparency were observed (Figure 4.9). These zones could correspond to hemolysis or to inhibition of growth, but it was difficult to determine to which of these two possibilities it corresponded. Also, most of the plates were contaminated with *Proteus*, which hindered the determination of possible inhibition (Annex, Table 2, 3 and 4).

Non-detection of antimicrobial activity in these samples does not preclude the absence of such activity. One factor that could have influenced is the loss of viability of bacterial populations due to the freezing of samples as evidenced by numerous studies (Bircher and al. 2018; Lin C and al., 2019). On the other hand, different results may be obtained if i*n vivo* assays could be performed, since not all bacteria present in the intestinal contents can grow under the tested conditions. Thus, there is a proportion of the intestinal microbiota that is not possible to recover and test in the conditions used.

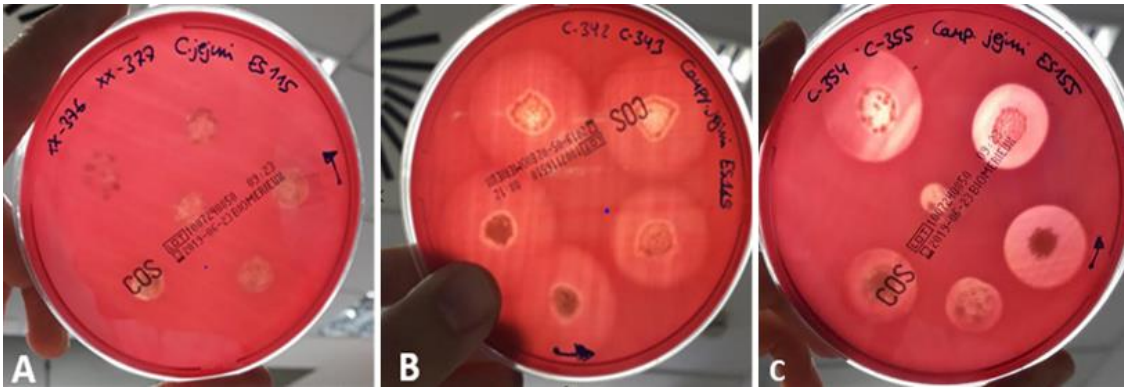

Figure 4.9_Inhibition zones for *C. jejuni* strains. A) No inhibition zone.; B) A weak inhibition zone; C) Strong inhibition zone.

4.2.3. Detection of *in vitro* antimicrobial activity of the ileum content of chickens fed with different animal diets

Since no antimicrobial activity was found in caecal samples (see section 4.2.2), a prospective screening of few ileum samples from the same birds was conducted. Thus, three ileum samples per group (overall 9 samples) were tested. As in caecal samples, no zone of inhibition was observed for *E. coli*, *Salmonella* (neither in the plates incubated in aerobiosis nor anaerobiosis), nor *Clostridium*.

For *C. jejuni*, in some of the samples from the 3 types of treatments, areas with different degree of transparency were observed. However, it was not possible to determine if there was growth inhibition or both hemolysis and inhibition of growth. Also, in some plates the presence of *Proteus* hindered the proper reading of the plates. In all these situations, the mixed growth was picked and re-streaked to store the fresh mixed culture to further retest them later on.

5. CONCLUSIONS

- *Y. enterocolitica* can produce AHLs under different growth conditions (aerobiosis, anaerobiosis; 30ºC and 37ºC). AHLs degradation occurs after 24 h.

- The indirect fluorescent method used for AHL detection allowed the assessment of the presence of these QS molecules in samples of intestinal contents.

- The concentration of the intestinal contents (dilution 1/2 vs 1/100) influence the production of AHL from *Y. enterocolitica*.

- The diffusion agar method allowed the qualitative assessment of the antimicrobial activity of intestinal microbiota.

- No AHL or antimicrobial activity could be detected in the intestinal content of animals fed the different diets (conventional diet, xylanase or xilooligosaccharides and xylanase), under the conditions tested. *In vivo* assays may lead to different results.

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ANNEXES

Table 1_Chikens sampled for caeca and ileum tested in this study.

Treatment	Pen number	Bird/sample number	Bird weight on day 35 (g)
Control	3	342	2898
Control	3	343	2422
Control	41	354	2600
Control	41	355	1942
Control	47	366	2140
Control	47	367	2222
Control	85	378	2333
Control	85	379	2830
Xylanase	5	346	2447
Xylanase	5	347	2036
Xylanase	43	358	2961
Xylanase	43	359	2489
Xylanase	46	364	2130
Xylanase	46	365	2470
Xylanase	88	384	2822
Xylanase	88	385	2790
Xylanase+XOS	1	338	2538
Xylanase+XOS	1	339	2772
Xylanase+XOS	42	356	2380
Xylanase+XOS	42	357	2560
Xylanase+XOS	50	372	2274
Xylanase+XOS	50	373	2718
Xylanase+XOS	84	376	3047
Xylanase+XOS	84	377	2906

	Bird nº	Sample dilution	*E. coli* EDL 933		*E.coli* K88		*S.* Typymurium CS34		*S. E*nteriditis CS35		*C.jejuni* ES84[a]	*C.jejuni* ES115[a]	*C. perfringens GP435*
			AE	ANA	AE	ANA	AE	ANA	AE	ANA			
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Control	**342**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Control	**343**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Control	**354**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I ++	I +++	NI
Control	**355**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I ++	I ++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I ++	I ++	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Control	**366**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -1	NI	NI	NI	NI	NI	NI	NI	NI	I+	I+	NI
Control	**367**	**Dil. -2**	NI	NI	NI	NI	NI	NI	NI	NI	I+	I+	NI
		Dil. -3	NI	NI	NI	NI	NI	NI	NI	NI	I+	I+	NI
		Dil. -1	NI	NI	NI	NI	NI	NI	NI	NI	I++	NI	NI
Control	**378**	**Dil. -2**	NI	NI	NI	NI	NI	NI	NI	NI	I++	NI	NI
		Dil. -3	NI	NI	NI	NI	NI	NI	NI	NI	I++	NI	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Control	**379**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI

Table 1_Results of the antimicrobial activity test in chicken from the control group. Dil.: dilution, NI: no inhibition of growth, I: inhibition of growth, + : weak inhibition, ++ : moderate inhibition, +++ : strong inhibition.

[a]: It could not be ruled out if the presumptive inhibition zone (either weak or strong) was an hemolysis halo.

	Bird nº	Sample dilution	*E. coli* EDL 933		*E.coli* K88		*S.* Typymurium CS34		*S. E*nteriditis CS35		*C.jejuni* ES84 [a]	*C.jejuni* ES115 [a]	*C.perfringens GP435*
			AE	ANA	AE	ANA	AE	ANA	AE	ANA			
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I ++	I ++	NI
Xylanase	**346**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
		Dil. -1	NI	NI	NI	NI	NI	NI	NI	NI	I+ d	NI	NI
Xylanase	**347**	**Dil. -2**	NI	NI	NI	NI	NI	NI	NI	NI	I+ d	NI	NI
		Dil. -3	NI	NI	NI	NI	NI	NI	NI	NI	I+ d	NI	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Xylanase	**358**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Xylanase	**359**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I ++	I +++	NI
Xylanase	**364**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I ++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I ++	I +++	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Xylanase	**365**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Xylanase	**384**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -1	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Xylanase	**385**	**Dil. -2**	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
		Dil. -3	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

Table 2_Results of the antimicrobial activity test in chicken from the xylanase diet group. Dil.: dilution, NI: no inhibition of growth, I: inhibition of growth, + : weak inhibition, ++ : moderate inhibition, +++ : strong inhibition.

[a]: It could not be ruled out if the presumptive inhibition zone (either weak or strong) was an hemolysis halo.

	Bird nº	Sample dilution	*E. coli* EDL 933		*E.coli* K88		*S.* Typymurium CS34		*S. E*nteriditis CS35		*C.jejuni* ES84[a]	*C.jejuni* ES115[a]	*C. perfringens GP435*
			AE	ANA	AE	ANA	AE	ANA	AE	ANA			
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Xylanase+XOS	**338**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Xylanase+XOS	**339**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -1	NI	NI	NI	NI	NI	NI	NI	NI	I++	NI	NI
Xylanase+XOS	**356**	**Dil. -2**	NI	NI	NI	NI	NI	NI	NI	NI	I++	NI	NI
		Dil. -3	NI	NI	NI	NI	NI	NI	NI	NI	I++	NI	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Xylanase+XOS	**357**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -1	NI	NI	NI	NI	NI	NI	NI	NI	I+	I+	NI
Xylanase+XOS	**372**	**Dil. -2**	NI	NI	NI	NI	NI	NI	NI	NI	I+	I+	NI
		Dil. -3	NI	NI	NI	NI	NI	NI	NI	NI	I+	I+	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
Xylanase+XOS	**373**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. -2	NI	NI	N.	NI	NI	NI	NI	NI	I +++	I +++	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Xylanase+XOS	**376**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
		Dil. 1/2	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Xylanase+XOS	**377**	**Dil. -1**	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
		Dil. -2	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

Table 3_Results of the antimicrobial activity test in chicken from xilanasa + XOS diet group. Dil: dilution NI: no inhibition of growth, I: inhibition of growth,+ : weak inhibition, ++ : moderate inhibition, +++ : strong inhibition.

[a]: It could not be ruled out if the presumptive inhibition zone (either weak or strong) was an hemolysis halo.