Control of the intestinal flora ecology in poultry for ensuring the products safety for human consumers

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POULTRYFLORGUT

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Summary

European consumers are becoming increasingly concerned about the safety and nutritional quality of their foods, which should be free of pathogens and antibiotic residues. At the same time, food production systems must meet consumers concerns for animal welfare, environment and public health issues, leading to, for instance, rapid changes in poultry management practices (e.g. ban of antibiotic feed additives) and rearing conditions (e.g. free range breeding). However, changes in management practices led an increase of the prevalence of enteric diseases among poultry flocks, linked to digestive bacterial dysfunctions. This could affect the colonisation of the animal intestinal tract by opportunistic bacteria, leading to a degradation of the hygienic quality of animal product meant for consumption.

There is a lack of data on the effect of the nutritional and rearing changes on prevalence of food-borne pathogens in poultry production. In other respects, the ecology of poultry intestinal flora is partially unknown due to the lack of accurate methodology for deeply characterisations. Fortunately, novel molecular techniques provide unique opportunities to investigate bacterial diversity.

The first objective of this project was to concentrate the efforts of several European scientists on the study of the effect of the new European regulations and new tendencies in breeding management on (1) prevalence of food-borne pathogens (already known and emerging) at the preharvest level and during products processing; (2) possible strategies to reduce these contaminations; (3) socio economic aspects of these changes. In parallel, a study of the whole intestinal flora of poultry and of its interactions with the food borne pathogens was undertaken, through the development of novel technologies providing tools to help in finding solutions to control intestinal flora and thus to reduce food-borne pathogens prevalence. The first part of the project was aimed to fill out information on different kinds of digestive diseases, like the specific, or necrotic, enteritis or cause of unspecific enteritis origins were collected. Moreover, some indicators of the unspecific enteritis were highlighted. The second part of the project was focused on the evaluation of occurrence and, whenever possible, of prevalence of food-borne pathogens in birds reared using different technologies as well as at the slaughterhouse level in different EU countries. Moreover, the effects of different feed alternatives on the reduction of the occurrence/prevalence of enteric pathogens were assessed. Selected strains collected in the broiler flocks tested were typed and the overall results were included in a database. The third part of the work dealt with the evaluation of molecular methods to monitor the changes in poultry gut microflora. Finally, socio-economical aspects regarding poultry productions in European countries and recommendations were discussed.

Characterisation of digestive disorders in poultry

Among the intestinal troubles of poultry, the necrotic diseases are the most common and financially devastating bacterial disease in broiler flocks. There are multifactorial (environment, pathogens…) diseases caused by several micro-organisms (*Salmonella* spp., *Escherichia coli, Enterococcus* sp and *Clostridium perfringens*) alone or combined with *Eimeria* spp. The bacteria infect poultry principally by the oral route, i.e. by ingestion of soiled litter, animal faeces and/or feed. If the diagnosis is made early enough, the symptoms can be controlled more or less easily by antibiotic treatment. However, due to possible antibiotic resistance, prevention of necrotic disease with good hygiene practices and the control of gut flora had to be preferred. Some other dietary factors as the changes in diet formulation, such as addition of a high level of viscous cereal grains and/or products like fish meal and meat meal, could contribute to an outbreak of necrotic disease. The sources of contamination are not specific, but the control of the hygienic quality of the environment and of the water could also limit the appearance of these conditions. Some necrotic disease indicators such as the presence of diarrhoeas and wet litter in the buildings, the quantitative evolution of the

digestive flora and the presence of lesions in the digestive tract were evaluated in this work. The identified risk factors were related to environmental or the rearing conditions (temperature, speed of air, lighting programs, heating), or they were related to the characteristics of food (food transitions, coccidiostat...) and water (pH, bacteriological quality).

Occurrence of pathogens at the farm level

Regarding the analysis of specific pathogens associated with poultry, the occurrence of *Campylobacter* was estimated in broilers in Germany, Italy and the Czech Republic, the occurrences of *Salmonella* and *L. monocytogenes* were analysed in broilers and laying hens in Italy, Germany, the Czech Republic and Lithuania, and new or re-emerging food-borne pathogens such as *H. pullorum* and *C. perfringens* were analysed in broilers in Germany, Lithuania, the Netherlands and France.

About a harmonisation process on pathogen detection methods was implemented early in the project, small differences between partners were still present. However, the overall number of flocks analysed was quite high. Therefore, the occurrence results give a good picture of the prevalence data.

Regarding broiler flocks:

- The occurrence of *Campylobacter* spp. ranged from 67.2% [59.8-74] to 96.1% [91.1-98.8] in Germany, Italy and the Czech Republic. The occurrence of *Campylobacter* spp. was rather low in Lithuania where only 28.6% [18.4-40.6] positive samples where found, which result what could be explained by climatic conditions less favourable to the survival of *Campylobacter* spp. in the environment, further investigations are needed. The majority of isolated strains of *Campylobacter* spp. were identified as *C. jejuni* in the Czech Republic, Germany and Lithuania. *C. coli* was identified in a majority of broiler flocks in Italy where *C. coli* seemed to represent the most prevalent species in broilers reared in conventional farms, whereas *C. jejuni* was mostly found in free range farms.
- The percentage of *Salmonella* isolates in broiler flocks from conventional housing systems was 29.6% [13.7-50.2] in Lithuania, 11.1% [1.4-34.7] in the Netherlands and 20% [2.5-55.6] in Italy, respectively. These figures differ from the results recently published in the EU baseline study on broilers (EFSA, 2007). However, the data from our investigations covered only a fraction of the totals presented in the mentioned study.
- The occurrence of *Clostridium perfringens* in conventional Italian broiler farms was higher than in the Czech Republic (91.3%[72.0-99.0] *vs* 64.7% [50.0-77.5], respectively). The percentages of *C. perfringens* positive samples, detected in Italian organic, conventional and free range broiler farms did not show any statistically significant difference. Moreover, they were not influenced by the parameters tested in the different flocks such as animal gender, skin colour, litter type, feeding type and ventilation type.
- The prevalence of *Listeria monocytogenes* observed in French broiler flocks (both conventional and free range) corresponding to 31% [23.6-39.2] was higher than expected, according to a previous investigation carried out in 1995 (2.4%). The occurrence of *L. monocytogenes* in conventional broiler farms was lower in Lithuania (i.e. 3.5% [0.4-12.1]) as compared with France. Organic poultry farms were not tested in Lithuania, as this production system has not been certified yet.
- In Italian broilers, *Helicobacter pullorum* had an estimated occurrence of 80.6% [75.2-85.4] and the percentage of positive samples among broilers

reared in conventional and organic farms seemed to be higher than that of broilers reared in free range farms. *H. pullorum* was present in 27.6% [12.7-47.2] of the conventional samples tested in the Czech Republic.

Regarding layer flocks:

- In conventional production in Lithuania and in the Netherlands, the occurrence of *Salmonella* was 35% [20.6-51.7] and 33.3% [9.9-65.1], respectively. The occurrence of *Salmonella* in Lithuanian alternative productions (enriched cages) was not significantly different from the occurrence found in conventional production. All the other types of production tested in Germany and the Netherlands were negative.
- In the dust samples from conventional cages, the occurrence of *L. monocytogenes* was 5% [2-10] and 1.4% [0.04-7.7] in France and Lithuania, respectively. In France, there was no statistical difference between caged and floor-reared hens, when taking into account all the flocks; however, in flocks tested positive, dust samples were more frequently contaminated in floor-reared hens than in caged hens. *L. monocytogenes* serotype 1/2a was frequently detected in broilers (30/74) and in laying hens (26/31).

The prevalence of *Listeria monocytogenes* in France in broiler and in laying hens and the prevalence of *Campylobacter* at the slaughterhouse level in Italy and in Germany were estimated. The prevalence depends on the number of individuals in the population. Because the data at the national level were not always available for all involved countries, the occurrences corresponding to the number of positive samples occurring at specified sample points during a defined period of time were estimated. The comparison between the occurrences of food-borne pathogens in farms applying different rearing systems in Europe was not possible due to the absence or to the low number of organic and free range farms in the Czech Republic and Lithuania. A second imitating factor was the inability to visit such farms in several other European countries during the course of the avian flu crisis. However, in Italy, the Netherlands and France where some comparison between conventional and organic broiler and in laying hen production systems was possible no significant differences were observed. Regarding the comparison between alternative production systems, the data collected in Italy within farms applying different rearing technologies seem to suggest that the occurrence of food-borne pathogens might be slightly lower in free range farms in comparison to organic and conventional farms. However, more data should be collected to confirm this observation.

Food alternatives to control the colonisation of poultry gut pathogens

Several alternatives were tested to control the colonisation of poultry gut by pathogens. Organic and inorganic acids were tested to control *E. coli*, *Campylobacter* and *Salmonella*. Competitive Exclusion (CE) was evaluated to control *Salmonella* under field and experimental conditions, but also to control *C. perfringens* under field conditions. Finally vaccination was tested to control *Campylobacter* and *Salmonella* under experimental conditions.

The effect of organic acids was tested *in vitro* on pathogens (*E. coli* O149K91 and *C. jejuni*), and on *Lactobacillus casei*, the latter used as a probiotic and a representative of the commensal flora. Efficacy against *E. coli* depended on pH and time of incubation. At pH=6, most of the tested acids could be used to control *E. coli*, the lethal effect on *L. casei* was generally lower. Fumaric and Benzoic acids showed some lethal effect against *L. casei* as

well. There was a large variety in the inhibition of *Campylobacter* by organic acids, Sorbic acid proved the most effective.

Corn silage was tested as a floor cover in experimental broiler houses to control *S.* Java and *C. jejuni.* Corn silage is a fermented mixture of the whole corn plant containing several organic acids such as Lactic acid, Acetic acid and Butyric acid. It was shown under these experimental conditions that the use of corn silage as a floor cover may contribute to the control of *Salmonella* contamination of broilers. However, there was no effect on *Campylobacter.*

Protected organic and inorganic acids were evaluated in broiler farms selected according to their previously positive status for *Salmonella*. The addition of protected organic and inorganic acids to the feed during the whole rearing period reduced the *Salmonella* colonisation at the caecal level, thus reducing the risk of cross contamination at slaughter.

Under Italian field conditions, in *Salmonella*-positive broiler farms, CE also proved a useful tool for reducing the incidence of *Salmonella* infection. Indeed, as early as three weeks post-hatch, *Salmonella* shedding in the broilers treated with CE was already reduced by approximately 60%, as compared with control broilers. However, under experimental conditions, CE treatment contributed in controlling *Salmonella* Java but could not eradicate the infection. The results concerning the control of *C. perfringens* showed a statistically significant reduction of lesion scores, especially during the first 21 days, in groups treated with CE, in the farms where necrotic enteritis had been observed. However there was no statistically significant difference in mortality at the end of the rearing period.

Under experimental conditions, vaccination of broilers with a live vaccine developed from *S. paratyphi* B biovar Java did not succeed in eradicating *Salmonella* neither from the homologous nor from heterologous serovars and the results showed no difference in the clearing of infection between vaccinated and non-vaccinated broilers.

Occurrence of pathogens at the slaughter house level

At the slaughterhouse level, the quantification of culturable bacteria by traditional culturing methods along the processing line is an important tool for the quality control or for the assessment of exposure to bacterial pathogens. Traditional bacterial cultures can be supplemented by fingerprint molecular biological methods like Single-Strand Conformation Polymorphism (SSCP) and sequencing, which allows the detection of both culturable and most non-culturable bacteria and may also be used to characterize complex microflora. For instance in this study, SSCP allowed distinguishing slaughter steps by looking at the changes of flora on the chicken surface.

The analysis of the prevalence and numbers of *Campylobacter* during poultry processing showed several factors that influenced the contamination levels of carcasses; these were the flock status, the initial colonisation levels and the season. Such an influence of the flock status on the contamination of carcasses could not be demonstrated for *L. monocytogenes* or *Salmonella* and no relationship was found between the strains of *L. monocytogenes* isolated from the flocks and those from the slaughterhouses. Interestingly, although *Campylobacter* is thought to be a sensitive organism, its total eradication was not achieved along the processing chain and the mean reduction in *Campylobacter* counts after processing varied from 3.2 $\log_{10}$ CFU/carcass in German samples to 2.7 $\log_{10}$ CFU/carcass in Italian samples. In some cases, *Campylobacter* – which is normally microaerophilic - was able to survive under aerobic conditions when co-cultured together with *Pseudomonadaceae*. Thus, the possible interaction of bacteria in the environment, as well as on the product, has to be considered when making assumptions about the survival of bacterial pathogens. In addition, the Cleaning and Disinfecting Procedures (CDPs) appeared

as important tools in food production to enhance the hygiene of the processing environment, hence of the product:

- In Italian slaughterhouse investigated, CDPs (including applications of foams, disinfectants and sodium hypochloride) applied on surfaces in contact with meat products at the end of processing were efficient against *L. monocytogenes* and *Campylobacter* and *C. perfringens.* The mean pathogen load on surfaces before cleaning was 1.8, 2.6 and 1.2 $\log_{10}$ CFU/10 cm^2, respectively, and the microorganisms were never detected on surfaces after cleaning.

- *C. perfingens* was the only pathogen isolated on slaughterhouse surfaces after cleaning, with a mean load of 0.9 $\log_{10}$ CFU/10 cm^2.

- *Salmonella* was not found on the studied surfaces, neither before nor after CDPs.

- The wide part of the strains having the best biofilm forming abilities was isolated from hydrophilic surfaces i.e. stainless steel. In fact the bacterial cells were always negatively charged and possessed hydrophilic surface properties. In particular, *L. monocytogenes* MTP22E, isolated from stainless steel cut module for poultry breast, resulted the best biofilm forming strain. However, if isolation source provides to select a certain number of *L. monocytogenes* strains potentially contaminants, their adhesion and persistence to 20 and 40 hours may be explained as the result of two further factors: formation of flagella and morphological changes of surface attached cells.

In addition to the decontamination of surfaces in the processing plant, several other technical options that could reduce the bacterial contamination of carcasses along the processing chain have also been investigated in the Poultryflorgut programme:

- Treatment of the product itself has recently been debated as an option to enhance food safety. Indeed, poultry carcasses are normally treated with water along the entire processing line, both to reduce surface contaminations and to avoid drying out, which would result in an adverse appearance of the product. Theoretically, treatment with different chemicals in aqueous solution could thus be applied at different stages. However, the potential effects of such chemicals are controversial. In the present project, when chemicals (a mixture of Chlorine dioxide, Acid sodium chlorite, Lactic acid and Lactoferrin) were applied through the internal/external washer, no additional lethal effect was seen on *Enterobacteriaceae* and *Salmonella*, while there was only a limited effect on *Campylobacter,* possibly because of too short exposure times.

- Another way of limiting contamination along the processing chain could be an early evisceration, in order to limit the amount of faeces disseminated along the subsequent slaughtering steps. However, no effect of an early evisceration could be demonstrated during this study, using commercial evisceration equipment.

- Finally, the kind of meat packaging applying different modified atmospheres has already proved useful to enhance the shelf-life, maintaining organoleptic properties. However, no additional lethal effect of different CO_2 / O_2 / N_2 mixtures on *Salmonella* or *Campylobacter* was seen in the present study.

Molecular methods to monitor intestinal microflora

Currently, little is known about the composition and the dynamics of the gut flora, since the proportion of non culturable bacteria is estimated to be up to 90 %. Most of the studies on poultry digestive microflora relied upon bacterial culture with selective media. These protocols excluded fastidious organisms and viable but not culturable bacteria.

One way to overcome this problem is to use molecular methods based on the detection of an ubiquitous gene, most often the 16S rDNA. Two main strategies for the direct investigation of the diversity of complex microbiota are currently available. The first is the direct detection and count of individual cells, by using fluorescently labelled 16S rDNA probes (*In situ* hybridization method, abbreviated as FISH), or by using quantitative or real time PCR. The second strategy consists in molecular approaches to study the composition of the total 16S rDNA, based either on the cloning of the 16S rDNA, or on numerous fingerprint methods including Denaturing Gradient Gel Electrophoresis (DGGE), Temporal Temperature Gradient gel Electrophoresis (TTGE), Temperature Gradient Gel Electrophoresis (TGGE), Restriction Fragment Length Polymorphisms (RFLP), Terminal Restriction Fragment Length Polymorphism (T-RFLP), and Single-Strand Conformation Polymorphism (SSCP). The common starting point for all these methods is the total extraction of DNA from the intestinal content or tissue. A variable region of the 16S rDNA gene is then amplified by PCR, using oligonucleotide primers that flank this variable region and are conserved among most bacterial genera. The amplified fragments (or amplicons) must then be differentiated according their specific characteristics, which depend on the bacteria from which they were amplified. In SSCP and CE-SSCP, PCR amplicons with the same length but different nucleotide sequences are separated on the basis of the conformation of single-stranded DNA. This is achieved using polyacrylamide electrophoresis (SSCP), possibly performed in a capillary DNA sequencer (CE-SSCP). With DGGE or TGGE, the separation of amplicons depends on the double-stranded amplified DNA melting; this separation is highly dependent upon the sequence and is achieved in gel containing DNA denaturants (DGGE) possibly associated with a temperature gradient (TGGE). With RFLP, the micro-organisms may be differentiated by the cleavage patterns obtained when the PCR amplicon is treated with restriction endonucleases. The T-RFLP is based on the cleavage of amplified genes by using one or more restriction enzymes. Following the cleavage reaction, the fragments are separated by using capillary or polyacrylamide gel electrophoresis in a DNA sequencer and the sizes of the different terminal fragments are determined by the fluorescence detector. T-RFLP was used more extensively than RFLP in the past ten years to investigate bacterial microflora.

The separation of amplicons according to their specific properties results in complex peak patterns (molecular fingerprint), where each peak or band should be correlated with the 16S rDNA sequence derived from at least one micro-organism. As it is usually impossible to identify each of these peaks individually, two complex microflora are compared by evaluating how similar their fingerprints are. Using Pearson, Jaccard or Dice correlation coefficients, similar patterns, or clusters are evaluated. Dendrograms are usually calculated by using the Unweighted Pair Group Method using Arithmetic average (UPGMA) method. In this method, the distance between two clusters is calculated as the average distance between all pairs of objects in the two different clusters. Other statistical methods such as Principal Component Analysis should be useful for the interpretation of profiles.

In this present study, fingerprint methods such as DGGE, SSCP, RFLP and their variants TTGE, CE-SSCP, T-RFLP as well as the FISH quantitative method have been evaluated and used to distinguish the digestive flora of chickens raised under different rearing systems, or to monitor under experimental and field conditions the impact of different feed (whole wheat), feed additives (organic acids, botanicals, prebiotic) or pathogens (*Salmonella, Campylobacter*) on the poultry gut microflora.

All tested methods revealed differences induced by production systems as well as by different intestinal compartments. T-RFLP, SSCP and CE-SSCP showed a more diverse caecal microflora as compared to ileal flora as it was shown in literature.

No effect of Salinomycin on a *C. jejuni* or *S.* Typhimurium infection in chicken was found by using T-RFLP, cage group and each intestinal compartment cluster together. Nevertheless, by using SSCP, the samples from *Campylobacter* naturally infected flocks cluster together, although no cluster was found with samples from the experimental *Campylobacter* infection. The effect of Salinomycin on microflora was shown by DGGE and some DGGE bands were identified.

Most methods weakly detected the effects of feed additives on the fingerprint of the gut microflora. Further, the CE-SSCP and TTGE evidenced some specific bands associated with dietary treatments. These bands will have to be characterised in an attempt to determine from which microorganism(s) they are linked. With chicken intestinal samples, RFLP showed significant differences for organic acid treatments used as alternative to feed antibiotic. Indeed, the negative control as well as feed supplemented with Sodium butyrate were clearly different to the other treatments. RFLP patterns associated with feed supplemented with Lactic acid and feed supplemented with Formic and Propionic acids and Ammonium formate clustered together as well as Salinomycin and Calcium formate. Moreover, differences between treatments were detected in microbial biodiversity related to the number of electrophoretic bands in the RFLP profiles.

Regarding quantitative methods tested in this study, existing FISH protocols were evaluated to investigate contribution of dominant bacterial groups to the chicken intestinal microflora in relation to production system and the feed additive Salinomycin. Results obtained by quantitative FISH method showed that Salinomycin in feed reduced counts of the *Lactobacillus* -, *Enterobacteriaceae* - and *Clostridium*-like bacteria in the lumen of ileum, as compared to the conventional control. The effect of Salinomycin on the bacterial counts in the caeca was unconsistent. Further design of probes including hybridization conditions will be needed before the FISH technique can be used for routine quantification of bacterial phylotypes in the chicken intestinal tract. The resolution at the species level of bacteria is probably more limited with this method compared to the molecular DNA fingerprinting methods. Indeed the detection threshold of the FISH technique limits its use for examining subdominant population.

In conclusion, neither can one outstanding superior method be chosen, nor can methods be excluded for further use due to serious limitations. If one methodology had to be selected to start up investigation or to screen treatment effects on the chicken GIT, the optimal choices seem to be CE-SSCP or T-RFLP, completed by DGGE or SSCP. Indeed, CE-SSCP and T-RFLP methods allow a high processing rate through automation and SSCP or DGGE allow a subsequent characterization of individual fragments by DNA sequencing. When specific groups of bacteria are identified by DNA sequencing in response to treatments, further investigation should be carried out by PCR with bacterial groups as specific targets.

Despite the advantages of the fingerprint methods, several limitations were identified: all procedures involved the extraction of DNA, this step being crucial as the extent of DNA extraction is not the same for all bacteria. Secondly, the main problems with these PCR-based techniques are that the single signals that are observed (band, peak) are not always caused by single PCR fragments, hence by single bacterial species. This is both related to divergent sequences migrating to the same length and to heteroduplex molecules formed as artefacts after PCR. Moreover, intra and interlaboratory variation in the patterns obtained from any given flora was also observed: slight differences were noticed between different series of runs by using these fingerprints methods. The change of polymer may influence the profile even when the protocol is strictly followed. Depending on the sensitivity of the

methods, the variability between individuals could mask the effect of the tested treatments. With CE-SSCP a “pool strategy” was implemented, consisting in mixing samples from 12 individual samples coming from the same treatment. This proved efficient to reduce the variability between individuals. Altogether, the interpretation of profiles and dendrograms will remain a real challenge because of the lack of reproducibility, the co-migration of fragments and the individual variability. The impact of feed or infection on the animal gut microflora is complex and related to multifactorial parameters; it is strongly suspected that mainly drastic changes could be demonstrated by the analysis of clusters.

Clone libraries do offer the highest degree of phylogenetic resolution available for culture-independent methodologies but can be cumbersome for analysis of the large numbers of samples that may be produced by studies on temporal or spatial variability of microbial communities. In addition, comparison of community composition between samples when using clone libraries can be problematic if the libraries offer incomplete coverage of a community. The current challenge of these techniques is to compile standardized patterns in a database for interlaboratory use and future reference but this could be difficult because of the high variability mentioned above.

Moreover, for all these molecular methods, one of the most significant unresolved obstacles appears to be the primer and probes design. The development of primers remains a significant challenge because of the high conserved nature of rDNA and the extensive diversity of microbial life.

Socio-economical impact of AGP ban in European poultry production

The socio-economic analysis of the relationship between growers and processors showed that the education and the feeding field offer some possibilities of enhancing the skills of growers in performing the production protocols. This could support the idea that the selection of growers and the plan of feeding tasks may facilitate the enhancements of the process organization. In the investigated sample, the disinfection tasks appeared to be critical to the expected improvements. The case studies showed that the companies invested a large amount of physical, technological, human and financial resources in food safety strategies. The common assessment of the companies about the AGPs was that the management could cope with the consequences of their withdrawal at large costs. The outcomes from a previous survey, which were complementary to the case studies, suggested that safety strategies require investment in specific assets and promote the adoption of tight coordination forms. Regarding the variety and the role of contract in poultry sectors, particularly with respect to the food safety strategies, a few models have been identified in literature as production contract in poultry sector. The aim of the analysis is to identify how the contract contributes to the food safety strategy. The most diffused poultry contract allows agent to cope with food safety issues as it basically represents a hybrid governance form, it includes enforcement procedures which secure the food safety objective against the potential opportunistic behaviours and finally, the contract in poultry sector can be thought as reliable organizational arrangements with respect to food safety objectives and to the related technical changes.

The key economic cost associated with the removal of AGPs from broiler production in four case study countries (United Kingdom, Spain, the Czech Republic and Lithuania) has been the increased amount of feed required to raise broilers to market weight. As feed is the single biggest input cost in broiler production, this is the largest cost to producers associated with the AGP ban. Other changes in production, as a result of the ban, include changes in stocking density, feed composition, increased use of coccidiostats and therapeutic antibiotics, type of litter used and increased frequency of topping up or changing litter. Such costs have been borne by the producers. The AGP ban may also have increased the incidence of diseases such as necrotic enteritis, dysbacteriosis, pododermatitis, hock burn and breast blisters in broiler flocks. The benefits of the AGP ban are less tangible - being more difficult to quantify. In broad terms, the AGP ban may have benefited human health and welfare through the food chain, the feed additives industry, and gained greater public trust in the domestic market and international trade for European produce.

Recommendations on where future EU policy spending should be focused with regard to best practice on the use of alternatives to AGPs were included in this report. They include the need for more field trials on alternative feed additives to AGPs and setting up systems to monitor the impact of regulatory changes in advance of any amendments.

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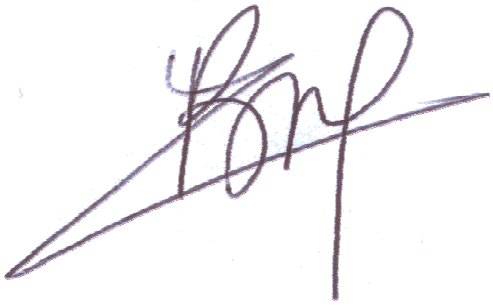

Final report

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List of abbreviations

AGP : Antibiotic Growth Promoters

AP : Alkaline Phosphatase

Approx. : Approximately

aw : Activity of water

BA : Brucella Agar

BGA : Brilliant Green Agar

BHI : Brain Heart Infusion Agar

BPW : Buffered Peptone Water

Ca : Calcium

CAT : Cefoperazone, Amphotericin B, Teicoplanin

CC : Colonal Complexes

CCDA : Charcoal Cefoperazone Deoxycholate Agar

CDP: Cleaning and disinfecting procedures

CE : Competitive Exclusion

CE-SSCP : Capillary Electrophoresis Single-Strand Conformation Polymorphism

CFU: Colony Forming Units

CI : Confidence Interval

ClO_2 : Chlorine Dioxine

CO : Carbon monoxide

CO_2 : Carbon Dioxine

cps. : Centipoise

DGGE : Denaturing Gradient Gel Electrophoresis

DNA : Deoxyribonucleic acid

DSM: strain collection

EFSA : European Food Safety Authority

EU : European Union

FCR : Feeds Conversion Ratio

FDA : Food and Drug Administration

FISH : Hybridization in situ

FOS : Fructo-olisaccharides

g : Gram

GIT: Gastro Intestinal Tract

GSP : glutamate starch phenol-red

h. : Hour

HACCP : Hazard Analysis Critical Control Point

HI : Heart Infusion

LAP : Leucine aminopeptidase

Log: Napierian Logarithm

M : Molar

MAP : Modified atmosphere

min. : Minute

MOS: Mannanoligosaccharides

MPCR : Multiplex PCR

MSRV : Modified Semi-solid Rappaport Vassiliadis

MTA : Stainless steel cut modules for poultry wings

MTP : Stainless steel cut modules for poultry breast

NAGM: Normal Avian Gut Microflora

ND: Not Determined

Nd: Not detected

NSPs: Non-Starch Polysaccharides

NTF : Teflon strips for transportation of broiler carcasses

OD : Optic Density

OIE : World organisation for animal health

OP : Organic-plus

OTU : Operational Taxonomic Unit

p.i. : Post Infection

PCR : Polymerase Chain Reaction

ppm : parts per million

PFGE: Pulsed field gel electrophoresis

PSS : Physiological saline solution

R : Reproduction Ration

RFLP : Restriction Fragment Length Polymorphism

RNA : Ribonucleic acid

SBA : Sheep Blood Agar

SD : Standard Deviation

SEM : Scanning Electron Microscopy

SID : Index of Discrimination

SO_2 : Sulfur Dioxine

SSCP : Single Strand Conformation Polymorphism

ST : Sequence Type

TGGE : Temperature gradient gel electrophoresis

T-RFLP : Terminal Restriction Fragment Length Polymorphism

TSA : Tryptic soy agar

TSI : Triple sugar iron

TSP : Tri sodium phosphate

TTGE : Temperature Gradient Gel Electrophoresis

UA: Ureum Agar

UK : United Kingdom

v. : Volume

w. : Weight

XLT4 : Xylose Lysine Tergitol 4

µM : micro-molar

Section 1: Introduction

1.1 - POULTRYFLORGUT project at a glance

POULTRYFLORGUT is a Specific Targeted Research Project supported by the European Commission for a three-year period (2005-2008). POULTRYFLORGUT aimed to enhance control of the intestinal flora ecology in poultry for ensuring the products safety for human consumers.

1.2 - Context

European consumers are becoming increasingly concerned about the safety and nutritional quality of their food, which must be free from chemical and biological contaminants. At the same time, food production systems must meet the concerns of consumers for animal welfare, the environment and public health.

This leads to, for instance, rapid changes in both poultry nutrition (e.g. banning of the use of in-feed antibiotics regulating the intestinal micro-flora, use of 100%-vegetal diets) and rearing (e.g. reduction of the number of available coccidiostats, development of out-door breeding and organic farming) context.

These drastic changes raised questions in the scientific community and the Agro-Food industry concerning an increasing risk of microbiologic contamination of poultry products (meat, eggs), and therefore demands a thorough and comprehensive investigation of all parts of the poultry industry.

1.3 - Project objectives

- Main objectives:

The over-all objective of the project was to provide a strong factual basis for the optimisation of the hygienic quality of the poultry products meant for human consumption, in compliance with the new European regulations concerning feed additives, and the changes occurring in poultry production in response to public concerns in terms of animal welfare, environment and health.

A special focus was done on the control of the intestinal flora of the broilers and laying hens, including the food-borne pathogens. Our approach to reduce microbial risks associated with consumption of poultry products (meat, eggs) addressed the different steps of poultry production from the farm to marketing. It included improvement of housing systems, poultry flocks management and feeding, slaughterhouse processing, and it can be applied whatever the poultry production system, in particular to new poultry housing systems (in-door, out-door…) independent of the size of the flock. Looking at the consequences of the new poultry production system on poultry products safety should provide supplementary information that could be used to adapt, if needed, the new EU regulations concerning feed additives but also food-borne pathogens control. Improving the safety of poultry products will contribute to satisfy European consumer demand for better and safer food products.

The other objective of the project was to develop methodologies, using mainly novel molecular techniques, allowing the global and integrative study of the intestinal flora of the poultry. These methodologies will make it possible to define and validate intestinal health or food safety criteria in poultry.

- ✔ Strategic objective

The POULTRYFLORGUT project carried on the following main objectives:

- Quantify the food-borne pathogens at the different steps of the food chain, from field to slaughterhouse, in different European countries;
- Determine how to improve management in order to decrease the prevalence of food-borne pathogens along the food chain;
- Assess novel techniques for the study of the intestinal flora and of the relationship between the whole flora and the food-borne pathogens in poultry;
- Estimate the socio-economic consequences of the changes occurring in poultry production in response to the new European regulations and to the (public) European citizens concerns.

1.4 - Work forces in presence

The project included 15 partners from 10 EU countries bringing together laboratory with a strong basic research activity in microbiology, food-borne pathogens and zoonosis. These laboratories are also involved in various areas, such as hygiene and quality for animal products, animal health, epidemiology, animal immunology, animal feeding, molecular typing, risk assessment and socio-economic study.

Figure 1: Logos of partners institutions involved in the POULTRYFLORGUT project

AFSSA (FR); ITAVI (FR); KVL [UOC] (DK); IRTA (SP); INRA (FR); VU (AU); TIHO (D) ; UNIBO (IT); IIVU (LT); VFU (CZ); ASG-WUR [CVI] (NL); VLA/DEFRA (UK); UNIPG (IT); DFVF [DTU-VET](DK); CreSA (SP)

1.5 - Total food-chain approach and framework

- Total food-chain approach:

The food-chain approach developed in the POULTRYFLORGUT project suits the need to study the different factors that can affect the poultry product safety.

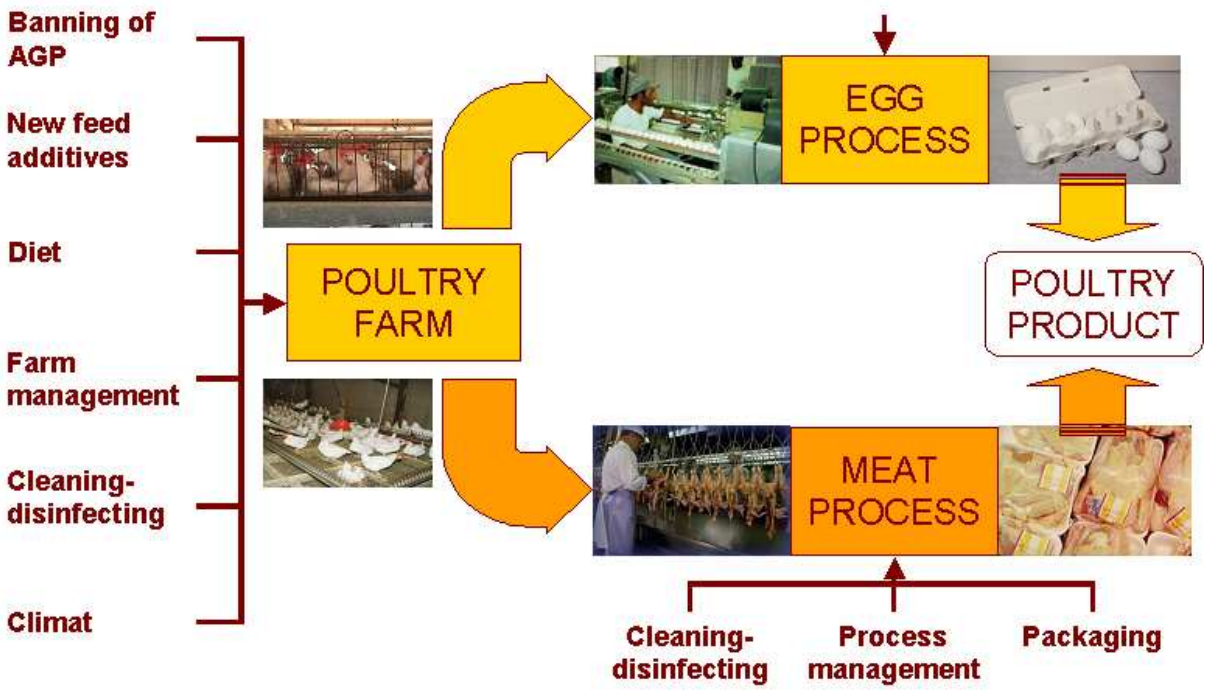


Figure 2: POULTRYFLORGUT Food chain approach

- Framework:

The project has been divided into five main work packages. The first work package (WP1) was dedicated to the management of the project and to the dissemination of the scientific results.

Work package 2 (WP2) and work package 3 (WP3) focus on the effects of the new European regulations and the new trends in breeding/management on the food-borne pathogens (traditional and emerging) along the food chain.

Work package 4 (WP4) aimed to validate new molecular methodologies allowing the study of the whole intestinal flora of poultry as well as poultry meat and to determine the effect of dietary factors and rearing types, and interactions with the food-borne pathogens. Work package 4 (WP4) provided novel molecular methodologies that will be also used in work package 2 (WP2) and work package 3 (WP3).

Work package 5 (WP5) provided an economic assessment of changes in poultry practice under the current pressure of the European regulatory and studied the effect of organizational arrangements in the poultry food chain affecting food safety.

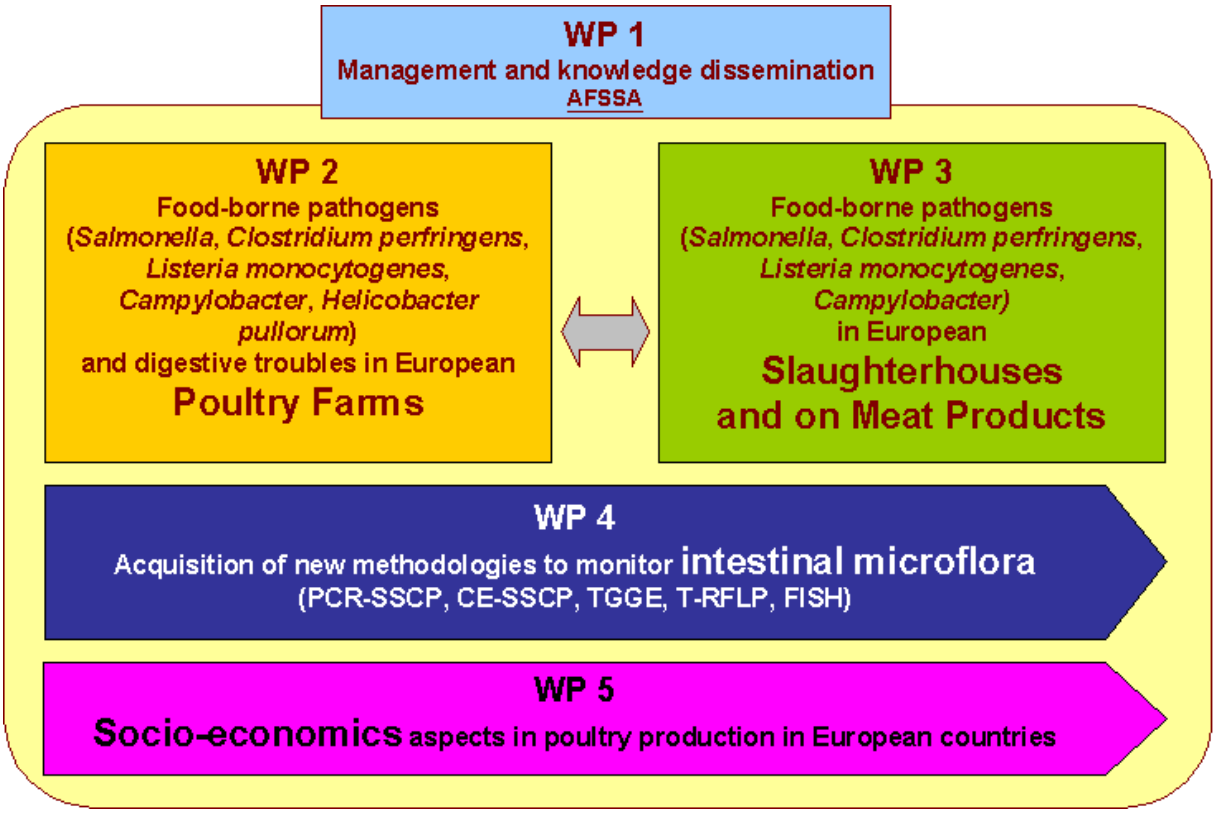


Figure 3: POULTRYFLORGUT Framework and Work Packages

1.6 - Poultryflorgut Website

The POULTRYFLORGUT Web site aims to increase awareness and to keep people informed on the research results developed within the consortium. It was designed to provide a single point of access to all relevant information in relation with the POULTRYFLORGUT project: www.poultryflorgut.org

This report is divided into 9 sections. Sections 2 and 3 are dedicated to the intestinal troubles; an inventory of the different kinds of digestive troubles like the specific or necrotic enteritis or unspecific enteritis origins was drawn. Moreover, indicators looking for the characterisation the apparent signs of unspecific enteritis emergence were searched. In Sections 3 and 5, the occurrences of food-borne pathogens in different rearing conditions and at the slaughterhouse levels respectively were established in different EU countries. Moreover, different food alternatives were tested to reduce pathogens in poultry gut (section 4). The molecular characterization of the collected strains was included in a database (section 6). Section 7 deals with the evaluation of molecular methods to monitor the poultry gut microflora. Finally, socio-economical aspects regarding the poultry production in European countries and recommendations are discussed in sections 8 and 9.

Section 2: Digestive troubles in broiler rearing farms

During the last decade, successive events including the ban of meat and bone meal and fat from animal origin, the ban of in-feed antibiotics, and the limitation of the number of available coccidiostats have promoted a phase of digestive instability in broiler rearing (Balloy, 2003), there are a recrudescence of dysbacteriosis in broilers, implying an increase of diarrhoeas episodes and wet litter. This involves an additional cost for the breeder due to the increasing of the mortality risks, feed conversion ratio and the use of therapeutic antibiotics. According to Van Der Sluis, 2005, 94% of the world producers would have declared on their stockbreeding a form of bacterial enteritis at least, necrotic or unspecific origin. In the same direction, Balloy, 2003 observed that the enteritis represented about half of the causes of veterinary surgeon consultation (Balloy, 2003). This rise of the enteritis cases results in increase of mortality, use of antibiotics into therapeutic. Moreover, enteritis can be related to an imbalance of the digestive flora, generating risks of intestinal colonization by opportunist bacteria and of viral origin super infections (Balloy, 2003) leading to potential microbiological contaminations of the poultry products. The dysbacteriosis monitoring allows to control the potential contamination in poultry flocks. This part of the report draws up an inventory of the different kinds of digestive troubles like multifactorial necrotic diseases, which origins are still misunderstood and seem to be caused by several alone or associated microorganisms such as *Campylobacter*, *Salmonella*, *Listeria monocytogenes*, *Escherichia coli*, *Enteroccocus* sp, *Clostridium perfringens* or *Coccidia*.

2.1 - NECROTIC ENTERITIS

The specific or NE is a serious digestive disease in poultry. It is associated with a rapid proliferation of *C. perfringens* types A and C. This disease has been increasing for the last few years, coinciding with drastic changes occurring in the feeding practice in poultry production (ban of products used as feedstuffs and ban of the AGPs such as Avoparcine) (Valancony, 2001).

2.1.1 - Clostridium perfringens

A) Description of the bacteria

C. perfringens is very common and is widely distributed in the environment where it acts as a “cleaner”, destroying the organic matter of dead animals or plants (Valancony, 2001). This

bacterium frequently occurs in the soil, the digestive tract of human and animals including poultry, and in the drinking water (Pedersen *et al*., 2003). Any raw food may contain spores or bacteria that are very resistant in the environment. Indeed, spores of *C. perfringens* resist to high temperatures (100 to 120°C), oxygen and dryness. They persist in soil, sediments, and areas subject to human or animal faecal pollution, such as the litter (Valancony, 2001).

C. perfringens is a Gram-positive bacillus, sporulated and strict anaerobic (Valancony, 2001). It appears as a squat stick of 1×4 µm, square-ended, and it is motionless. *C. perfringens* type A and C secrete the α-toxin but the development of NE in chickens is not only dependent on *C. perfringens* producing a functional α-toxin. The association with other predisposing factors such as nutrition and/or concomitant diseases is also involved in pathogenic mechanisms. In favourable conditions, the bacterial population can be duplicated every 3 to 5 minutes, which explains the suddenness of infections with *Clostridium* in poultry farms, and the need for quickly correction actions (Figure 4) (Valancony, 2001).

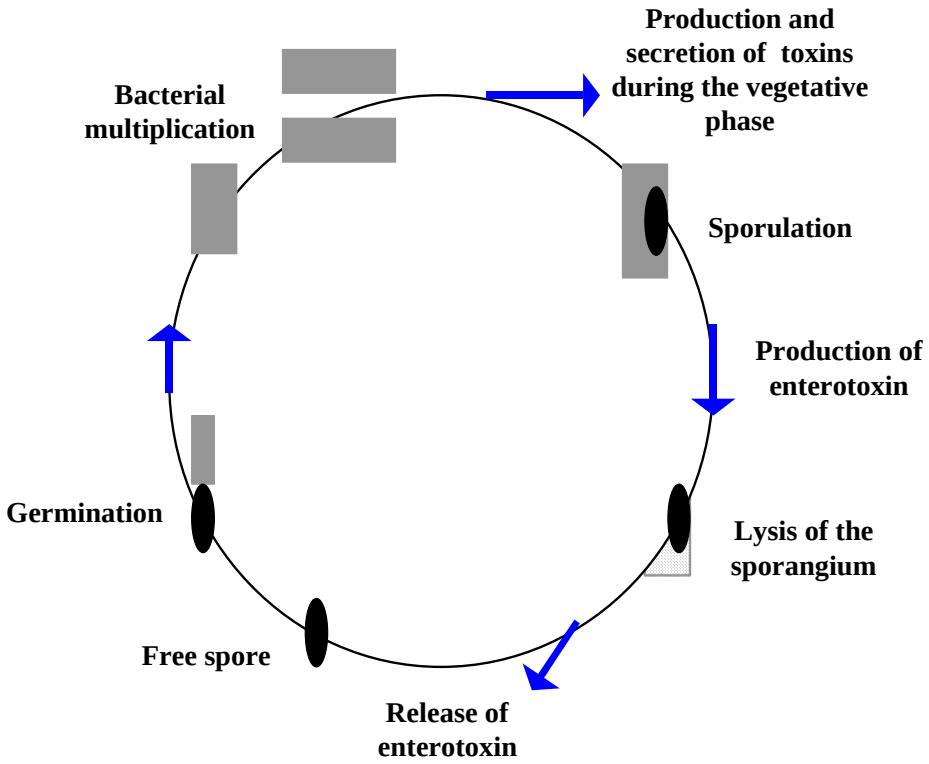


Figure 4: Cycle of *Clostridium perfringens*

Adapted from Valancony, 2001

B) Symptoms and lesions

The NE is an acute disease with a very fast evolution. *C. perfringens* can also be associated with hepatitis and cholangio-hepatitis, leading to reduction of the performances in poultry production (Perdersen *et al.*, 2003). It occurs in birds in apparent good health, but presenting symptoms of prostration, loss of appetite, and low growth rate, sometimes followed by bloody and blackish diarrhoea (Valancony, 2001). This disease affects broilers between 15 and 25 days of age, period when the level of maternal antibodies is reduced. The rate of mortality varies between 5 and 10% (Pedersen *et al*., 2003).

Lesions, mainly caused by the production of α toxin (Lovland *et al*., 2001), sit principally at the level of the small intestine (jejunum ileum). According to the severity of the disease, small ulcers or small yellow points on the mucous membrane appear in the intestine. Usually, the

most severe form observed is: a membrane covers all the mucus membrane of long segments of the small intestine. The content of the small intestine is dilated by gases and looks like “bread crumb”. The intestinal wall is thinned or velvety. The gizzard is often filled with food. The muscles are faded, and sometimes, intramuscular bleedings appear. The liver presents zones of degeneration, and it can take the aspect of a cooked liver. Biliary retention is possible. The putrefaction of the corpses is fast. Lesions of this disease must be differentiated from those of coccidiosis and ulcerative enteritis with *Clostridium colinum* (Valancony, 2001).

C) Sources of contamination

C. perfringens usually uses the oral way to penetrate into the poultry. It can be through ingestion of soiled litter, dejections of infected animals, or contaminated food. The disease is not transmitted by direct contact between animals. At the opposite of other bacteria which settle on the walls of the digestive tract or which penetrate them (*Salmonella*), *Clostridia* remain in the alimentary bolus. Usually, the ingestion of *Clostridia* has no consequence. To induce disease, several risk factors must occur simultaneously and Clostridia concentration must reach 10^6 bacteria per gram of digestive content to induce lesions. The disease is especially related to the production of toxins in large quantity, which are responsible for intestinal wall necroses and mortality (Valancony, 2001).

Several factors promote the multiplication of *C. perfringens*:

- Sensitive birds that are more or less weakened (immunodeficient, having undergone a thermal stress, etc.) (Valancony, 2001).
- Low bacteriological and chemical quality of water (especially an alkaline pH).
- Low speed of the intestinal transit. Indeed, undigested dietary components (starch, protein) that stagnate in the small intestine are an ideal substrate for the growth of *Clostridia*.
- Unbalance of the digestive flora caused by an inappropriate use of feed additives such as AGP, coccidiostats, and antibiotics.
- Abrupt food transitions.
- High dietary levels of energy and protein (a high proportion of wheat and/or barley in the diet increases the risk of necrotic enteritis).
- Diseases such as Gumboro and Marek diseases, the virus of avian infectious anaemia (immune-depressing factors).
- Presence of mycotoxins in the feed (immuno-depressing factors).
- Use of fast-growing broilers (Pedersen *et al*., 2003).

D) Treatments and prevention

Treatments

When the diagnosis is made sufficiently early, clinical disease manifestations can be managed more or less easily by an antibiotic treatment. *C. perfringens* has a high sensitivity to some antibiotics (β-lactamins, macrolids, etc.). Coccidiostats can be used also against some parasite infections (Valancony, 2001).

C. perfringens presents however resistances to some antibiotics (Colistin, Tetracyclin, etc). According to the study of Mitsch *et al.* (2004), phyto-therapy may be an alternative method to solve this problem. Indeed, essential oils would stimulate the activity of the digestive enzymes, stabilize the intestinal flora and deactivate toxins produced by *C. perfringens*, thus allowing the decrease the intestinal colonization by this germ and consequently the risk of necrotic enteritis. However, further studies on the efficiency of essential oils on the toxins of this bacterium are necessary.

According to the study of Lovland *et al.* (2004), maternal vaccination seems to be also an alternative for the control of the necrotic enteritis in broiler farms. Indeed, broiler breeder hens were inoculated by intramuscular injection with different candidate vaccines prepared with toxins (types A and C) produced by *C. perfringens* and aluminium hydroxide as adjuvant. Vaccination induced an important serologic response with the production of immunoglobulins (IgG) against the α toxin produced by *C. perfringens,* and specific antibodies could be transferred to the line of descent. Vaccination could allow a compensation of the maternal α toxin antibodies reduction.

Prevention

a- Sanitary measures

The best prevention remains a good hygiene in farms:

- Use of recognized disinfectants at the concentration allowing the destruction of the bacterial spores.
- Decontamination of the water pipelines using alkaline detergent followed by acid-scaling product.
- Chlorination and/or acidification of the drinking water.
- Control of the water's pH and sulphite-reducing anaerobic flora in water.
- Cleaning and adequate disinfections of the silos used for the feed storage.
- Decontamination of the clay soils and of the hen housing system by spreading of quicklime (0.5 kg/m^3) or using caustic soda (Valancony, 2001).

b- Control of the balance of the intestinal flora

To prevent necrotic enteritis is also to control the balance of the intestinal flora, using:

- Acidification of the litter (using formic or propionic acid and sodium lignosulfate) in order to reduce the horizontal transmission of pathogenic bacteria such as *C. perfringens*.
- Acidifying agent in the drinking water.
- Feed additives sold as regulators of the intestinal flora.
- Adequate light program to avoid a too fast growth rate of the chicks.
- Balanced diet compositions.
- Progressive food transitions (Novoa Garrido *et al*., 2004).

2.2 - UNSPECIFIC ENTERITIS

Until now, the causes of unspecific enteritis are still misunderstood. However, the origins of unspecific enteritis seem to be multifactorial, and it seems to be caused by several micro-organisms, alone or associated.

2.2.1 - *Campylobacter* spp.

A) Description of the bacteria

Campylobacter spp. is a Gram-negative bacterium, it is helicoidal sticks (1.5-6.0 $\times$ 0.2-0.5 µm) very mobile, curved with frayed ends and one or two polar flagella.

C. jejuni, *C. coli* and *C. lari*, the most common species, are microaerophilic micro-organisms, i.e. they require a level of oxygen for growth under the air level (O_2: 3-15% and CO_2: 3-5%) (Denis *et al*., 2001). These bacteria are also defined as being thermophilic, i.e. they are able to grow under the range of temperature 37-42°C, with an optimum of growth at 42°C.

However, they are sensitive to temperatures higher than 60°C, and to cold temperatures (Keener *et al.*, 2004). These last characteristics reflect a good adaptation to their usual habitat: homeotherms' intestine, including birds' intestine (Denis *et al*., 2001).
Campylobacter spp. is regarded as a commensal organism of the avian species. When infected chickens are introduced into a farm, *Campylobacter* spp. is propagated very quickly (Keener *et al*., 2004). At the opposite of *Salmonella* spp., *Campylobacter* spp. colonizes mainly the digestive tract of chickens. They spread over the caeca, the distal jejunum and the cloaca by their polar flagella that facilitate the penetration through the layer of mucus of the intestinal epithelium, as well as the adhesion to the walls of the caeca. Moreover, these bacteria are adapted to live in the mucus of the caeca and cloaca.
Under certain conditions (stationary phase, exposure to atmospheric oxygen), *Campylobacter* spp. can become spherical or coccoidal. This change is associated with a transition from a culturable viable form to an unculturable viable form. This latest form is an adaptation to survive in a hostile environment (Denis *et al*., 2001).

B) Symptoms and lesions

The disease is generally either asymptomatic or induces very discrete symptoms in the animals. The chicks are uninfected at birth but often from the second or the third week of age, for the most part, animals are infected through the environment or water. The symptoms are diarrhoeas and microscopic lesions at the levels of ileum and caeca (Mead, 2002).

C) Sources of contamination

During the period of rearing, the contamination of the poultry flocks occurs generally between the second and the fifth week of age, but rarely before the second week (Laisney *et al*., 1999). The factors of contamination are numerous and various.

a- Vertical transmission

Vertical transmission of *Campylobacter* spp. from the hen to the chick via egg is an improbable way of transmission. Several studies demonstrate that the eggs and the chicks coming from hens contaminated by *Campylobacter* spp. are *Campylobacter* free. In addition, the penetration of *Campylobacter* spp. in eggs seems to be very difficult (Denis *et al*., 2001). However, a study highlighted the possibility that *Campylobacter* spp. could be transmitted from one generation to the following one. Thus, the assumption of a vertical transmission in the contamination of the chicken flocks by this bacteria cannot be completely excluded, but seems however unlikely (Réfrégier-Petton *et al*., 2001a, 2001b).

b- Horizontal transmission

The horizontal transmission plays a major role in the contamination of the chicken rearing. The risk factors are various, such as:

- The summer/autumn period is a factor increasing the risk of contamination.
- A static system of ventilation (or natural ventilation) can induce problems of circulation/renewal of air inside the building, as well as temperature, in particular during hot seasons. Low oxygen contents and high temperatures are favourable conditions for growth of *Campylobacter* spp.
- The acidification of the drinking water could be favourable to the contamination by *Campylobacter* spp. This change could favour the growth of *Campylobacter spp.* compared to the other micro-organisms present in the natural intestinal flora of chickens.
- The number of people working in the building, humans being the vector of these bacteria from the external environment into the building.

- The number of buildings on the site. The management of several hen houses on the same site implies a regular passage from one building to another. Thus, the farmer can also be a vector of contamination from one building to another. A number equal or higher than three building is a factor increasing the risk. (Refregier-Petton *et al*., 2001).
- The presence of tenebrio is related to the contamination by *Campylobacter* spp.
- The surrounding soil is a reservoir of *Campylobacter* spp.
- The dirty litter
- The presence of rodents, wild birds and insects in the buildings.
- Other animals present on the exploitation.
- Lack of both cleaning and disinfections between two flocks (Réfrégier-Petton *et al*., 2001a, 2001b).
- The process of reduction of bird density. The partial removal of the chickens can be responsible for a contamination by *Campylobacter* spp. Indeed, material insufficiently cleaned and disinfected is a vector for the introduction of *Campylobacter* spp. (Laisney *et al*., 1999).
- The coprophagy (Denis *et al*., 2001).
- Stress periods.
- Boxes used for the transportation of chicks, when insufficiently cleaned and disinfected (Vienot, 2004).

The main vectors of *Campylobacter* spp. in the chicken rearing farms are drinking water and the environment, in particular, the surrounding soil around the buildings (Denis *et al*., 2001).

D) Treatments and prevention

Means to reduce the contaminations by *Campylobacter* spp. are various, such as:

- Disinfections of the building between two flocks.
- Fight against the vermin (rat extermination, elimination of the insects, etc.)
- Regular disinfections of the distribution system of drinking water.
- Chlorination of drinking water.
- Adjustment of the accesses of the hen house (cement, treat the soil, etc.) (Denis *et al*., 2001).
- Respect of the sanitary barriers (clean zone/dirty zone, change of behaviour, foot bath, etc.) (Vienot, 2004).
- Cleaning and disinfections of the material used for birds transportation, in particular at the time as density reduction.
- Antibiotic administration. An antibiotic treatment given to the birds following a disease decreases the risk of contamination by *Campylobacter* spp. (Refregier-Petton *et al*., 2001)

  Two hypotheses can be given:
  - the objective of the antibiotic treatment is to stop the development or to destroy the pathogenic agents. Like other micro-organisms, *Campylobacter* spp. could be sensitive to this treatment.
  - an antibiotic treatment modifies the ecology of the intestinal flora of chickens, which becomes unbalanced. This unbalanced environment would not be favourable to the implantation of *Campylobacter* spp.

In addition to these means of fight against *Campylobacter* spp., there are new possibilities which are considered, such as:

- Vaccination (Vienot, 2004).
- The use of organic acids to treat the drinking water. Chaveerach *et al.* (2002) studied the bactericidal activity of four organic acids (Formic, acetic, Propionic and Hydrochloric acids) on *Campylobacter* spp., at various levels of pH (pH 4; 4.5; 5; 5; 5.5). They highlighted that all these organic acids, administered alone or in combination, had a strong bactericidal capacity on *Campylobacter* spp. at pH 4. Thus,

a regular utilisation in the drinking water of chickens would prevent and/or decrease the transmission of *Campylobacter* spp.

- Administration of a barrier flora. According to the study of Laisney *et al.* (2003), the administration of a barrier flora to young chicks (because they seem to be less sensitive to the contamination by *Campylobacter* spp. during the first two weeks of rearing) could limit the implantation of these bacteria. However, a genetic factor has to be taken into account. Indeed, this flora is less effective when the inoculum comes from different chicken species to the producing species.

2.2.2 - ***Salmonella* spp.**

A) Description of the bacteria

Salmonella are bacteria Gram negative from the *Enterobacteriaceae* group. They are bacilli of 3 microns long for 0.5 micron wide, not sporulated, not capsulated, mobile or motionless (Villate, 2001). They are able to grow at temperature ranging from 7°C to 46°C, the optimal a_w (activity of water) for their growth is higher than 0.92 (Beroff *et al*., 1998). *Salmonella* could be destroyed by high temperature (at least 60°C), light, Formol, Hypochlorites, Phenolic and Iodophorous disinfectants in the absence of abundant organic matter like plant and animal material.

Two species of *Salmonella* can be pointed out: *S. bongori* including only one species, and *S. enterica* is subdivided in 6 subspecies (including *enterica*). Among all these subspecies, it is possible to distinguish different serovars (or serotype) characterized by their somatic antigens O and their flagella antigens H. In France, 99.8% of *Salmonella* isolated from humans and homeothermic animals belong to the subspecies I (*enterica*), while the pathogenic role of subspecies II, IIIa and IIIb are exceptional and those of subspecies IV, V and VI is unknown. Up to now, these last subspecies have been found only in the environment. The Enteritis and Typhimurium serovars, including subspecies are the most frequently found and are ubiquitous, they can colonize indifferently various animal species.
The reservoir for *Salmonella* is very broad, and many animals, including poultry, are likely to take, multiply and excrete these bacteria. Most of *Salmonella* present in the environment (soil, water, feedstuffs, etc.) or in food products meant to humans come from a faecal or caecal contamination. These various host constitute secondary reservoirs where *Salmonella* can survive a very a long time but multiply only accidentally. The principal reservoirs in which *Salmonella* multiply actively are all the digestive tracts of their potential hosts.

B) Symptoms and lesions

In birds, more than 200 serotypes of *Salmonella* have been identified, and the disease is expressed clinically, according to the date of infection and of the age of the individuals, by extremely various genital, digestive or organic disorders (Lecoanet, 1992).

Clinical aspects

- **In young subjects**

- Antenatal disease with embryonic mortalities and hatching disorders.
- Postnatal disease with white and chalky diarrhoeas in the case of pullorose (*Salmonella pullorum* Gallinarum), or an Enteritis associated with lesions of either pericarditis or perihepatitis in the case of other salmonellosis (*Salmonella* Enteritis and Typhimurium). In all cases, mortality exists with a strong heterogeneity.

- **In adults**

- The chronic form is often the prolongation of a pullorose and appears especially through genital disorders: a delay of ovulation with a fall of laying rate, an ovaro-salpingitis (eggs containing blood necrotic or stained fragments because of a prolonged stay on the nests), and an attack of the shelly gland (absence of shell). A stenose or obstruction of the oviduct is often at the origin of abdominal laying and

peritonitis, quickly fatal. Abdominal cysts or inversions of the cloaca are also observed.

- The acute form of salmonellosis in adults corresponds to the typhose characterized by serious general symptoms (prostration, fever, severe cyanositis of the limbs), digestive symptoms with greenish yellow and blood striated diarrhoeas causing an inextinguishable thirst, respiratory and nervous disorders that can be also observed in some subjects (Lecoanet, 1992).

Lesions

Lesions observed in poultry depend on the age of the animals. In young birds, the lesions are: persistency of the vitellin bag, catarrhal inflammation of the caeca, hepatic necrosis seats, and nodular lesions much more characteristic of the heart, the lung and the liver in the chronic forms (Lecoanet, 1992). As for the adults, the apparent lesions are green bronze and hypertrophied liver, hypertrophied spleen, casein plug in the caeca, and enteritis sometimes haemorrhagic or membranous.

C) Sources of contamination

Salmonella Enteritidis and *S.* Typhimurium are able to transgress the digestive barrier, to extend to the sub-mucosa and to infect the deep organs (liver, spleen, ovary). Indeed, even after phagocytosis by monocytes and macrophages, they are able to survive and to multiply by preventing the adhesion of the primary lysosomes to the secondary lysosomes. A vertical transmission is then possible.
However, the serotypes of *Salmonella* “distributed” by the hatcheries tend to naturally vanish with time, the relay being taken by the serovars of the rearing farm. The presence of *Salmonella* in the environment of the buildings (water, food, materials, etc.) plays a major role in the propagation and transmission of the affection. The sensitivity of the birds is correlated to the rearing stress and to the aggressiveness of the bacterial strain. The modifications of the intestinal flora caused by an inappropriate antibiotic administration during the first days of life of the chicks promote the fixation of *Salmonella,* because of the delaying or unbalancing of the fixation of the beneficial intestinal flora, reducing competitive exclusion (Villate, 2001).

D) Treatments and prevention

Treatment

The treatment of *Salmonella* utilises the whole therapeutic arsenal used against the negative Gram germs (quinolones, aminosids, betalactamins, tetracyclins). It must be applied as soon as possible, during at least 4 to 5 days, at a sufficiently high dosage (Villate, 2001). *Salmonella* have the property to multiply and concentrate in two organs in poultry: caeca and liver. Antibiotics having an entero-hepatic cycle, as quinolones, should be used for an effective action. However, in spite of the resistance to many antibiotics there are several active antibiotics available (Lecoanet, 1992).

Prevention

- **Vaccination**

Vaccines are prepared from killed or living strains spontaneously attenuated or worked out in laboratory (physical or chemical mutagenic agents, molecular biology targeted on a precise gene). The living vaccines are more effective and are prepared from the auxotroph mutants (failure in the synthesis of certain essential elements for the life of the bacterium), but these mutant bacteria can sometimes recover a virulence (Villate, 2001).

- **Competitive exclusion (flora of barrier)**

The principle of Competitive Exclusion (CE) consists in providing the animals, as soon as possible, with a non-pathogenic balanced flora that will colonize the intestinal light of chicks.

Being established the first, this flora will prevent the adhesion and thus the later implantation of germs coming from the external medium, therefore not very controlled, and likely to be pathogenic or undesirable. This flora constitutes a “barrier” between exogenic germs and the intestinal mucous membrane.

- **Feed**

During the manufacture of animal feed, it is necessary to be ensured of the high bacterial quality of the materials, of the control of heat treatment, and of general hygiene (Beroff, 1998). The acidification of feed can considerably reduce the danger of contaminated feed (Lecoanet, 1992). At the poultry farm, the silo is the main reservoir of *Salmonella*. It must be cleaned then disinfected using a bactericidal product (smoke-bombe) at the time of each “sanitary depopulated period”.

- **Environment of the rearing house and sanitary prevention**

It is very difficult to establish the mode, the origin and the infection level with *Salmonella*. The elementary rules of sanitary prophylaxis can be applied:

- Insects and rats extermination: tenebrios and rodents are the elements of persistence of *Salmonella* in the rearing housing system. Rodents frequently pollute the litter stored. *Salmonella* are present in and/or on the tenebrios, and poultry become infected by ingesting either the larva or the adult tenebrio.
- Cleaning, disinfections of the building and the material.
- “sanitary depopulated period” between two successive flocks.
- Presence of a “buffer room” at the entrance of the building.
- Silos easy to clean.
- Absence of wild or domestic animals.
- Litter preferably composed with shaving.

Good hygiene of the poultry farmers and technicians. They must change shoes and wash their hands before entering the housing system.

2.2.3 - Listeria monocytogenes

A) Description of the bacterium

L. monocytogenes is a Gram-positive bacillus, appearing as a regular stick (0,5-2.0 µm length and 0.4-0.5 µm diameter) and round-ended. Several bacteria are associated among themselves in parallel in short chains, or in pairs with a V shape. This bacterium is no sporulated. It is mobile within 20-25°C, thanks to peritrich flagella, and motionless at 37°C. It can grow under aerobic and anaerobic atmosphere. *L. monocytogenes* is able to colonize many ecological niches. The *Listeria* bacteria include 5 other non-pathogenic species for human: *L. innocua, L. ivanovii, L. seeligeri, L. welshimeri* and *L. gravi*.

The optimal temperature for growth ranges between 30 and 37°C, but growth is possible between -2 and +45°C. *L. monocytogenes* is not considered as heat-resisting and is quickly destroyed at 60°C. However, a thermal pre-treatment can influence its thermotolerance. *L. monocytogenes* multiplies between pH 4.6 and 9.6 with an optimum at pH 7.1. The minimal a_w for the growth of *L. monocytogenes* is 0.90. This bacterium generally does not develop in medium containing more than 10-11% NaCl. This bacterium has moderate nutritive requirements allowing its growth in adverse conditions.
L. monocytogenes is ubiquist. It is commonly found in soil, water and on plants, particularly those in decomposition. Soil is considered as the source of contamination. *L. monocytogenes* can survive several weeks in soil. The survival of *L. monocytogenes* depends on the nature of the soil and its moisture degree. This bacterium is found in excrements of a large number of healthy animal species. It was isolated mainly from excrements of herbivores, but also of pig and poultry. The faecal excretion is often related to

the type of poultry farms and animal feeding. *L. monocytogenes* is found in intensive poultry rearing, but it is an emergent bacterium.

B) Symptoms and lesions

Listeriosis is essentially an animal infection, but accidentally human. It occurs sporadically in birds, but it can evolve in an endemic way in certain exploitations according to the techniques of rearing. *L. ivanovii* and *L. monocytogenes* are recognized as pathogenic for the animal.
Animal contamination happens most generally through plant ingestion, herbivores are the main targets. However, birds can also be infected by listeriosis. Indeed, poultry with a good physiological state can ingest large quantities of *L. monocytogenes* without any clinical symptom. However, they excrete germs, mainly in faeces.

When listeriosis occurs in poultry farms, the disease is frequently associated with an affection that weakens the immune system: salmonellosis, coccidiosis, etc. Young birds seem more sensitive than older subjects (Bailey *et al.*, 1990). The mortality is very variable, generally low but can reach 40%. The symptoms are relatively unknown. Thus, in numerous wild or domestic birds, no symptom is observed, at the exception of some prostrated chickens, in some cases. They were easily captured and exhibited a high reduction of growth rate (anorexia). A mucous membranes cyanosis and some potential diarrhoea can also be observed. Nervous symptoms sometimes sign a meningo-encephalic form, such as stiff necks, shaking, and lack of coordination of the movements. In other cases, a septicemic form can also be observed as the origin of a sudden death. In young birds, lesions of spleen, kidneys, heart] and liver are also noticed (Bailey *et al*., 1990).

C) Sources of contamination

In poultry rearing, the birds are frequently contaminated by *L. monocytogenes*. The main sources of contamination are:

- Feed and drinking water.
- Litter and droppings.
- High presence of rodents and arthropods.
- High presence of wild and semi-domestic birds (vector role of healthy carriers).
- Soil contaminated by healthy carriers dejections.

2.2.4 - Escherichia coli

A) Description of the bacterium

Escherichia coli is a Gram-negative bacterium, not sporulated, 2.5 microns long by 0.6 microns wide, most often mobile (Villate, 2001). This bacterium belongs to the family of the faecal coliforms. This germ is a commensal host of the digestive tract of the poultry and most of the strains are not pathogenic. The most important reservoir of the avian *E. coli* is the digestive tract of the animal where 10 to 15% of the colon bacillus population belong to potentially pathogenic serotypes. In chicken, the concentrations are about 10^6 bacilli per gram of faecal matter. Greatest concentrations are found in young chickens of less than 3 weeks old, mainly at the level of the posterior digestive tract (Stordeur and Mainil, 2002). The colon bacilli usually considered as pathogenic for poultry are O78 K80, O1 and O2 and the aerobactin positive strains. The pathogenic capacity of bacilli from the colon is related to various virulence factors that allow them to multiply, to leave the intestinal environment and to produce toxins.

B) Symptoms and lesions

E. coli causes few enteritis in birds: 10 to 15% of the bacilli considered as pathogenic are normal hosts of the avian digestive tract and settle down on pre-existent lesions or on a weakened organism. Colibacillic diseases affect essentially young birds because of their

immature immune system and the absence of barrier effect of the incomplete intestinal flora. The single intervention of the bacillus in avian diseases is rare and is only due to very virulent strains. This disease is often the result of rearing mistakes worsened by the intervention of infectious agents (Villate, 2001).

C) Sources of contamination

Colibacillic contamination occurs essentially through air. The falling apart of dry droppings and litter causes the emission of dust which constitutes an important source of contamination (Villate, 2001). It has been demonstrated that dust present in the rearing building can contain up to 10^6 bacilli per gram. These germs can also be found in the food and drinking water (Stordeur and Mainil, 2002). The drinking water can be soiled by faecal matter and can become a real “culture medium” (Vilate, 2001).

The direct vertical transmission, starting from the infected ovary or oviduct is rare. The major infection source of eggs seems to be the contamination of the surface (egg shell) while crossing the cloaca or while falling on a dirty litter. Bacteria are imprisoned during the drying of cuticles. They are restored during the hatching in true aerosols (Villate, 2001). The transmission of the pathogenic strain to the whole set after hatching is fast (Stordeur and Mainil, 2002).

D) Treatments and prevention

Treatments

Treatments are mainly based on antibiotherapy. The most used antibiotics are sulphamids, betalactamins and quinolons. However, it is necessary to take care to the use of antibiotics because recent studies showed an increasing number of resistant strains (Stordeur and Mainil, 2002). It is advisable to treat colibacilloses after a rational antibiogram and the duration of the treatment should be long enough (5 days as minimum), in order to avoid antibiotic resistance (Villate, 2001).
Alternative treatments to antibiotics exist, such as treatment with ascorbic acid that contributes to intensify the activity of phagocytes (Stordeur and Mainil, 2002).

Prevention

Prevention aims to control the environmental contaminations, the animated and unanimated vectors, in order to reduce to a maximum the predisposing factors. One of the methods consists in decreasing and better controlling the faecal contaminations, by reducing the transmission of the *E. coli* from the hen to the chick thanks to a fumigation of the eggs within the 2 hours following the laying, in collecting them as quickly as possible and in turning down those in bad condition or presenting faecal stains at their surface.
Infections of the respiratory tract of the animals can be reduced by rearing mycoplasm free animals and by a better control of certain environmental factors, such as humidity, ventilation, and dust production and ammonia levels in the air. Rodents, parasitic, coprophagous, and necrophagous insects are also potential reservoirs of colon bacilli and must be systematically destroyed (Stordeur and Mainil, 2002).

General measures of prevention can be applied, such as disinfections of the building and of the water pipelines, the regular cleanings of the materials of watering, and others are at present recommended on the field, such as acidification of feed and water, and supply of barrier flora (lactic flora), probiotics or exogenous digestive enzymes.

2.2.5 - Enterococcus spp.

The general Classification of faecal *Streptococcus* was modified in the Eighties by the creation of a new type, *Enterococcus*. In this context, several species belonging before to the

Streptococcus type were transferred towards the type *Enterococcus* corresponding more or less to the *Streptococcus* of the serologic group D in the classification of Lancefield.

A) Description of the bacterium

Enterococcus spp. are Gram-positive coccus, found alone, in pairs or in short chains. Their morphology can vary according to culture conditions. They are not sporulated bacteria, sometimes mobile, aero-anaerobic and homofermenting (the glucose fermentation leads to the production of lactic acid). Their optimum of temperature is 35°C. *Enterococcus* are able to survive under hostile conditions, at temperatures ranging between 10°C and 45°C, and a pH of 9.6. Moreover, some species can resist to a heating of 60°C during 30 min.

Enterococcus spp. is ubiquist bacteria that are in human intestine and animal intestine, in wastewater, fresh water, seawater, soil and on plants. Most of the *Enterococcus* species take part in the composition of the intestinal flora and some *Enterococcus* strains spp. would have a favourable effect on the growth of the animals because of the competition with the pathogenic germs. Several species can cohabit within the same ecological niche, but there is a relative specificity of the host. In poultry, the most observed species are *Enterococcus durans*, *E. faecium* and *E. faecalis* in young animals and *E. cecorum* in animals older than 12 weeks.
The persistence of *Enterococcus* in various types of water can be higher than the other indicating organisms, in particular because of their resistance to the disinfecting agents. Thus, they are privileged indicators to the effectiveness of the water treatments. Moreover, owing to their high resistance to desiccation, *Enterococcus* is a good indicator for the control of the pipes during repairing of the water distribution system requiring a dry out. In addition, usually *Enterococcus* are not detected in the pipes, their detection indicates a recent faecal pollution. The interest of *Enterococcus* compared to the coliforms (including *Escherichia coli*) is due to their higher resistance to environmental conditions (cold temperature and small quantity of nutritive elements) and to their longer persistence in water.

B) Symptoms and lesions

In poulty, *E. faecalis* have been associated with cases of amylosis. Animals present a delay of growth and a deposition of amyloidal substance in many organs and particularly in the articulations, leading to polyarthritises and lameness. A lack of hygiene during the chick vaccination against the disease of Marek can lead to a contamination of the needles and vaccine suspensions by strains of *E. faecalis,* which could explains the occurrence of the affection in some poultry farms. The possibility of a vertical contamination by eggs has been shown experimentally. *E. hirae* spark off some delay of growth in poultry. This pathogenic species was shown to be responsible for nervous disorders in 3 to 8 days old chicks. The disease that usually affects 1% of the animals, results in stiff necks. At the autopsy, small yellowish spots are visible in the brain and the histology reveals the presence of small centres of necrosis.

2.2.6 - Coccidia

A) Description of the protozoa

Coccidiosis, frequently observed in poultry farming, is a parasitic disease caused by intracellular protozoa called coccidia that multiply in the intestinal tract of the birds (Repérant, 1998). In chicken, there are five main species of coccidia (*Eimeria acervulina*, *E. brunetti, E. maximum, E. necatrix and E. tenella*), more or less pathogenic, which can be identified according to their intestinal localization, to the induced lesions, and to the size of their oocysts (Figure 5) (Gregrio Rosales, 1998). The two species the most frequently observed are *Eimeria acervulina* and *E. tenella.* They survive very easily in the environment, whatever the conditions of temperature and moisture and even after meticulous disinfections of the

housing system. Non-optimal breeding conditions and birds with a bad health status are favourable conditions for the multiplication of coccidian.

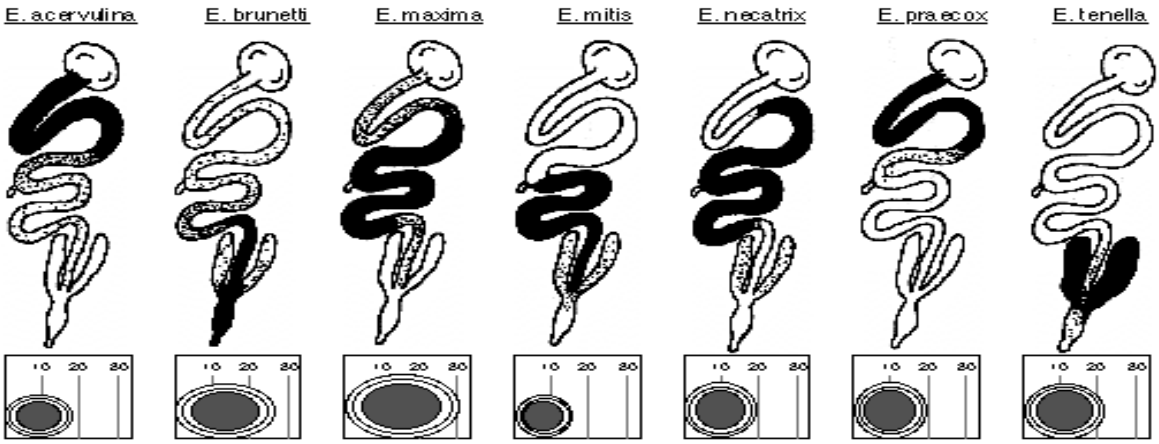


Figure 5: **Localization and size (µm) of coccidia**

Adapted from Crevieu-Gabriel, 2001

The duration of the cycle of the intracellular parasite in the host is 4 to 6 days according to the species (Figure 6) (Crevieu-Gabriel, 2001).

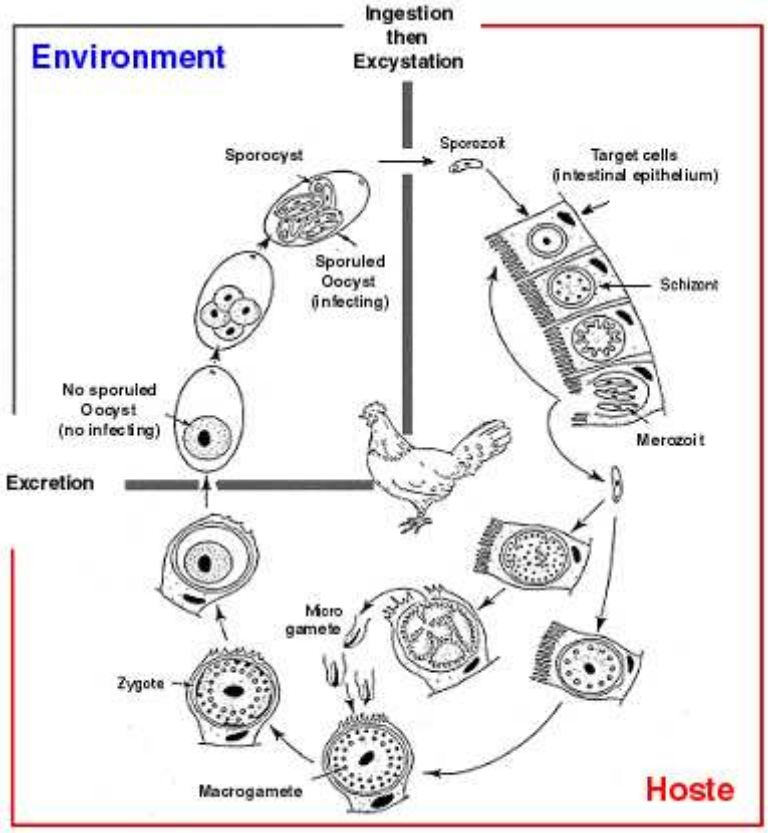


Figure 6: **Cycle of life of *Eimeria* spp.**

Adapted from Crevieu-Gabriel, 2001

B) Symptoms and lesions

Infected birds look chilly and prostrated and the intensity of these symptoms is related to the severity of the disease. They tend to huddle, they curl up, their eyes are half-closed or

closed, their feathers are dirty and dishevelled, and they present hanging wings. This condition is accompanied or not, depending on the species of coccidia causing the problem, by a loss of appetite, a loss of weight, and hemorrhagic diarrhoeas. These symptoms are specific to coccidiosis only if they occur in chickens at 3-4 weeks of age.

Two types of coccidiosis can be observed:

Caecal coccidiosis is caused by *E. tenella*. As caecum does not play a major role in the digestive function, caecal coccidiosis become prejudicial for the birds only when they cause clinical symptoms. They are characterized by a loss of appetite, anaemia and bloody diarrhoeas leading to high rates of mortality.

Intestinal coccidiosis is caused by other species and is generally less serious. Clinical intestinal coccidiosis is characterized by a loss of appetite, lower growth performances, and diarrhoeas, these symptoms varie with the level of infection. The parasitic development can disturb the digestive function. The absorption of nutrients is disturbed all along the intestine but mainly at the duodenum and jejunum levels. Intestinal coccidiosis can also alter certain metabolisms (protein synthesis for instance) and to have consequences on the poultry production (decrease of the feed efficiency, increase of the heterogeneity of the flocks, development of pathogenic bacteria in the digestive flora).

When efficient coccidiostats are used, mortality due to coccidiosis is rare. The disease is characterized by a low growth rate, a low feed efficiency, and intestinal lesions that are sometimes difficult to be identified.

Sensitivity of the host

Among the factors related to the host, it can be mentioned:

- Age: coccidiosis is a disease affecting young birds. In poultry breedings, excretion of oocysts is observed between 28 and 35 days of age, when the number of oocysts present in the litter is maximum, then this number declines.
- Race/strain: some races or strains are less sensitive to coccidiosis than others. However, the absence of symptom does not mean the absence of parasite. The excretion of oocysts by a resistant strain can be comparable with that of a sensitive strain, with the same consequences on the contamination of the environment.
- Immune potential: According to the degree of protection, the immune response contributes to a reduction of the severity of the lesions, of the production of oocysts and to an improvement of the birds' performances.
- Feeding: nutrition plays a major role on the health status of the animal. The lesion's scores due to coccidiosis are higher in chickens fed a wheat-based diet compared to chicken fed a maize-based diet.
- Coccidiosis protection.
- Intercurrent diseases: coccidial lesions are worsened when certain bacteria, virus or mycotoxins are present. Coccidia and bacteria seem to have a reciprocal action on their development. Thus, lesions due to *E. tenella* exist only in the presence of the bacterial flora of the caecum, while *E. tenella* promotes the development of *Salmonella*. The intestinal flora does not seem to have any effect on the development of *E. acervulina,* while it promotes the development of germs such as *Clostridium perfringens*. Intercurrent diseases promote coccidiosis by decreasing the resistance of the host, the food consumption and consequently the intake of coccidiostats.

Risk factors

- Coccidiosis are infections occurring most often in autumn and winter (Graat *et al*., 1996). Coccidiosis are very prolific and generally do not kill their host. This balance between the parasite and its host can be altered by various factors. Indeed, a bad control of either the breeding conditions (density, temperature, hygrometry, ventilation, and lighting) or of the feeding (unexpected breaks in the distribution, low

levels of incorporation of coccidiostats into the feed, presence of mycotoxins, deficiency in proteins or vitamins) stress the chickens, causing reactions of the animals which increase their sensitivity to coccidia and promote the development of these parasites. Moreover, birds affected by other diseases, like Gumboro or Marek, they have too weak immune defences to be able to resist to coccidiosis.

- It is important to stress that contamination with coccidia is inevitable and permanent in poultry farming even in farms where prevention and hygiene measures are correctly applied. The contamination appears during first band of chickens in a new building ("new building syndrome"). This initial contamination of the rearing buildings is not yet completely elucidated. However, chickens can live in harmony with their coccidia if the breeder takes care to maintaining the balance between the host and its parasites by an optimal management of the breeding (choice of the coccidiostat, rotation or alternation of the products to avoid the emergence of resistance, respect of the programmes of vaccination against the intercurrent diseases…).

C) Treatments and prevention

Coccidiostats

Coccidiostats are used in poultry feeding and are subject to the legislation for additives in animal feeding. Although their properties and use are related to the prevention of the diseases caused by the development of coccidia, they are not subject to the legislation concerning veterinary drugs.

Coccidiostats, as food additive, are incorporated at well-established dosages into the complete food. Their use follows precise rules, in particular concerning target species, the posology, periods of utilisation, delays of withdrawal before the slaughtering of the chickens or the marketing of the meat-products, the incompatibilities of drugs, etc. Since 2004, two coccidiostats are still used in European broiler farms. They belong to the ionophor family. All coccidiostats used on the field can lose all or part of their effectiveness, because of the emergence of resistance in the populations of coccidia. In-feed, coccidiostats are the preventing measures the most used in poultry farming (Repérant, 1998).

Alternative methods

- Alternative methods to coccidiostats can be considered, such as homeopathy, isotherapy, and phytotherapy.
- Homeopathy is therapy based on the practice of treating like with like. A disease is treated using agent/substance that produces in a healthy individual similar symptoms to those experienced by a sick individual. Homeopathic medicines contain very, very small quantities (high dilutions) of the agent/substance prepared in a special way.
- Isotherapy is a specialized application of homeopathic theory and therapy. A homeopathic remedy is prepared from blood or secretion of an infected individual in the same manner of dilutions as regular homeopathic medicines.
- Phytotherapy is the treatment of a disease using natural plant-substances.

Although according to certain actors of the poultry production, these alternative methods give satisfying results in the field for the control of coccidiosis, their efficiency have never been demonstrated in controlled experimentation (Repérant, 1998).

Control of the rearing conditions

The control of the rearing conditions remains a good means for limiting the problems of coccidiosis in broiler farms. The limitation of the contact between the birds and the oocysts present in their environment allows to stopping the cycle of the parasite. Consequently, it should be made sure that the litter is not degraded and that the working material, possibly in

contact with the animals, is disinfected. Moreover, control oocysts entry from the outside of the building allows to limiting the contamination of the environment of the birds.
The good sanitary follow-up of the birds with respect to all the affections is crucial in the control of coccidiosis. Indeed, the presence of other diseases among the flock promotes the contamination by *Eimeria* spp. because of a reduction of the immune defences of the birds. The elimination of the coccidia at the end of the rearing period, i.e. after the departure of the chicken, allows to start a new flock with a low parasitic pressure, promoting thereby a good performance for the birds. Therefore, it is necessary:

- To remove the litters at the end of the flock,
- To wash the building conscientiously,
- To dry the building (allows to eliminate mechanically the majority of the oocysts),
- To respect the crawl space,
- To start a flock with new litter and clean material.
- To insist on the mechanical cleaning, because disinfections do not have any effect on the oocysts (Repérant, 1998).

To make sure of the correct functioning of the feeding and drinking devices.

2.3 - Conclusion

Regarding the intestinal troubles, among the necrotic diseases, the NE is the most common and financially devastating bacterial disease in broiler flocks. The necrotic diseases are multifactorial. The sources of contamination are not specific, but it seems that the control of the hygienic quality of the environment (litter, feed, faeces…) and of the water could limit the appearance of these troubles. If the diagnosis is made soon enough, the symptoms can be controlled more or less easily by a treatment with antibiotics. However, because of problems of antibiotic resistance, the best ways to prevent necrotic diseases are a good hygiene and the control of digestive tract bacteria. Some other dietary and husbandry factors can contribute to the outbreak of necrotic enteritis. For example, damage to the intestinal mucosa through coccidial infection or a change in the normal intestinal microflora as a result of a change in diet formulation, such as inclusion of a high level of viscous cereal grains and/or animal by products like fish meal and meat meal, can predispose birds to the rapid proliferation of *C. perfringens*.

Section 3: Bacterial contamination in broiler and laying hens farms

Poultry is a known source of human pathogenic bacteria with the potential in causing gastrointestinal infections. The most important bacteria in this context are *Salmonella* and *Campylobacter*. Poultry is frequently found colonized and products can become contaminated during production, carrying the pathogenic germ to the consumer by means of contaminated meat. Other poultry associated bacteria are *C. perfringens*, *H. pullorum* and *L. monocytogenes*. These bacterial species can occur in the chicken gastrointestinal tract itself or be introduced to products during processing.

A major drawback in controlling these bacteria is their usually clinical unapparent colonization of broilers and laying hens. Poultry doesn't suffer from infection but is shedding the bacteria posing a risk to the consumer, who is often not aware of the presence of pathogenic bacteria. Improper kitchen hygiene can lead to the uptake of infectious numbers of pathogenic bacteria, finally inducing human illness.

The main sources and ways of contamination of these pathogens are not fully explained. Therefore, in a farm to fork approach, it is necessary to assess the incidence of these pathogens along the entire production chain and particularly at the primary production step (broiler and laying hen flocks), taking into account that it could be one of the sources of introduction of this pathogen into food plants.

During the different tasks of the project, microbial indicators to improve the characterisation of unspecific enteritis were followed (section 3.1). Moreover, the occurrence (the number of positive samples occurring at specified sample points during a defined period of time) of *Campylobacter* was estimated in broilers (section 3.3.1), the occurrence of *Salmonella* and *L. monocytogenes* was analysed in broilers and laying hens in Italy, Germany, the Czech Republic and Lithuania (sections 3.3.2 and 3.3.4), new emerging food-borne pathogens such as *C. perfringens* and *H. pullorum* were analysed in broilers in Germany, Lithuania, the Netherlands and France (section 3.3.3 and 3.3.5).

3.1 Characterisation of the unspecific intestinal troubles

3.1.1 - Introduction

Indicators allowing a better knowledge of the unspecific enteritis constitute a major stake for the farmers. This study characterized the apparent signs of unspecific enteritis emergence: qualitative modifications of the intestinal flora, deteriorations of the digestive tract, diarrhoeas and wet litters (Van Der Sluis, 2005). The links between these criteria were studied, as well as their impact on the rearing factors.

Three main objectives were:

- To take bearings on the notion of digestive troubles, to establish a classification through a list of the clinical symptoms of the illnesses, to determine which bacteria are involved, to list and to form into a hierarchy (according to experts) the different causes for the appearance of the digestive troubles.
- To follow about fifty broiler farms in order to quantify the digestive troubles, according to the list of clinical symptoms. Some indicators were followed at the critical period of breeding: water consumption, state/appearance of the droppings (use of Elancobox), injury index, breeding practice, and a final evaluation of the flock.
- To study the health status of the intestinal tract (ileum) by studying its morphology and the intestinal bacterial flora. The intestinal tract of some birds from 20 flocks out of 50 was sampled at the critical period. Selected samples from intestinal tract were analysed by classical counts, the histology of ileum samples was performed by microdissection in order to detect correlations between the histological lesions and the gut microflora population.

3.1.2 - Material and methods

Experimental trial and sampling

The study was carried out in 2005-2006 in West of France. It is related to batches of standard broilers, aged 28 days old. During the visits, a questionnaire relating to various topics (general information on the exploitation, the building, medical practises, management of the starting, water and medical history) was filled out with the stockbreeder, in order to specify his practices of breeding. Drinking water was analysed, in order to characterize its bacteriological quality. Collection of information relating to the batch characteristics, the rearing practices and the medical history was carried out at 28 days using a questionnaire. A clinical examination of the digestive tract of four animals taken randomly in each batch was carried out after autopsy, comprising a study of lesions of the various digestive segments. Four animals per flock were taken for autopsy. Content of the bacteriological tract was analysed to quantify the flora, and also to histological dissection of the tract. Samples of the intestine were also taken at the jejunum level and histological technique was used to realise morphometrical measures of villi (height, width) and crypts (depth, width) by picture analysis.

Different levels of lesions have been defined: the level 0 is applied to the farm with no intestinal lesions observed, the level 1 for the farm where birds present only lesions on the duodenum mucosa, and the level 2 concerns more extended lesions on the mucosa of two intestinal segments, duodenum and jejunum.

Cultivation and isolation

The intestinal microflora counts of jejunum and caeca contents (*Coliform* bacteria, *Lactobacillus,* Sulfito-reducing anaerobic bacteria) were performed according to the regulation (subcontracted analysis).

Moreover, dust and droppings were collected in order to test the presence of *L. monocytogenes* from 336 samples (5 samples of faeces and 2 samples of dust) taken from 48 of the 50 flocks. The samples have been analysed according to the standard NF EN ISO 11290-1. Characteristic colonies are identified biochemically then serotyped (see section 6).

Statistical analysis

The collected data were coded in nominal data and analysed. Four independent data associated with the presence of digestive disorders were created: diarrhoeas, wet litter, lesions of the digestive tract, and characterization of the digestive flora. These data were crossed between them and also with the rearing in order to show the different links and to carry out a first selection of the explanatory data.

3.1.3 - Results

A) Gut microflora

Lactobacillus is the main bacterial group in jejunum and caeca (approx. 90% of the total bacteria) (Table 1). There are significant differences between *Lactobacillus* and *Coliform* counts in jejunum and caeca, CFU range from 8.3 ± 3.1 to 9.2 ± 3.2 Log CFU/g. The number of sulfito-reducing bacteria is significant higher in caeca (3.8 ± 1 Log CFU/g) than in jejunum.

Table 1: Bacterial counts in jejunum and caeca

	Jejunum		**Caeca**	
	Mean Log CFU/g of content	**Standard Deviation**	**Mean Log CFU/g of content**	**Standard Deviation**
Lactobacillus	8.3	3.1	9.2	3.2
Coliform	6.1	2.3	8.7	2.6
Sulfito-Reducing anaerobic bacteria	Log CFU≤10	Log CFU≤10	3.8	1.0

These results are in accordance with the literature; in chickens, a large proportion of bacteria are Gram positive and mainly include facultative anaerobes from the crop to the terminal ileum, while caeca additionally contain strict anaerobes which are dominant. The crop bacteria are principally *Lactobacillus* attached to the epithelium and forming an almost continuous layer, and *Enterococci*, coliforms and yeasts Garbriel *et al*. 2006.

A statistically significant link (p=0.0095) was found between a high bacterial counts and digestive tract deteriorations. These deteriorations were characterized by the lesions of the mucus membrane (16% of the duodenum, 4% of the caeca) and atypical contents (22% of the duodenum, 38% of the jejunum, 12% of the caeca) (Table 2). These deteriorations were also significantly linked to the presence of wet litter (p=0.08).

Table 2: Lesions of the digestive tract

		Duodenum	**Jejunum**	**Caeca**
Membrane	Inflammation	38%		100%
	Petechies	37%		
	Scraps	25%	100%	
Content	Orange	54%	52%	
	Mucus	17%	4%	
	Liquid	29%	44%	83%
	Solid			17%

Significant histological differences were observed between flocks. Regarding the level of lesions according the definition described in section 3.2, no significant differences (p>0.05)

were observed for crypt depth or crypt surface, but a tendency (0.1>p>0.05) for higher crypt depth and surface for the animals of level 2 lesions compared to those of level 0. No difference was noticed for the ratios villus length/crypt depth and villus surface/crypt surface.

According to Lan *et al*. (2005), some microflora variations could deteriorate the functional characteristics of the digestive tract. Gabriel *et al.* (2003), noticed indeed that the intestinal flora and the mucous membrane have symbiotic and competitive relations, which generate structural and digestive modifications.

B) Occurrence of diarrhoeas

Fifty two per cent of farmers declared at least one episode of diarrhoea between 10 days and removal. These episodes appear during 4 distinct periods: around 10 days (period 1), between 12 and 18 days (period 2), between 20 and 32 days (period 3), after 32 days of age (period 4).

The results showed also that some rearing practices could be associated with the enteritis presence. A significant statistical link was found between the destocking of animals and the increase of the intestinal flora (p=0.006) and the lesions of the digestive tract (p=0.02), and wet litters (p=0.07).

A linear logistic regression between intestinal morphology and gut flora was found showing a positive significant statistical link between villi size and the *Lactobacillus* counts. The same link was observed between crypts depth and *Lactobacillus* counts.

C) Rearing factors

The results of this study indicated that some rearing practices would be associated with the enteritis presence. Regarding the influence of the drinking water on digestive pathologies described a pH higher than 6.5 is significantly linked with wet litters (p=0.07) and enteritis (p=0.005), by creating a proliferation of Gram-negative bacteria, and deposit constituting the biofilm.

Table 3 : Data linked with unspecific enteritis criteria

Data	**Diarrhoea**	**Wet litter**	**Digestive tract lesion**	**High intestinal bacterial count (*Lactobacillus* ≥ 8 Log CFU/g, coliforms ≥ 2 Log CFU/g)**
Rearing practices				
Dynamic ventilation (/static)	p = 0.076	p = 0.0002		p = 0.043
Animal destocking		p = 0.0004	p = 0.02	p = 0.006
Clair building (/dark)		p = 0.0031		
Ambient heating (/located)		p = 0.0013		p = 0.016
Starting				
Temperature < 32.5℃	p = 0.11	< 0.0001		
Drinking water				
Ph > 6.5	p = 0.07		p = 0.05	p = 0.1
Total coliforms < 5				p = 0.03

The results showed also the influence of the environmental factors, a significant link between a dynamic type of ventilation and the presence of wet litters (p=0.0002), diarrhoeas (p=0.076) and of an important digestive flora (p=0.043). This type of ventilation generates a bad distribution of air through the building, and also temperature variations (Mayne, 2005) resulting in a thermal stress increasing the harmful bacteria at the expense of the beneficial bacteria (Suzuki *et al*., 1989) and generating diarrhoeas. The results also shown an influence of the ligthing program, clear buildings (where the animals would be more nervous) constituting a risk factor of wet litters (p=0.003). Eventually, use of heating spot, not optimising the temperature and animals distribution, also generates wet litter (p=0.0013) and increases digestive flora numeration (p=0.016).

3.1.4 - Conclusion

This study showed unspecific enteritis indicators such as the presence of diarrhoeas and wet litter in the buildings, the quantitative evolution of the digestive flora, and the presence of lesions on the digestive tract. The relationship between these indicators confirmed the multi-factorial characteristics of unspecific enteritis. A link was found between the numeration of the flora and digestive tract deteriorations. Furthermore, these deteriorations are related to the appearance of wet litters and diarrhoeas.

The environmental factors (temperature, speed of air, lighting programs, heating) have also an influence on the unspecific enteritis appearance. The management of water quality and the environmental factors, the management of hygiene should allow a maintenance of the microbial balance, and thus to limit appearance of unspecific enteritis in batches.

3.2 - Definition of broiler rearing technologies in different EU countries

This section aims to draw an inventory of the broiler production systems from different European countries involved in the project, in order to compare the results collected from the different tasks on a homogeneous basis.

3.2.1 - Methodology

In March 2005, two questionnaires were set up to achieve the goal: the first concerned broiler flocks and the second laying hen flocks. The questionnaires included six criteria related to the local definitions, genetics, poultry houses and farming practices, sanitary practices, quality control and economic data. Data from 8 countries (United Kingdom, France, Denmark, Lithuania, Austria, the Czech Republic, Germany and the Netherlands) out of 10 are available. The data from Spain and Italy are not available because of the lack of centralisation of this kind of data by a specific institution.

3.2.2 - Results

Globally, the response rate was of 84 % for broilers distributed between 50 and 100% for the corresponding criteria (Table 4). The 4th criterion dealing with quality control was not fully filled in because in the majority of the countries, there is no additional regulation other than that imposed by the EU.

Table 4: Response rate of the countries regarding the six criteria of the questionnaires

	Definitions	***Genetics***	***Poultry houses***	***Sanitary practices***	***Quality control***	***Economic data***
Broilers	75 %	100 %	100 %	100 %	50 %	81 %

The responses collected about the local definitions allowed to draw an inventory concerning the different types of poultry production practiced in each country (Figure 7). For the broiler production, the intensive system exists in all the countries. The extensive system (without outdoor access) is practiced in France and consisted of broilers which are kept inside the buildings with density of 18 birds/m²; they are slaughtered at the age of 56-64 days. In the Netherlands, the Volwaard production very similar to the extensive system in France, is specific of this country; broilers are kept inside normal size buildings with a maximal density of 17 birds/m^2, with outdoor access in a covered area, with concrete floor. Slaughter age is minimum 56 days of age.

Six countries out of 10 had organic farms (France, Netherlands, United Kingdom, Austria, Germany and Denmark). In Lithuania and the Czech Republic, the organic farming is not practiced.

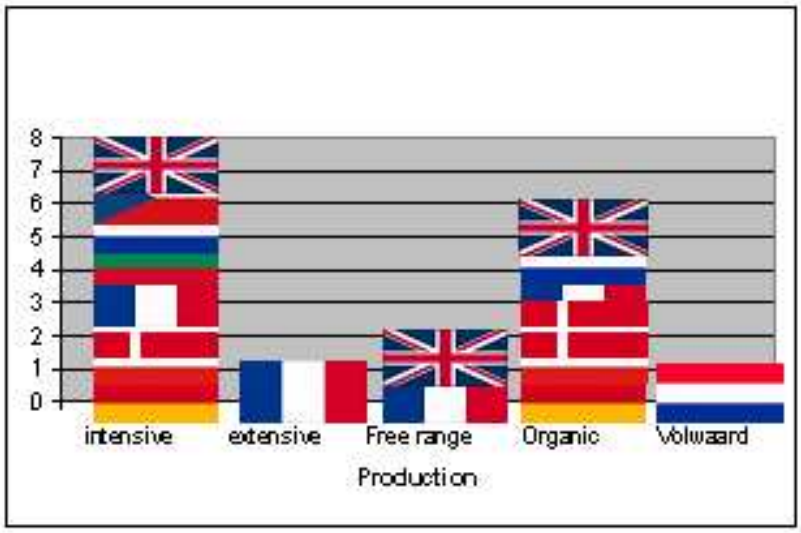


Figure 7: **Types of poultry production within the eight European countries participating to the investigation: UK, France, Denmark, Lithuania, Austria, the Czech Republic, Germany and the Netherlands. Occurrence of pathogens in broiler rearing farms in different EU countries**

The information collected about the genetics showed that Ross 308 avian strain is widely used within the broiler production in the 8 EU countries monitored, followed closely by Cobb 500. Some countries are alone to use specific strains (Figure 8).

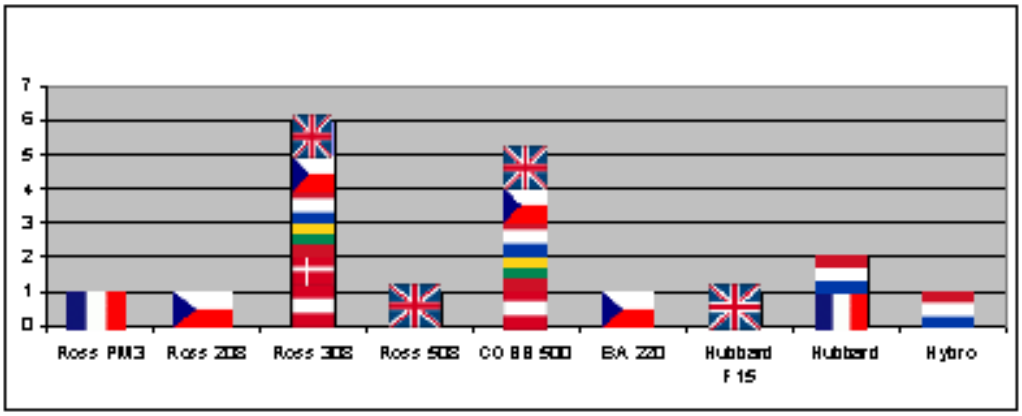


Figure 8: **Types of poultry strain within the eight European countries participating to the investigation: UK, France, Denmark, Lithuania, Austria, the Czech Republic, Germany and the Netherlands: broiler production**

More details about rearing characteristics in conventional and organic broilers are provided in tables 5 and 6. Data about sanitary practices collected from EU countries regarding the broiler production are provided in table 7.

Table 5: **Rearing characteristics collected from EU countries regarding the broiler intensive production**

		Denmark	UK	Lithuania	NL	Germany	France	CR	Austria
National mean size		40000	No data	No data	No data	20000-40000	No data	No data	6000-140000
Density	Number (birds/m^2)	22,1	17-19	17.8-19.2	21,8-25,8	22-24	20-22	15-30	15-17
	Weight (kg/m^2)	41,9	34-38	38	42	35	38-42	<45kg	30-34
Range period (number of cycle/year)		7	6-7	6-6.5	7	No data	6	6	6
Length of rearing period (days)		37,5	42	35-45	38-48	No data	38-41	38-42	42
Other animal production within the farm		Very seldom	No data	No data	No data	No data	No data	No data	No data
Species and frequencies		No data	No data	No data	No data	No data	No data	No data	No data
Animal feeding	Commercial diets	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Feed composition	No data	No data	No data	No data	No data	No data	No data	No data
	Utilisation of animal meals	No	No	No	No	No data	No	No	No
	Utilisation of whole seeds	No data	Sometimes	No	Yes	Sometimes	No data	No	Sometimes
	Starter diet's presentation	Pellets	Crumb or mash	Pellets or meals	Pellets	Pellets	Crumb	Pellets	Meals
	Growing diet's presentation	Pellets	Pellets	Pellets or meals	Pellets	Pellets	Pellets or crumb	Pellets	Meals
	Function diet's presentation	Pellets	Pellets	Pellets or meals	Pellets	Pellets	Pellets	Pellets	Meals
	Thermal treatment	Yes	Yes	Sometimes	Yes	No	No	Sometimes	Yes
	Other treatment	No	Yes	Yes	No data	No	No data	No	Yes

Table 6: **Rearing characteristics collected from EU countries regarding the broiler organic production. This production system is not practiced in Lithuania and the Czech Republic. In Denmark the data is not available**

		UK	NL	Germany	France	Austria
National mean size		No data	No data	No data	No data	1000-9000
Density	Number (birds/m^2)	10-16	9	Max 10	10	10
	Weight (kg/m^2)	21-30	20-22	Max 21	20-22	21
Range period (number of cycle/year)		4	2,3	No data	3	4,3
Length of rearing period (days)		73-81	81-91	70-90	81-91	81
Other animal production within the farm		Yes	No data	No data	No data	No data
Species and frequencies		Cattle, pigs	No data	No data	No data	No data
Animal feeding	Commercial diets	Yes	Yes	Yes	No data	Yes
	Feed composition	No data	Certified	No data	No data	No data
	Utilisation of animals meal	No	No	No	No data	No
	Utilisation of whole seeds	Sometimes	Sometimes	Yes	No data	Yes
	Starter diet's presentation	Crumb or mash	Meals	No data	No data	Shredded corn
	Growing diet's presentation	Pellets	Meals	No data	No data	Shredded corn
	Function diet's presentation	Pellets	Meals	No data	No data	
	Thermal treatment	Yes	Yes	No data	No data	Sometimes
	Other treatment	No	No	No data	No data	Yes

Table 7: **Data about sanitary practices collected from EU countries regarding the broiler production: (a) intensive, (b) organic. The organic production system is not practiced in Lithuania and the Czech Republic. In Denmark the data is not available**

(a)		Denmark	UK	Lithuania	NL	Germany	France	CR	Austria
Regulation national program		Yes	Yes	Yes	Yes	No data	Yes	Yes	Yes
Disinfections methods		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
All in-all out principle		Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Floor type		Litter	Concrete	Litter, concrete	Concrete	Solid floor with litter	80% litter, 20% concrete	80% litter, 20% concrete	Concert, wood shaving, chaffed straw
Water	Public	Yes	Yes	No	Yes	No data	Yes	Yes	Yes
	Private	No	Yes	Yes	Yes	No data	No	Yes	Yes
	Treatment	No	Yes	Yes	No	No data	Yes	Yes	Yes
Animal treatment	Vaccinations	No	Yes	Yes	Yes	No data	Yes	Yes	Yes
	Preventive treatment	No	No	Yes	Yes	No data	Yes	Yes	No
	Curative treatment	No	Yes	Yes	Yes	No data	Yes	Yes	Yes

(b)		UK	Netherlands	Germany	France	Austria
Regulation national program		Yes	Yes	No data	Yes	Yes
Disinfection methods		No data	Yes	No data	Yes	Yes
All in-all principle		No	No	No data	Yes	Yes
Floor type		Concrete	Litter on concrete floor	No data	80% litter, 20% concrete	Concert, wood shaving, chaffed straw
Water	Public	Yes	Yes	No data	Yes	Yes
	Private	Yes	Yes	No data	No	Yes
	Treatment	Yes	No	No data	Yes	Yes
Animal treatment	Vaccinations	No data	Yes	No data	Yes	Yes
	Preventive treatment	No data	Yes	No	No	No
	Curative treatment	No data	Yes	Yes	Yes	Yes

3.3 - Occurrence of pathogens in broiler farms in different EU countries

3.3.1 - Occurrence of *Campylobacter* in broiler rearing farms in Germany, Italy, the Czech Republic and Lithuania

Foods of animal origin, in particular poultry, have been identified as significant sources of this enteropathogen as a result of infection and contamination at the preharvest and harvest levels, though *Campylobacter* spp. seems to be rather sensitive and appears to have substantial requirements for survival and replication in food. In recent years, in the EU an incidence of 47.6 cases per 100 000 population was reported (EFSA, 2006). The reasons for constantly increasing occurrence are not fully understood, though raw poultry meat and inadequately heat-treated poultry products are considered to be a significant source of infection. A number of poultry farms and chicken being slaughtered were found to be contaminated by *Campylobacter* spp. and during treatment in the slaughterhouse surface contamination could occur. Unhygienic handling of raw poultry meat results in contamination of other foodstuffs. The risk arises also from insufficient treatment of poultry meat and semi-finished poultry products (Berrang *et al.,* 2004). The frequency of *Campylobacter* spp. incidence in poultry varies significantly at particular farms and depends on a number of factors, primarily of zoo-hygienic circumstances (see section 2).

In this study, the methods were validated and optimised for the monitoring of the occurrence of *Campylobacter* spp in Germany, Italy, the Czech Republic and Lithuania with respect to the various types of broilers rearing technologies used in different EU countries. Moreover, the typing of *C. jejuni* and *C. coli* species was performed.

A) Material and methods

Experimental trial and sampling

The sampling was performed from February 2005 to November 2006.

In Germany, for the evaluation of the quantitative exposure assessment of *Campylobacter* in broiler farms ceacal samples were collected at the slaughterhouse. 18 flocks (12 different farms) were tested with 10 caeca sampled and analysed individually for each flock, thus 180 caeca were collected and their contents analysed. Additional samples were collected from another 22 flocks, where 5 caeca per flock were collected and analysed as pooled samples.

In Italy, *Campylobacter* occurrence has been estimated on broiler caecum contents collected in 44 broiler flocks reared in biological (N=7), intensive (N=26) and extensive (N=11) farms. In each flock up to 5 caeca were sampled and tested for *Campylobacter* presence. However, due to the expected high occurrence of *Campylobacte*r in the sample tested, the enrichment step has been performed only for those samples which turned out as negative after direct plating. From each presumptive positive sample one *Campylobacter* like colony was purified and identified by Polymerase Chain Reaction (PCR) (Manfreda *et al*., 2005).

In the Czech Republic, 20 caeca per flock were collected in 50 conventional (intensive) broiler farms.

In Lithuania, the samples from 16 flocks were collected from 6 randomly selected broiler farms with intensive production. Cloacal swabs, faeces, caecum content were taken at broiler slaughterhouse and also dust and water were examined for *Campylobacter* spp.

Isolation and cultivation

The samples were analysed and *Campylobacter* counts were estimated according to ISO 10272-2002, 1g of caecal intestinal content of each broiler was transferred to tubes containing 10 ml of peptone water, homogenized and analysed.

Typical greyish – white colonies with trailing growth were selected for the next confirmation. Genomic DNA was isolated using commercial DNeasy®Tissue Kit (Qiagen, UK).

Identification and typing

In Germany, confirmation of the presumptive colonies was performed by Gram-staining, phase contrast microscopy, oxidase and catalase reactions. Isolates were biochemically differentiated to species level by API Campy (Bio Mérieux). The diversity of the isolates and their relationship was analysed by PFGE according to the „CAMPYNET" Prototype standard protocol (http://campynet.vetinst.dk/PFGE.html).
In Italy, The *Campylobacter* spp. presumptive isolates were identified by using a multiplex PCR (mPCR) protocol (Manfreda *et al.,* 2003) able to identify *Campylobacter* spp. as genus, *C. jejuni* and *C. coli* during the same reaction. The expected PCR amplicons were at 857, 589 and 462 bp corresponding to the genus *Campylobacter* spp., to the species *C. jejuni* and to the species *C. coli*, respectively*. C. jejuni* ATCC 33560 and *C. coli* NCTC 11350 were used as positive controls. *Staphylococcus aureus* ATCC 51749 was used as negative control.
In the Czech Republic, each *Campylobacter* spp. strain was identified to the species level by using biochemical tests and Restriction Fragment Length Polymorphism (RFLP). Biochemical profiling system was based on the detection of hippurate hydrolysis and on the detection of cytochrome oxidase according to manufactures instructions for MIKRO-LA-TEST® (PLIVA – Lachema, CZ). The PCR technique involved the amplification of a 491 bp amplicon of a highly polymorphic part of the *23S rRNA* gene and further species differentiation was accomplished by digestion of the PCR product using two restriction enzymes *Alu*I and *Tsp*509I (Fermér and Engvall, 1999).
In Lithuania, the *Campylobacter* spp. was identified using the oxidase test, catalase production, natrium hippurate and indoxyl acetate hydrolysis, hydrogen sulphite production on Triple sugar iron (TSI) agar, the nalidixic acid and cephalotin sensitivity test.

Statistical analysis

A 95% Confidence Interval (CI) contains the selected pathogen 95 out of every 100 samples. The 95% CI for the observed occurrence of pathogen-positive samples were estimated according to the binomial distribution. The CI can be given by the interval whose lower (CI-) and upper (CI+) limits are as follows:

CI-=p-z[p(1-p)/n]0.5, CI+=p+z[p(1-p)/n]0.5

p = number of positive samples/number of tested samples;

z = (95%) 1.96;

n= number of tested samples.

B) Results

In Germany, the number of 40 flocks (from 32 various farms) was investigated for the presence of *Campylobacter* spp (Table 8). The results show a high occurrence of *Campylobacter* spp. in different samples (67.2 % [59.8-74]). The species distribution showed as the most often isolated species *C. jejuni* followed by *C. coli* and in some cases *C. lari* (Table 8). The enumeration revealed a concentration of *Campylobacter* spp. in caeca of about Log 7.0 CFU of *C.* / g intestinal content.

In Italy, a total of 128 caecum samples were tested for *Campylobacter* spp. and 123 (96.1% [91.1-98.8]) were positive in conventional (intensive) farms. The isolates collected were identified as *C. jejuni* (22.7% [15.7-30.9]), *C. coli* (54.7% [45.6-63.5])).

The *Campylobacter* occurrence in the 11 extensive broiler farms was 91.1% [80.4-97.0]. The 50 *Campylobacter* colonies isolated from the positive caecum samples were identified as *C. jejuni* (60.7% [46.7-73.5]), *C. coli* (26.8% [15.8-40.3]). Seven out of 10 positive farms were infected as a single *Campylobacter* species, whereas in 3 farms both thermophilic species were detected.

The *Campylobacter* occurrence in the 7 biological farms was 100 %. All biological farms were *Campylobacter* positive as well as all the 35 caeca collected from broilers reared in these farms (Table 8). The *Campylobacter* colonies isolated in the positive biological samples were identified as *C. coli* (48.6% [31.4-66.0]), *C. jejuni* (48.6% [31.4-66.0]). In 9 out of 26 farms identified *Campylobacter* spp. strains belonged to a single species, whereas in the other farms both thermophilic species were detected.

In the Czech Republic, the 78.9% [76.4-81.2] of samples from farms with intensive production were thermophilic *Campylobacter* spp. positive. These positive samples contained 80% [77.2-82.6] and 10.7% [8.7-12.8] of *C. jejun*i and *C. coli* respectively. Samples from farm environment were positive nearly in all farms (N=38). In the Czech Republic, the enumeration get the concentration of *Campylobacter* spp. of about Log 8.3 CFU of *C.*/ g of caecal content.
In Lithuania, from 70 tested samples, *Campylobacter* spp. was found in 20 samples (28.6% [18.4-40.6]) (Table 8). Infectious agent was found in cloacal swabs, faeces and caecum and distributed as follows: faeces 38.5 %, cloacal swab 37.5 % and caecum content 29.2%. The distribution of the *Campylobacter* species was: *Campylobacter jejuni* 95% [5-72.1] and *Campylobacter coli* 4.8% [0-9.8]. The dust and water were negative for *Campylobacter* spp. The mean load of *Campylobacter* spp was Log 5.2 CFU of *C.*/g of caecal content.

Table 8: *Campylobacter* occurrences in conventional and organic farms in Germany, Italy, the Czech Republic and Lithuania

Country	Type of production	N tested flock	N positive flock	Samples	N tested samples	N positive samples *C.* spp.	*C.* spp. (%) [CI (95%)]	N positive samples *C. jejuni*	*C. jejuni* (%) [CI (95%)]	N positive samples *C. coli*	*C. coli* (%) [CI (95%)]
Germany	Conventional	40	28	Caecum	180	121	67.2 [59.8-74]	21 (flocks)	52.5 [36.1-68.5]	10 (flock)	25.0 [12.7-41.2]
Italy	Conventional	26	25	Caecum	128	123	96.1 [91.1-98.8]	29	22.7 [15.7-30.9]	70	54.7 [45.6-63.5]
	Extensive	11	10	Caecum	56	51	91.1 [80.4-97.0]	34	60.7 [46.7-73.5]	15	26.8 [15.8-40.3]
	Biologic	7	7	Caecum	35	35	100.0 [0 - 54.3]	17	48.6 [31.4-66.0]	17	48.6 [31.4-66.0]
The Czech Republic	Conventional	50	45	Caecum	1135	895	78.9 [76.4-81.2]	716	80* [77.2-82.6]	95	10.7* [8.7-12.8]
Lithuania	Conventional	16	7	Cloacal Swabs	8	3	37.5 [8.5-75.5]	ND	ND	ND	ND
				Faeces	26	10	38.5 [20.2-59.4]	ND	ND	ND	ND
				Env. dust	8	0	Nd	ND	ND	ND	ND
				Water	4	0	Nd	ND	ND	ND	ND
				Caecum	24	7	29.2 [12.6-51.1]	ND	ND	ND	ND
				Total	70	20	28.6 [18.4-40.6]		95 [5.0 - 72.1]		4.8 [0-9.8]

(%) of *C. jejuni* and *C. coli* positive of *C.* spp positive samples

Env. Environmental, Nd, not detected; ND, Not determined

C) Discussion

During the last decade, the occurrence and spread of *Campylobacter* in broiler flocks has been intensively studied in several countries. Occurrence of *Campylobacter* in flocks ranging from 18% to 90% had been reported in Europe (Evans and Sayers, 2000; Refregiers-Petton *et al.*, 2001). In the Netherlands about 30% of broiler flocks were contaminated with *Campylobacter* (Bouwknegt *et al.*, 2004); while in Belgium 73% (Rasschaert *et al*., 2007). However, the occurrence of *Campylobacter* showed higher rates in mid and southern Europe, where up to 91% positive flocks was found (e.g. in Italy) (EFSA, 2006). According to this study, the occurrence of *Campylobacter* spp. in broiler samples (caecum content) is higher in Italy than in the Czech Republic and in Germany, these results are in agreement with the EFSA report (2006). The occurrence of *Campylobacter* spp. ranges from 67.2% [59.8-74] to 96.1% [91.1-98.8] in Germany, in Italy and in the Czech Republic. The occurrence of *Campylobacter* spp. is rather low in Lithuania where was found only 28.6% [18.4-40.6] positive samples.

Majority of isolated strains of *Campylobacter* spp. were identified as *C. jejuni* in the Czech Republic, in Germany and in Lithuania. *C. coli* was identified in majority of broiler flocks in Italy. The method of typing has to be harmonised between the different countries and laboratories to compare these data. In Italy, the percentages of positive samples detected among caeca collected from broilers reared in intensive, extensive and biological farms did not showed any statistically significative difference. Animal skin colour and gender influenced the percentage of positive caeca collected from broilers reared differently, whereas ventilation type as well as animal strain did not. *C. coli* seems to represent the prevalent species in broilers reared in intensive farms, whereas *C. jejuni* is most in extensive farms. Animal skin colour, gender and strains as well as ventilation type influenced the species distribution on broilers reared in extensive farms. The results from Lithuania showed no significant difference between contamination by *Campylobacter spp* in cloacal swabs, faeces and caecum respectively.

3.3.2 - Occurrence of *Salmonella* in broiler in rearing farms in Germany, Italy, Lithuania and The Netherlands

Broiler chickens, especially if intensively reared, are considered to be an important reservoir of *Salmonella* infections for humans (EFSA, 2006). Various risk factors can be responsible for *Salmonella* infection and its spread in poultry farms: housing system, flock size, different age of chicken and season of the year. (Rose *et al*. 1999, Heyndrickx *et al*., 2002). Many consumers assume that the organic chickens shed less *Salmonella* than conventional broilers because of the particular system management (low density stocking, access to outside and special diets) (Bailey and Cosby, 2005) (see section 2). On the other hand in organic farming the access to outside may increase the risk of the infection with *Salmonella* through the contact with faeces of wild birds and other animals. Cui *et al*. (2005) found that organically raised broilers had higher occurrence of *Salmonella* than broilers raised conventionally. In several studies the incidence of *Salmonella* was lower in organic than in conventional broiler farms (Heuer *et al*., 2001, Wolf-Reuter *et al*., 2002). However, Van Overbeke *et al.* (2006) reported no significant differences in the occurrence of *Salmonella* between organic and conventional broilers at slaughter. The subject of this study was to assess the occurrence of *Salmonella* spp. in broiler farms of 4 EU countries: Italy, Germany, The Netherlands and Lithuania. In Italy and in the Netherlands besides the conventional farms, organic flocks were investigated in order to preliminary determine the occurrence of *Salmonella* spp. in this rearing condition. The typing of *Salmonella* was also performed to get knowledge about the possible geographical distribution of the organism.

A) Material and methods

Experimental trial and sampling

The sampling was performed from 2005 to 2006.

In Italy and in the Netherlands both organic and conventional farms could be investigated. In Italy the investigations were performed in 11 organic flocks and in 10 conventional flocks over a period of two years. During the production cycle environmental samples consisting of 5 pools of litter, 2 pools of dust and 1 water sample were collected. At slaughterhouse 60 caecal samples per farm, subdivided in 2 pools, were collected.

In Lithuania and in Germany the investigations were performed in 27 and 22 conventional flocks respectively. In Lithuania flock samples consisting of 5 pools of litter, 2 pools of dust and 1 water sample were collected during the production cycle. Litter samples consisted of boots swabs. Five pooled samples of caecum per flock were taken from a broiler slaughterhouse. In Germany only caecum samples could be collected and analysed as one pooled sample per flock at the broiler slaughterhouse. One pool of 30 caeca was collected per flock.

In the Netherlands 18 conventional flocks and 108 organic flocks were examined over a period of two years. Different numbers of dust, litter samples, water and caecal pool samples were collected from each flock at the farm level

Isolation and identification

Salmonella strains were isolated by standard methods (ISO 6579-1998). From each pool of litter, dust and caecal content, 10 g were homogenised with approximately 1:10 Buffered Peptone Water (BPW) for 60 seconds; 25 ml of each water sample were poured into a container followed by adding 225 ml of BPW and cultivated. One loopful of inoculum was used for each plate. Presumptive colonies were inoculated onto Mc Conkey agar (Standard I agar in Germany and Brilliant Green Agar in the Netherlands), incubated at 37℃ for 24h and biochemically checked with Ureum Agar (UA), Triple Sugar Iron Agar (TSI) using commercial tests (API 20E). Identification was performed by agglutination testing with agglutination sera.

Statistical analysis

See section 3.3.1A

B) Results

In Italy 2 out of 10 conventional farms were positive for *S.* Hadar and *S.* Heidelberg (20%) while 2 out of 11 organic flocks were positive for *S.* Hadar (18.2% [5.2-40.3]). In particular, in conventional farms *Salmonella* was isolated from 5 out of 50 litter samples (10% [3.3-21.8]) and from 4 out of 20 caecal samples (20% [5.7-43.6]). In one farm both litter and caecal samples were *Salmonella* positive, while in the other farms it was detected only in the intestinal samples. The dust and water samples were always *Salmonella* negative.

In Lithuania, 8 out 27 samples from conventional flocks were positive for *Salmonella*.The results obtained in conventional broilers from Lithuania showed that the most infected samples were litter and caecum (25.9 % [18-35.2] and 25 % [17-34.4] respectively) while *Salmonella* presence was lower in dust and water (9.6% [3.2-21.0] and 3.8% [0.1-19.6], respectively) (Table 9). The prevalent serovars were *S*. Enteritidis and *S.* Typhimurium.

In the Netherlands the analysis for *Salmonella* was performed in 11 conventional farms with a total of 18 flocks and in 108 flocks from 16 organic broiler farms during 2 years. Two out of 18 conventional flocks (11.1%) and 5 out 108 organic flocks (4.6%) were postive for

Salmonella, respectively. In Dutch conventional flocks the most infected samples were caeca (4 positive out of 36 =11.1% [3.1-26.1]). The serotype isolates were *S.* Infantis in the samples from one farm (dust, litter and caeca) and *S.* Java from litter samples in one other farm (Table 9). With respect to organic farming a total of 181 litter samples were taken; from which 4 (2.2% [0.06-5.6]) were *Salmonella* positive. All samples from dust and environmental samples proved to be *Salmonella* negative. *S.* Infantis was isolated in 1 organic feed sample from one flock, while *S.* Senftenberg was found, both in a feed sample and in litter samples from another flock. No pools of caecal samples from organic chickens were *Salmonella* positive at slaughter. *Salmonella* serotypes isolated from organic flocks were: *S.* Infantis, *S.* Senftenberg, one isolate from the B group and one from the C group could not be typed completely (Table 9).

In Germany caecal samples from the 22 conventional flocks, collected over a period of one year gave negative results for *Salmonella* in all cases (Table 9).

Table 9: *Salmonella* spp. occurrence in conventional and organic farms in Italy, Lithuania, The Netherlands and Germany

Country	Type of production	Sample	N. of tested flocks	N. of positive flocks	N. of tested samples	N. of positive samples	*Salmonella* spp (%) [CI (95%)]	Prevalent serotype
Italy	Conventional	Caeca	10	2	20	4	20.0 [5.7-43.6]	S. Hadar
		Dust	10		20	0	Nd	S. Heidelberg
		Litter	10		50	5	10.0 [3.3-21.8]	
		Water	10		10	0	Nd	
		Total:	10	2	100	9	9 [4.2-16.4]	
	Organic	Caeca	11	2	22	4	18.2 [5.2-40.3]	S. Hadar
		Dust	11		22	0	Nd	
		Litter	11		55	0	Nd	
		Water	11		11	0	Nd	
		Total:	11	2	110	4	3.6 [1.0-9.0]]	
Lithuania	Conventional	Caeca	27	5	104	26	25.0 [17.0-34.4]	S. Enteritidis
		Dust	27	8	52	5	9.6 [3.2-21]	S. Typhimurium
		Litter	27	8	108	28	25.9 [18.0-35.2]	
		Water	27	1	26	1	3.8 [0.1-19.6]	
		Total:	27	8	290	41	14.1 [10.3-18.7]	
The Netherlands	Conventional	Caeca	17	2	36	4	11.1 [3.1-26.1]	S. Infantis,
		Dust	18	1	320	1	0.3 [0.01-1.7]	S. Java
		Litter	18	2	386	3	0.8 [0.2-2.2]	
		Water	16	0	29	0		
		Total:	18	2	771	8	1 [0.04-2]	
	Organic	Caeca	16	0	160	0	Nd	S. Infantis
		Dust	108	0	37	0	Nd	S. Senftenberg
		Litter	108	4	181	4	2.2 [0.6-5.6]	S. Group B
		Feed	61	2	61	2	3.3 [0.4-11.3]	S. Group C
		Total:	108	5	439	6	1.4 [0.5-2.9]	
Germany	Conventional	Caeca	22	0	660	0	Nd	

N. Number; Nd, Not detected; CI, Confidence Interval

C) Discussion

Various risk factors exist for infection with *Salmonella* spp. and its spread in poultry farms: housing system, flock size, different age of chicken and season of the year (Angen *et al.*, 1996; Skov *et al*. 1999). The presented results indicated that the occurence of *Salmonella* spp. varies in the countries investigated. The percentage of positive flocks from conventional housing system was 25.0% [17.0-34.4] in Lithuania, 11.1% [1.4-34.7] in the Netherlands and 20% [2.5-55.6] in Italy respectively.

In the EU Baseline study on broilers (EFSA, 2007) the results for the four countries involved in this study deviated from the results of the present report, on the flock basis, Germany, Italy, Lithuania and the Netherlands had respectively 15%; 28.3%; 2.9% and 7.5% of *Salmonella* spp. positive flocks. However the data from our investigations covered only a fraction of the totals presented in the baseline study. In Germany *Salmonella* spp. in conventional broiler flocks was not detected but it should be underlined that the data is based on limited sampling, though it consisted of caecal samples, always considered the best source for the *Salmonella* spp. recovery. *Salmonella* spp. isolation was also slightly inferior to expectations in Italy. In Lithuania and in the Netherlands a higher occurrence for *Salmonella* spp. was detected in comparison with the EU baseline study.

Salmonella spp. in organic broiler flocks was observed both in Italy (2 out of 11 flocks) and in the Netherlands (4 out of 108 flocks). The data is not enough extensive to make a comparison between the two rearing systems but they may give an idea in relation to the distribution of *Salmonella* in organic broiler farms. In the literature there is controversial data about the influence of housing systems for *Salmonella* infection. In several studies the occurrence of *Salmonella* was lower in organic than in conventional broiler farms (Heuer *et al*., 2001, Wolf-Reuter *et al*., 2002, Van der Hulst *et al.*, 2004). Italian data indicated that the presence of *Salmonella* in organic broiler flocks is comparable with conventional broiler flocks, and are in agreement with those reported by Van Overbeke *et al*. (2006), who did not find significant differences in the occurrence of *Salmonella* between organic and conventional broilers at slaughter. Baily and Cosby (2005) also did not agree in assuming that the free range or organic conditions can influence the *Salmonella* presence in the chickens. The explanation of the lower occurrence of *Salmonella* in Dutch organic broilers could be that organic broilers grow longer (8 weeks) than conventional broilers (5-6 weeks) and are mostly slow growing brands. The occurrence at the age of 8 weeks may have dropped below the detection level of the survey. *Salmonella* infection experiments in conventional broilers showed that even after relatively heavy *Salmonella* challenge at the early age, broilers may have cleared the infection at approximately 6 weeks of age (Bolder *et al*., 1999, Linton et al., 1985).

These results showed that the most infected samples were from litter and caecum in comparison with those of dust and water. Some investigators have determined that the contents of the caeca constitute the best single sample site for the search of *Salmonella* (Barrow *et al.*, 1988). Others have compared sampling of litter and the use of drag swabs for detection of *Salmonella* in poultry flocks (Kingston, 1981). Concerning the dust samples, *Salmonella* spp. was positive in conventional farms in Lithuania and in the Netherlands and always negative in organic farms. Although dust can be considered an excellent vector for *Salmonella*, survival in dust is limited (Davies *et al.,* 2003; Gast *et al*., 1998).

Different *Salmonella* serovars were identified in chicken. At the EU level the most common serovar was *S.* Enteritidis which represented approximately half of the isolates (EFSA, 2007). Our results indicated that *S.* Enteritidis and *S.* Typhimurium dominated in Lithuanian broiler flocks, while in the Netherlands *S.* Infantis and *S.* Java were isolated. In Italy *S.* Hada*r* and *S.* Heidelberg were found to be prevalent. These results confirm those of the EU baseline study in broilers (EFSA, 2007) where a wide variety of prevalent *Salmonella* serovars were isolated in different countries. Besides, other investigators (Byrd *et al.*, 1997; Roy *et al*., 2002) found

that *S.* Kentucky and *S.* Heidelberg were predominant serotypes isolated from poultry litter or poultry meat products.

3.3.3 - Occurrence of *Clostridium perfringens* in broiler rearing farms in Italy and in the Czech Republic

C. perfringens is capable of causing a broad spectrum of diseases in both humans and animals. It is widespread in the environment and commonly found in the intestines of animals, including humans, where it is pathogenic in certain circumstances (Petit *et al*., 1999, see section 2.1). *C. perfringens* negatively impacts the integrated system of poultry production (Craven *et al.*, 2003). It is frequently found in the faeces of livestock and poultry at high levels. However, its overgrowth in fowl can be considered an imbalance of the gut ecosystem at the microbial level, resulting in gastrointestinal dysbacteriosis and necrotic enteritis (see section 2.1). Occurrence of *C. perfringens* in chickens is increasing since the ban of AGP, therefore the aim of this task was to estimate the occurrence of *C. perfringens* in the caecal contents of broilers coming from intensive, extensive and organic systems and collected at the slaughterhouse in Italy and in the Czech Republic.

A) Material and methods

Experimental trial and sampling

The sampling was performed from October 2005 to September 2006.

In Italy, the *C. perfringens* occurrence has been evaluated in 42 broiler flocks reared in conventional (intensive) (N=23) and extensive (N=9) and in biologic (N=9) farms.

In the Czech Republic the *C. perfringens* occurrence has been evaluated in 52 broiler flocks reared in intensive (N=51) farms.

In Italy up to 5 caeca were sampled in each flock between for a total 194 caecum contents, whereas in the Czech Republic 5 up to 30 caeca were sampled in each flock for a total of 1385 caecum contents tested during the same period. Samples for the determination of *C. perfringens* occurrence were collected once a week and processed according to the same protocol described below.

Cultivation and isolation

The culture method used to detect *C. perfringens* in broiler caecum contents was that recommended by the Food and Drug Administration (FDA) (Bacteriological Analytical Manual, 7th Ed). Briefly, each ceacum content collected at the slaughterhouse was diluted 1:10 in physiological water and homogenised by using the stomacker at normal speed for 1 min. Then, the samples were inoculated from 1 ml of the neat and further dilutions. At the end of the incubation period the positive samples were considered those containing *C. perfringens* like colonies, characterised by black colour and surrounded by an opaque zone around the colony.

Statistical analysis

See section 3.3.1A

B) Results and discussion

In Italy, the *C. perfringens* occurrence in the 23 conventional (intensive) broiler farms was 91.3% [72.0-99.0]. In fact 21 out of 23 farms were *C. perfringens* positive. Overall, 64 out of 104 (61.5% [51.5-71.0]) caecum contents were colonised by the pathogen (Table 10). The *C. perfringens* occurrence in the 9 organic (biologic) farms has been 88.9% [51.7-99.7]. Overall

27 out of 45 (60% [44.3-74.3]) caecal contents were *C. perfringens* positive. In fact, all farms but one were *C. perfringens* positive, which means with at least one out of five caecum contents colonised by more than 9 Log CFU of *C. perfringens* /g of caecum content.
The *C. perfringens* occurrence in the extensive broiler farms was 88.9% [51.7-99.7]. In fact, all but one flock tested were positive. Overall, 23 out of 45 (51.1% [35.7-66.3]) caecum contents had more than 9 Log CFU of *C. perfringens* /g of caecal content (Table 10).

In the positive samples *C. perfringens* was enumerated and the mean pathogen load within organic, intensive and extensive broiler farms was 4.5, 4.6 and 3.4 Log CFU of *C. perfringens*/g of caecum content, respectively. The mean loads detected among broiler caeca of birds reared in intensive farms was statistically significant higher than those detected among birds reared in extensive farms (Table 10).

In the Czech Republic a total of 1385 caecum samples were tested for *C. perfringens* and 169 (12.2% [10.5-14.0]) were positive, i.e. contaminated beyond 9 CFU of *C. perfringens* per gram of caecum content. A total of 33 out of 51 (64.7% [50.0-77.5]) intensive farms were *C. perfringens* positive as well as the organic farm tested (Table 10).

Table 10: *Clostridium perfringens* occurrence in conventional and organic farms in Italy and in the Czech Republic

Country	Type of production	N of tested flock	N of positive flock	Type of samples	N of tested samples	N of positive samples *C. perfringens*	*C. perfringens* (%) [CI (95%)]
Italy	Conventional	23	21	Caeca	104	64	61.5 [51.5-70.9]
	Extensive	9	8	Caeca	45	23	51.1 [35.7-66.3]
	Organic	9	8	Caeca	45	27	60.0 [44.3-74.3]
The Czech Republic	Conventional (+1 organic)	51	33	Caeca	1385	169	12.2 [10.5-14.0]

N. Number; CI, Confidence Interval

The *C. perfringens* occurrence in Italian intensive broiler farms was higher than in the Czech Republic (91.3%[72.0-99.0] vs 64.7% [50.0-77.5]).

The percentages of *C. perfringens* positive samples, detected in Italian organic (60% [44.3-74.3]), conventional (61.5% [51.5-70.9]) and extensive (51.1% [35.7-66.3]) broiler farms did not show any statistically significant difference. Moreover, they were not influenced by the parameters tested such as animal gender, skin colour, litter type, feeding type and ventilation type. The *C. perfringens* mean counts in the Italian positive samples were 4.4 Log CFU/g, with values ranging between 0.7 and 5.7 Log CFU/g. In the Czech Republic, no comparison between the *C. perfringens* occurrence in farms using different rearing technologies was performed because only one organic farm was tested.

3.3.4 - Occurrence of *Listeria monocytogenes* in broiler rearing farms in France and Lithuania

L. monocytogenes is a zoonotic microorganism commonly isolated in foodstuffs of animal origin. Previous studies associating listeriosis with consumption of contaminated food underlined *L. monocytogenes* as one of the major human food-borne pathogens. Listeriosis is a serious illness, which may lead to death, serious cases of meningitis and encephalitis associated with abortion. There has been an unexplained significant increase in human listeriosis cases in Europe since 2004 (Doorduyn *et al.*, 2006*,* Hedberg *et al*., 2006*,* Jalava *et al*., 2006). This ubiquitous microorganism is common in the environment of food industries because of its ability to colonise contact surfaces and to grow at refrigeration temperatures

(Chasseignaux *et al.*, 2002*,* Giovannacci *et al*., 2000). The occurrence of *L. monocytogenes* in processed poultry products varies between 20% (Gudbjörnsdottir *et al*., 2004) and 60% (Toquin *et al.*, 1990). Studies of contamination at primary production have provided some insight into this environmental contamination, as in other meat production systems (Beloeil *et al.*, 2003). In a study carried out in 1995, 10 French broiler farms were investigated. The results showed that 2.4% of the sampled flocks were contaminated by *L. monocytogenes* (Toquin *et al.*, 1995). In other studies (Cox *et al*., 1997, Fenlon *et al.*, 1996), low numbers of positive samples associated with the primary production were detected; the authors reported that virulent strains of *L. monocytogenes* do arise on farms and become established in the processing environment, and are consequently responsible for contamination of the food available to consumers. More recently, in Denmark, 71 broiler flocks yielded an estimated prevalence of 3 % (Doorduyn *et al*., 2006). However in these studies, the culture media used were specific for the genus *Listeria* but not for the species *L. monocytogenes.* This may have generated false negative results (Capita *et al*., 2005) due to overlapping of *L. monocytogenes* by other *Listeria* species on culture media.

The aim of this investigation was to assess the occurrence of *L. monocytogenes* in poultry production in France and in Lithuania, with the objective of improving sensitivity of bacteriological methods and assessing the input of bacteria in the production chain. This study was performed in agreement with a national survey to assess to the occurrence of *L. monocytogenes* corresponding to the total number of positive samples in the population divided by the number of individuals in the population. The study of *Listeria* in France was a prevalence study as the sampling is representative of the national production. The investigation has been conducted in parallel to the EU baseline study for *Salmonella* (same samples).

Experimental trial and sampling

The sampling was performed from 2005 to 2006.

In France, the sampling scheme was representative of the French broiler production. A total of 145 flocks were sampled (85 conventional flocks, 60 free-range flocks). Sampling consisted of 5 boot swabs per flock. The boot swabs were purchased from Sodibox, France. One pair of boot swabs, wrapped individually with sterile gloves, was used as one sample. A total of 725 samples were collected. In France, the same samples as the EU baseline study for *Salmonella* namely: faeces, bootswabs and dust for laying hens and bootswabs for broilers.

In Lithuania, a total of 57 broiler flocks (conventional flocks) were sampled. A total of 126 samples was collected (faeces swabs (N=54), dust (N=13), caecum (N=32) and water (N=13)). Sampling consisted of 5 faeces swabs and caecum content per flock.

Cultivation and identification

All the samples were analysed for *L. monocytogenes* presence according to a modified protocol based on standard ISO11290-1. The samples were suspended in 150 ml of buffered peptone water to form a suspension; 25 ml of this suspension was added to 225 ml (1/10) of half-strength Fraser broth and was incubated for 24h ± 2 hours at 30°C for the first enrichment. A first isolation was carried out on ALOA agar (AES, France) and 5 characteristic colonies were purified on Tryptic Soy Agar (TSAYe) before biochemical identification and serotyping. For the second enrichment, 0.1 ml of the half-strength Fraser broth suspension were transferred into Fraser broth (AES, France) and incubated for 48 ± 2 hours at 37°C. A second isolation was carried out on ALOA agar and 5 characteristic colonies were isolated on TSAYe for identification and serotyping. A maximum of ten characteristic colonies for each positive sample were identified according to a miniaturisation of the biochemical tests for the identification of presumptive *L. monocytogenes* (Toquin *et al.*,

1987): Identifications were performed by xylose and rhamnose fermentation and catalase tests. The hemolysis and the camp tests were also carried out to complete the identification of isolated colonies. All the confirmed strains were serotyped with sera purchased from Eurobio.

Statistical analysis

See section 3.3.1A

B) Results

In France, *L. monocytogenes* was present in broiler flocks at a rate of 31% [23.6-39.2] (46 positive out of 145) flocks. The prevalence was respectively 12.7% [9.7-16.2] and 18.3%[14.1-23.2], in conventional and in free range systems. In Lithuania, *L. monocytogenes* was detected at rate of 3.5% [0.4-12.1] (2 positive out of 57 broiler flocks) (Table 11).

Table 11: *Listeria monocytogenes* occurrence in broiler farms in France and in Lithuania

Country	Type of production	N tested flock	N positive flock	Samples	N tested samples	N positive samples *L. monocytogenes*	*L. monocytogenes* (%) [CI (95%)]
France	Conventional	85	24	Boots swabs	425	54	12.7 [9.7-16.2]
	Free Range	60	22	Boots swabs	300	55	18.3 [14.1-23.2]
Lithuania	Conventional	57	2	Caeca	32	2	6.3 [0.8-20.8]
				Faeces	54	0	Nd
				Dust	13	0	Nd
				water	13	0	Nd
				Total	126	2	1.6 [0.2-5.6]

N. Number; Nd, Not detected; CI, Confidence Interval

Serotyping was only performed with samples from France. In France, 443 *L. monocytogenes* isolates were collected from 109 positive samples. The proportion of isolates was again heterogeneous among samples and flocks, and one serovar per flock was kept for data analysis: we analysed the distribution of 74 serovars isolated from these 443 isolates. The serotyping showed that 40.5% belonged to serotype 1/2a, 20.2% to serotype 1/2b, and 9.5% to serotype 4e. Serotyping highlighted the dominance of serovar 1/2a in conventional production (30%). In free-range production, serovar 1/2a was also dominant over the others (34%); however, serogroup 4, containing serotype 4e and 4e/4b together (40 %), may be dominant, but this result requires confirmation by genotyping the isolates. Statistical analysis showed no significant difference in the distribution of serovars (1/2a (p=0.15), 1/2b (p=0.91), 4^e (p=0.59), 4e, 4b (p= 0.075)) between standard and free-range systems.

Table 12: **Frequency of isolation of *L. monocytogenes* serotypes in broiler flocks**

Serotypes	N. of positive samples (Conventional)	N. of positive samples (Free range)	Occurrence (%)
1/2a	18	12	40.5
1/2b	8	7	20.2
4e	3	4	9.5
4e, 4b	5	10	20.2
Untypable	5	2	9.5
Total	39	35	100

N. Number

C) Discussion

In France, the prevalence was estimated on the basis of a nationally representative number of flocks. However, the comparison between France and Lithuania is not possible since the sampling methods (number and type of samples) were different. The level of contamination is dependent on the type of samples, and thus standard sampling is required to compare the rearing production systems between countries.

The prevalence observed in French broiler flocks (conventional and free range) (31.7% [24.2-40.0]) was higher than that expected from an investigation carried out in 1995 on broiler flocks, i.e. 2.4% (Toquin *et al*., 1995). However, the sampling was different and the number of sampled flocks was lower. This difference could be attributed to the improvement in the detection methods that have become more suitable for samples taken from primary productions in which competing flora may interfere with *L. monocytogenes* detection (Bruhn *et al.*, 2005).

The occurrence of *L. monocytogenes* was lower in Lithuania than in France in conventional broiler flocks. Various risk factors exist for infection with *L. monocytogenes* and its diffusion in broiler farms: housing system, flock size, different age and geographical zone. In Lithuania the climate is more severe than in France and it may influence the occurrence of *L. monocytogenes* in poultry. Organic poultry farming in Lithuania has not been certificated yet.

These results must be taken into account because of the possible introduction of *L. monocytogenes* in the poultry/meat production chain and should be considered for achieving better control of these bacteria at the end of the process. Further studies on sampling and better bacteriological methodology should be conducted to further improve the detection in faecal samples.

3.3.5 - Occurrence of *Helicobacter pullorum* in broiler rearing farms in Italy and in the Czech Republic

H. pullorum was classified as a new species of *Helicobacter* by Stanley *et al.*, 1994. This organism has been isolated from the livers and intestinal contents of laying hens with vibrionic hepatitis and from the caeca of broiler chickens (Stanley *et al.*, 1994; Burnens *et al*., 1996; Atabay *et al*., 1998). *H. pullorum* has also been isolated from faeces of humans with gastroenteritis (Burnens *et al.*, 1994; Steinbrueckner *et al.*, 1997; Melito *et al*., 2000; Ceelen *et al*., 2005a) and its DNA has been detected in livers from patients with primary sclerosing cholangitis, cirrhosis and hepatocellular carcinoma (Ponzetto *et al*., 2000; Pellicano *et al.*, 2004; Rocha *et al.*, 2005), although it was unclear if the organism had a causal role in these infections (Gibson *et al.*, 1999). As poultry carcasses can be contaminated by *H. pullorum* (Atabay *et al.*, 1998) during slaughtering, the potential role of these bacteria as an emerging foodborne human pathogen needs to be evaluated. There is no ideal method for the isolation of all *Helicobacter* species (On *et al.*, 2005) and an accurate identification of these bacteria is known to be a difficult task (On, 1996). In particular, the two traits that can be used as phenotypic markers for *Helicobacter* spp., i.e. the presence of sheathed flagella and resistance to polymyxin B, are absent in *H. pullorum*. Therefore, *H. pullorum* strains may be easily misidentified as *Campylobacter* spp., in particular *C. coli* and *C. lari* with which several key phenotypic traits are shared (Atabay *et al.*, 1998; Kuijper *et al.*, 2003). At present, it is well known that the recovery of *H. pullorum* can be optimised by examining the freshest possible samples (On *et al*., 2005) and by the use of non-selective methods or of selective media without polymyxin B (Atabay *et al.*, 1998). However studies on the occurrence of *H. pullorum* in poultry in Europe are scarce (Burnens *et al.*, 1996; Atabay *et al*., 1998; Ceelen *et al.*, 2006).

The aims of this task were to set up a specific and sensitive method for isolation and identification of *H. pullorum* in broiler faeces and to estimate the occurrence of *H. pullorum* in the caecal contents of Italian and Czech broilers collected at the slaughterhouse. The birds tested were reared using intensive, extensive and organic systems.

A) Material and methods

Experimental trial and sampling

The sampling was performed from September 2005 to September 2006.

In Italy, the occurrence of *H. pullorum* was estimated on samples collected in 50 broiler flocks reared in organic (N=8), conventional (N=34) and extensive (N=8) farms. In Italy up to 5 caeca were sampled in each flock, with a total of 238 caecum contents tested.

In the Czech Republic, the occurrence of *H. pullorum* was estimated on samples collected in 30 broiler flocks reared in intensive (N=29) and organic (N=1) farms. In the Czech Republic 5 up to 30 caeca were sampled in each flock, with a total of 423 caecum contents tested.

Cultivation and identification

In order to set up a protocol suitable to isolate *H. pullorum* from caeca, the recovery ability of different culture media, selective agents and filtration membranes was evaluated along with different isolation protocols. The culture media tested included Brucella Agar (BA), Brain Heart Infusion Agar (BHI) and Sheep Blood Agar (SBA) supplemented with 5% sheep blood.

Brucella Agar (BA) and Brain Heart Infusion Agar (BHI) were also tested supplemented with cefoperazone 32 mg/L and CAT (Cefoperazone, Amphotericin B, Teicoplanin).

Finally Brucella Agar was tested with filters of 0.65 µm. In relation to the filters the recovery ability obtained by using 0.65 µm membranes was quite higher in comparison to that achieved by using 0.45 µm membranes. In relation to the different protocols tested to isolate *H. pullorum* from caecum contents, the first protocol described below was selected because a caecum content enrichment in CAT broth allows the *Campylobacter* population to overgrown *H. pullorum* cells and the use of selective agents such as cefoperazone or CAT in the agar plates improves the growth of bacteria belonging to the intestinal flora reducing the number of *H. pullorum* colonies identified. Filtration of 300 µl of a mixture obtained adding 100 µl of faecal suspension and 400 µl of a sterile mixture, containing 25 ml of Brain Heart Infusion (Difco), 75 ml of inactivated horse serum and 7.5 g of glucose (Ceelen *et al*., 2005) on BA supplemented with 5% sheep blood, using the filter technique described by Steele and Mcdermott., 1984. Briefly, a sterile cellulose acetate membrane filter 0.65 µm (Millipore) was applied directly on the agar surface and 300 µl of the sample were spread on the filter. After one hour of incubation at 37℃ in an increased H_2-microaerophilic atmosphere obtained making a preliminary vacuum until -720 mm of mercury and then injecting a gas mixture (approx. H_2 10%, CO_2 10%, N_2 80%) the filter was removed and the agar surface streaked with a loop. Plates were then incubated in the same conditions as described above for 2-5 days and examined daily to check growth of small, greyish-white colonies of Gram-negative, gently curved, slender rod bacteria referable to *H. pullorum*.

One suspected *H. pullorum* colony from each sample was identified as it follows:

Molecular analysis: The molecular identification of the suspected colonies was performed in two steps. In the first one the isolates were submitted to the PCR assay described by Stanley *et al*., 1994 using the REDExtract-N-Amp Tissue PCR Kit (Sigma) and the following primers: 5' ATGAATGCTAGTTGTTGTCAG 3' and 5' GATTGGCTCCACTTCACA 3'. On the positive samples the PCR protocol followed by RFLP described by Fox *et al*., 2000 was performed in order to distinguish *H. pullorum* and *H. canadensis*. The PCR products amplified using primers C97 (5' GCTATGACGGGTATCC 3') and C05 (5' ACTTCACCCCAGTCGCTG 3') were submitted to restriction fragment length polymorphism analysis (RFLP) by using the restriction enzyme *ApaLI* (Fermentas, International INC, Burlington, ON, Canada)

Biochemical tests. The biochemical characterization of the isolates was performed using the following tests: cytochrome oxidase, urease, catalase, γ-glutamyltranspeptidase and alkaline phosphatase production, hippurate and indoxyl acetate hydrolysis, hydrogen sulphide production in triple sugar iron (Oxoid), nitrate reduction, growth under an increased-H_2 microaerobic atmosphere in the presence of 1% (w/v) glycine and 1% (w/v) bile, at 25℃, 42℃ and on Difco-MacConkey Agar (BD), growth at 37℃ in aerobic and anaerobic conditions and under microaerobic atmosphere with and without hydrogen, susceptibility to nalidixic acid and cephalotin determined by standard disk diffusion method with a 30 µg disk (BBL, Becton, Dickinson and Company). Gamma-glutamyltranspeptidase production was carried out as recommended by Chevalier *et al.*,1999 using *H. canis* ATCC 51401 and *H. pullorum* ATCC 51801 as quality control strains. The growth under a microaerobic atmosphere without hydrogen was performed as described by Atabay *et al.*, 1998.

*1D SDS-PAGE analysis of whole cell proteins***.** Whole cell proteins of two-day-old bacterial cultures were extracted at 95℃ for 10 minutes in NuPAGE^{tm} LDS. Sample Buffer (Invitrogen Life Technologies, Carlsbad, CA 92008 USA) supplemented with NuPAGE^{tm} Sample

Reducing Agent (Invitrogen) and separated by electrophoresis in polyacrylamide gel. In brief, 7-8 µg of each extract were loaded on NuPAGE^{tm} Novex 4-12% Bis Tris Gelformat 1.0 mm 10 wells (Invitrogen) and were run for 140 minutes at constant current (30 mA) and temperature (5°C). Protein bands were stained by Si mply Blue Safestain^{tm} (Invitrogen); the profile was digitized by FLUOR-S MultiImager (Bio-rad Laboratories, Hercules, CA 94547 USA) and subjected to comparative numerical analysis using Diversity Database (Bio-rad). The similarity among protein profiles was calculated by the Pearson product moment correlation coefficient using UPGMA as a clustering method (Costas, 1992).

Statistical analysis

See section 3.3.1A

B) Results

As shown in table 18, in Italy a total of 248 caecum samples were tested for *H. pullorum* and 200 (80.6% [75.2-85.4]) were positive. The occurrence of *H. pullorum* in the 34 conventional (intensive) broiler farms was 100% [91.6-100] and 142 out of 169 (84% [77.6-89.2]) caecum contents were colonised by the pathogen. The occurrence of *H. pullorum* in the extensive broiler farms was 62.5% [24.5-91.5]; in fact, 5 out of 8 extensive farms were *H. pullorum* positive. Overall 23 out of 40 (57.6% [40.9-73.0]) caecum contents were *H. pullorum* positive. The *H. pullorum* occurrence in the 8 organic farms was 100% [68.8-100]. In fact, all organic farms were *H. pullorum* positive, i.e. at least one out of 5 caecum contents was contaminated with the pathogen. Overall, 35 out of 39 (89.7% [75.8-97.1]) caecum contents were colonised by *H. pullorum*.

In the Czech Republic, any *H. pullorum* has been isolated by using the culture method. However, 8 out of 29 (27.6% [12.7-47.2]) intensive farms tested as well as the organic farm turned out as positive using PCR.

Table 13: *Helicobacter pullorum* occurrence in conventional and organic meat chicken farms in Italy and in the Czech Republic.

Country	Type of production	N of tested flock	N of positive flock*	Type of samples	N of tested samples	N of positive samples *H. pullorum*	*H. pullorum* (%) [CI (95%)]
Italy*	Conventional	34	34	Caeca	169	142	84.0 [77.6-89.2]
	Extensive	8	5	Caeca	40	23	57.6 [40.9-73.0]
	Organic	8	8	Caeca	39	35	89.7 [75.8-97.1]
				Total	248	200	80.6 [75.2-85.4]
The Czech Republic	Conventional	29	8	Caeca	443	0	Nd

N. Number; Nd, Not detected; CI, Confidence Interval; * In Italy positive flocks were detected by culture method whereas in the Czech Republic by PCR

In Italy, the *H. pullorum* occurrence in broilers reared in extensive farms was statistically significant lower than that observed within intensive and organic farms. This trend was confirmed also when testing each rearing parameter (Table 14), among which animal gender seems to influence the *H. pullorum* occurrence. In fact the percentage of positive caeca collected from male birds was statistically higher than positive caeca from female birds except for the organic ones.

Table 14: Percentages of positive samples in intensive, extensive and organic farms in relation to the different parameters considered

Parameters considered	% of positive samples		
	Intensive	Extensive	Organic
Male	94.7	100	100
Female	70.7	43.4	86.7
Vegetable feeding	81.6	57.5	89.8
Animal feeding	85.6	-	-
Litter type:			
Straw	79	57.5	95.8
Wood shaving	100	-	80
Rice hulls	60	-	-
Chaff	60	-	-
White skin	82.8	66	-
Yellow skin	86.5	50	89.9
Forced ventilation	86	90	-
Natural ventilation	70	46.7	89.8

***=Values with different letters superscript in the same column and for the same parameter are statistically significant different for p<0.05**

C) Discussion

There is little information regarding the diffusion of *H. pullorum* in the literature. Atabay and Corry, 1997 and Atabay *et al.*, 1998 isolated *H. pullorum* from the caeca of 8 out of 15 broilers collected in two different farms. More recently, Ceelen *et al*., 2006 carried out a study on the intestinal contents of 110 broilers coming from 11 flocks and found that 33.6% of the animals were positive for *H. pullorum* using PCR, and only 16 animals from two different flocks using the cultural method. Our results show a very high occurrence of *H. pullorum* in Italian broilers. A similar level of occurrence was recently reported in France by Pilon *et al.*, 2005, who found all caecal contents of 10 chickens positive *H. pullorum* using real-time PCR. The reason why we found higher percentages of positive samples in comparison to Atabay *et al*.,1998 might be attributed to sample processing protocol. In fact, in this study fresh materials were tested, avoiding the use of frozen samples. Moreover, in comparison to the

study published by Celeen *et al.*, 2006, filters of 0.65 mm were used instead of the enrichment technique based on CAT broth.

In Italian broiler caeca, the *H. pullorum* occurrence was 80.6% [75.2-85.4] and the percentage of positive samples among broilers reared in intensive and organic farms was found to be higher than that of broilers reared in extensive farms. The isolation protocol used in this study was more sensitive than those published in previous papers. Moreover, it seems highly specific. In fact all isolates collected on presumptive positive samples were identified as *H. pullorum* by PCR and PCR-RFLP. The high *H. pullorum* occurrence among Italian healthy broilers along with the few cases of isolation of this pathogen in humans might suggest that *H. pullorum* has its natural habitat in broiler caeca and it can be rarely transmitted to humans. However, more studies should be performed to clarify the pathogenic rule of this bacterium for human health.

Following the application of the culture method optimised during the project, the estimated occurrence of *H. pullorum* in Italian broiler farms was 90%, in comparison to the percentage of positive caecum samples which was 80.6%. Within each farm the percentage of positive samples ranged between 20 and 100%. (In relation to the Czech Republic any positive sample was obtained in broiler farms using the culture method. This result might be due to: 1) the low number of *H. pullorum* cells in comparison to *Campylobacter* that might overgrowth *H. pullorum* on plates especially when the percentage of hydrogen in the incubation atmosphere is lower than 7-10%; 2) the low viability of H*. pullorum* in the samples, especially when they are not tested the day of sampling.

3.4 - Definition of laying hen production in different EU countries

This task aimed to draw an inventory of the laying hen production systems from different partner countries of the project, in order to compare on a homogeneous basis the results collected from the different tasks.

3.4.1 - Methodology

The methodology is described in section 3.1.

3.4.2 - Results

Globally, the response rate was of 80 % for laying hens distributed between 25 and 100% for the corresponding criteria (Table 15).

Table 15: **Response rate of the countries regarding the six criteria of the questionnaires**

	Definitions	*Genetics*	*Poultry houses*	*Sanitary practices*	*Quality control*	*Economic data*
Laying hens	75 %	100 %	100 %	100 %	25 %	88 %

For the laying hen production, the conventional system is practiced in all the countries (Figure 9). The free-range system is conducted in the United Kingdom, France, Denmark and Austria. The alternative and organic systems are practiced in six countries out of eight: Denmark, France, United Kingdom, Austria, Netherlands and Germany.

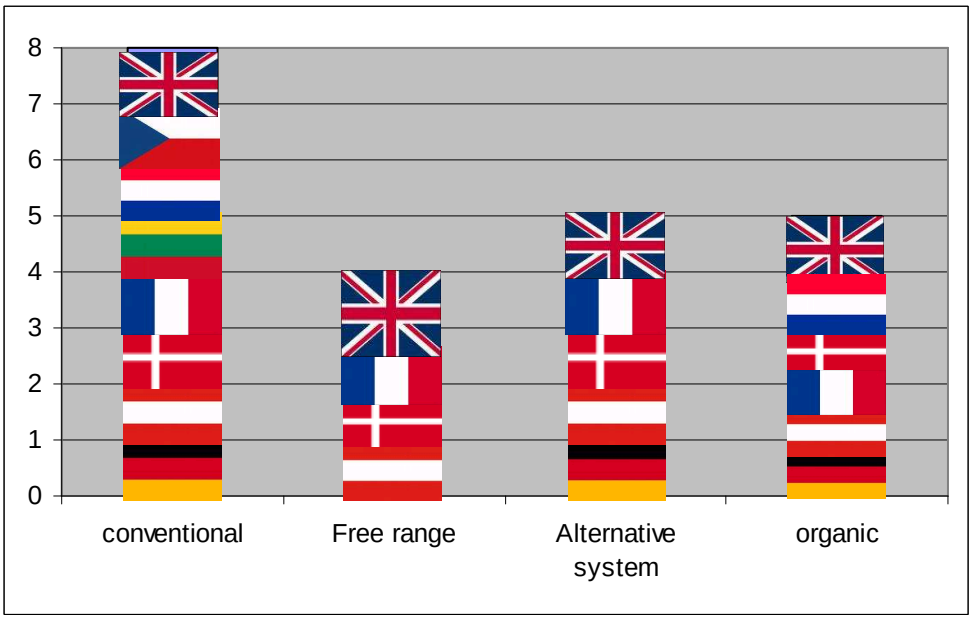


Figure 9: **Types of poultry production within the eight European countries participating to the investigation: UK, France, Denmark, Lithuania, Austria, the Czech Republic, Germany and the Netherlands. Laying hen production**

In the laying hen production, the strains are quite specific for each country although 3 of them (LSL, Lohmann tradition and Lohmann brown) are used in several countries (Figure 10). The other data collected are listed in the tables 13 and 14.

More details about rearing characteristics in conventional and organic laying hens are provided in tables 16 a and 16 b. Data about sanitary practices are provided in tables 17 a and 17 b.

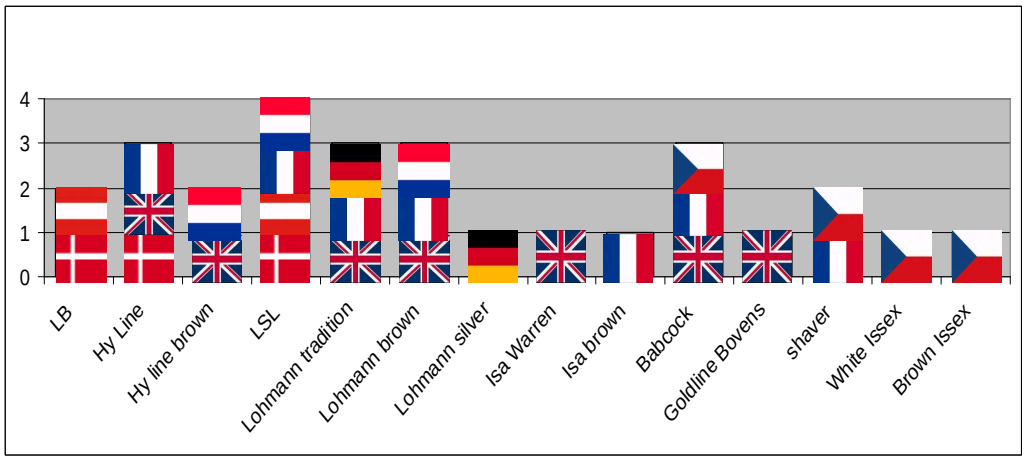


Figure 10 : Types of poultry strains within the eight European countries participating to the investigation: UK, France, Denmark, Lithuania, Austria, the Czech Republic, Germany and the Netherlands. Laying hen production

Table 16 : Rearing characteristics collected from EU countries regarding the laying hen production: (a) conventional, (b) alternative systems

		Denmark	UK	Lithuania	NL	Germany	France	CR	Austria
National mean size		20000	20000	No data	No data	10000-30000	35000	No data	6365
Density	Number	No data	550-750 cm^2/hen	No data	550-750	No data	550-750	550-600	550
	Weight	No data		No data		1.6-1.8 kg			
Range period (duration in weeks)		No data	72-80	No data	70-80	44-80	69	72-80	70
Length of the production (in weeks)		56	52-60	No data	55-65	No data	50	75	52
Other animal production within the farm		Yes	No	No data	No data	No data	No data	No data	No data
Species and frequencies		20-30% PIG	No data	No data	No data	No data	No data	No data	No data
Animal feeding	Commercial diets	Yes	Yes	No data	Yes	Yes	Yes	Yes	Yes
	Break trimming	Yes	Yes	No data	No	Demand authorisation	Allowed	Mostly not	Allowed but not used
	Feed composition	No data	No data	No data	No	No data	No data	No data	No data
	Thermal treatment	Yes	Sometimes	No data	Yes	No data	Sometimes	Mostly not	Yes
	Other treatment	No	No data	No data	Yes	No data	No data	No	Yes

		Denmark	UK	NL	Germany	France	Austria
National mean size		9000	10000	No data	2000-20000	5000-12000	3189
Density	Number	No data	9-12 birds/m^2	No data	Max 9hens/m^2	Max 9hens/m^2	7
	Weight	No data		No data	1.6-1.8 kg	No data	No data
Range period (duration in weeks)		No data	72-80	No data	48-60	67	70
Length of the production (in weeks)		52	52-60	No data	18-20	47	52
Other animal production within the farm		Yes	No data	No data	No data	No data	No data
Species and frequencies		20-30% PIG	No data	No data	No data	No data	No data
Animal feeding	Commercial diets	Yes	Yes	No data	Yes	Yes	Yes
	Break trimming	Yes	No data	No data	No data	No data	No data
	Feed composition	No data	No data	No data	No data	No data	No data
	Thermal treatment	Yes	Sometimes	No data	No data	No data	Yes
	Other treatment	No	No data	No data	No data	No data	Yes

Table 17 : Data about sanitary practices collected from EU countries regarding the laying hen production: (a) conventional, (b) alternative systems

(a)		Denmark	United Kingdom	Netherlands	Germany	France	The Czech Republic	Austria
Regulation national program		Yes	Yes	Yes	No data	Yes	Yes	Yes
Disinfection methods		Yes	Yes	Yes	Yes	Yes	Yes	Yes
All in-all principle		No	Yes	Yes	Yes	Yes	Sometimes	Yes
Floor type		No data	Concrete	Litter on concrete	Concrete	No litter	Bedding	Wire mash, astro turf mats
Water	Public	Yes	Yes	Yes	No data	No data	Yes	Yes
	Private	No	Yes	Yes	No data	No data	Yes	Yes
	Treatment	No	Yes	No	No data	No data	No	Yes
Animal treatment	Vaccinations	Yes	Yes	Yes	Yes	No data	Yes	Yes
	Preventive treatment	No	No	Yes	No data	No data	Yes	Yes
	Curative treatment	Yes	No	Yes	No data	No data	Yes	Yes

(b)		Denmark	United Kingdom	Netherlands	Germany	France	Austria
Regulation national program		Yes	No data	No data	No data	No data	Yes
Disinfection methods		Yes	Yes	No data	Yes	No data	Yes
All in-all principle		No	Yes	No data	Yes	No data	Yes
Floor type		Deep litter	Concrete	No data	Concrete	No data	Concrete wood shavings
Water	Public	Yes	Yes	No data	No data	No data	Yes
	Private	No	Yes	No data	No data	No data	Yes
	Treatment	No	Yes	No data	No data	No data	Yes
Animal treatment	Vaccinations	Yes	Yes	No data	Yes	No data	Yes
	Preventive treatment	No	No	No data	No data	No data	No
	Curative treatment	Yes	No data	No data	No data	No data	Yes

Eight countries (UK, France, Denmark, Lithuania, Austria, the Czech Republic, Germany and the Netherlands) out of 10 were able to fill in the questionnaire and provide data by a national organism. The data from the two remaining countries (Spain and Italy) were not available because of the lack of centralisation of this kind of data by a specific institution.

3.5 - Occurrence of pathogens in laying hens in different EU countries

3.5.1 - Occurrence of *Salmonella* spp. in laying hens in Germany, Lithuania and The Netherlands

Food-borne pathogens (*Salmonella* spp) contamination during the rearing phase of laying hen production is an area that receives increased attention. Two pieces of European legislation aimed to control the prevalence of *Salmonella* in poultry and eggs across the EU have recently been adopted (European commission, 2006). The first new piece of legislation sets targets for the reduction of *Salmonella* in commercial laying flocks, which should in turn lead to less *Salmonella* contamination of eggs. Every member state will have to reduce the number of laying hens infected with *Salmonella* by a specific minimum percentage each year, with bigger reduction targets for member states with higher levels of *Salmonella.* The second piece of legislation sets rules on the methods to control *Salmonella*.

There is a lack of information on the effect of the nutritional and rearing conditions on the occurrence of food-borne pathogens in laying hen production. The aim of this study was to investigate the *Salmonella* spp. occurrence in laying hens in three European countries (Germany, Lithuania and The Netherlands) in consideration of the different rearing conditions (organic vs conventional).

A) Material and methods

Experimental trial and sampling

The sampling was performed over the years from March 2004 to October 2006.

In Germany, the samples were collected in 6 flocks, in two different rearing systems (aviary with escape, enriched cages) until February 2007. The samples collected were eggs, water samples, swabs from enriched cages and litter from the aviary. Caecal contents were sampled during routine slaughtering of selected hens. The samples were analysed as pooled samples, one pool consisted of 10 eggs, 4 swabs, or 4 water samples per system. Samples were collected from a total of 6 flocks during 1 full and 2 partial laying periods (Table 18).

In Lithuania, the laying hens were grouped according to the rearing systems in conventional and enriched cages. 37 laying hen flocks (10 rearing in conventional cages and 27 in enriched cages) were investigated (Table 18). The samples were faeces swabs, dust and water.

In the Netherlands, conventional cages, traditional barn free range and organic laying hen systems were sampled (8 flocks). The samples were faeces and caecal contents.

Cultivation, identification

See section 3.3.2

Statistical analysis

See section 3.3.1A

B) Results

As shown in table 18, in Germany, *Salmonella* was not found in the samples collected from the laying hens on any occasion (Table 18).

In Lithuania, there was no significant difference of *Salmonella* occurrence was found between enriched and conventional cages. Regarding the different type of samples, 28.7% [20.6-37.9] - 35% [20.6-51.7] of faeces as well as 31.6% [17.5-48.6] - 40% [16.3-67.7] of dust samples were infected with *Salmonella* spp., whereas water samples were *Salmonella* free (Table 18).

In the Netherlands, since the number of farms with furnished cages is very small, and at the time of the monitoring Avian Influenza threat appeared, these farms were not sampled, and only one traditional barn type and one barn type with free range were sampled. No postive flock was found amoung conventional, traditional and free rage productions. Overall, only one flock from organic system was positive, the occurrence of *Salmonella* spp. in faeces or caeca was 33.3% [9.9-65.1].

Table 18: *Salmonella* spp. occurrence in laying hen farms in Germany, Lithuania and the Netherlands

Country	Type of production	N of tested flock	N of positive flock	Type of samples	N of tested samples	N of positive samples *S.* spp	*S.* spp (%) [CI (95%)]
Germany	Aviary with escape	3	0	Eggs (shell)	448	0	
				Yolk	448	0	
				Water	14	0	
				Swabs (cages)	0	0	
				Litter	15	0	
				Caeca	81	0	Nd
				Total	1006	0	Nd
	Enriched cages	3	0	Eggs (shell)	614	0	
				Yolk	614	0	
				Water	26	0	
				Swabs (cages)	119	0	
				Litter	0	0	
				Caeca	140	0	Nd
				Total	1513	0	Nd
Lithuania	Conventional cages	10	3	Faeces swabs	40	14	35% [20.6-51.7]
				Dust	15	6	40% [16.3-67.7]
				Water	5	0	
				Total	60	20	33.3% [21.7-46.7]
	Enriched cages	27	7	Faeces swabs	115	33	28.7% [20.6-37.9]
				Dust	38	12	31.6% [17.5-48.6]
				Water	15	0	
				Total	168	45	26.8% [20.3-34.1]
The Netherlands	Conventional cages	3	0	Faeces/caeca	18	0	Nd
	Traditional barn	1	0	Faeces/caeca	6	0	Nd
	Free Range	1	0	Faeces/caeca	12	0	Nd
	Organic	3	1	Faeces/caeca	12	4	33.3% [9.9-65.1]

N. Number; Nd, Not detected; CI, Confidence Interval

C) Discussion

Regarding these results, it is difficult to compare the frequency of *Salmonella* between countries because of the lack of standardization between traditional and alternative laying hen systems used in the different European countries. For this reason, it was difficult to harmonize sampling in each country. All German investigated samples were negative for *Salmonella* spp. although the European baseline study for *Salmonella* in laying hens reported a prevalence of nearly 30% *Salmonella* positive holdings for 2004-2005 in Germany

(Report on the Analysis of the baseline study on the prevalence of *Salmonella* in holdings of laying hen flocks of Gallus gallus, (EFSA Journal (2007). The farm that was sampled during the project had a known *Salmonella* positive history in the years before 2005. After that biosecurity measures were established that showed to be effective. Concerning the comparison between systems in each country, no significant difference was found between conventional and enriched cages in Lithuania. In the Netherlands, *Salmonella* spp was only found in organic systems, the occurrence was 33.3% [9.9-65.1].

In cages, laying hens are subjected to restricted movement and lack of ability to fulfil most of their natural behaviours. On the other hand, providing chickens with access to an outdoor area may increase the risk of poultry becoming infected with *Salmonella* due to the contact with wild birds and other animals and their faeces. Flocks kept in a cage system with wet manure had a significantly lower chance of infections with *Salmonella* Enteritidis compared with a cage system with dry manure (Mollenhorst *et al*., 2005). A flock with deep litter also had a significantly lower chance of infection with *Salmonella* compared with the cage system with dry manure. A possible explanation could be that the manure in the cage system with dry manure was air-dried and that through this airflow *Salmonella* was transported.

3.5.2 - Occurrence of *Listeria monocytogenes* in laying hens in France and in Lithuania

The aim of this investigation was to assess to the occurrence of *L. monocytogenes* in conventional cage and free range laying hen systems from faecal and environmental samples in France and in Lithuania. These results were compared with those obtained in broilers (see section 3.3.4).

A) Material and methods

Experimental trial and sampling

The sampling was performed during a one year period between 2004 and 2005.

In France, a total of 200 laying hen flocks were sampled: 88 were caged flocks and 112 were floor-reared flocks (including both total confinement and free-range flocks). Samples taken from hens reared in cages consisted of dust and faeces (4 or 5 pools of faecal materials picked up from the faecal conveyor belt or from the manure pit). The sampling scheme for floor-reared hens was limited to dust material. A total of 840 samples were collected. Sixty-six samples were pooled in order to reach the weight defined by the protocol (25 g for each sample). Finally, 784 samples from laying hen flocks were analysed for *L. monocytogenes* detection.

In Lithuania a total of 74 laying hen flocks from conventional cages was analysed for L. *monocytogenes* infection. A total of 285 samples was collected (faeces swabs (184), dust (70) and water (31)). Sampling consisted of 5 faeces and dust specimens per flock.

Cultivation and Identification

For laying hens: for the first enrichment, 25 g of faeces or dust were diluted (1/10) in half strength Fraser broth and incubated for 24h ± 2 hours at 30℃ and analysed according to the protocol described in section 3.3.4

Statistical analysis

See section 3.3.1A

B) Results

A flock was considered positive if at least one sample was positive for *L. monocytogenes*. In France, among 200 flocks, 31 tested positive, i.e. a prevalence of 15.5% [10.1-21.3]. The dust samples, in common between both types of production (caged- and floor-reared hens), were used to compare the respective contamination of each production type. No feces were sampled in floor reared systems. When all the flocks were considered (Table 19), no significant (p=0.31) difference was observed between caged- and floor-reared hens. Faecal samples were found to be more contaminated by *L. monocytogenes* than dust samples (10.3 % vs 3.8 %), but the difference was not significant (p=0.053).

In Lithuania, among 74 flocks, 5 were positive, i.e. an occurrence of 6.8% [2.2-15.1] and 1.1% [0.2-3] of samples were postive (Table 19).

Table 19: *Listeria monocytogenes* occurrence in laying hen farms in France and Lithuania

Country	Type of production	N tested flock	N positive flock *	Samples	N tested samples	N positive samples *L. monocytogenes*	*L. monocytogenes* (%) [CI (95%)]
France Laying hen flocks	Conventional cage	88	26	Faeces	439	45	10.3 [7.6-13.5]
				Dust	140	7	5.0 [2-10]
				Total	579	52	9 [6.8-11.6]
	Floor reared	112	5	Dust	205	6	2.9 [1.1-6.3]
Lithuania	Conventional cage	74	5	Faeces	184	2	1.1 [0.1-3.9]
				Dust	70	1	1.4 [0.04-7.7]
				Water	31	0	
				Total	285	3	1.1 [0.2-3]

N. number

L. monocytogenes serotypes in laying hen flocks

The 61 positive samples provided 180 isolates of *L. monocytogenes*. The proportion of isolates was heterogeneous among the samples and the flocks. The dust samples, in common between both types of production (caged- and floor-reared hens), were used to compare the respective contamination of each production type.

One serovar per flock was selected. The final analysis thus included 35 of the total of 180 isolates and was representative of the distribution of serovars among positive flocks (Table 20). Serotype 1/2a represented a large proportion (74.2 %) of the isolates. Of the remaining isolates, 5.7% were attributed to serotype 1/2b and 8.6% to serotype 4e (Table 20). Some strains (5.7%) required a molecular discriminating method to distinguish 4e from 4b. No significant difference was found in the distribution of serovar 1/2a between caged hens and floor-reared hens (p=0.73) or between faeces and dust samples (p=0.075).

Table 20: **Frequency of isolation of *L. monocytogenes* serotypes in laying hens in France**

Serotypes	N. of positive samples (Caged hens)	N. of positive samples (Floor reared hens)	Occurence (%)
1/2a	23	3	74.2
1/2b	2	0	5.7
4e	3	0	8.6
4e, 4b	2	0	5.7
Untypable	0	2	5.7
Total	30	5	100

N. Number; CI, Confidence Interval

C) Discussion

To compare frequency of *L. monocytogenes* in laying hens in France and in Lithuania harmonized sampling should have been used.

In France, when all the flocks are taken into account, there was no statistical difference between caged and floor-reared hens; however, dust samples were more frequently contaminated in floor-reared hens than in cage hen flocks in flocks. This may be attributed to dust samples being polluted as a result of contact with faecal materials shed on the floor (boot swabs). Data obtained from caged hens indicated that faeces were more contaminated than dust samples, but when both types of samples (dust and faeces) are considered, the contamination level is greater. This indicates that floor-reared hen flocks may have similar

contamination levels if faecal samples were analysed, but this requires further investigation. The occurrence of *L. monocytogenes* is also a risk to consumers buying eggs, as it may be transferred to the eggshells and occasionally the egg products. Some studies have described the contamination of egg products by *L. monocytogenes* (Protais *et al*., 2007). Bacteria may be introduced to the production chain *via* the contaminated eggshell. Regarding the comparison of the contamination by *L. monocytogenes* between laying and broilers in France, serotype 1/2a, frequently detected in broilers (30/74) and in laying hen flocks (26/31), is involved in listeriosis cases (33%) but less than serogroup 4. This highly prevalent serogroup within broiler production (29.7%) is still responsible for the majority of human cases (42%), especially in single origin grouped cases (Goulet *et al.*, 2001*,* Salvat *et al*., 1995). In other investigations, serovar 1/2a was also found to be prevalent in poultry products (Hellström *et al*., 2007*,* Jay, 1997).

The level of contamination in dust and faeces samples is also lower in Lithuania than in France but the number of analysed samples is lower in Lithuania than in France and a comparison is not possible.

3.6 - Conclusion

Data on the occurrences of the main food-borne pathogens in broilers and laying hens in different European countries and the effect of management factors on their presence were collected. The reason why occurrence instead of prevalence results are available, as final project results, is because, due to the avian flu crisis, in many EU countries involved in the project the sampling plan initially organised to achieve prevalence data was then modified excluding geographical area at high poultry farms, in order to answer the mandatory biosecurity procedures in force in the EU. For example, some partners performed samplings at farms and other at slaughterhouses. The occurrence data collected in the project are summarised in Tables 21 and 22. Since *Campylobacter* occurrences estimated in Germany, the Czech Republic and Italy are high the results of this project are useful for a future baseline study of *Campylobacter* prevalence in Europe.

The comparison between occurrences of food-borne pathogens in farms applying different rearing systems (i.e. organic and free range) was not possible in all EU countries participating in the project. In fact, in the Czech Republic and Lithuania, for example, farms applying alternative rearing systems are few or none. However, in Italy, the Netherlands and France where some comparison between conventional and organic broiler and in laying hen production systems was possible no significant differences were observed.

In relation to the emerging food-borne pathogens, during the POULTRYFLORGUT project was set up of a very sensitive microbiological procedure to isolate *H. pullorum* from broiler caecum contents. The occurrence of this organism was slightly lower than 90 and 30% in Italy and the Czech Republic, respectively. However, according to Italian data collected out of this project, *H. pullorum* does not seem to represent a bacterium causing human diseases. The occurrences of *L. monocytogenes* were 31% and 15.5% in French broilers and in laying hens respectively. Because of the differences of sampling and typing protocols, the comparison with lithiuanian occurrences was not possible.

Table 21 : Occurrence of pathogens in broilers in different European countries

Broilers			
***Campylobacter* spp**			
	N. of tested flocks	**N. of positive flocks**	**Occurrence (%) [CI (95%)]**
Germany	40	28	70 [53.5-83.4]
Italy	44	42	95.4 [84.5-99.4]
The Czech Republic	50	45	90 [78.2-96.7]
Lithuania	16	7	43.7 [19.7-70.1]
***Salmonella* spp.**			
Italy	21	4	19 [5.4-41.9]
Lithuania	27	8	29.6 [13.7-50.2]
The Netherlands	126	7	5.6 [2.3-11.1]
Germany	22	0	Nd
Clostridium perfringens			
Italy	41	37	90.2 [76.9-97.3]
The Czech Republic	51	33	64.7 [50.0-77.6]
Listeria monocytogenes			
France	145	46	31.7 [24.2-40.0]
Lithuania	57	3	5.3 [1.1-14.6]
Helicobecter pullorum			
Italy	50	47	94 [83.4-98.7]
The Czech Republic	29	8	27.6 [12.7-47.3]

N. Number; Not detected; CI, Confidence Interval

Table 22 : Occurrence of pathogens in laying hens in different European countries

Laying hens			
***Salmonella* spp.**			
	N tested flock	**N positive flock**	**Occurrence (%) [CI (95%)]**
Germany	6	0	Nd
Lithuania	228	65	28.5 [22.7-34.8]
The Netherlands	48	4	8.3 [2.3-20.0]
Listeria monocytogenes			
France	200	31	15.5 [10.8-21.3]
Lithuania	74	5	6.8 [2.2-15.1]

N. Number; Nd, Not detected; CI, Confidence Interval

Section 4: Food alternatives to reduce pathogens in the intestinal tract

Over the years, a number of strategies for improvements in animal health, productivity, and microbial food safety that not involve antibiotic have been explored. It is not easy to eradicate pathogen bacteria. A subsequent problem is the increasing resistance to antibiotics, especially to the fluoroquinolones. Resistance to flumequine increased from 3% between 1996-1999 to 20% between 2000-2002 whilst that of other serotypes in poultry remained about 7%. *S.* Java is also quickly becoming less sensitive to ciprofloxacin which is the antibiotic of first choice in serious cases of human salmonellosis and to the modern third-generation cephalosporins (Mevius, 2005). *C. perfringens* presents however resistances to some antibiotics (colistin, tetracyclin, etc).

Different alternatives to antibiotic to prevent intestinal bacterial pathogens in broilers can be used such as control of feed matters and poultry feed contamination, control of farm contamination and control of animal infection. Probiotic, Competitive Exclusion (CE) and vaccination are mainly used in poultry production. Maternal vaccination is also an alternative for the control of the necrotic enteritis in broiler farms Lovland *et al.* (2004).The addition of enzymes was found to be beneficial depending on the feed ingredients used. Cytokines were shown to act as growth promoters in immuno-enhancers for broiler chickens (Joerger, 2003). Treatments of feed or drinking water are also developed as alternatives. Numerous nutritional additives such as prebiotic, organic acids, essential oils… are used or were proposed as means to reduce or eliminate pathogens or as to improve growth and feed conversion. In particular, according to the study of Mitsch *et al.* (2004), essential oils would stimulate the activity of the digestive enzymes, stabilize the intestinal flora and deactivate toxins produced by *Clostridium perfringens*. However, further studies on the efficiency of essential oils on the toxins of this bacterium are necessary. Other agents as bacteriocins, antimicrobial peptides, and bacteriophages were proposed as potential animal therapeutics (Joerger, 2003).

This work is focused on the use of organic acids, CE and vaccination to reduce pathogens in the intestinal tract. Short chain fatty acids such as Acetic acid and Propionic acid or its derivates have already been used as feed and water additives for the control of *Salmonella* in poultry (Chaveerach *et al* 2004). Data on the effect of butyric acid in poultry are limited. *In vivo* studies have given variable results (Van Immerseel *et al*., 2003, 2004, 2005; 2006). In the search for intervention methods to control pathogens in broilers and other poultry

species, organic acids are widely used in feed and drinking water. It has proven difficult finding measures for effective control of *S*. Java and *C. jejuni* and *C. perfringens* in the poultry industry. In a first part of this study, the effect of short chain fatty acids acids on pathogens (*E. coli* O149K91, *Campylobacter jejuni*) and on substantial gut bacteria such as (*Lactobacillus casei*) was investigated by an *in vitro* model. In a second part, the effect of organic acids against the pathogen colonisation was studied by an *in vivo* model in field conditions. Competitive Exclusion (CE) was tested to control *Salmonella* in field and experimental conditions, CE was also tested in experimental model to control *C. perfringens*. The development of live vaccines could offer an opportunity to use vaccines against *Salmonella* in broilers. In the last part of this study, a vaccine was tested to prevent *Salmonella* colonization.

4.1 - In vitro essays

4.1.1 - Organic acids against pathogen contaminations

A) Material and methods

Effect of organic acids on E. coli O149K91 *and L. casei bacteria*

Ten organic acids (**Formic acid, Acetic acid, Propionic acid, Butyric acid, DL Lactic acid, Valeric acid, Caproic acid, Fumaric acid, Benzoic acid and Citric acid**) were tested in a concentration of 50 mM in buffers at pH 4 or pH 6. These pH values could be representative for the stomach (pH4) or the gut (pH6). Buffer at pH4 contained Citric acid and Na_2HPO_4 (4:1ww) and the buffer at pH 6 Na_2HPO_4 and KH_2PO_4 (1:2 w:w). The choice and concentration of acids was concluded from a preliminary study.

The bacterial culture was a mixed culture of ***E. coli* (O149 K91)** (10^7 CFU/ml) and ***L. casei*** (10^7 CFU/ml). Immediately after mixing and before addition of the organic acid (t=0), a sample was analysed for CFU counts of *E. coli* and *L. casei.* The acids were mixed into the buffers and samples were analysed at 30 min, 1h, 3h, 6h and 24h after mixing. The cultures were homogenized before taking the samples of 10 ml. This sample was diluted in 90 ml Peptone Physiological Saline solution (PPS). After homogenizing this mixture a decimal dilution was prepared from which CFU counts were made on MacConkey Agar no3 (Oxoid CM), that was incubated at 37ºC for 24h and Rogosa Agar (Oxoid CM), that was incubated at 37ºC for 72h.

Effect of organic acids against Campylobacter jejuni

In another experiments, the lethal effect of organic acids and mixtures was tested on ***C. jejuni*** *in vitro*. The experiments were carried out with the agar diffusion method and the fluid titration method. Hundred microliters of the acid were inoculated on the surface of one agar plate (experiment 1), in experiment 2 only one disc was used per agar plate. The agar of choice was Heart Infusion Agar (HI), without antibiotics or other bactericidal components. A larger number of organic acids (**Formic, acetic, Propionic, Butyric, DL Lactic, Valeric, Caproic, Fumaric, Benzoic, and Citric acids)** were tested twice (50 mM) (experiment 1)). The results were the mean value of 3 discs that were placed on one agar plate.

In experiment 2 the fluid titration method was tested. In a microtiter plate with 2.5 ml wells a decimal dilution series of the **organic acids** (**Sorbic acid, C8/C18, C8/C10**) was prepared in a PPS solution. The *Campylobacter* culture (24h in HI broth at 37ºC) was added. In all experiments a control group without acid was included. Sampling started immediately after inoculation (<10 min) and was repeated after 4h, after 6 h.

B) Results

Effect of organic acids on E. coli O149K91 and L. casei bacteria

In this study four acids (Formic acid, Butyric acid, Lactic acid and Caproic acid) were tested in their lethal ability against *E. coli* and *L. casei* in two different buffers (pH 4 and pH6) and at different acid concentrations (25 mM, 50 mM and 100 mM), depending on the acid type.

On basis of these results at pH4, Formic acid, Propionic acid, Butyric acid, DL Lactic acid, Valeric acid and Citric acid could be used in controlling *E. coli* (grey colour in table 23). Although Caproic and Benzoic acid are effective against *E. coli*, they are also lethal for *L. casei*, especially at low pH, so they could damage part of the gut microflora (no colour in table 23)

At pH 6, which is the upper and lower gut pH range, most acids could be used in controlling *E. coli*, although Fumaric and Benzoic acid show some lethal effect against *L. casei* as well (Table 23).

Table 23: Decrease of *E. coli* and *L. casei* after exposure to organic acid

Strain	*E. coli*				*L. casei*			
pH	**pH4**		**pH6**		**pH4**		**pH6**	
Time of incubation (h)	**3**	**6**	**3**	**6**	**3**	**6**	**3**	**6**
Formic	≥4	≥4	1.5	2	0	0	0	0.5
Acetic	1.5	3	1	1	0	0	0	0
Propionic	3	≥4	0.5	1	0	0	0	0
Butyric	3	≥4	1.5	2	0	0	0	0
DL Lactic	0.5	≥4	1	≥4	0	0	0	0
Valeric	2.5	≥4	2	2	1.5	2	0	0
Caproic	≥4	≥4	≥4	≥4	≥4	≥4	0	0
Fumaric	≥4	≥4	2	3.5	1	3	2	2
Benzoic	≥4	≥4	2	2	2	≥4	2	2
Citric	3	≥4	3.5	3.5	0.5	1	0	0

Decrease of Log CFU/ml after addition of acids (50 mM)

From this preliminary *in vitro* experiment some information may be generated on the effect of organic acids on pathogens (represented by *E. coli* O149K91) and on a representative commensal bacteria of the gut microflora (*L. casei*).

Effect of organic acids against Campylobacter jejuni

From the experimental data with a variety of organic acids and mixtures on agar diffusion tests, it is clear that there was a large variety in inhibition against *Campylobacter*. Mainly the 2% concentration of acids was effective and occasionally 1%.

Sorbic acid results in the most effective inhibition, even at low concentrations. However in the fluid titration test system, Sorbic acid was less effective than C8/C10 and Selko pH (Table 24). The consistency of the titration method was high, so it might make it the method of choice for *Campylobacter*. Testing a larger variety of acids should confirm this supposition.

Table 24: Inhibition of organic acids against *C. jejuni* by titration model

Acid concentration	**2%**	**1%**	**0.5%**	**0.25%**	**0.125%**	**0.0625%**
*Control (10 min)	+	+	+	+	+	+
Control (4h)	+	+	+	+	+	+
Sorbic acid (10 min)	+	+	+	+	+	+
Sorbic acid (4h)	-	-	-	+	+	+
C8/C18 (10 min)	-	-	-	-	-	-
C8/C10 (4h)	-	-	-	-	-	-
Selko pH (10 min)	-	-	-	-	+	+
Selko pH (4h)	-	-	-	-	-	-

*** growth in CCDA agar after 48h at 37℃ microaerobi c incubation**

4.2 - In vivo essays

4.2.1 - Evaluation of the efficacy of organic acids (corn silage) on *Salmonella* Java and *Campylobacter jejuni* colonization in broilers

In practical situations the application of corn silage could be a good alternative in the control of *Salmonella* in general and *S.* Java specifically. Corn silage is a fermented mixture of the whole corn plant, and contains several organic acids such as Lactic acid, Acetic acid and Butyric acid (Driehuis *et. al.*, 1999). In this study, Nalidixic acid and Novobiocin resistant strain of *Salmonella* Java 506 and *C. jejuni* 18887 were tested. Both strains were originally isolated from commercial broilers in the Netherlands and S. Java was typed by the Dutch NRL for *Salmonella* and *C. jejuni* by ASG-Lelystad respectively.

A) Material and methods

Experimental design and sampling

The study was performed at the experimental facilities of ASG at the Runderweg in Lelystad. One-day-old Ross broiler chickens, mixed gender from a commercial broiler hatchery, were placed in three separated groups:

Group 1: Corn silage

- Housed on corn silage,
- Challenged with *Salmonella* Java and *C. jejuni* with approx 3 x 10^8 CFU of S. Java and *C. jejuni*, at day 13 (day 14 of age) directly into the crop
- Fed non pelletized nor heat treated feed
- 25 animals (Ross)
- Housed in cages
- Orally challenged with a nalidixic acid and novobiocin

Group 2: Control group

- Housed on wood shavings
- Inoculated with a placebo (peptone physiological saline solution) at day 13 (day 14 of age)
- Fed non pelletized nor heat treated feed
- 25 animals (Ross)
- Housed in cages
- Inoculated with placebo at day 13 (day 14 of age)

Group 3: Positive control

- Housed on wood shaving
- Challenged with *Salmonella* Java and *C. jejuni* with approx 3 x 10^8 CFU of S. Java and *C. jejuni*, at day 13 (day 14 of age) directly into the crop
- Fed non pelletized nor heat treated feed
- 25 animals (Ross)
- Housed in cages
- Orally challenged with a nalidixic acid and novobiocin at day 13 (day 14 of age)

Inoculum:

For preparation of inocula for the oral challenge, the *Salmonella* Java strain was grown in 100 ml HI broth for 24 hours and the *C. jejuni* in HI also for 24 h but micro aerobically. Animals were inoculated with a dose of 0.5 ml of the mixed cultures per animal. At inoculation every seeder chick was wing tagged.

Sampling:

Before the challenge, samples of feed, faeces and paper inlays were checked for the absence of *Salmonella* and *Campylobacter*. After the animals were killed, caeca and liver samples were collected. From each animal 1 g of caecal content was added to 9 ml peptone saline solution and decimal dilutions were prepared (10^{-2} to 10^{-7}).

Cultivation and isolation

Campylobacter analysis in faeces swabs was by direct plating of swabs on CCDA, and from livers by enrichment in Preston enrichment broth (Nutrient broth Oxoid CM0067; plus selective supplement SR0204 and SR0232). After 24h micro aerobic incubation at 41.5ºC a sample was plated on Charcoal Cefoperazone Deoxycholate Agar (CCDA) (Oxoid CM0739 plus selective supplement Oxoid SR0155), that was subsequently incubated micro aerobically at 41.5ºC. Suspect colonies were confirmed by microscopic examination and Latex agglutination (Oxoid DR 0150).

For the recovery of *Salmonella* Java from cloacal swabs and livers, direct plating as well as enrichment (1:10 w:v) was performed. BGA with the addition of 25 mg/L Novobiocin and 25 mg/L Nalidixic acid (BGA+novo+nal) (BioTrading, Mijdrecht) was used as a selective plate. Enrichment broth was incubated aerobically for 24 hours at 37℃. Plates were incubated aerobically at 37℃ for 48 hours and checked for the presence of *Salmonella* after 24 and 48 hours of incubation. Suspect colonies were confirmed by agglutination with *Salmonella antisera* for confirmation of S. Java (Prolab, UK).

B) Results and discussion

In this study, the application of corn silage as a floor cover for broilers had a controlling effect on the excretion of *Salmonella* Java until 12 days after inoculation. No difference could be detected with regard to caecal counts at 19 days after inoculation, the chick age was 32 days (Table 25).

The percentage of animals from which liver samples were culture positive for *Salmonella* as well as the weight gain was similar in the groups that were either where housed on corn silage or on wood shavings.

Campylobacter colonized perfectly in all chicks. No protective effect of corn silage could be found. In relation to *Campylobacter*, no difference in colonization was found between the seeder group and the susceptible contact chicks.

Liver samples from the seeders in the corn group were all free of *Salmonella.* This indicates that the organic acids in the substrate have inhibited the invasiveness of the *Salmonella* (Table 25).

For *Campylobacter* this protective effect could not be measured.

Table 25: Percentage of samples positive for *Salmonella* Java or *C. jejuni*

			N. of day post incubation						
Group	**Strain**		**Day 2**	**Day 5**	**Day 8**	**Day 12**	**Day 19**	**Caeca**	**Liver**
1 Corn	*Salmonella*	Seeder	80%	90%	70%	30%	80%	80%	0%
		Contact	14%	79%	77%	54%	33%	83%	42%
		Total	42%	83%	71%	42%	50%	82%	23%
		Log CFU						**1.4**	
	Campylobacter	Total	42%	100%	100%	100%	100%	100%	36%
		Log CFU						**8.0**	
2 Negative control	*Salmonella*	Total	0	0	0	0	0	0	0
	Campylobacter	Total	0	0	0	0	0	0	0
3 Positive control	*Salmonella*	Seeder	100%	100%	100%	40%	30%	30%	30%
		Contact	53%	93%	87%	47%	33%	33%	13%
		Total	72%	96%	92%	44%	32%	32%	20%
		Log CFU						**0.6**	
	Campylobacter	Total	38%	100%	100%	100%	100%	100%	32%
		Log CFU						**7.8**	

N. Number

The effect could be ascribed at the organic acids in the corn silage, which presumably will contribute to the lethal effect on *Salmonella*. It is surprising that there is no effect on *Campylobacter*. This result is in agreement with Chaveerach *et al*., 2004, who found no inhibitory effect for *Campylobacter* by organic acids in drinking water.

Short chain fatty acids such as acetate and propionate have been widely used as feed and water additives for the control of *Salmonella* in poultry. The lethal effect of organic acids is not the same for all types of acids. However, the effect of short-chain fatty acids in the feed on the colonization of *Salmonella* in chickens seems to be dependent on several factors:

- the age of the animals at which infection occurs may be an important factor. Van Immerseel *et al*., 2005 found a reduction in the early colonization, but only in very young animals. Van Immerseel *et al*., 2005 infected 5-day-old Ross broiler chickens and found that coated Butyric acid significantly decreased caecal colonization 3 days post-infection compared with control chickens. However caecal colonization at slaughter age was equal for both groups, although the group of broilers receiving coated Butyric acid had a lower number of broilers shedding *Salmonella* Enteritidis by culture of cloacal swabs. (Van Immerseel *et al*., 2005). In our experiment we found similar results for caecal *Salmonella* carriage, however we found a significant difference in *Salmonella* positive caeca between positive, negative and “corn” groups.

- the effect of corn silage can primarily be attributed to the organic acids, present in the product (Driehuis *et al.,* 1999). They found significant proportions of Lactic acid, Acetic acid and Propionic acid in the silage. In our experiments we did not analyse the composition of the corn silage we used, but random samples for the same batch showed the following organic acid composition. Butyric acid: 0.06-0.2 g/kg; Lactic acid: 20 g/kg and Acetic acid: 4.6-5.7 g/kg. Mean pH value was 4.2.

- the antibacterial effect in the crop by concentration of acids may lead to a decrease in horizontal transmission. Another mechanism is that the fatty acids have an effect on the bacterial flora of the litter itself and may reduce the amount of *Salmonella* and *Campylobacte*r.

In this experiment the *Salmonella* challenge was relatively high and not representing the practical situation. Corn silage therefore may has a good effect against *Salmonella* under practical conditions. In conclusion it can be stated that application of corn silage as a floor cover in broiler houses may contribute to the control of *Salmonella* contamination of broilers.

4.2.2 - Efficacy of protected organic and inorganic acids on *Salmonella* colonisation in broiler farms

A) Material and methods

Experimental trial and sampling

Three farms of newly hatched chicks received standard broiler rations supplemented with 0.5% (farm A, 23500 birds) and 1% (farm B, 25320 birds) of the protected acids continuously starting at one day of age, while a control group (farm C, 23180 birds) received unsupplemented feed. The trial was performed in three broiler farms selected according to their previously positive for *Salmonella*. Each trial farm consisted of single age broilers, hatched from a single breeder flock and reared sexed or as hatched. All farms were characterised by the same type of broilers houses in terms of size, ventilation system, feeding and watering equipment.

Procedure 1: In the **farm A** the disinfecting procedures included an all in-all out period of 2 weeks, a **disinfection** with water containing **sodium hypochloride** and a fumigation before housing of chicks.

Procedure 2: In the **farms B and C** the disinfecting procedures included an all in all out period of 2 weeks, a disinfection with water containing **sodium ammonium quaternary** and a fumigation before housing of chicks.

Sampling:

A total of 30 paper lines collected in the farms at the housing time and 30 caecal droppings (about 10 g. each) randomly collected at 28 days of age in each farm, were located in sterile plastic boxes and transferred to the laboratory under refrigeration conditions. Moreover, 30 caecal contents were aseptically collected at the slaughterhouse and located into stomacher bags transferred to the laboratory under refrigeration conditions.

Cultivation and isolation

Samples were directly plated onto brilliant green agar (BGA) and Xylose Lysine Tergitol 4 (XLT4) plates. The remaining sample was diluted (1:5) with Selenite Cystine (SC) broth incubated at 37℃ for 24h. At the end of the incuba tion period each broth was streaked onto BGA and XLT4 plates. All plates were incubated at 37℃ for 24 h and the *Salmonella*-like colonies were enumerated. The *Salmonella* presence in the analysed samples was evaluated as it follows: 0=any colony on plates; 1=isolation of *Salmonella* on plates after enrichment in SC broth; 2= isolation of 1-10 colonies on plates without enrichment in SC broth; 3= isolation of 10-100 colonies on plates without enrichment in SC broth ; 4= isolation of more than 100 colonies on plates without enrichment in SC broth.

Statistical analysis

The mean values of the results obtained for each group of birds were compared using the Student Newman-Keuls test.

B) Results and discussion

All birds included in the experimental trial were *Salmonella* free at day 1 confirming the efficacy of the sanitary practices applied in the breeder flocks as well as in the hatchery. On the contrary at day 28 and 49 all groups were *Salmonella* positive. In particular, farm B showed a *Salmonella* colonisation score statistically significant lower than control group (i.e. 0.35 vs 1.30 at day 28 and 1.26 vs 2.2 at day 49). On the contrary, the farm A showed a *Salmonella* colonisation score significantly lower only at 28 days of age (i.e. 0.60 vs 1.30) (Table 26). Furthermore, all samples belonging to farm B at day 28 were *Salmonella* negative using direct plate count, whereas the majority of control samples were *Salmonella* positive using this procedure. At day 49 all samples belonging to farms A and B were positive after the enrichment step, whereas the control samples showed a *Salmonella* load between 1 to 10 colonies using direct plating. Even if in farm A it was used sodium hypochloride considered more efficacious than sodium ammonium quaternary for the sanitary procedures, the *Salmonella* reduction seems to be related to the use of acids added to the feed.

Table 26: Average of *Salmonella* colonization scores in broilers receiving feed containing different concentrations of organic and inorganic acids

Farms*	Sanitary procedure	N. of samples collected	1 Day	28 Days	49 Days
A (0.5 ppm)	1	30	0	0.60±0.68	1.4±1.50
B (1 ppm)	2	30	0	0.35±0.48	1.26±1.33
C (Control)	2	30	0	1.30±0.97	2.2±1.74

N. Number; *=Means with different letters in the same column and in the same row are statistically significant (p<0.05)

4.2.3 - Efficacy of CE to control *Salmonella* colonisation in broiler farms

A) Material and methods

Experimental trial

The trial was performed in broiler farms located in North of Italy. Each trial farm consisted of single age broilers, hatched from a single breeder flock and reared sexed or as-hatched. A total of 658.000 broilers (331900 females and 326100 males) were treated with the CE product Aviguard® (Bayer Animal Health) whereas 665900 controls (389600 females and 276300 males) were used as controls. Forty-four houses on a total of 26 farms and 45 houses on 16 farms were stocked with treated and control broilers, respectively. The farms selected were *Salmonella* positive during the last three previous cycles. All animals were fed ad libitum with the same commercial feed. During eight successive weeks one-day old commercial broiler chicks (Cobb and Ross strains) were treated by coarse spray in the same hatchery with one dose/bird of Aviguard®(Bayer) (treated) or with water only (placebo controls) on a week-on-week-off basis.

Cultivation and isolation

ISO 6579 standard method was used for *Salmonella* detection in each sample. The Rappaport Vassiliadis (RV), as enrichment broth, and Brilliant Green Agar (BGA) and Xylose Lysine Tergitol 4 (XLT4) as selective media were used. The suspect colonies (red colonies on BGA, red colonies with black centre on XLT4) were inoculated into Trypticase Soy Agar plates for purification and subsequently were confirmed by serological tests, using *Salmonella* O and H antiserum.

Statistical analysis

Pearson's chi-square analysis was used to determine significant differences between treatments in Salmonella isolation with Statgraph software (v. 5.1 STSC, Maryland). All statements of significance were based on P≤ 0.05. Means were compared for significant (P≤ 0.05) differences by using the Newman Keuls test.

B) Results and discussion

In this field investigation all the 89 surface swabs, collected from each of the 42 farms before the housing were *Salmonella* spp. negative. The data of table 25 show that, in comparison to the controls, the *Salmonella* shedding was significantly reduced in the Aviguard® (Bayer) treated flocks despite a numerically higher initial *Salmonella* exposure at one day-old (11.5% treated *vs* 6.2% control). In fact at 21 days of age 68.7% of the control farms and 26.3% of the treated farms respectively, were *Salmonella* positive using XLT4 media. These results show a reduction of the occurrence of *Salmonella* spp. of approximately 60%, as already reported by other investigators (Deruyttere *et al*., 1997). Regarding the total amount of samples, a statistically significant reduction of *Salmonella* spp. colonization was observed at 21 days in the treated animals, in comparison to the control birds (10.85 % for treated *vs* 20.30 % for control).

The results obtained with the different isolation media (BGA and XLT4) were comparable. The detection rate for caecal dropping was statistically significant higher than from all the other samples collected at each time. In particular among the samples (surfaces, swabs and samples related to the chicks) collected within the 1st day of life any statistically significant differences was observed on *Salmonella* detection (Table 27). Otherwise among the sample collected at the 21st day of treatment the detection rate from caecal droppings was statistically significant higher than from surface swabs (16.5 *vs* 6.5).

Table 27: Ratio of *Salmonella* spp. isolation from treated broiler farms and corresponding controls

Groups	**N. positive*/examined farms (percentage± SD)**		**N. of positive*/examined samples (percentage ± SD)**	
Media	BGA	XLT4	BGA	XLT4
Treated	8/26 (30.7 ± 45.9)	8/26 (30.7 ± 45.9)	36/332 (10.8 ± 31.1)	36/332 (10.8 ± 31.1)
Control	11/16 (68.7 ± 47.8)	11/16 (68.7 ± 47.8)	54/267 (20.3 ± 40.3)	53/267 (19.9 ± 40.0)
Chi-square value	0.01633	0.01633	0.0014	0.0020

N. Number; *Each farm was considered positive in presence of one single positive sample

The results of this research confirm the high contamination level of commercial broilers during the growing phase (Hayes *et al*., 2000) and that early treatment of chicken in the hatchery with Normal Avian Gut Microflora could reduce subsequent persistent broiler infection.

C) Conclusion

In conclusion the results obtained in these studies, conducted under Italian field conditions, support earlier observations that CE cultures like Aviguard® (Bayer) are a useful tool for reducing the occurrence of *Salmonella* spp. in commercial broiler production. In fact already after three weeks post hatch the *Salmonella* shedding in the broilers treated with Aviguard® was reduced of about 60% in comparison with control broilers. Furthermore, the addition of protected organic and inorganic acids to the feed during the whole rearing period reduces the

Salmonella spp. colonisation at the caecal level reducing the risk of cross contamination during slaughtering.

4.2.4 - Effect of CE on transmission of *Salmonella* Java in experimental model

A) Material and methods

Experimental design and sampling

For the experiment commercial Ross broiler from a commercial hatchery were placed in groups of 23 chicks each. In table 24 the experimental setup is given. The chicks were *Salmonella* free at hatch and on arrival at the experimental facilities. CE treatment of the groups took place individually orally immediately after hatch with Aviguard® (Bayer).

The oral *Salmonella* Java infection with **10^5 CFU/chick** of the seeders took place at day 2 after separation of the susceptible contact chicks into a clean room. One day after colonization of the *Salmonella* Java, the contact chicks were placed back in their original group of 23 animals. Swab sampling of every individual chick started at day 5 after infection and reunion of the groups until day 27.

Table 28: Experimental setup CE flora on transmission of *S.* Java

Treatment	Number of chicks	Number of chicks
Infected with CE	10	10
Contact with CE	13	13
Infected without CE	10	10
Contact without CE	13	13

Cultivation and isolation

See section 3.2.2

B) Results and discussion

All seeders were shedding *Salmonella* spp. from the day after infection. The susceptible contact animals appeared to be *Salmonella* spp. shedders from the starting point of monitoring at day 8. Even part of the seeder chicks refuse to shed *Salmonella* throughout the experimental period. Contact chicks become infected with *Salmonella* spp., although there is a delay of 2-3 days (data not shown). The effect of the CE treatment is reproducible in the two groups (Table 29). The data show that all chicks were infected but they were shedding *Salmonella* intermittently in contrast with the control group, where all chick were shedding at every occasion. From these experiments it can be concluded that *S*. Java is an efficient spreader in broilers. CE treatment can contribue in controlling *S.* Java, but cannot eradicate the infection.

Table 29 : Proportion of *Salmonella* shedders in CE treated groups

		CE Group 1	CE Group 2
Seeders	Shedders	67	93
	%	33.5	46.5
Contact	Shedders	102	130
	%	39.2	50.0

4.2.5 - Effect of CE to control *Clostridium perfringens* in broiler farms

A) Material and methods

Experimenatl design and sampling

A total of 14 farms located in the Veneto region (Italy), characterised by the same type of broilers houses in terms of size, ventilation system, feeding and watering equipment and management. The birds tested (337.510 treated and 272.080 controls) were selected in the hatchery according to the criteria "week off-week on" and divided in 2 groups (Table 30). The broiler farms included in the trial were chosen on week off-week on basis:

- **4 control groups:** non-treated selected from all groups hatched during the **first week**

- **4 groups treated** with Aviguard® at hatchery level (via spray) selected from all groups treated during the **second week;**

- 3 control groups: non-treated selected from all groups hatched during the **third week;**

- **3 groups treated** with Aviguard® at hatchery level (via spray) selected from all groups treated during **the fourth week.**

All the broilers will be housed in "problem" farms i.e. presence of NE in the previous growth outs. All the broilers included in the trial were vaccinated with Paracox 5 according to standard scheme (via spray at hatchery).

Table 30: Experimental design CE against *C. perfringens*

Group	Farms	N. animals (n°houses)	Rearing period (days)	Thinning (days)	% animal depopulated
Treated	A	43.385 (2)	43 and 41	ND	ND
	B	56.985 (2)	51 and 52	41 and 42	32.10 and 32.15
	C	52.415 (2)	51 and 52	38 and 40	36.44 and 40.21
	D	51.260 (2)	50 and 51	37 and 39	37.87 and 37.62
	E	64.380 (2)	55	42	44.02 and 45.11
	F	48.585 (1)	37	ND	ND
	G	20.500 (1)	38	ND	ND
Control	H	33.900 (2)	40 and 41	ND	ND
	I	42.760 (2)	50	37 and 38	43.21 and 41.93
	L	45.620 (2)	51 and 52	39 and 40	40.61 and 40.33
	M	43.425 (2)	50	38	36.84 and 35.17
	N	54.175 (2)	51 and 56	42	35.51 and 39.40
	O	25.700 (1)	56	42	36.09
	P	26.500 (1)	40	ND	ND

N. Number; ND, Not Determined

Clinical evaluations:

They were carried on 7, 14, 21, 28, 35 days of age following this classification: 1 = normal appearance; 2 = few birds (<5%) are affected by enteric disorders; 3 = birds between 5-50% are affected by enteric disorders; 4 = more than 50% of the birds are affected by enteric disorders

Post mortem examination:

Until 7 days on 5% of dead or clinical ill birds/broiler house post mortem examinations were performed to identify lesions referable to NE. Then it was performed at 21 and 35 days on 20 birds/house (maximum 60 birds/farms) at random among birds preferably with clinical signs. The gross lesion of the jejunum and ileum was evaluated with the following schema: 0 = No lesions; 1 = Friable and distended intestine; 2 = Focal necrosis and ulcers; 3 = Large necrotic areas.
Gross lesions are frequently confined to jejunum and ileum and include yellow or green pseudomembranes adherent to the mucosa intestines may be friable with a thickened wall distended by gas and contained bile-stained fluid or a granular core of fibrinonecrotic debris and litter.

Cultivation and identification

On the same birds will be collected about 5 cm of intestine (Meckel's *diverticulum*) and tested for *C. perfringens* culture isolation at 7, 21 and 35 days of age. On the isolates will be performed a PCR technique to identify the toxigen strains.

Statistacal analysis

The mean values of the results obtained for each group of birds were compared using the Student Newman-Keuls test.

B) Resuts and discussion

The results concerning the control of *C. perfringens* showed a statistically significant reduction of lesions score, in particular during the first 21 days, in the groups treated with Aviguard® in comparison to control groups. In the treated farms the lesions score index was constant on time whereas in the control group at 35 days a sensible reduction was observed, except for one farm in which necrotic enteritis was detected (Table 31).

Table 31: Mean values of the lesions score according to the bird age

Groups	Farms	N. samples	Lesions score index	
			21 Days	35 Days
Treated	A	40	0.5	0.925abc*
	B	40	0.725	1.475cd
	C	40	0.925	0.550ab
	D	40	0.475	0.525ab
	E	40	0.550	0.10a
	F	40	0.5	0.35ab
	G	20	1.05	0.75abc
	Total	260	0.646	0.661
Control	H	40	0.925	0.150a
	I	40	0.725	1bc
	L	40	1.150	0.40ab
	M	39	1	0.61ab
	N	20	1.175	-
	O	20	0.50	0.45abc
	P	20	1.20	2.20d
	Total	219	0.966	0.698

N;, Number; *= Different letters in the same column show statistically significant differences for p<0.05

Any statistically difference was observed in the different groups in relation to the mortality at the end of the rearing period (3.73 *vs* 3.01).
The *C. perfringens* loads on litter samples didn't show statistically significative differences among groups tested at different ages. In particular, the level of contamination observed was very low with values ≤ 3 Log_{10}.
In table 32 the toxins classification of *C. perfringens* isolates were reported. No toxins β, ε and enterotoxins but only toxin α were detected. These results are agree with the results reported by other researchers In particular the percentage of *C. perfringens* type A was of 65% in the treated group in comparison with 70.4% in the control group.

Table 32 : Toxins classification of *Clostridium perfringen*s

Group	Farms	N. of samples	% of toxin α positive
Treated	A	5	60
	B	7	71.5
	C	3	100
	D	6	50
	E	4	66.6
	F	6	50
	G	9	77.7
	Total	40	65
Control	H	4	75
	I	3	66.6
	L	2	100
	M	6	66.6
	N	4	50
	O	6	83.3
	P	2	50
	Total	27	70.4

N., Number

In database (section 6) the ribotypes for genetic characterisation of *C. perfringens* isolates were reported. The results obtained showed a high genetic diversity. Different ribotypes were identified not only from different farms but also coming from the same farms.

4.2.6 - Vaccination to control *Salmonella* paratyphi B Java

A) Material and methods

Experimental design and sampling

One day old broilers from a commercial Dutch hatchery were set after individual oral vaccination into the crop with 0.5 ml LAH vaccine against S. Typhimurium (TAD-Salmonella vac®-T) with a so-called crop needle. After homogenizing chicks were treated with the vaccine within one hour. The control groups were treated with a physiological saline solution following the same procedure.

Twenty-five chicks were placed in cages of approximately two square meters with ad libitum feed and water supply. The different groups were randomly scattered in the room, so at least one-meter space was between the groups. Table 33 shows the groups and the number of chicks per treatment.

Table 33: Treatment groups

Group	Vaccination	*Salmonella* infection	N. of chick
1	Yes	S. Typhimurium	25
2	Yes	S. Java	25
3	No	S. Java	25
4	Yes	S. Java	25
5	No	S. Typhimurium	25
6	No	No	25
7	Yes	S. Typhimurium	25

N., Number

After 24 hours 10 chicks from each group were individually orally infected with 0.5 ml of an overnight culture of either *Salmonella* Java or *Salmonella* Typhimurium, as indicated Table 33). Both *Salmonella* strains were originally isolated from broilers. After inoculation the seeder chicks were wing banded. The chicks were fed a commercial broiler diet without coccidiostat or antimicrobial feed additives.

Sampling

Before and after inoculation with both the vaccine and the *Salmonella* cultures samples were taken for bacteriological CFU counts. Fresh faeces sampling of the chicks started at day 2 post infection (p.i.) daily by taking a pooled sample from every treatment group. Starting at day 13 p.i. the cloaca of every individual chick was sampled by swab, and from the seeders the wing band number was registered. The control group was sampled first followed by the other groups in random order. On day 34 p.i. the broilers were slaughtered and caeca and livers were sampled. After suspension of the vaccine, CFU *Salmonella* counts were made immediately (0.5 hour pre vaccination) and within one hour after vaccination.

Vaccination

Application of the oral TAD-Salmonella vac®-T vaccine was individually orally by direct injection into the crop, although the product sheet only mentions drinking water application. In our small-scale experiment individual vaccination was relatively easy to perform and guarantees that every individual chick is vaccinated.

Cultivation and isolation

The vaccine strain from the **TAD-Salmonella vac®-T** was resistant against Rifampicin and Nalidixic Acid (Schröder *et al*., 2004). CFU counts were made on Brilliant Green Agar (BGA-RIF) (LabM Lab34) plus 100 µg/L Rifampicin (Sigma R3501) and 5 µg/L Nalidixic Acid (Sigma N8878) and Heart Infusion agar with 5% sheep blood (48h at 37℃).

Faeces samples for detection of the vaccine strain were cultured on BGA-RIF agar by incubation at 37℃ for 48h, on (24h at 37℃), on BG A-NAL agar and Modified Rappaport Vassiliadis Agar (MSRV) (24h at 42,5℃) after pre-e nrichment in Buffered Peptone Water (BPW) (Oxoid CM 509) (24h at 37℃). For identificat ion control, confirmation of isolates from each selective agar medium 5 individual colonies were pure cultured on BGA-RIF, BGA-NAL and MSRV. The serological typing of these *Salmonella* isolates was done by slide agglutination with *Salmonella* anti-sera from Pro-Lab diagnostics. The results should be either S. Java (antigen structure O:4, H_1:b, H_2: 2) or S. Typhimurium (antigen structure O:4, H_1:i, H_2: 2) or the vaccine strain (antigen structure O:4, H_1:i, H_2: -).

Cloacal swab samples were incubated on BGA-NAL after direct plating of the swab and by plating after 24 h enrichment of a 10^{-1} dilution in BPW for 24h at 37℃.

Caecal contents were diluted in BPW a decimal dilution series, which was inoculated on BGA-NAL plates for CFU count estimation. The 10^{-1} serial dilution was enriched in BPW for 24 h at 37℃, followed by plating on BGA-NAL (24h, 37℃).

Livers were homogenised in BPW (1:9 w:w) and after enrichment (24 h, 37℃) these samples were plated on BGA-NAL (24h, 37℃).

Statistical analysis

Anova of the data on shedding of the *Salmonella* groups was performed; and differences between the groups was determined with t-test where the significance level was set at p=0.05.

Results and discussion

At slaughter day 34 p.i., cloacal swabs, caeca and liver of every individual chick were tested for *Salmonella* status (Table 34). Caeca and livers from every individual broiler could be assigned the same chick and since the sentinels were not wing tagged, cloaca swabs could only be assigned to the seeders. In the S. Java groups only a very limited number of livers was *Salmonella* positive, whereas these groups were all infected with a relatively high dosage: 6.2 10^6 CFU *S.* Java per chick. The isolates from one individual chick in group 2 and in group 4 appeared to be a mix of *S.* Typhimurium and S, Java. In the groups most of the isolated *Salmonella* were either *S.* Typhimurium only or mixed infections of both *Salmonella.* Four isolates from liver appeared to be only S. Java: group 3, 4 (1 sample) and group 6 (2 samples). No vaccine strain could be isolated from livers.

Table 34: *Salmonella* isolates from liver, caecal and cloacal swabs of broilers (at day 34 p.i)

Liver	**Seeders**										**Sentinels**														
Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
group 6	1	1	0	0	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0	1	1	0	0	1	0
group 3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
group 2	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
group 4	0	0	0	0	0	*	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
group 5	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
group 1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	*
group 7	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	*	*	*
Caeca	**Seeders**										**Sentinels**														
Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
group 6	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1
group 3	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	0	0	0
group 2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
group 4	0	0	0	0	0	*	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0
group 5	0	1	1	0	0	1	0	1	1	1	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1
group 1	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	*
group 7	1	1	1	0	1	0	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	1	*	*	*
Swabs	**Seeders**										**Sentinels**														
Samples	1	2	3	4	5	6	7	8	9	10	*														
group 6	*	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	1	1	1	1	0	0
group 3	0	0	0	*	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0
group 2	*	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
group 4	0	0	0	0	0	*	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	*
group 5	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
group 1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	1	1	1	1	0	*
group 7	1	1	1	0	1	0	1	0	1	1	1	1	0	0	1	0	0	1	1	0	1	0	*	*	*

(1, > 200 CFU/g, 0,<200 CFU/g) * Swabs could not be assigned to the same sentinel chick number as for caeca and liver

The result clearly showed that *S.* Typhimurium is more pathogenic (=invasive) for broilers than S. java. Broilers with *Salmonella* positive livers however were not always shedding *Salmonella* (only for seeders) nor were they *Salmonella* positive in their caeca, vice versa. In group 5, 6 and 7 only one chick proved *Salmonella* positive in swab, caeca and liver. Here again always *S.* Typhimurium was involved. In group 6 the largest number of chicks showed *Salmonella* positive caeca and livers, respectively 19 and 11 out of 25. Eight out of 25 samples were positive in both caeca and liver, and from 7 of these both S. Java and *S.* Typhimurium were isolated. From other sample only S. Java could be isolated.

In group 6, where the *Salmonella* infection was introduced at day 23, four out of 25 caecal contents were above the detection level, respectively $2.0.10^2$, $3.0.10^2$, $1.3.10^4$ and $1.7.10^5$ CFU/g. These samples there did not show a correlation between a high CFU level and infection of liver.

From these experiments it can be concluded that vaccination of broilers with live vaccine did not eradicate *Salmonella* of homologous nor from heterologous origin. This can be explained by the relatively highly challenged environment where the broilers stayed. Another explanation might be the short time between vaccination and slaughter, moreover there was no booster treatment after treatment with the live vaccine. The results showed no difference in clearing an infection between vaccinated and non vaccinated broilers. Cross contamination between the groups appeared to have no effect on the experimental results.
From the *S.* Java inoculated broilers only a very small proportion was *Salmonella* positive and no difference was found between vaccinated and control group.

There was a significant difference in cloacal swab result between both vaccinated groups that were infected with *S.* Typhimurium. This discrepancy was also in caecal samples, but not in livers.
The vaccine strain could be isolated from faecal droppings until the end of the experiment, when the broilers were 35 days of age. No transmission of the vaccine strain between vaccinated and control groups was observed. The vaccine strain could be found in the regular monitoring, which is undesired.

4.3 - Conclusion

In vitro, at pH 4 most **organic acids** established a lethal effect on *E. coli*. At pH 6 a substantially smaller effect of many organic acids on *E. coli* was found, but the effect after 6h of Caproic acid, DL lactic acid, Fumaric acid and Citric acid were fair. The lethal effect on *Lactobacillus casei* was generally lower in comparison with the effect on *E. coli*. At pH 6 only Fumaric acid and Benzoic acid proved to be lethal and at pH 4 four acids show lethal effects.

From the experimental data *in vitro* with a variety of **organic acids and mixtures** on agar diffusion tests, a variation in inhibition against *Campylobacter* was found. Mainly the 2% concentration of acids was effective and occasionally 1%. Sorbic acid resulted in the most effective inhibition, even at low concentrations. However in the fluid titration test system, sorbic acid was less effective than C8/C10 and Selko pH. The consistency of the titration method was high, so it might make it the method of choice for *Campylobacter*.

In vivo, the application of **Corn silage** as a floor cover in broiler houses may contribute to the control of *Salmonella* contamination of broilers. However, there was no effect on *Campylobacter.*

The addition of **protected organic and inorganic acids** to the feed during the whole rearing period of broilers reduced the *Salmonella* colonisation at caecal level, thus reducing the risk of cross contamination during slaughtering.

Under field conditions, **CE** cultures were a useful tool for reducing the incidence of *Salmonella* and *Clostridium perfringens* infection in commercial broiler production. Moreover, this treatment does not interfere negatively with immune response. Under experimental conditions, CE treatment can contribue in controlling *Salmonella* Java, but cannot eradicate the infection .

Under experimental conditions, **vaccination** of broilers with live vaccine did not eradicate *Salmonella* of homologous nor of heterologous origin. The results show no difference in clearing an infection between vaccinated and non vaccinated broilers. The vaccine strain could be isolated from faecal droppings until the end of the experiment, when the broilers were 35 day of age. No transmission of the vaccine strain between vaccinated and control groups was observed. Indeed there is no or hardly any information on the efficacy of these vaccines in broilers. Lohmann Animal Health Cuxhaven Institute in Germany provides information on field trial they have performed, but scientific proof is not available.

Section 5: Bacterial contamination at the slaughterhouse level

The consumption of poultry meat is world wide increasing (Speedy *et al*., 2003). Nevertheless, poultry meat products are a main source of human food-borne disease (Cogan and Humphrey, 2003; Corry and Hinton, 1997; Fischer *et al*., 2004). Food of animal origin becomes readily contaminated with the infectious agents by contamination either during food processing and handling or directly by a pathogen or commensal the food producing animal is hosting. Both contamination routs are frequent for food-borne pathogens *Salmonella* spp. and *Campylobacter* spp. If broilers harbour the pathogens in their intestine and thus contaminate and/or infect other animals by the faecal-oral route. According to the results at the farm level, the occurrence of *Campylobacter* spp. ranged from 43.7 to 95.4% in Lithuania and Italy respectively. The occurrence of *Salmonella* spp. ranged from 5.6% to 29.6% in Lithuania and in the Netherlands respectively (see section 3).

At slaughterhouse level the pathogens may contaminate slaughter carcasses as well as slaughter facilities. Even though, slaughter technology for broilers is highly automatized contamination through intestinal content is frequent. Public health and quality assurance plans for safe meat are based on the overall concept “from stable to table” (Anderson *et al*., 2006; Anonymous, 2006; Kasbohrer and Heckenbach, 2006). Thus, monitoring of slaughter processing steps is becoming increasingly important e.g. for defining critical control points, cross contamination, logistic slaughter efficiency and to define the overall microbiological status of processed poultry. Quality assurance plans, new slaughter technologies, advices for product handling and to establish correct shelf lives must be based on a sound understanding of the composition of the microbial flora of poultry carcasses (Evers, 2004; Nauta *et al*., 2005). Not only a fast and reliable diagnosis regarding diverse pathogens on the carcass but also information on the overall carcass flora is important to draw conclusions on hygienic standards, new slaughter technologies or risk assessment studies.

During the slaughtering and processing operations, the contamination can spread to other birds and flocks by soiling of the slaughter environment. The highly industrialised and automated broiler slaughtering may enhance this further. The scalding and plucking process are considered as major sites for cross contamination, because faeces is pressed out of the cloacae in these early steps and spilled over the carcasses. In the later process of evisceration the rupture of the gut can lead to the contamination of the following carcasses (Bryan and Doyle, 1995). Scalding is said to be the most important step on hygienic means in poultry slaughter (Barros *et al*., 2007). Defeathering is most often technically combined

with scalding in the slaughter process; nevertheless this is the first step where microorganisms from feathers and skin are inseminated in the slaughter line. The mechanical action of defeathering machines has even been defined by using marker organisms as being most important for dispersal of microorganisms on the poultry meat surface (Allen *et al.*, 2003). Hence, this was defined as a critical processing step where samples have been drawn. The process of evisceration is most important for contamination of poultry meat with pathogens and intestinal commensal bacteria (Barros *et al*., 2007). After the evisceration and a final washing step, the carcasses are transferred into a chilling operation. The immediate cooling of carcasses after slaughter is regarded as an absolute essential step not only to store and transport meat but also to minimise growth of pathogens and spoilers (James *et al*., 2006). After the evisceration and a final washing step, the carcasses are transferred into a chilling operation. In particular, while the immersion chilling in a spinchiller is well known and evaluated in regards to the changes of numbers of *Campylobacter* on the chilled broilers, information on the effect of air chilling on numbers of surface *Campylobacter* are yet scarce. Finally the packaged product is of upper importance because that is what consumers get into their hands. A schematic overview of a broiler slaughterhouse is shown in figure 11.

In a first part of this study the most important slaughter processing steps were defined, intestinal samples and carcasses were analysed at different slaughterhouses using conventional microbiological methods and alternative molecular-based methods such as Single Strand Conformation Polymorphism (SSCP) and sequencing analysis. The contamination by *Salmonella* and/or *Campylobacter* in regards to the flock status was also studied in Austria.

In a second part, *Campylobacter* was monitored along the broiler slaughtering and processing in a German and Italian slaughterhouses, the seasonal effect was also evaluated. The analysis were performed to acquire quantitative data about pathogens of broiler meat production.

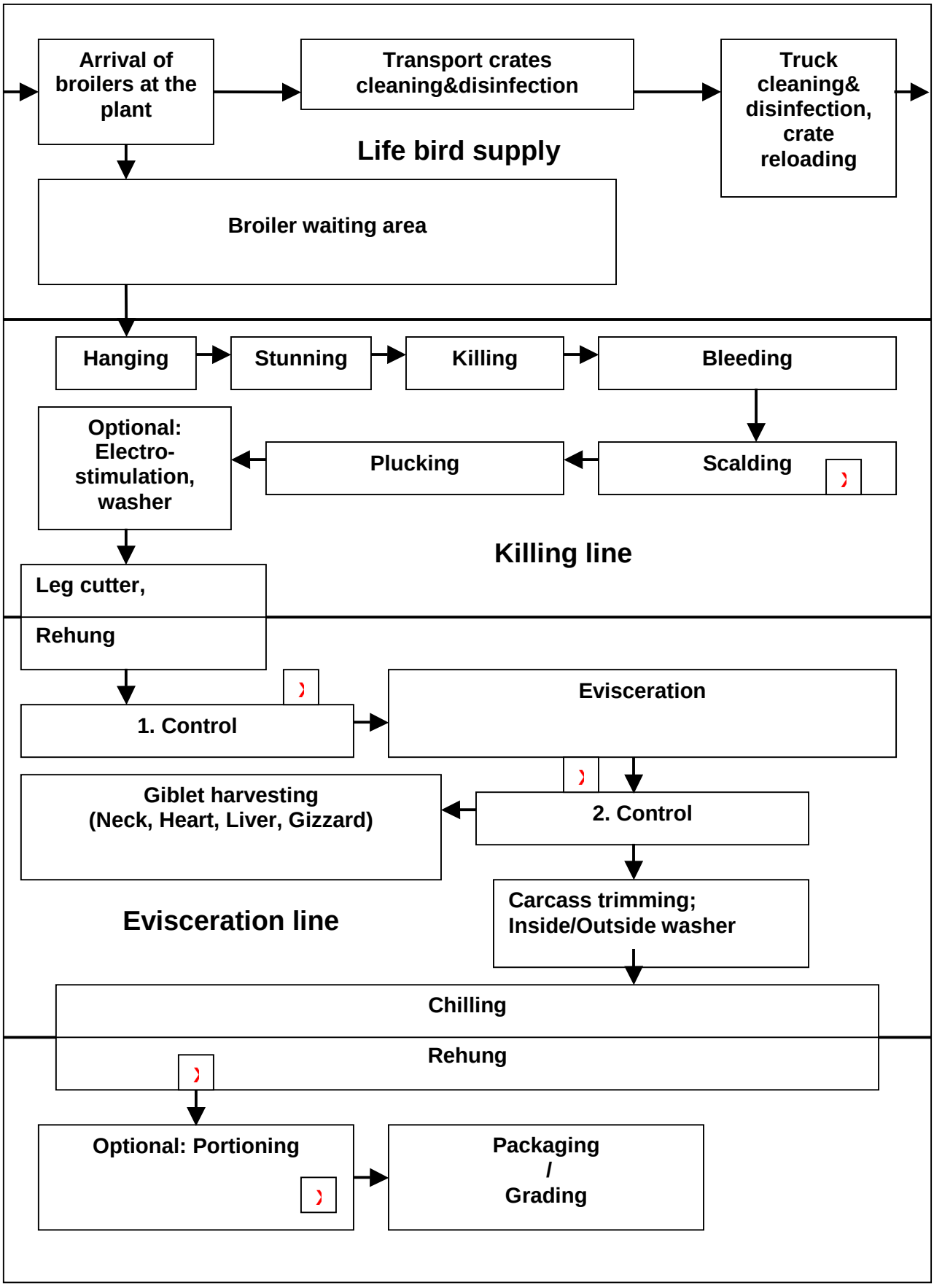
In the third part, the contamination by *L. monocytogenes* in French broiler slaughterhouses in regards to the flock status was evaluated. The relationship between isolates of *L. monocytogenes* was studied by genotyping.

Isolates collected in these tasks were serotyped and included in the database (section 6).

In the other part, the synergism and antagonism of spoilage bacteria and pathogens on poultry meat was evaluated.

In the last part, different alternative intervention strategies implemented in the broiler processing were studied in the Netherlands for their potential impact on the reduction of *Campylobacter* and *Salmonella*.

Figure 11: Schematic overview of broiler slaughterhouse



5.1 Monitoring of slaughter processing steps regarding food-borne pathogens and commensal bacteria by traditional and molecular methods (Austria)

Rapid and efficient detection methods to study microbial communities based on molecular methods are becoming increasingly important. As cultural based methods used in food microbiology are often time consuming and do not reflect the diversity of the microbial flora that might be important for defining safe meat. Rapid microbiological methods, useful for controlling the slaughter process are either based on fast and efficient detection of a specific microorganism – most often a certain pathogen – or the detection of a broad mircoflora community – both qualitative and quantitative. For analysing microbial communities different molecular methods are commonly used and most are based on the analysis of 16S rRNA gene fragments (section 7).

In this part of the work, pooled faecal and intestinal samples and carcasses of poultry flocks were analysed at different processing steps in two different slaughter facilities in Austria for the main foodborne pathogens, *Salmonella* sp, *Campylobacter* spp., and for the commensal *Escherichia coli*, *Lactobacilli*, *Staphylococci*, *Enterococci*, and *Enterobacteriaceae*. Statistical analysis of colony counts was explored at different processing steps and relevant significant differences are discussed. Additional microbial community analysis of fecal samples and slaughter carcasses were done by molecular methods based on a 16S rRNA gene specific primer set, using SSCP in order to find specific maker of slaughter contamination.

5.1.1 - Material and methods

A) Experimental trial and sampling

In two different poultry slaughterhouses in Austria samples were drawn. All four seasonal samplings were performed as planned, slaughterhouse were sampled four times through the project. Samplings were performed from 8.2.2005 – 24.3.2005 (spring sampling) five flocks, 3 conventional and two organic flocks and from 12.12.2005 – 23.2.2006 (winter sampling) 4 conventional and 2 organic flocks, from 1.9.2006 - 3.10.2006 (autumn sampling) 4 conventional flocks and a final sampling was performed from 27.02.07 – 12.04.07 (spring-summer sampling) where 18 conventional flocks were sampled. Each week during the fattening period from each flock faecal and intestinal content (caecal and ileal content) were drawn and analysed.

At the slaughterhouse level, different processing steps were identified to give important information on cross contamination, logistic slaughter efficiency and general hygiene. Five different processing steps were included as are after scalding, after defeathering, after evisceration, after cooling and packed products. At each processing step at least three carcasses were sampled from each flock, 10g of pooled faecal samples were also analysed. Carcasses were immediately transported to the laboratory for further analysis. Carcasses were swabbed using 300ml sterile physiological sodium chloride solution.

B) Cultural methods

A dilution series of the rinsate was made in peptone water and spread onto specific agar plates: For counting *E. coli* -2/-4 dilutions were plated on Coli-ID agar plates (Bio Merieux 42017), aerobically incubated for 24h at 37°C and typical colonies (pink / violet) were counted. A random sample of typical colonies was biochemical verified as *E. coli* by using the Api-20E (Bio Merieux 20100). *Lactobacilli* were enumerated by plating on Rogosa-agar plates (Merck Art. Nr. 1.05413.0500 and 1.00062.1000) incubated anaerobically at 30°C for 5 days (Rogosa, et al., 1951). *Staphylococc*i were enumerated using dilutions on Kranep-agar (Oxoid Art.Nr. CM 441 and SR 47) after an aerobic incubation time of 48h at 37°C, typical

colonies were counted (Sinell and Baumgart, 1967). *Enterococci* were enumerated by plating dilutions on Chromocult® Enterococci agar (Merck Art. Nr. 1.13991.0100/0500) and aerobic incubation for 24h at 42°C and counting blue colonies (Manafi and Sommer, 1993). Enterobacteriaceae were determined by plating on VRBD (Violet-red bile dextrose agar acc. to Mossel et al., 1962) agar after incubation for 18h at 37°C and counting red colonies. Colony counts of bacteria were converted in $\log_{10}$ (CFU per millilitre of rinse) for statistical analysis.

Salmonella: a portion of the rinse (50 ml) was analysed according to ISO 6579-1998 followed by sero agglutination (Hoechst-Behring, Marburg, Germany, Polyvalent I; Anti-O6, 7, 8; Anti-O3, 10, 15; Anti-O4, 5; Anti-O9).

Thermophilic *Campylobacter* spp: a portion of the rinsate (50 ml) was analysed according to ISO 10272-2002.

C) Molecular-based method: SSCP

For analysing microbial communities molecular methods commonly rely on the analysis of 16S rRNA gene fragments. The variable region V4-V5 of the 16S RNA gene was used to analyse slaughter processing steps (after scalding and defeathering, after evisceration, after cooling). DNA-Isolation: 100 ml of the rinsate was centrifuged at 1700xg for 10min. The pellet was extracted using the QIAamp® Stool Mini Kit (Cat. No. 51504) according to manufactures advise. PCR: The 16S RNA gene variable region V4-5 was amplified and analysed according to the SSCP protocol described in Annex 2.

D) Sequencing analysis

Sequencing analyses were done using a newly developed protocol (SSCP protocol Annex 2).

E) Cluster and statistical analysis

Cluster analyses were done according to the SSCP protocol described in Annex 2. Statiscal analysis was completed by SPSS version 14.0 for windows. Differences between slaughter processing steps were analysed using Kolmogorov-Smirnov Test and Kruskal-Wallis test. Differences between slaughterhouses at processing steps and difference between rearing conditions were analysed using the Kolmogorov-Smirnov Test and Mann-Whitney-U-test.

5.1.2 - Results and discussion

A) Composition of microbial flora of carcasses at slaughter processing step: cultural method

Colony counts of *Enterococci, Staphylococci*, *Lactobacilli*, *E. coli*, and *Enterobactericeae* differed significantly between slaughter processing steps after scalding and all other processing steps (higher number were counted at the processing step after scalding). Colony counts of *Enterococci* were highest at the first processing step scalded and were continuously reduced for about 2 Logs during the whole slaughter process. Colony counts for *Staphylococci* were highest at the first processing step scalded and they were continuously reduced for about 3 Logs during the slaughter process. Colony counts of *Lactobacilli* were highest at the first processing step scalded and they were continuously reduced for about 2 logs during the slaughter process but slightly increased again during cooling. Colony counts of *E. coli* and *Enterobacteriaceae* were highest during the first three processing steps scalded, defeathering and eviscerated and they were mainly but rapidly reduced during cooling. Colony counts of *Pseudomonadaceae* were highest at the first processing step scalded stay at an about 1 Log reduced level for the following processing steps and

decreased another Log at cooling but increased again slightly during cooling. Only counts of *Pseudomonadaceae* did not differ significantly between processing steps after scalding and after defeathering. Between slaughter processing steps after defeathering and after evisceration colony counts of *Staphylococci* differed significantly (higher numbers were seen for *Staphylococci* at slaughter processing steps after defeathering (Table 35), but not for other determined colony counts. Enumerated colony counts differed significantly between slaughter processing steps defeathered and cooled as well as packaged and also between slaughter processing steps eviscerated and cooled as well as packaged. No difference of colony counts was seen between slaughter processing steps cooled and packaged using statistical analysis. To test the impact of different production systems, two organically reared flocks at all processing steps were examined and statistically analysed data from both rearing systems. No significant difference was observed between both rearing systems.

No correlation was found between high or low colony counts of faecal samples and high or low colony counts of eviscerated carcasses (p values for *Enterococci* p=0.29, *Staphylococci* p=0.26, *Lactobacilli* p=0.55, *E.coli* p=0.63 and *Enterobacteriaceae* p=0.57). Mean values of logarithmic colony counts were investigated after the slaughter processing step eviscerated and the logarithmic colony counts of faecal samples before slaughter. The reduction of bacterial counts between faecal and eviscerated samples could be too high to find a correlation between these steps, other correlations had to be prospected between two other more linked processing steps.

Table 35: Mean values of logarithmic colony counts at the slaughter processing steps

Log CFU	*Enterococci*	*Staphylo cocci*	*Lactobacilli*	*E. coli*	*Entero bacteriaceae*	*Pseudo monacea*
Faeces (/g)	6.84 ± 0.75	7.50 ± 0.62	7.43 ± 0.54	6.34 ± 0.39	6.67 ± 0.55	
Scalded (/ml)	5.95 ± 0.58	6.59 ± 0.70	6.16 ± 0.33	5.91 ± 0.76	5.86 ± 0.66	5.94 ± 0.52
Defeathred (/ml)	5.15 ± 0.48	5.37 ± 0.58	5.29 ± 0.39	5.16 ± 0.42	5.27 ± 0.37	5.71 ± 0.57
Eviscerated (/ml)	4.71 ± 0.28	4.84 ± 0.25	5.59 ± 0.52	5.56 ± 0.59	5.14 ± 0.51	5.57 ± 0.56
Cooled (/ml)	3.92 ± 0.36	4.2 ± 0.59	4.58 ± 0.28	3.83 ± 0.38	4.01 ± 0.38	6.64 ± 0.31
Packaged (/ml)	3.27 ± 1.27	3.76 ± 1.3	4.05 ± 1.56	3.52 ± 1.32	3.62 ± 1.33	4.2 ± 1.59

Mean values of colony counts from pooled faecal samples and carcasses at the time of slaughter ± Standard Deviation (SD) between flocks

Results revealed that slaughter technology has an influence on the overall carcass microbiota (Table 35). Highest colony counts were seen at the first slaughter processing step (after scalding). After this processing step most examined microorganisms decrease during the following processes. Hence a reduction at this first step would be most effective to reduce the overall carcass microorganisms. Different articles suggest spry scalding as a possible method to reduce the overall carcass flora (Dickens *et al.*, 1999). Nevertheless, this method is not accepted by the industry because change of skin colour, less efficient defeathering, skin lesions and higher costs for water are accompanying this slaughter technology. However, research to find new technologies regarding the scalding process of poultry seems to be a demand to reduce the overall microflora as well as pathogens. After eviscerating we expected an overall increase of intestinal bacteria e.g. *E. coli* and *Enterobacteriaceae*. Results show no overall rise of colony counts of these commensal bacteria but some flocks and some samples clearly show an expansion which might be due to mechanical lesions of intestines caused by the evisceration process.

B) Composition of microbial flora of carcasses at slaughter processing step: molecular method

SSCP analysis:

A typical pattern for each slaughter processing step was found by cluster analysis (Figure 12). Next to the typical cluster formed by each processing step every flock revealed a cluster but less strong so that clustering of slaughter processing steps could be defined in each sample despite the flock differences. As the faecal and intestinal flora has an influence on the carcass flora (Huis in 't Veld *et al.*, 1994) and the flora of organically and conventionally kept broilers has been shown to differ. Carcasses were analysed from flocks fattened under different rearing conditions. One flock was kept under conventional conditions and another flock under organic conditions (data not shown). The influence of rearing conditions did not inhibit the clusters of slaughter processing steps.

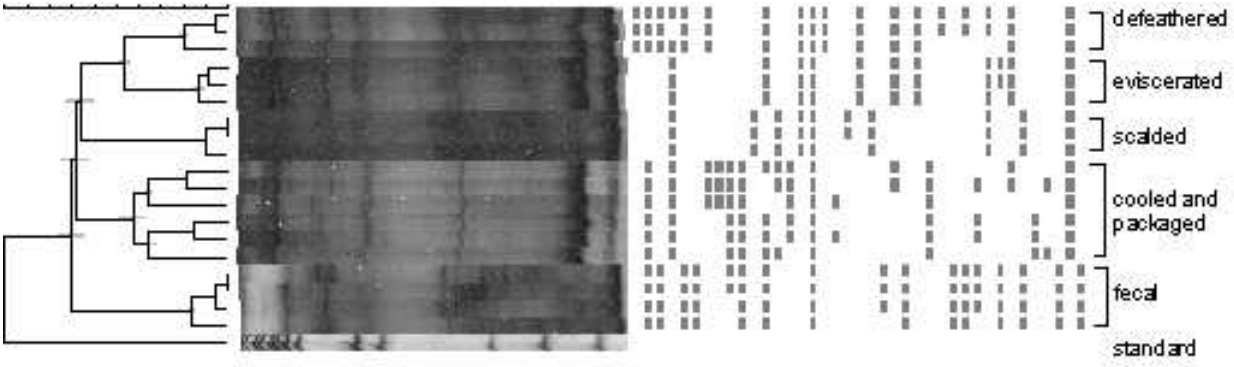


Figure 12: Different slaughter processing steps as are scalded, defeathered, eviscerated, cooled and packaged.

***SSCP of carcass rinsing water drawn at each processing step. All samples are from the same flock.**

Clusters of these processing steps occurred and were stronger than clusters by rearing conditions. Slaughter processing steps could be distinguished using two different slaughter technologies. Nevertheless, slaughter technology had an influence on patterns detected and on the overall colony counts.

Sequencing analysis:

By sequencing analysis using a new method to analyse bands directly from SSCP gels we wanted to define specific indicator bacteria for each processing step. The results showed that there are certain bands that can be detected from faecal samples as well as from all processing steps of a flock. To determine if these bands correspond to a single microorganism we sequenced gel cut outs, but were not able to determine a certain species even though sequencing data confirmed a single PCR product, no alignment with known sequences could be detected. However, sequencing analysis produced sequencing data that could not be verified as belonging to a specific bacterial species but produced new sequences or sequences similar to unidentified bacterial species when compared with the sequencing data bank. In general SSCP might be a tool for advanced hygienic controls and to study microbiology in the development of new slaughter technologies. Nevertheless, profound data for indicator bacteria for certain slaughter processing steps have to be based on sequence analysis of bacterial species that were identified and thus, cultured by traditional culture based techniques.

To compare the new molecular methods with cultural based techniques, samples were analysed at different processing steps for *Escherichia coli*, *Lactobacilli*, *Staphylococci*, *Enterococci*, and *Pseudomonadaceae*. Using statistical analysis of colony counts most slaughter processing steps could be distinguished despite different slaughter technology. Exceptional none of the upper mentioned colony counts differed between slaughter steps cooled and packaged and only *Staphylococci* colony counts differed significantly between slaughter steps defeathering and evisceration. Thus, both methods, SSCP and traditional culture methods showed consistent results on equally slaughter processing steps and packaged products.

C) *Salmonella* and *Campylobacter* contamination in slaughterhouses in regards to the flocks status (Austria)

To see if slaughter of a *Salmonella* or *Campylobacter* naturally infected flock takes an influence on the numbers of contaminated carcasses from flocks, the number of carcasses on which the pathogen was detected were statistically analysed. Taking into account the number of faecal samples in which the pathogen was found and the number of carcasses drawn in total.

Salmonella spp. were found in 5 of the 30 flocks from organic production (16.7% [5.7-34.7]) (Table 36). The serovars were defined as Montevideo, Agona, Indiana and Senftenberg, whereas, most often the Serovar Montevideo was detected followed by Agona. Tables 37 shows the numbers of carcasses contaminated with *Salmonella* and the flock status in regards to *Salmonella*.

Table 36: *Campylobacter* and *Salmonella* occurrences in flocks at the slaughterhouse level in Austria

Country	Type of production	Sample	N. tested flocks	N. positive flocks	*Campylobacter* spp (%) [CI (95%)]	*Salmonella* spp (%) [CI (95%)]
Austria	Organic	Caeca	30	6	20 [7.7-38.6]	
	Organic	Caeca	30	5		16.7% [5.7-34.7]

Table 37: *Salmonella* occurrence in samples and the flock status at the slaughterhouse level in Austria

Country	Flock status	Sample	N. tested samples	N. positive samples	*Salmonella* spp (%) [CI (95%)]
Austria	Positive	**Carcasses** (after air cooling)	108	18	16.7 [10.2-25.1]
	Negative	**Carcasses** (after air cooling)	260	26	10.0 [6.6-14.3]

Table 38: *Campylobacter* occurrence in samples and the flock status at the slaughterhouse level in Austria

Country	Flock status	Sample	N. tested samples	N. positive samples	*Campylobacter* spp (%) [CI (95%)]
Austria	Positive	**Carcasses** (after air cooling)	205	195	95.1 [91.2-97.6]
	Negative	**Carcasses** (after air cooling)	124	27	21.8 [14.9-30.1]

There was no significant difference between flock status and the number of carcasses contaminated with *Salmonella* (p=0.089). It might be due to low colony counts of *Salmonella*

detectable in the intestinal tract, as *Salmonella* of the isolated serovars seem only to colonize the intestinal tract of broilers in rather low numbers and hence, a contamination of carcasses without prior replication seems low. Nevertheless, the contamination of slaughter material on which a replication is possible is a hazard for contaminating poultry meat with high numbers of *Salmonella* cell counts.

Tables 38 shows the numbers of carcasses contaminated with *Campylobacter* and the flock status in regards to *Campylobacter*. *Campylobacter* spp. where found in 6 of the 30 flocks (20% [7.7-38.6]) (Table 36). There is a significant difference in the number of carcasses contaminated with thermophilic *Campylobacter* spp. in dependence on the flock status (p=0.03). *Campylobacter* spp. cell counts found in the intestine of broilers are rather high (as high as 10^6CFU/g).

5.2 - *Campylobacter* carcasses contamination at the different slaughter processing steps (Germany and Italy)

In the course of this study conventional broiler flocks were sampled throughout the slaughtering process over a period of 18 months. In Italy, this allowed to assess seasonal variation of *Campylobacter* contamination of carcasses. The samples were analysed qualitatively after an enrichment step and quantitatively by direct plating of aliquots of carcass and meat rinse dilutions spread on agar surfaces. Finally the results were compiled and statistically analysed to show the impact of slaughter operations and season and the influence of the *Campylobacter* status in slaughtered broiler flocks. For answering epidemiological questions regarding the distribution of *Campylobacter* at the slaughterhouse, selected isolates were typed by Pulsed Field Gel Electrophoresis (PFGE) (Germany) and Ribotyping (Italy). These data were put into the database (see section 6)

5.2.1 - Material and methods

A) Experimental trial and sampling

In Germany, the samples were collected at a modern broiler slaughterhouse in northern Germany in the period from April 2005 to September 2006. Eighteen flocks in total were sampled on a monthly basis. The sampling was performed in the course of slaughtering and processing. The samples collected on each slaughtering day were taken after about one third of the daily slaughtering schedule has gone through the slaughterhouse. This allowed analysing the amount of occurring cross contamination. The size of the sampled broiler flocks was between 18,500 and 38,000 birds. The flock size was taken into account in the sampling plan by evenly distributing the sampling over the flocks´ whole slaughtering process. The slaughter capacity at the slaughterhouse was between 10,000 and 12,000 birds per hour. The sampled flocks were all reared conventionally, which is the representative production system for broilers in the German market. For the evaluation of the flock status 10 caecal samples were collected from each flock. This has been shown to be an appropriate sampling size. Statistically, a flock could be identified as colonised with 10 caecal samples, when the inner flock prevalence of *Campylobacter* is about 30%, with a level a of confidence at 95%. The initial contamination level at the slaughterhouse was acquired by the sampling of scalding water. Furthermore five carcasses were collected at each of the following processing steps: after scalding/plucking (one enclosed operation), after evisceration and after air chilling. After the chilling the fresh carcasses were portioned directly at the processing facility. Cut poultry parts were collected before packaging: 5 breastcaps (breast pieces with bone and skin) and 5 pairs of breast fillets.

In Italy, *Campylobacter* was enumerated on 213 broiler carcasses from 22 broiler flocks. All carcasses were collected at the end of slaughtering after the air cooling operation. During each sampling, 8 to 10 carcasses with a mean weight ranging between 2.1 and 2.7 kg (i.e.

average age of 56 days), processed as first group of the day, and 9 to 10 carcasses with a mean weight ranging between 1.2 to 1.9 kg (i.e. average age of 46 days), processed as second to fifth group of the day, were investigated. This allowed analysing the amount of occurring cross contamination. The relative contribution of different slaughtering steps to the *Campylobacter* count on carcasses was calculated testing 126 carcasses from 7 different flocks. In particular, the relative contribution of evisceration, carcass washing and air cooling was evaluated in 5 flocks, whereas the contribution of an additional carcass washing with chlorine dioxide after defeathering was evaluated in 2 flocks. During each sampling, 3 carcasses were analysed before and after each target step (i.e. evisceration, washing, air cooling, washing with chlorine dioxide). All tested flocks were reared within conventional farms.

B) Cultivation and detection

In Germany and Italy, the samples were analysed qualitatively and quantitatively for thermophilic *Campylobacter* according to ISO 10272-2002. After the incubation plates were observed for colonies presumptive for *Campylobacter*. The mean *Campylobacter* count per carcass was calculated according to the number of colonies on plate and dilution factor. 1g of caecal content was prepared 1:10 in Preston-broth (Oxoid) for enrichment and in 0.85% NaCl / 0.1% peptone-water for the initial dilution for quantification. Carcasses and portioned poultry meat were rinsed by hand for 90 seconds in 500ml of 0.85% NaCl / 0.1% peptone-water and 1ml of each rinse was inoculated in 9ml of Preston broth for enrichment. From the initial carcass rinse and the samples of scalding water tenfold dilutions were prepared and direct plating was performed by spreading 0.1ml of each dilution to duplicates of mCCDA (Oxoid) and Karmali (Oxoid) agar plates. Enrichment cultures were held at 42℃ for 48-72h under microaerobic conditions. After that, the enrichment culture was inoculated onto mCCDA and Karmali agar plates.

In Italy, each carcass was aseptically placed in a sterile plastic bag and rinsed with 300 or 200 ml of sterile water according to its weight. Bags were shaken by hand for one minute and a half, then the carcass was removed and the rinse water was aseptically transferred to a smaller sterile bag and stored refrigerated at 4°C to be transferred to the laboratory within three hours. In the laboratory the rinse waters were serially diluted and spread plated onto CCDA plates incubated as previously described. Suspect colonies were presumptively identified through phase-contrast microscopical observation for typical morphological aspects and corkscrew movement and then enumerated.

C) Identification and typing

In Germany, the confirmation of presumptive colonies was performed by Gram-staining, phase contrast microscopy, oxidase- and catalase reactions. Isolates were biochemically differentiated to species level by API Campy (Bio Mérieux). The diversity of the isolates and their relationship was analysed by PFGE according to the “CAMPYNET“ Prototype standard protocol (http://campynet.vetinst.dk/PFGE.html).

In Italy, the *Campylobacter* spp. presumptive isolates were identified by using a multiplex PCR protocol (Manfreda *et al.,* 2003) able to identify *Campylobacter* spp. as genus, *Campylobacter jejuni* and *Campylobacter coli* during the same reaction*. C. jejuni* ATCC 33560 and *C. coli* NCTC 11350 were used as positive controls. *Staphylococcus aureus* ATCC 51749 was used as negative control.

5.2.2 - Results and discussion

A) *Campylobacter* spp. detected on carcasses in regards to the flock status

In Italy, all 213 carcasses tested at the end of processing after the air cooling operation were *Campylobacter* positive. The *Campylobacter* mean count per broiler carcass resulted in 5.2 ± 0.80 Log_{10} CFU. Any evidence supported the assumption that processing plant cross-contamination increases *Campylobacter* mean loads on carcasses through the processing day. In fact, the levels per carcass among the first flocks sampled each day were, on average, higher than the counts observed later in the processing day. The *Campylobacter* mean values per carcass in the 22 broiler chicken flocks were examined are shown, they ranged from 3.9 to 6.1 Log_{10} CFU per carcass. Some flocks were statistically significant less contaminated than other flocks.

In Germany, *Campylobacter* spp. where found in 13 of the 18 flocks (72.2 [46.5-90.3]) (Table 39). The *Campylobacter* mean load in caeca from positive flocks is 6.2 ± 2.2 log_{10} CFU/g, the *Campylobacter* mean load from carcasses after chilling from positive flocks is 4.8 ± 1.3 log_{10} CFU/g. The numbers of *Campylobacter* in positive flocks is correlated with the initial *Campylobacter* in the caecal contents. This lead to high counts of *Campylobacter* on carcasses and breastcaps when the initial flock colonisation levels were high and a lower contamination when the levels of *Campylobacter* in the caecal content of the appropriate flock were low. In flocks without evidence of *Campylobacter* the amount of positive samples seemed to be correlated to the status of the preceding flock, although, this could not be statistically underlined because of the low number of negative flocks sampled.

Table 39: *Campylobacter* occurrence in flocks at the slaughterhouse level in Germany and Italy

Country	Type of production	Sample	N. tested flocks	N. positive flocks	*Campylobacter* spp (%) [CI (95%)]
Germany	Conventional	Caeca	18	13	72.2 [46.5-90.3]
Italy	Conventional	Caeca	22	22	100 [87.3-100]

Table 40 *Campylobacter* occurrence on carcasses and the flock status, after processing steps at the slaughterhouse level in Germany and Italy

Country	Flock status	Sample	N. tested samples	N. positive samples	*Campylobacter* spp (%) [CI (95%)]
Germany	Positive	**Carcasses** (after chilling)	65	62	95.4 [87.1-99]
	Negative	**Carcasses** (after chilling)	25	11	44 [24.4-65.1]
Italy	Positive	**Carcasses** (after air cooling)	213	213	100 [98.6-100]

B) *Campylobacter* spp. detected at different processing steps during slaughtering (Italy and Germany)

In Germany, the occurrence at the individual sampling sites and the number of *Campylobacter* in carcasses and portioned broiler meat were influenced by the flocks´ status. The results of the quantification for *Campylobacter* positive flocks are shown in table 39. The occurrence of the samples at the individual slaughtering operations was above 90%, or just below 90% in the case of breast fillets. The numbers of *Campylobacter* decreased in the course of the broiler processing from carcasses after scalding/plucking to carcasses after chilling. This reduction was 0.7 Log_{10} / cycles, it was statistically significant (p=0.0083).

In Italy, in the first 5 flocks tested evisceration and air cooling were able to produce a significant reduction of *Campylobacter* counts on carcasses (Table 41). However, these reductions may change according to the initial load of *Campylobacter* on carcasses before slaughtering as well as according to the physiological properties of the contaminating strains. Overall the reduction achieved after the air cooling tunnel was around 2.7 Log_{10} CFU/carcass. However, considering each single flock tested, this reduction ranged between 1.5 and 3.5 log_{10} CFU/carcass. These values were calculated by the difference between carcass loads before evisceration (i.e. after defeathering) and carcass loads after the air cooling, mathematically corrected in order to minimise the effect of differences between initial carcass loads before evisceration. In the 2 flocks where the carcass washing with chlorine dioxide after defeathering was performed, the *Campylobacter* contamination on processed carcasses decreased from about 4 to 1.6 Log_{10} CFU/carcass. Therefore, this step may reduce the *Campylobacter* mean load on processed carcasses to acceptable level for human exposure.

Table 41: *Campylobacter* mean counts on carcasses during slaughtering in Italy and Germany

Italy		**Germany**	
Slaughtering step	**Log_{10} CFU/carcass**	**Slaughtering step**	**Log_{10} ± SD CFU/carcass**
Caeca	ND	**Caeca***	6.2 ± 2.2 (N=130)
Before Evisceration	6.9 (N=5)	**Scalding water**	3.3 ± 1.1 (N=48)
After evisceration	5.9 (N=5)	**After scalding/plucking***	5.5 ± 1.9 (N=65)
Before washing	5.6 (N=5)	**After evisceration***	5.1 ± 1.8 (N=65)
After washing	5.2 (N=5)	**After chilling***	4.8 ± 1.3 (N=65)
Before air cooling	5.2 (N=5)	**Breastcaps***	4.1 ± 1.4 (N=65)
After air cooling	5.1 (N=5)	**Breast fillets***	3.0 ± 1.6 (N=65)

*** from positive flocks**

C) Seasonal effect (Italy)

Three farms were tested along a one year period to investigate the influence of the season on the *Campylobacter* load on carcasses. In these flocks the *Campylobacter* mean values per carcass detected in autumn and summer were significantly higher than those detected in winter and spring (Table 42). According to other authors, seasonal variation in *Campylobacter* contamination levels demonstrates the importance of conducting long-term surveys on chicken flocks, rather than short-term surveys, which may give erroneous results, dependent on the particular single season sampled (Meldrum *et al*., 2004).

Table 42: Influence of season on the *Campylobacter* mean load per carcass (Italy)

Sampling season	**Log_{10} CFU/carcass**
Autumn	5.6^{b}
Summer	5.3^{b}
Winter	5.0^{a}
Spring	4.7^{a}

One to five *Campylobacter* colonies were isolated from the rinse waters of carcasses belonging to 16 out of 22 flocks examined for identification by using the PCR protocol previously described. Overall, 678 isolates were tested and 50% were classified as *C. jejuni*, 47% as *C. coli* and 3% as mix cultures of both species.

5.3 - *Listeria monocytogenes* contamination at the slaughterhouse level (France)

The aim of this part is to check the status of animals regarding *L. monocytogenes* and to trace the contamination between the farms and the slaughterhouse. The results obtained at the farm level were presented in section 3.2.4.

5.3.1 - Material and methods

A) Experimental trial and sampling

Three volunteer slaughterhouses in France were visited and sampled.

The day of sampling, samples were collected from each batch slaughtered:

- From the environment of the slaughterhouses and the cutting room using bootswabs and swabs, before and after cleaning and disinfecting procedures (CDP), and after each batch.

- From each batch: 5 pools of 5 caeca, 5 pools of 5 neck skins 5 pools of 5 fillets.

B) Identification and typing

See section 3.2.4

5.3.2 - Results and discussion

Three slaughterhouses were included in this study. In the first one, the samples from the farms were all negative to *Listeria monocytogenes*. At the slaughterhouses, 20 samples were positive out of 78 (25.6% [16.4-36.8]): six samples from the environment before CDP procedures (7.7% [2.9-16]) and 12 positive during the activity (15.4% [8.2-25.3]) (Table 43). Two products (neck skins) were positive out of 10. The genotyping of the isolates showed that 9 positive samples had the pulsotype AC105; these samples were taken after CDP procedures, from environmental samples during the activity and from the products. The pulsotypes AC107, 108, and 110 were also found in common between samples taken after CDP procedures and during the activity. This result clearly indicated the residual contamination as *L. monocytogenes* was found after CDP and the cross contamination spread over the slaughterhouse during the activity in the environment and on the products. In the second, three flocks at the farm level were positive before the slaughtering. Seven samples out of 195 (3.6% [1.5-7.3]) (Table 43) were positive within the slaughterhouse; all these samples were from the environment during the activity. At the farm level, one pulsotype AC76 was found and was different from those at the slaughterhouse. The isolates identified within the slaughterhouse were not similar.

In the third, one flock at the farm level was positive before the slaughtering. At the slaughterhouse, seven environmental samples were positive out of 171 (4.1% [1.7-8.3]). The serovar 1/2c was identified in the positive sample from the environment (sawbs and bootswabs) of the slaughterhouse and the cutting room. Here again, the pulsotype found at the farm level was different from those found at the slaughterhouse.

Table 43: *L. monocytogenes* occurrence in environmental samples from slaughterhouses after CDP in France

Country	Flocks status	N. tested/ samples	N. positive samples	*L. monocytogenes* (%) [CI (95%)]
France	Negative	78	20	15.4% [8.2-25.3]
	Positive	195	7	3.6% [1.5-7.3]
	Positive	7	171	4.1% [1.7-8.3]

In this investigation, any relationship was found between the strains isolated from the flocks and those from the slaughterhouses. In the first slaughterhouse, inefficient CDP procedures and cross contamination could be the main sources of contamination.

5.4 - Conclusions

According to results, it is even more important to use logistic slaughter in a *Campylobacter* infected flock than for a *Salmonella* harbouring flock, where logistic slaughter is already a common tool to avoid carcass contamination. Evisceration is the most critical step for contaminating the broiler carcass with intestinal content and thus, is also responsible for contaminating the carcass with intestinal pathogens. Regarding the other bacteria, colony counts for *Enterococci*, *Staphylococci*, *Lactobacilli, E. coli*, and *Enterobactericeae* differed significantly between slaughter processing steps after scalding and all other processing steps. *Pseudomonadaceae* did not differ significantly between processing steps after scalding and after defeathering.

The occurrence of *Campylobacter* in broiler flocks is high in most European countries beginning at the farm level, where the initial colonisation of broilers with *Campylobacter* occurs. Besides the status of the broiler livestock in the country, the occurrence and numbers within the colonised flocks is important for slaughtering and processing. The correlation between flocks and the initial *Campylobacter* in the caecal contents depends on the mean load of bacteria. The higher the occurrence and numbers of *Campylobacter* within a flock, the higher is the possibility of significant surface contamination by shedding of faeces and subsequent surface contamination of carcasses at different steps of the broiler processing.

The *Campylobacter* contamination monitored in Italian slaughterhouses depends on the seasons, the contamination in autumn and summer were significantly higher than those detected in winter and spring.

Regarding *L. monocytogenes*, no relation was found between the studied farms and the slaughterhouses in France when comparing genetically the isolates of isolates.

Intensive rearing makes up the most part of broiler processed for the markets in Europe. At the moment, the status of the flocks delivered to the slaughterhouse is dependent on farm hygiene management. The slaughterhouse has some influence on rearing conditions in the course of vertically integrated broiler production.

5.5 - Synergism and antagonism of certain spoilage bacteria and pathogens on poultry meat (Austria)

Commensal bacteria, spoilers and pathogens readily contaminate meat products during meat production and processing. Next to the intestinal flora, broiler feathers, skin, air, scalding water, and the overall slaughterhouse flora lead to an average of $10^{4-6}/cm^2$ bacteria on the carcass using traditional microbial methods (Voidarou *et al*., 2007; Arnold, 1998; Berndtson *et al.*, 1996). Thus, it is of upper importance to understand not only the environmental viability of a single species under certain conditions but to evaluate the interaction (commensalism, parasitism, cooexistance) of different bacterial species or even different microorganisms to consider new strategies for food safety. Thus, most foodborne pathogens have been studied as single bacterial species to survive in vitro and in different food related environments. Especially for *Campylobacter* spp. it seems to be essential to investigate other ways of survival. Hence, commensal bacteria and spoilers were analysed most often found on meat and in slaughterhouse surroundings to permit/and or support the survival and growth of *Campylobacter* spp. under different laboratory conditions.

For initial experiments 10 different *Campylobacter* poultry meat isolates and two type strains (DSM 4688 and 4689) were tested under different conditions (aerobe, anaerobe, low temperature, high temperature, different pH, low and high water activity) in combination with strains of *Citrobacter* spp., *Enterococcus* spp., *Pseudomonas* spp. *Proteus* spp. *Staphylococcus* spp. and *Lactobacillus* spp. type strains and wild type isolates from poultry meat and meat products for growth and/or survival. Most prominent was the experimental conditions in which certain strains of *Pseudomonas* supported different *Campylobacter* strains and isolates. Thus, research was focused on aerobic growth of *Campylobacter* in commensalism/parasitism with *Pseudomonas*.

5.5.1 - Material and methods

A) Bacterial stains

In total 143 thermophilic *Campylobacter* spp. isolates were tested. These included 47 isolates from poultry meat slaughtered or purchased in Austria, 54 isolates of faecal samples from chickens and 42 human isolates from Austria (isolated in the year 1998). Two *Campylobacter* type strains (*Campylobacter jejuni* and *Campylobacter coli*) were included as controls (DSM 4688 and DSM 4689). Two *Pseudomonas* strains one type strain DSM 50198 (*P. putida*) and one meat isolate were included.

B) Cultivation and detection

Campylobacter strains were cultured on CCDA agar plates under microaerophilic conditions (10% CO_2 and 5%O_2) at 42℃ for 48h. A number of colonies were inoculated in Mueller-Hinton broth and optical density was determined and diluted to an OD_{600} of 0.3. These strain suspensions were diluted 1:100 for inoculation in microtiter plates. *Pseudomonas* strains were grown on LB-agar. A number of colonies were inoculated in Mueller-Hinton broth and diluted to an OD_{600} of 0.4. These strain suspensions were diluted 1:100 for growth experiments in Mueller-Hinton broth in microtiter plates. Growth experiments were performed in microtiter plates in a total volume of a maximum of 150 µl. After inoculation the plate was incubated at 35℃ for 32h under aerobic conditions. As blanks *Pseudomonas* and *Campylobacter* strains were inoculated separately in a double sample within each experiment. After the incubation time each well was streaked in parallel onto CCDA agar and glutamate starch phenol-red (GSP)-Agar according to Kielwein. mCCDA agar plates were incubated at 42℃ for 48h under microaerophilic conditions and GSP-Agar plates at 25℃ for 24h aerobically. After numerous growth controls of *Pseudomonas* on GSP-Agar plates,

growth control of *Pseudomonas* was only performed by OD_{600} measurements of microtiter plates. Each strain combination (*Pseudomonas*/*Campylobacter*) was analysed in duplicate per experiment. Each experiment was performed at least three times.

Level of growth on mCCDA was determined by colony counts as is aerobic growth, high reduction (only a small number of colonies, less then 10) and no aerobic survival (no colonies).

C) Statistical analysis

Statistical analysis was completed by SPSS® version14.0 for Windows®. Differences were analysed using the Chi-Square-Test and Fishers exact test.

5.5.2 - Results and discussion

Analysis of different thermophilic *Campylobacter* spp. wild type isolates showed that the ability of growth in commensalism/parasitism with *Pseudomonas* strains varied. Some isolates were able to survive more than 72 hours others were not able to survive less than eight hours. Thus, the first aim of the study was to set up an experimental condition in which two *Campylobacter* type strains (DSM 4688 *C. jejuni* and DSM 4689 *C. coli*) served as positive and negative controls, respectively. To see if the observed results were dependent on the *Pseudomonas* strain that supported growth a type strain (DSM 50198 *Pseudomonas putida*) and a wild type poultry meat isolate was used in the experiments. Optimal time and temperature conditions were designed experimentally (32h 35°C).

To see if thermophilic *C. jejuni* of different origin vary according the ability to grow under the described conditions aerobically we analysed *C. jejuni* isolates previously isolated from broiler faeces, from poultry meat and of human origin. Table 44 shows the results in total numbers and percentages. Statistical analysis was performed on numbers. Faecal isolates from broilers differed significantly from poultry meat isolates and human isolates but no significant difference was detected between poultry meat isolates and human isolates in regards to their ability to grow aerobically in commensalism with *Pseudomonas*. Results for statistical analysis were similar for both *Pseudomonas* strains used.

Table 44: Parasitism/commensalism with a *Pseudomonas putida* type strain and a wild type isolate

	Aerobic growth^{+++}		**no aerobic survival^{+}**		**high reduction^{++}**	
Total	DSM50198	FP56bII	DSM50198	FP56bII	DSM50198	FP56bII
Broiler faeces (N= 54)	39% (N= 21)	52% (N= 28)	39% (N= 21)	22% (N= 12)	22% (N=12)	26% (N=14)
Poultry meat (N=47)	72% (N= 34)	92% (N= 43)	4% (N= 2)	2% (N= 1)	24% (N= 11)	6% (N= 3)
Human faeces (N= 40)	88% (N= 35)	100% (N= 40)	-	-	12% (N= 5)	-

***growth conditions: aerobic growth at 35°for 32h**
*******Campylobacter* **spp. without *Pseudomonas* could not survive under these conditions. After incubation of one streak on CCDA agar plates $^{+++}$numerous colonies of *Campylobacter*. $^{++}$a maximum of 10 colonies. $^{+}$no colonies.**

Even though, thermophilic *Campylobacter* species are in most European countries the leading cause of foodborne disease in humans they are most sensitive to environmental stress, e.g. oxygen pressure, water activity, pH and high and low temperature. But nevertheless, most studies regard poultry meat as the main source of campylobacteriosis in humans mainly caused by *C. jejuni* (Harris *et al*., 1986). *Campylobacter* must survive in different environments to cause foodborne infections in humans e.g. the chicken intestinal tract, the slaughterhouse environment, the acidic environment of the human stomach but also specific and unspecific immune response in the human intestine. Research has focused on *Campylobacter* resistance due to the cell phase e.g. “viable but not culturable”. Another possibility to withstand environmental stress is to parasite within or close to other resistant microbes e.g. in biofilms. Recent research has shown that *Campylobacter* cells are able to form biofilms (McLennan *et al*., 2008; Guerry, 2007) and hence must have communication systems such as “quorum sensing”. The intestine, meat and carcass surfaces are environments that harbor a number of bacterial species and most of them are in close contact. Nevertheless, not much work was done to elaborate the interaction of foodborne pathogens e.g. thermophilic *Campylobacter* spp. with commensal bacteria or spoilers found on food. On poultry meat different spoilers can be identified in dependence on storage temperature and packaging. In this study different spoilers were tested to support growth of *Campylobacter* under aerobic conditions. One of the most dominant genera on meat surfaces under cooling conditions is *Pseudomonadaceae*. Certain *Campylobacter* isolates can survive under aerobic conditions cultured with *Pseudomonas* strains for 72 hours and even longer. *Pseudomonadaceae* express high levels of oxidase and thus, may reduce oxygen tension close to their micro-environment. Human *Campylobacter* isolates were tested, broiler faecal isolates and isolates from poultry meat for the ability to survive under aerobic conditions in parasitism / commensalism with *Pseudomonas*. Results show that most isolates from meat as well as human isolates are able to replicate under the upper mentioned conditions but only 60% of the faecal isolates from broilers. Genetic studies have to identify genes, gene expression and quorum sensing abilities necessary for this phenomenon and to force the importance for survival in different environments.

5.6 - Efficacy of common cleaning and disinfecting procedures to reduce bacterial contamination on food contact surfaces

The presence of pathogenic bacteria such as *L. monocytogenes*, *Campylobacter, C. perfringens* and *Salmonella* on live poultry can lead to the introduction of these pathogens into the processing plant where the contamination of equipments can result in the contamination of the final products. However, few data are available on the relative contribution of food contact surfaces in poultry products contamination. Adhesion of microorganisms to food processing equipment surfaces and the problems it causes are a matter of concern to the food industry. Biofilms have the potential to act as a chronic source of microbial contamination which may compromise food safety and represent a significant health hazard.

The main aims of this study were:

- to investigate the efficacy of the most common CDPs applied in the Italian slaughterhouses to reduce or eliminate *L. monocytogenes*, *Campylobacter*, *C. perfringens* and *Salmonella* on food contact surfaces

- to screen the biofilm forming ability of 28 *L. monocytogenes* strains isolated on the surfaces under study and to asses the *L. monocytogenes* adhesion to the surfaces commonly used in food plants: stainless steel and Teflon.

5.6.1 - Efficacy of cleaning and disinfecting procedures used in Italian slaughterhouse against *Salmonella, Campylobacter, Clostridium perfringens and Listeria monocytogenes*

A) Material and methods

The samples were collected in winter (from 14/11/2006 to 20/01/2007) and summer (from 12/06/2007 to 21/07/2007).

A total of 32 sample surfaces were collected before (N=16) and after (N=16) the application of CDPs. Samples were collected before and after cleaning with Foam A and Disinfectant 1, before and after cleaning with Foam A, Disinfectant 1 and sodium hypo-chloride; before and after cleaning with Foam B and Disinfectant 2 (quaternary ammonium compounds); before and after cleaning with Foam B, Disinfectant 2 and sodium hypo-chloride.

The environmental sites directly in touch with poultry products were analysed along with manhole covers because the target bacteria might colonise this site characterised by high relative humidity. Overall, the surfaces investigated were Teflon strips for transportation of broiler carcasses (NTF); Teflon strips for transportation of poultry breasts (NTP); Stainless steel cut modules for poultry breast (MTP); Stainless steel cut modules for poultry wings (MTA); Manhole covers (TSA). A total of 10 cm^2 of each sample surface were sampled using a pre-moistened sponge then placed in a sterile stomacher bag refrigerated at 4℃ during the transportation to the laboratory. Then, 10 ml of physiological water were added to each sponge, homogenised in the stomacher at normal speed for 1 min. In the cell suspension, *Campylobacter*; *Salmonella C. perfringens* and *L. monocytogenes* were both tested using the enrichment procedure as well as direct plating, performed according to the ISO regulations: 10272-2002, 6579-1998 for *Campylobacter* and *Salmonella* respectively. C. was isolated perfringens recommended by the FDA (Bacteriological Analytical Manual, 7th Ed) and *L. monocytogenes* according to microtiter plate assay standardized by Djordjevich *et al*., 2002.

B) Results and discussion

The procedures used in the Italian slaughterhouses tested were quite similar and based on a weakly rotation of foam and a disinfectant and the application of a second foam and sodium hypochloride once a week. According to the results achieved on the different surfaces tested before and after the application of the most common CDPs.

Table 45: Occurrence of pathogens in environmental samples at the slaughterhouse level before and after CDPs

	Before CDPs			After CDPs		
Strain	**N. tested samples**	**N. positive samples**	**% [CI (95%)]**	**N. tested samples**	**N. positive samples**	**% [CI (95%)]**
L. monocyogenes	80	8	10% [4.4-18.8]	80	0	0% [0-3.7]
Campylobacter	80	39	48.8% [37.4-60.2]	80	0	0% [0-3.7]
C. perfringens	80	57	71.3% [60-80.8]	80	17	21.3% [12.9-31.8]
Salmonella	80	0	0% [0-3.7]	80	0% [0-3.7]	0% [0-3.7]

L. monocytogenes was isolated on 8 out of 80 (i.e.10%) (Table 45) surfaces tested before the application of the CDP, but it was never detected on clean environments. All isolates but one were obtained on stainless steel cut modules. For all positive samples both enrichment as well as direct plating turned out as positive.
Campylobacter was detected on 39 out of 80 (i.e. 48.8%) dirty environmental surfaces but never after the application of the CDP. The majority of positive samples (i.e. 54%) were represented by stainless steel cut modules, 36% by Teflon strips and 10% by manhole covers. For all positive samples both enrichment as well as direct plating turned out as positive.
C. perfringens was detected on 57 out of 80 (i.e. 71.3%) dirty surfaces, mainly represented by Stainless Steel cut modules and Teflon strips (i.e. 35 and 40%, respectively) but also by manhole covers (25%). *C. perfringens* was the only target bacteria isolated also on 17 sample surfaces after CDPs. The colonised clean sites were mainly represented by manhole covers (i.e. 52%) but also by stainless steel cut modules (24%) and Teflon strips (24%).
Salmonella was never isolated before as well as after the application of CDPs by using both enrichment as well as direct plating.
L. monocytogenes, *Campylobacter* and *C. perfringens* mean loads on dirty surfaces were 1.8, 2.6 and 1.2 $Log_{10}CFU/10cm^2$, respectively. *C. perfringens* was the only pathogen isolated on clean surfaces, with a mean load of 0.9 Log_{10} CFU on 10 cm^2.

5.6.2 - Biofilm forming ability of *Listeria monocytogenes* on different sample surfaces

L. monocytogenes frequently colonise processing plants due to its high ability to form biofilm and persists in the environment even after the application of CDPs. In this study, the biofilm forming ability of *L. monocytogenes* on different surfaces was studied.

A) Material and methods

The biofilm forming ability of 28 strains of *L. monocytogenes* isolated on stainless steel cut modules (N=12) and Teflon strips (N=16) was assessed by the microtiter plate assay standardized by Djordjevich *et al.*, 2002. Furthermore, direct observation of adhesion of *L. monocytogenes* on stainless steel cut modules and Teflon strips was performed by Scanning Electron Microscopy (SEM).

B) Results and discussion

The *L. monocytogenes* isolates further investigated showed different biofilm forming abilities. In particular, growth performances of planktonic cells in BHI after 20 and 40 hours did not show significant differences, except for the three strains isolated from NTP having an optical density 50% higher than the strains isolated from all the other surfaces. On the other hand all the strains considered presented a biofilm forming ability ranging between low and medium and this ability seemed to be influenced by the isolation sites. Whatever was their density in the planktonic state, the biofilm forming performances were strongly heterogeneous both after 20 and 40 hours of contact. In particular, the three strains isolated from NTP surface were characterised by a high density of planktonic cells associated to a low density of adhered ones. The strain labelled as MTP22E, isolated from stainless steel cut modules for poultry breast, was the more adhesive and persistent strain along the 40 hours of contact and it adhesion performances were studied on an hydrophilic surface (i.e. stainless steel chips) as well as on an hydrophobic surface (i.e. Teflon chips). The data collected evidenced a greatly higher affinity of MTP22E strain to Teflon surface. In fact the biofilm layer formed on this surface was thicker and denser than that formed on stainless steel. Moreover a very interesting ability to form flagella of adhered cells was observed in particular on Teflon after 20 hours of contact. After 40 hours of contact to stainless steel, *L. monocytogenes* adhered cells evidenced a stronger persistence in this surface and a great morphological heterogeneity in cell length. The formation of flagella depends on environmental conditions including nutrients and surfaces characteristics. They were reported to serve as an adhesive structure; however, no evidence has demonstrated that flagella alone are involved in attachment. In fact, according to Lemon *et al.*, 2007, the primary role of flagella in *L. monocytogenes* biofilm formation is in generating motion, and that if there is any role for *L. monocytogenes* flagella as surface adhesins in biofilm formation it is either minimal or is dependent upon motility. However, the same author suggested that, likewise other bacteria, the primary role of flagellum in surface-associated biofilm formation is to provide the force necessary to overcome repulsive forces that might exist between the bacteria and the surface (Pratt and Kolter, 1998) and thereby increasing the probability of encountering a surface. The higher adhesion in Teflon in the first 20 hours of contact may be attributable to a result of this overcoming action induced by the hydrophobic surface. During biofilm development in many other motile bacteria, motility and extracellular matrix production are inversely regulated, such that once motile cells contact a surface they switch to producing matrix. According to this approach, after 40 hours the higher affinity for stainless steel that provided to a stronger persistence may be ascribed to a higher matrix production or to some other strategies. One of these alternative strategies may be represented by the morphological differentiation. In fact, unlike the growth of bacteria in liquid cultures, produce homogeneous populations of genetically identical cells, growth in biofilms generates a large amount of genetic diversity. Therefore morphological differentiation may be one of the bases of the ability of *L. monocytogenes* cells to persist in biofilm and differentiate morphological, physiological and genetic traits including the resistance to sanitizers.

5.6.3 - Conclusions

The CDP mostly used in the Italian slaughterhouses includes a weakly rotation of a foam and a disinfectant and the application of sodium hypochloride and a second foam once a week. These common CDPs eliminate *L. monocytogenes* and *Campylobacter* but were not always effective against *C. perfringens*. *Salmonella* does not seem to represent an environmental contaminant for surfaces under study. The wide part of the strains having the best biofilm forming abilities was isolated from hydrophilic surfaces i.e. stainless steel. In fact the bacterial cells were always negatively charged and possessed hydrophilic surface properties. In particular, *L. monocytogenes* MTP22E, isolated from stainless steel cut module for poultry breast, resulted the best biofilm forming strain. However, if isolation source provides to select a certain number of *L. monocytogenes* strains potentially contaminants, their adhesion and

persistence to 20 and 40 hours may be explained as the result of two further factors: formation of flagella and morphological changes of surface attached cells.

5.7 - Alternatives procedures to reduce bacterial contamination on poultry products

5.7.1 - Chemical carcass decontamination to control *Salmonella* and *Campylobacter*

In the EU Regulation 853/2004 of the European Parliament and of the Council of 29 April 2004 (Anon, 2004) removal of surface contamination from products of animal origin is allowed under certain restrictions, with substances that are approved. The use of decontamination shall however not be used without fulfilling the requirements of hygienic food production. In the EU legislation concerning carcass decontamination is being prepared. Late 2005 EFSA published an opinion (Anon, 2005a) with results of a study for food safety aspects for the treatment of poultry carcasses with Chlorine dioxide (ClO_2), Acid Sodium Chlorite (ASC), Tri Sodium phosphate (TSP). The evaluation of the efficacy of Peroxy acids is written in another opinion of EFSA (Anon, 2005b) which was published 14 December 2005. March 2006 EFSA published an opinion on the efficacy of Lactic acid for carcass decontamination (Anon 2006b). The EU draft Regulation (Anon, 2006a) laying down specific conditions for the antimicrobial treatment of fresh poultry carcasses contains details on the approval of substances (including a draft list) and on the use of the approved substances. An outline of labelling requirements is also included.

Implementation of carcass decontamination in poultry slaughter plants for controlling the *Salmonella* or *Campylobacter* contamination of carcasses demands an effective and practically applicable system. Additional measures during processing were mentioned as necessary contributions in this respect (Havelaar *et al*., 2005).

During broiler processing carcasses are sprayed with water intermittently in order to remove undesired residues such as feathers, faeces and blood. The number of micro-organisms is only slightly reduced; approx. one Log cycle. *Salmonella* and *Campylobacter* bacteria may occur on carcasses in CFU numbers up to 1000 per gram skin and treatment with water under the given practical conditions is not sufficient to eradicate pathogens completely.

Research in the past not only shows clearly that the effect of a treatment under practical conditions cannot guarantee eradication of pathogens, but also that decontamination chemicals and process conditions play a role (Bolder, 1997). Repeated treatment during one processing operation could be more effective, since a permanent fluid layer (with chemicals) on the skin is present (Snijders *et al*., 2004).

In the present project, the decrease of different bacterial counts was measured as a consequence of carcass decontamination under practical conditions.

A) Material and methods

Commercial flocks were treated in the final carcass washer and chilled with air. The final washer was equipped with an experimental dosage system through which the chemicals could be applied in the desired concentration. In the experiments water was used as a control treatment and the chemicals of choice were: Chlorine dioxide, Acid Sodium Chlorite, buffered Lactic acid and activated Lactoferrin. The decontamination treatment was applied in the final washer, where the carcasses are cleaned with water both on the outside and the inside of the carcass just before entering the air chiller. In this equipment chemicals can be dosed very precise (Table 46).

Table 46 : Dosage of chemical couponds

Treatment Group	Dosage in the final washer
Control (water)	37.5 l/min
Chlorine dioxide (Halox, Ecolab, Düsseldorf , D)	4.25 ppm (9g Cl_2/h)
Acid sodium chlorite (ASC, Ecolab, Düsseldorf, D)	1250 ppm (2.8g Cl_2/h)
Lactic acid (Purac, Biochem, Gorinchem, NL)	2% (45 l/h)
Lactoferrin (ALF; DMV International, Veghel, NL)	1% (22.5 l/h)

Salmonella and *Campylobacter* counts were performed according ISO regulations, 6579-1998 and 10272-2002 respectivelly. CFU counts of *Enterobacteriaceae* were made on 3M Petrifilm™.

B) Results and discussion

Results showed approx. one Log reduction of enterobacteria in all treatments. The lethal effect on *Campylobacter* was less; 0.7 Log, but in the control groups there was only 0.3 Log reduction. *Salmonella* could only be isolated from samples of the third flock and there was approx 50% reduction in *Salmonella* positive skin samples. A semi-quantitative analysis showed approx. one Log reduction of the *Salmonella* CFU counts. Carcass decontamination with chemicals tested here showed no additional lethal effect on *Salmonella* or *Enterobacteriaceae* when compared with water treatment (controls); the additional lethal effect of chemicals on *Campylobacter* was only 0.5 Log reduction. The application of chemicals in the washer implies a very short exposure time for chemicals. The procedure might be improved by using an additional spray tunnel or a short time immersion.

5.7.2 - Forced defecation of broilers under practical conditions in a processing plant

Reducing pathogens in broiler slaughter plants can be obtained by application of different interventions. One of those is forced defecation of broilers just after killing. Recently one slaughter equipment manufacturer designed a machine that can do this operation in line, the Preventer (Heemskerk, 2004). This machine can be applied at the very beginning in the slaughter line, i.e. just before the carcasses encounter the scalding tanks. It pushes out faeces by applying pressure on carcasses with a curved metal bar. The faeces then are removed. It is claimed that the treatment results in less organic matter in the scalding tanks, and consequently reduces contamination that occurs during evisceration, and reduces the amount of bacteria, including pathogens on the retail product.

In this study the efficacy of early defecation was tested under practical conditions in a processing plant.

A) Material and methods

In two parallel lines, one with a Preventer and one without a Preventer, birds from the same flock were slaughtered as the first flock on a production day. The bacterial load of scalding water and of carcasses at different points during the process was determined according protocols previously described. *Salmonella* and *Campylobacter* were counted with classical cultural methods according to protocols previously described. CFU counts of *Enterobacteriaceae* were made on 3M Petrifilm™.

Practical scale experiments were carried out with birds from *Salmonella* and *Campylobacter* positive flocks in processing plants. Procedures that were investigated at the various stages in a slaughter line (Table 47).

B) Results and discussion

The effect of defecation on bacteriological conditions of carcasses could not be determined. Immediately after the treatment, there was a marginal effect on *Enterobacteriaceae* CFU counts, but this difference did no longer exist after chilling (Table 47). There was no difference in bacterial load of the scald water, although the dry matter contents after treatment were lower.

Summarized an effect of early defecation on the pathogen load of broilers could not be demonstrated.

Table 47 : Average CFUs (Log N/ml) on carcasses taken from different locations

Experiment 1		*Enterobacteriaceae*	Total CFU	*Pseudomonas*	*Campylobacter*
Location	**Preventer**				
pre Preventer	+	4.8 a	5.7	*	<1
	-	4.2 b	6,1	*	<1
post preventer, pre scalding	+	5.5 p	6.7	*	<1
	-	4.6 q	6.7	*	<1
post scalding pre chilling	+	3.0	3,7	*	<1
	-	2.8	3.6	*	<1
post chilling	+	3.2	4,2 a	3.8	<1
	-	2.7	4.0 b	3.8	<1
Experiment 2		***Enterobacteriaceae***	**Total CFU**	***Pseudomonas***	***Campylobacter***
pre Preventer	+	4.8	6.6	4.3	4.4 p
	-	4.7	6.4	4.2	3.0 q
post preventer, pre scalding	+	5.0	6.8	5.2 p	5,1
	-	4.6	7.1	4.3 q	4,7
post scalding pre chilling	+	3,0	4.5	3.5	<1
	-	3.0	4.2	3.3	<1
post chilling	+	3.4 p	4.5 p	4.6 p	<1
	-	2.7 q	4.1 q	4.3 q	<1

All figures are an average from 10 measurements. Symbols: a,b: significant difference p<0,05; p,q: significant difference p<0,01; * many *Pseudomonas* CFU counts are below the level of detection (5) for which an average can not be given.

5.7.3 - Effects of Modified Atmosphere Packaging (MAP) on survival of *Salmonella* and *Campylobacter* on chicken meat products

The present state of the art in controlling *Salmonella* in broilers in The Netherlands shows that there is a steady decrease of the prevalence. This decrease however slows down since new and persistent *Salmonella* serotypes have appeared in broiler farms, such as *Salmonella paratyphi* B Java. For *Campylobacter* the situation is different, since there is hardly any progress in controlling or eradication *Campylobacter* in live animals. Problems show best during summer, when the *Campylobacter* prevalence is extremely high. On the other hand, effective interventions during or after slaughter are not (yet) available.

An intervention to influence the bacterial load on poultry meat is the application of Modified Atmospheres for Packaging (MAP).

Packaging food products under MAP is being applied for many years already. Many applications use elevated CO_2 levels in order to suppress Gram-negative spoilage bacteria (Sutherland *et al*., 1997; Erichsen and Molin, 1981). CO_2 increased the growth phase and generation time, which prolongs the shelf life (Bohnsack *et al.*, 1988; Thys *et al.*, 1994). The three main gasses used in MAP are O_2, CO_2 and N_2. The choice of gas is very dependent on the food product being packed and the pathogen that needs to be inhibited. Used single or in combination, these gasses are commonly used to balance safe shelf life extension with optimal organoleptic properties of the food. Noble or 'inert' gasses such as argon are in commercial use for products such as coffee and snack products; however, the literature on

their application and benefits is limited. Experimental use of Carbon Monoxide (CO) and Sulphur Dioxide (SO_2) has also been reported (Mullan, 2002).

A) Material and methods

For *Campylobacter* the optimum growth environment consists of a micro-aerobic gas mixture containing 5% O_2, 10% CO_2 and 85% N_2, so, gas mixtures used for packaging should be different from this composition when *Campylobacter* should be eliminated. In the experiments described here extreme gas mixtures sometimes not very practicable were chosen to investigate the effect on both *Salmonella* and *Campylobacter* on inoculated chicken wings. CFU were performed according to protocols previously described (Table 48).

Table 48 : Experimental design of MAP assay

CO_2 level (%)	N_2 level (%)	O_2 level (%)	CFU counts at day:
Experiment 1			
0	80	20	3, 7, 10, 14
35	30	35	3, 7, 10, 14
65	25	10	3, 7, 10, 14
100	0	0	3, 7, 10, 14
Experiment 2			
0	80	20	0, 5, 8, 12, 15
0	50	50	0, 5, 8, 12, 15
20	10	70	0, 5, 8, 12, 15
50	0	50	0, 5, 8, 12, 15

B) Results and discussion

Many applications use an elevated CO_2 level, in order to suppress Gram-negative bacteria, which are mainly responsible for spoilage of the products. For *Campylobacter* optimum growth usually a micro-aerobic gas mixture with 5% O_2, 10% CO_2 and 85% N_2, is used. Results in this study with alternate gas mixtures using chicken wings inoculated with *Salmonella* and *Campylobacter* showed that there were no lethal effects of high levels of CO_2, also not when combined with high levels of O_2.

These experiments prove that high levels of CO_2, separate or in combination with high levels of O_2, have no additional lethal effect on *Campylobacter* or *Salmonella*. In a further investigation the combined effect of carcass decontamination and MAP on the survival of *Campylobacter* and *Salmonella* should be tested. The effect of MAP under commercial conditions with lower contamination rates, and without inoculation should be performed as well. The experiments with pre-chilling by immersion could not be carried out since the company where the equipment was available no longer exists.

5.8 - Conclusion

The quantifiaction of bacteria, performed by traditional cultural methods, along the processing line is an important tool for quality control in case of hygiene parameters or for exposure assessments of bacterial pathogens. These can be supplemented by molecular biological methods like SSCP and sequencing, which allows distinguishing between flocks, slaughter steps and possibly the analysis of the bacterial composition on the chicken surface (unclear sentence).

The analysis of the occurrence of *Campylobacter* during poultry processing showed different factors influencing the contamination levels of carcasses; these can be the flocks´ status, initial colonisation levels and season. Such an influence of the flock status could not be shown for *L. monocytogenes* or *Salmonella* on contamination of carcasses.

Campylobacter is thought to be a sensitive organism, a reduction in numbers was shown along the processing, but the total eradication does not seem possible at the moment. Analysis showed that in some cases, *Campylobacter* was able to survive under aerobic conditions when cultured together with *Pseudomonadaceae*. Thus, the possible interaction of bacteria in the environment, like on the product, has to be considered when making assumptions about the survival of bacterial pathogens.

Cleaninig and Disinfecting Procedures (CDPs) are important in food production to enhance the hygienic properties of the processing environment and finally of the product. Especially pathogenic bacteria are concerned. Tested CDPs proved to be effective in eliminating *L. monocytogenes* and *Campylobacter* from the slaughter environment. *C. perfringes* occurrence was reduced after CDPs, but it was still present with 21.3% of positive surface samples, with half of them being potential food contact surfaces. *Salmonella* was not found at all on surfaces under study, neither before nor after CDPs.

Biofilm formation is an important feature of bacteria, which allows growth even under unfavourable conditions. Tested strains of *L. monocytogenes* with highest biofilm forming ability were isolated from hydrophilic surfaces like stainless steel.

Besides decontamination of surfaces in the processing plant, also treatment of the products itself is an option to enhance consumer safety. Poultry carcasses are treated with water along the entire processing line to reduce surface contaminations and to avoid drying out which would result in adverse appearance. Treatment with different chemicals in aqueous solution can be applied at different stages. Though, the impact of potential effects is controversial. In this study there was no additional lethal effect of chemicals on *Enterobacteriaceae* and *Salmonella*, while a limited effect was seen for *Campylobacter*, when applied through the internal/external washer. This might be because of too short exposure times. Another way of limiting contamination could be early defecation, thus limiting the amount of faeces distributed in the slaughterhouse. An effect could not be confirmed during this study using commercial defecation equipment. Finally the kind of packaging can enhance the product quality by applying differently composed atmospheres. MAPs have already proven themselves in the elongation of shelf-life while maintaining acceptable organoleptic properties. An additional lethal effect on *Salmonella* or *Campylobacter* was not seen in this study.

Section 6: Molecular characterisation of food-borne pathogens

All avian bacteria isolated in broilers and laying hens in different European countries along the project were purified, classified and stored in the laboratories of the POULTRYFLORGUT Partners. Moreover, many of them were typed by using different genotyping methods and the results were sorted in a molecular database. This pathogens collection and the molecular database will be available to the public and they represent the first source of broiler and laying hen European isolates and genotypes.

For the storage and use of typed isolates a database was set-up in form of an EXCEL sheet with important data like classification, origin, identification and typing of isolates collected during the course of this project. It was designed in a way to allow the later use of isolate properties for epidemiological studies. The database set-up and information included allows the usage for different types of epidemiological investigations. These are small-scale investigations on individual slaughter days. Here the distribution and diversity of *Campylobacter* isolated from different samples from slaughtered broilers and from the slaughter environment can be compared and flock specific strains can be looked for as their distribution along the slaughter line. Second there are medium scaled investigations, where isolates from broiler flocks of different farms can be compared for similarities within a defined area of interest. Third, large scale analysis can be performed by exchanging isolates between partners to allow for additional typing methods to be carried out and then compare the results and profiles of isolates from an European point of view.

6.1 - Methodology

In Germany, 98.1% of the total number of isolates (814 out of 830) were subtyped using Pulsed-field Gel Electrophoresis (PFGE) according to the CAMPYNET prototype standardised protocol using the DNA restriction enzyme *Sma*1. The similarity of the restriction fragment patterns was compared by unweighted-pair group method analysis (UPGMA) based on the construction of matrices with help of the BioNumerics software. A similarity cut-off at 90 % separated the different *Sma*1 restriction pattern clusters from each other.

A total of 190 *Campylobacter* strains collected during the POULTRYFLORGUT project in different EU countries were submitted to *Hae*III automated ribotyping. In particular, 90 strains were isolated in Italy from the caecum contents of broilers; 46 were isolated in the Czech Republic from the caecum content of broilers; 54 were isolated in Germany from different

samples (ex. caecum contents, poultry meat, carcasses) collected within conventional farms (i.e. 1 strain per sample). The strains tested were previously identified by the different project Partners as *C. coli*, *C. jejuni*, *C. jejuni ssp jejuni* and *C. jejuni ssp doylei*.

A total of 93 *Clostridium perfringens* strains collected during the POULTRYFLORGUT project in Italy and in the Czech Republic were submitted to *Eco*RI automated ribotyping and the Italian strains to toxinotyping too. The 55 strains were isolated in Italy from the caecum contents of broilers reared within conventional, extensive and biologic farms and 38 were isolated in the Czech Republic from the caecum content of broilers reared within conventional farms.

A total of 89 *H. pullorum* strains collected during the POULTRYFLORGUT project in Italy were submitted to *Hae*III automated ribotyping as well as PFGE analysis. The isolates were collected from the caecal contents of broilers reared within conventional, extensive and biologic farms. The ribotyping profiles collected in this project were labelled with an alphanumeric code (ex. PFGEHaeIII 153-328-S-5) that defined the ribogroup o ribotype (RG) of the isolate. All ribotypes, toxinotypes and pulsotypes obtained along the whole project were sorted and added in the molecular database.

In table 49 the isolates available at the individual laboratories for epidemiological investigations are shown. The table gives information about the production system, the pathogen species and the number of isolates per species and the production system.

Table 49: Isolates recorded during the project and stored

Country (Partner)	Production system	Pathogen	N. of isolates collected and stored the laboratory
Germany (TIHO)	Broilers intensive	*Campylobacter*	843
Lithuania (IIVU)	Broilers Intensive	*Salmonella*	270
Lithuania (IIVU)	Laying hens	*Salmonella*	200
Lithuania (IIVU)	Broilers Intensive	*Campylobacter*	20
Lithuania (IIVU)	Broilers Intensive	*L. monocytogenes*	7
Lithuania (IIVU)	Laying hens	*L. monocytogenes*	3
France (AFSSA)	Broilers (intensive and free range)	*L. monocytogenes*	725
France (AFSSA)	Laying hens (cages and at the floor)	*L. monocytogenes*	774
Italy (UNIBO)	Broilers intensive	*Campylobacter* spp.	337
Italy (UNIBO)	Broilers extensive	*Campylobacter* spp.	45
Italy (UNIBO)	Broilers organic	*Campylobacter* spp.	45
Italy (UNIBO)	Poultry slaughterhouse (environment)	*Campylobacter* spp.	72
Italy (UNIBO)	Broilers intensive	*H. pullorum*	166
Italy (UNIBO)	Broilers extensive	*H. pullorum*	23
Italy (UNIBO)	Broilers organic	*H. pullorum*	38
Italy (UNIBO)	Broilers intensive	*Clostridium perfringens*	124
Italy (UNIBO)	Broilers extensive	*Clostridium perfringens*	50
Italy (UNIBO)	Broilers organic	*Clostridium perfringens*	42
Italy (UNIBO)	Poultry slaughterhouse (environment)	*L. monocytogenes*.	36
The Czech Republic (VFU)	Broilers intensive	*Campylobacter* spp	39
The Czech Republic (VFU)	Broilers intensive	*Clostridium perfringens*	37
Italy (UNIPG)	Laying hens conventional cages	*Salmonella spp*	3
Italy (UNIPG)	Laying hens organic farm	*Salmonella spp*	2

6.2 - Database set up

The database was designed to show the origin and properties of selected bacterial isolates for later molecular biological analysis on different scales of interest, therefore the isolates were typed by at least one molecular biological method. Furthermore, selected isolates were exchanged between partners to allow typing by additional methods.

The set-up of the database is shown in figure 13. The main properties are the Isolate classification, the origin of the isolates, the species identification and the molecular typing methods carried out for the particular isolate in question.

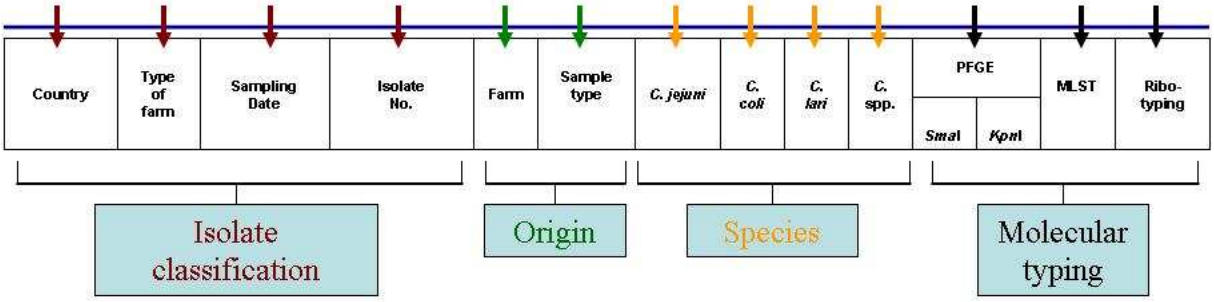


Figure 13: Overview of the set-up of the database

Isolate classification:
This section shows the data that classifies an isolate and allows for the tracing back of isolates together with the information in the section “origin”. It consists of four properties that show the “name” of the individual isolate.

Country: The memberstate where the isolate was collected.

Type of farm: The type of farming from which the samples were collected is shown here. In Europe the intensive rearing of broiler is still the most prevalent production system. But as in the laying hen sector the consumer demands for more animal welfare have led to an increase of alternative production systems not only in the laying hen sector. Therefore it is interesting to see whether there are differences in the types of bacterial strains isolated from different types of farming and production systems.

Sampling date: This column shows the specific day of sample collection. It gives information about the season in which the sample was collected and allows the analysis of seasonal changes and the inclusion of officially available weather data and to look for influences of the temperature and weather situation on the diversity of strains.

Isolate No.: The isolate number is a unique code that is used for one particular isolate only and should allow the tractability.

Origin: This section strongly corresponds to the isolate classification and gives two special properties of the isolate.

Farm: Here is shown a number that allows the direct allocation of the farm from which the slaughtered flock came from together with the knowledge of the country. This information is important for medium scale analysis, when several flock rotations from one farm can be analysed for persistent strains. Another possibility is to analyse the strains for diversity between farms in geographical neighbourhood and of farms farther away from each other.

Sample type: The isolates were collected from different kinds of samples. They originated from the intestinal tracts of broilers or laying hens (cacum or cloacal

swabs) taken at different stages of the poultry processing, the rearing environment itself and from samples of the slaughter and production sites, where environmental samples carcasses and products were taken. Especially when small scale comparisons are of interest for example during one processing cycle, the type of sample shows the presence of flock specific strains in intestinal contents and on carcasses and shows ways of cross contamination during processing. This can give information about critical processing steps. Also the diversity between flock specific strains in different populations can be shown.

Species: The species collected and implemented into the database are thermophilic *Campylobacter* spp., *C. perfringens*, *H. pullorum* and *L. monocytogenes*. The species identification is shown as performed by the partners´ methods.

Molecular typing: This section shows the molecular typing methods performed for each isolate. The methods used are PFGE, Ribotyping and MLST. The combination of several typing procedures either by combination of different endonucleases/enzymes or typing methods can compensate weaknesses or deficiencies of a single method and give more thorough results of higher epidemiological value.

PFGE: The PFGE or pulsed field gel electrophoresis is a typing method where chromosomal DNA embedded in small gel blocks is cut by restriction endonucleases. These are enzymes digesting the DNA at defined nucleotide sections, resulting in fragments of high but different molecular weights, which are then separated by a pulsed field in an agarose gel. This will result in a number of bands which give the isolate a unique fingerprint. The PFGE has a good discriminatory power and good reproducibility. But it suffers from genomic changes of the bacteria analysed and is therefore best used in small to medium scaled epidemiological analyses. It is considered as “gold-standard” in outbreak analysis (Fitzgerald *et al.*, 2001). The enzymes used for PFGE typing in this project were *Sma*I and *Kpn*I.

Ribotyping: In this method the ribosomal RNA is used for typing of bacteria. This RNA has highly conserved regions and is therefore usable in molecular typing. Digested genomic DNA after electorphoresis is processed in southern blot technique and is hybridized with rRNA specific probes. After that, labelled probes containing ribosomal genes are highlighted. This is resulting in a fingerprint for this tested isolate. Ribotyping by itself is a technique of low discriminatory power but this can be enhanced by automated ribotyping.

MLST: In Multi Locus Sequence Typing (MLST) so-called “housekeeping genes” are sequenced on the nucleotide level. These genes are highly conserved as they play a major role in the bacterial metabolism. Even small rates of mutation do not lead to a significant change in the overall profile therefore this method is of high value in long term or large scale epidemiological studies (Djordjevic *et al*., 2007). The sequencing of the mentioned genes results in a unique Sequence Type (ST) which can be grouped in Colonal Complexes (CC). The CC represents bacterial lineage and derives from a common ancestor. Selected isolates typed by PFGE and or Ribotyping were also typed by MLST and STs as CCs were generated to study the large-scale relationship of poultry related pathogenic bacteria.

6.2.1 - Campylobacter spp

In Italy, a total of 93 different ribogroups were associated to the 190 *Campylobacter* strains ribotyped in this project using *Hae*III as restriction enzyme. Overall, 60 profiles were associated to single strains, whereas 33 were shared between 2 to 14 isolates. Both unique as well as common ribotyping profiles were characterised by 5 to 8 bands, with a molecular weight ranging between 1 to 18 kbp. The overall similarity between ribotyping profiles ranged between 16 and 91%. The 90 Italian strains were classified in 41 different ribogroups. Overall, 17 were ribogroups shared between 2 and 11 strains, whereas 24 were unique ribogroups. Ten out of 17 common ribogroups (i.e. 59%) were associated to isolates from

broilers reared in the same type of farm, whereas 7 (i.e.41%) were associated to isolates collected from broilers reared using different types of farming. In particular, 6 ribogroups were associated to strains collected from broilers reared within conventional and biologic farms and one ribogroup was associated to strains isolated from broilers reared using the three different farming technologies tested in this project. Six out of 7 ribogroups identified for only 2 strains coming from the same type of farm were associated to isolates from the same caecum content. The majority of the unique ribogroups were associated to strains from conventional farms that represented the majority of the isolates ribotyped. The Simpson's Index of Discrimination (SID) achieved by *Hae*III ribotyping among Italian strains was 0.960.

The 46 *Campylobacter* strains isolated in the Czech Republic were classified in 23 different ribogroups. Overall, 8 were ribogroups shared between 2 and 11 strains, whereas 15 were unique ribogroups. The SID achieved by *Hae*III ribotyping among strains from the Czech Republic was 0.926.

The 54 German strains were classified in 35 different ribogroups. Overall, 11 were ribogroups shared between 2 and 4 strains, whereas 24 were unique ribogroups. The SID achieved by *Hae*III ribotyping among strains from Germany was 0.980. The ribotypping profiles of *Campylobacter* strains were characterised by 5 to 8 bands, with a molecular weight ranging between 1 to 18 kbp.

Overall, the most common ribogroup detected among EU *Campylobacter* isolates was PFG HaeIII 153-328-S1, shared between 14 *C. coli* strains isolated in Italy (N=11) within conventional and biological farms and in Germany (N=3) within conventional farms. In Italy that was the most common ribogroup, whereas in Germany it was PFG HaeIII 153-352-S1 and in the Czech Republic PFG *Hae*III 153-38-S6.

6.2.2 - Helicobacter pullorum

A total of 49 different ribogroups were associated to the 89 *H. pullorum* strains ribotyped using *Hae*III as restriction enzyme. Overall, 35 profiles were associated to single strains whereas 14 ribotyping profiles were shared between 2 to 7 isolates. Both unique as well as common ribotyping profiles were characterised by 2 to 5 bands, with a molecular weight ranging between 1 to 48 kbp. The overall similarity between ribotyping profiles ranged between 5 and 92%. The SID achieved by *Hae*III ribotyping among the *H. pullorum* strains was 0.972. The PFGE results obtained by using a protocol set up during this project confirmed the presence of the similar genotypes among the isolates tested.

The 38 strains isolated in the Czech Republic were classified in 12 different ribogroups. Overall, 4 were ribogroups shared between 2 and 14 strains, whereas 8 were unique ribogroups. The SID achieved by *Eco*RI ribotyping among these strains was 0.818. Both unique as well as common ribotyping profiles were characterised by 9 to 15 bands, with a molecular weight ranging between 1 to 45 kbp. The overall similarity between ribotyping profiles ranged between 20 and 90%.

6.2.3 - Listeria monocytogenes

The genotyping of 130 isolates in broilers generated 58 patterns obtained with the combination of the two restriction enzymes *Apa*I and *Asc*I. Within the serovars 1/2a, the pulsotype AC105 was predominant and represented 11 % of the isolates. It was followed closely by AC76 which was represented by 10 % of the isolates. The other types grouped between 1 and 5% of the isolates. Within the serovars 4e4b, the pulsotypes AC 30 and AC 69 were predominant and represented each 12 % of the isolates. The other types grouped between 1 and 4% of the isolates. Within the serovars 1/2b, the pulsotypes grouped between 1 and 4% of the isolates.

The genetic diversity of the isolates having the serovar 1/2a was high 0.975 similar to the first observation from laying hen flocks. For the serovar 1/2b, the genetic diversity was also high 0.964 and confirmed that the low diversity observed among the isolates from laying hen flocks was in relation with the low number of isolates (Table 50). The serogroup 4 (4e,4b and 4e) seemed more clonal than 1/2a or 1/2b as the genetic diversity observed is lower 0.86. Regarding the production system, the genetic diversity of the isolates from conventional and free range productions was comparable 0.98 and 0.94 respectively (Table 50).

Table 50 : Variability among the different serovars and samples from laying hens in France calculated according to The Simpson discrimination index (SDI)

Serovars/ samples/system	1/2a	1/2b	4e,4b	4e	Conventional	Free range
Number of samples	65	24	27	14	66	64
SDI	0.97	0.96	0.86	0.86	0.98	0.94

6.2.4 - Clostridiuim perfringens

A total of 42 different ribogroups were associated to the 93 *Clostridium perfringens* strains ribotyped in this project using *Eco*RI as restriction enzyme. Overall, 29 profiles were associated to single strains, whereas 11 ribotyping profiles were shared between 2 to 16 isolates. Both unique as well as common ribotyping profiles were characterised by 9 to 15 bands, with a molecular weight ranging between 1 to 45 kbp. The overall similarity between ribotyping profiles ranged between 20 and 90%.

The 55 Italian strains were classified in 31 different ribogroups. Overall, 10 were ribogroups shared between 2 and 9 strains, whereas 21 were unique ribogroups. Eight out of 10 common ribogroups (i.e. 80%) were associated to isolates from broilers reared in the same type of farm, whereas 2 (i.e.20%) were associated to isolates collected from broilers reared using conventional and extensive farming. Only 2 out of 10 common ribogroups were shared between different isolates from the same sample confirming the importance to test up to 5 isolates per sample due to great genetic heterogeneity associated to the *Clostridium perfringens* population contaminating broilers. The SID achieved by *Eco*RI ribotyping among Italian strains was 0.96.

The 38 strains isolated in the Czech Republic were classified in 12 different ribogroups. Overall, 4 were ribogroups shared between 2 and 14 strains, whereas 8 were unique ribogroups. The SID achieved by *Eco*RI ribotyping among these strains was 0.818. Both unique as well as common ribotyping profiles were characterised by 9 to 15 bands, with a molecular weight ranging between 1 to 45 kbp. The overall similarity between ribotyping profiles ranged between 20 and 90%.

A total of 3 different ribotyping profiles were identified between broiler strains isolated in Italy and in the Czech Republic. The ribogroups RIBO1::KHA::EcoRI 153-309-S5 and 153-322-S4 were associated to isolates from conventional farms, whereas the ribogroup RIBO1::KHA::EcoRI 153-322-S5 to isolates from conventional and biologic farms.

Toxinotyping results obtained for the Italian *Clostridium perfringens* strains showed that all isolates investigated were type A, but anyone showed the CPE gene. All strains were positive for the α toxin and negative for β, ε and enterotoxin.

All ribotyping, toxinotyping and pulsotyping results obtained for the 372 strains tested during the project, belonging to the genus *Campylobacter* (N=190), *Clostridium perfringens* (N=93) and *H. pullorum* (N=89) and *L. monocytogenes* (N=130).

6.2.5 - Conclusion

The typing of isolates of pathogenic bacteria is an important part in understanding infection routes. Not only does it allow to follow the paths of distribution, their origin and finally their way into the product ready for consumption, but also to gain knowledge about their appearance within the population. This is interesting for food processors in the course of their daily work of quality control and also for scientists when trying to assess a “big picture” for the distribution of pathogenic bacteria within Europe. Especially when the marketing of products not only within the own country is considered, leading to the potential distribution of pathogenic bacteria beyond the border.

The collection of bacteria into the database comprised the most important pathogens correlated to foodborne infections: *Campylobacter, Clostridium perfringens, L. monocytogenes* and *Salmonella*. *Helicobacter pullorum* was included as “emerging pathogen”. Six partners from five countries were involved in the collection of isolates. The isolates were collected in broiler and laying hen production systems representative for the systems used in each country.

A systematic collection of data for each isolate was the fundamental task for being able to use typing results in epidemiological analyses. Each isolate was classified, its origin and species identification included in an EXCEL data sheet. The molecular biological typing methods used for epidemiological analysis were: PFGE, MLST and Ribotyping.

The typing of *Campylobacter* revealed that there were mostly distinct types found in the Czech Republic, Germany and Italy. One particular type was found in isolates from both Italy and Germany. Although, distinct types were found in single isolates within each country, also a grouping was possible for several isolates. This was also true for the typing of *Clostridium perfringens* and *Helicobacter pullorum* and *L. monocytogenes*, where isolates from one farm or one production system shared the same molecular type.

Section 7: Evaluation of molecular methods to monitor the gut microflora

7.1 - Introduction

The bacteria of the gut microflora interact with each other and directly with the lining of the gastrointestinal tract which may alter the physiology of the tract and the immunological status of poultry (van Leeuwen *et al.*, 2004). The bacterial flora is involved in the digestion and metabolisms process and thus in animal growth, and protection against pathogenic bacteria as reported in the review of (Gabriel *et al.*, 2006). It has effects on the well being of the broilers, on the production economy and on the public health following poultry meat consumption.

The luminal flora appears to be different from those of the mucosa (Gong *et al*., 2002; Lu *et al*., 2003). The bacteria adhering to the mucus layer and the mucosa have closer interactions with the host, and might have more consequences on the host (Gabriel *et al.*, 2006). They might be involved in the global effect of bacteria on the digestive immune system. However the mucosal bacterial community is less studied because of the difficulty to obtain mucosal bacteria without contamination by luminal flora (Zhu *et al*., 2002).

The most abundant bacteria in the poultry digestive tract are Gram-positives which have also been observed in other animals and humans (Franks *et al.*, 1998; Gabriel *et al.*, 2006; Leser *et al*., 2002). The microflora appeared to be different from one compartment to the other (Salanitro *et al.*, 1978). In the crop, *Lactobacilli* are predominant (Fuller, 1984; Gong *et al.*, 2007). In the jejunum, mostly facultative anaerobes such as *Lactobacilli*, *Streptococci,* and *Enterococci* were isolated (Lu *et al.*, 2003). In the caeca, the bacterial population was quite different with the presence of strict anaerobes such as *Eubacterium*, *Bifidobacterium, Clostridii* and facultative anaerobes (Barnes, 1979; Gong *et al.*, 2002; Salanitro *et al.*, 1978). The composition of these microbiota varies with age (Amit-Romach *et al.*, 2004; Knarreborg *et al*., 2002).

The balance of this ecological niche is involved in the growth of the birds and probably influences their sensitivity to infectious diseases and is conditioned by various factors as rearing condition (rearing density, stress, rearing farm, ...), diet (feed ingredients, antibiotics

as AGP or their alternatives such as probiotic, prebiotic or plant extract. (Knarreborg *et al.*, 2002, Apajalahti *et al.*, 2001, Netherwood *et al.*, 1999). It was shown that the commensal microflora of the digestive tract of broilers plays a role in exclusion of enteropathogenic bacteria (Rantala and Nurmi, 1973). The caeca were the subject of numerous analysis since the microbiota of the digestive tract is mainly concentrated in these organs; it may contain 10^{11} bacteria / g instead of 10^{9}/g in the ileum (Apajalahti *et al*., 2004) and potential human pathogens, e.g. *Salmonella enterica* and *Campylobacter*, frequently are most numerous in this gut compartment (Zhu *et al.*, 2002). The aim of the use of some antibiotic as AGP was to regulate the microbial flora and mostly the Gram-positive bacteria (Butaye *et al.*, 2003). Among them, Avilamycin reduces the clostridia populations (Butaye *et al.*, 2003). With the ban of the AGP in poultry, at the beginning of the year 2006, this balance is now to be changed, an increase of unspecific digestive troubles may appear with the emergence of new pathogens. In consequence, the knowledge of gut microbiology is increasingly important to evaluate these changes and to study putative alternatives to AGPs.

The information about the composition and the dynamics of the gut flora is limited since the number of non-culturable bacteria is estimated to be up to 90 % (Lan *et al*., 2002). Most of the studies on poultry digestive microflora were performed by using culturing techniques with selective media. These practises excluded fastidious organisms and viable but not culturable bacteria. The way to overcome this problem is to use molecular methods based on the detection of a ubiquitous gene, the 16S rDNA. Two main strategies for the direct investigation of the diversity of complex microbiota are currently available. A direct detection and count of individual cells by using fluorescently labelled 16S rDNA probes (*In situ* hybridization method, FISH, q-PCR) or a molecular approach for studying the composition of the total 16S rDNA. Among them, the cloning of 16S rDNA and fingerprint methods (Denaturing Gradient Gel Electrophoresis DGGE, Temporal Temperature Gradient gel Electrophoresis (TTGE), Temperature Gradient Gel Electrophoresis (TGGE), Restriction Fragment Length Polymorphisms RFLP, Terminal Restriction Fragment Length Polymorphism (T-RFLP) Single-Strand Conformation Polymorphism (SSCP)) have been used successfully to investigate the gut microbiota (Johansen *et al*., 2006; Knarreborg *et al*., 2002; Lan *et al*., 2002; Lu *et al*., 2003; Torok *et al*., 2005; Zhu *et al*., 2002)***.***

Analysis of 16S rDNA libraries showed that the biodiversity of the caecal flora is represented by 50 phylogenic groups or subgroups and that the 4 major groups are composed of *Clostridium leptum*, *C. coccoides*, *Sporomusa* sp. and enteric and remaining sequences belong to new species or even new genera (Zhu *et al*., 2002). The microbial diversity of flora of the small intestine tends to be is less diverse than those of the caeca (Lu et al, 2003; Amit-Romach et al, 2004; Dumonceaux et al, 2006; Bjerrum et al, 2006). It is mainly composed of Clostridiales as *Clostridiaceae*, but also Sporomusa, and *Ruminococcus*. This flora also contains Bacteroidetes, Enterobacteriales as *E. coli,* and also *Enterococcus* and *Streptococcus*. The flora of small intestine is less diverse than those of the caeca (Lu *et al*, 2003; Amit-Romach *et al*, 2004; Dumonceaux *et al*, 2006; Bjerrum *et al*, 2006). It is mainly composed of lactobacilli (mainly *Lactobacilli*), although it also contains *Enterococcus*, *Streptococcus*, Clostridiaceae as *Clostridium* sp., and Enterobacteria as *E. coli* (Lu *et al*, 2003; Bjerrum *et al*, 2006). While no change was detected by culturing, community hybridization of amplified 16S ribosomal DNA demonstrated that the bacterial flora of the gastro-intestinal tract changed significantly in response to probiotic treatments (Netherwood *et al.*, 1999).

DGGE and other related techniques as TTGE, are a powerful screening method for evaluation of microbial diversity and one of the advantages of these molecular based techniques is the ability to monitor temporal and spatial changes in community structure in response to changes in environmental parameters (Simpson *et al.*, 2000; Cocolin *et al.*, 2001), and the bacteria can be identified. Recently, the DGGE technique was used to investigate the intestinal microbial communities of chickens (van der Wielen *et al.*, 2002; Knarreborg *et al*., 2002). For example, consequences of feed composition or bacterial

infection on the microflora could be detected (Johansen *et al*., 2006; Knarreborg *et al.*, 2002). Infection of young chicks with *Campylobacter jejuni* modified the caecal microflora of young chicks on the contrary to that of the ileum as demonstrated by DGGE (Johansen *et al*., 2006). Chemical gradients such as those used in DGGE are not as reproducible, it is difficult to establish and often do not completely resolve heteroduplexes. In order to minimize some of the problems encountered with DGGE, TGGE and TTGE were developed as suitable, more reliable techniques. These two techniques provide a temperature gradient instead of a chemical gradient. These fingerprinting methods present several advantages. Microflora dynamics can be registered, profile modifications detected and bacteria can be identified by subsequent cloning and sequencing. However, most of the molecular techniques currently used to study gut microbiota are unable to characterize the bacterial community in a single assay or are not conducive to high-throughput analysis. Other fingerprint alternative methods such as SSCP or the derivate capillary-SSCP method (CE-SSCP), RFLP or T-RFLP could be conductive to high-throughput analysis.

SSCP method allows the separation of single stranded DNA molecules according to conformation on a non-denaturing polyacrylamide gel. This technique was first developed for detection of gene polymorphisms in the human genome and later applied for detection of mutations (Hayashi, 1991; Orita *et al*., 1989). In addition, it is now developed to study the composition and dynamics of different ecological niches such as soil, anaerobic digestors and food (Dabert *et al*., 2005; Delbes *et al*., 2001; Duthoit *et al*., 2005; Lee *et al.*, 1996; Schwieger and Tebbe, 1998). By using the SSCP method Ott *et al.*, (2004) reported reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel diseases. Recently, the variant CE-SSCP was described as a simple and effective technique to obtain the molecular signature of an ecological complex matrix (soil matrix) (Zinger *at a*l., 2007).

RFLP profiles are variation in DNA fragment banding patterns of electrophoresis restriction digests of DNA from different individuals of a species. Often due to the presence of a restriction enzyme cleavage site at one place in the genome in one individual and the absence of that specific site in another individual. Terminal Restriction Fragment Length Polymorphism (T-RFLP) works by PCR amplification of DNA using primer pairs that have been labelled with fluorescent tags. The PCR products are then digested using RFLP enzymes and the resulting patterns visualized using a DNA sequencer.

Despite to the advantages of these fingerprints methods, these techniques present some limitations. Indeed, the level of DNA extraction from complex biological matrix is not the same for all bacteria. Moreover, these techniques are PCR-based methods, and several inhibitors present in biological samples may inhibit PCR.

The results from fingerprint methods are analysed either by simply counting and comparing bands or peaks in the profiles. From these images, using universal primers, these methods are not used to quantify bacteria, bands or picks are applied to estimate dynamics and diversities assuming that each bands or peak refers to a unique sequence called phylotype, ribotype or Operational Taxonomic Unit (OTU) but the pictures of bacterial community is given by discrete bands or peaks which emerge from the signal subband or subpeak background. Discrete bands or peaks have been associated to numerically dominant sequences whereas the subband from a large number of subdominant sequences (Loisel *et al*. 2008).

The in situ Hybridation method (FISH) has been used extensively for the analysis of human faecal flora (Langendijk *et al.*, 1995; Franks *et al*., 1998; Harmsen *et al*., 1999; Harmsen *et al.*, 2000; Schwiertz *et al*., 2000; Zoetendal *et al*., 2002; Rigottier-Gois *et al.*, 2003; Barc *et al.*, 2004), only few studies have dealt with the quantification of bacteria in chickens (Mbuthia et al., 2001; Moreno *et al.*, 2001; Bojesen *et al.*, 2003).

One of the most significant unresolved obstacle appears to be primer design. The development of primers remains a significant challenge because of the highly conserved nature of rDNA and the extensive diversity of microbial life (Valinsky *et al*., 2002).

The choice of the analysis methods depends on the objective of the study and, in particular it depends upon the complexity of the community, the expertise of personnel in laboratory, the required depth of analysis, availability of instrumentation, and the budget and time contains. According to these parameters, the objective of this part of the study was mainly to optimise and to assess these molecular methods to distinguish different rearing systems, to evaluate the effect of AGP and feed additives on the intestinal microflora, and on the course of an infection with pathogens.

7.2 - Optimisation of molecular methods

Culture independent approaches are used to characterize the bacterial community of the chicken gastro-intestinal tract (GIT). The starting point is total DNA extracted from intestinal material or tissue. The 16S rRNA gene is in most cases the target for PCR amplification to link information directly or indirectly to bacterial taxonomic groups. Total DNA can be extracted using kits (e.g. QIAamp DNA Stool Mini Kit) with additional steps (see Annex 2). 'DNA fingerprinting' is then performed by size either following digestion with restriction enzymes (RFLP) or by utilizing differences in the mobility of double stranded DNA under denaturing conditions (DGGE) and single stranded 'melted' DNA under non-denaturing conditions (SSCP). A sample-specific profile is obtained. The approach is cost and time beneficial and allows comparison of samples from different treatments in the same experiment. However, the optimal conditions for the molecular techniques had to be determined. The variability linked to the method or the kind of sample (individual or pool) had to be studied. Different parameters such as age, space distribution and profiles of the microflora of different gut compartments needed to be studied. In a first part of this work, DGGE, CE-SSCP and FISH methods have been adapted and optimised in order to monitor the poultry gut microflora.

7.2.1 - DGGE- TTGE

In DGGE as well as the variant temperature gradient gel-electrophoresis (TGGE), one of the primers for PCR amplification of bacterial 16S rDNA genes has an extra GC rich 5' end acting as 'clamp' that hinders the separation of the two DNA strands. PCR products are loaded on a polyacrylamide gel and with increasing 'gradient' of denaturing agent the movement of 'clamped' fragments stops in the gel due to formation of single-strands only attached at the clamp. The first use of the method with microbial communities was reported by Muyzet *et al.*, (1993).

DGGE technique was implemented and optimised to chicken intestinal samples. The primers HDA1 and HDA2 (Walter *et al.*, 2000), which are universal for all bacteria and amplifies a fragment of 200 bp of the 16S rDNA gene, were found very suitable for our purpose. The 8% polyacrylamide gel containing denaturant (urea and formamide) in a gradient from 30%-55% was used. The technique has subsequently been used on a variety of samples collected from chicken ileum and caecum in order to optimise the method, standardise conditions and on the basis of the results, construct the final protocol (Protocol Annex 2).

DGGE method was run on samples from an infection study with *C. perfringens* where broilers suffered from experimentally induced NE (see section 7.4). Similar investigations were carried out on samples from the infection experiment with *C. jejuni* and Salinomycin (see section 7.4). TGGE was used to detect the impact of feed additive (see section 7.5).

7.2.2 - CE-SSCP

SSCP was originally performed by analyzing PCR fragments in a polyacrylamide gel (gel-SSCP) where the concentration of denaturant contributed to the differences between movement of double stranded and partly single stranded (heteroduplex) or fully single stranced DNA fragments. The SSCP method was automated by running samples in a capillary DNA sequencing machine. Capillary electrophoresis single strand conformation polymorphism (CE-SSCP) was developed by Lee *et al.*, (1996) and later used by Delbes *et al.*, (2000) on complex anaerobic bacterial communities. However, the CE-SSCP molecular method had still to be optimised, particularly to estimate inter-individual variability in poultry gut microflora.

The optimisation of the method required several steps. The first one concerned the extraction of the DNA of the gut microbiota. The PCR reaction was performed by using the W49F-Hex and W104R-6Fam primers direct towards the V3 region of the 16S rDNA that appeared to be the most informative one. The two strands of the amplified DNA were labelled in order to obtain a pattern for each strand. The calibration of electrophoresis was performed by using an internal standard. The electrophoresis conditions were optimised. Reproducible patterns were observed within and between runs. However, slight differences were noticed between different series of runs. Since the CE-SSCP method is very sensitive, we noticed that the change of polymer might influence the profile even when the protocol is strictly followed. These results point out the technical variability and the limit of this technology over time. The patterns of the flora of different gut compartments and droppings were analysed. The fingerprints of caeca, ileum and droppings exhibited from about 25 to 40 bands whatever the labelling (Hex or 6-Fam). The pattern of the ileum was closer to the cloacal content than to caecal one. Variability was noticed between individuals and it was reduced by pooling the samples. The highest individual variability was noticed for the droppings.

The evolution of similarity according to the pool complexity was studied. Pools of 3, 6 and 9 individuals were performed. A higher degree of similarity was observed between pools than between individual samples. For the droppings, more variability was observed. Pools of 6 individual samples appeared to be a satisfactory compromise between variability and representative information for all the gut compartments and droppings.

Single strand conformation polymorphism CE-(SSCP) is used as the molecular principle for separation of fragments either by capillary electrophoresis (CE-SSCP) or by polyacrylamide gel electrophoresis (and gel-SSCP) techniques. These principles of separation were used to study the consequence of rearing conditions on the gut microflora (section 7.3) and to demonstrate perturbation in the intestinal flora affected by alternatives to AGPs (section 7.5).

7.2.3 - RFLP-T-RFLP

RFLP, the 16S rDNA gene was amplified with general primers and the product was digested with a four-base cutter and fragments resolved by agarose-gel electrophoresis. RFLP has been used with uncultured bacterial communities from the early 1990es (Pukal 2006). Fragment length profiles can be compared directly by cluster analysis or length profiles can be compared to a database for identification of bacteria (Pukal 2006). In T-RFLP the 16S rDNA gene is also amplified with general primers like in RFLP but the 5' primer was fluorescently labelled. The product is then also digested with a four-base cutter and fragments resolved on an automated DNA sequencing machine (Pukal 2006). The detection level was quoted to be 0.1 - 1.0% of the bacterial community (Pukall 2006). T-RFLP was first described by Clement *et al*., (1998) using univeral 16S rRNA primers for amplification and HaeIII, Sau3A or MspI for restriction. T-RFLP has been set up by using the universal 16S rDNA primers S-D-Bact_0008-a-S-20 forward and S-D-Bact-0926-a-A-20 reverse with the forward primer being FAM labelled. The purified PCR products were cut with the restriction enzyme HhaI before separation of the terminal fragments on a sequencer (e.g. ABI 3100). Samples are mixed with an internal standard (GS-500 ROX, PE Biosystems) before the run (Annex 2).

RFLP and T-RFLP were used to distinguish different rearing systems (see section 7.3), to monitor the effect of AGPs on the intestinal microflora and on the course of an infection with pathogens (see section 7.4.2) and to detect the impact of feed additive (see section 7.5).

7.2.4 - FISH

FISH was originally developed for simultaneous microscopic observation and taxonomic characterization of bacteria in environmental samples without culture (De Long *et al*., 1989). The method was optimized for the detection of bacteria in the GIT of chicken (Olsen *et a*l. 2008). The objective was, in a first part, to develop a method for the detection of bacteria in faeces and intestinal material. Tested parameters included load of material per sample, permeabilisation of bacteria, conditions and group-specific probes and comparison between manual and automatic counting were tested.

Optimisation of the FISH method was performed with birds obtained from a commercial source. Procedures were trained for dissecting the birds and removing the intestinal content. The amount of intestinal content per sample was adjusted according to microscopic counting. In parallel experiments, the amount of Cy3 labelled oligonucleotide probe was adjusted and different procedures for fixation and permeabilisation of bacteria were compared. The last part of the optimisation included testing a range of reference strains against a panel of probes. Based on the test, 12 probes were selected for the final protocol (Protocol Annex 2).

The developed method was used with intestinal material from three experiments in order to characterize intestinal flora of organic free range rearing systems (see section 7.3), to demonstrate perturbation in the intestinal flora affected by alternatives to AGP (see section 7.5).

7.3 - Possible use of molecular methods to distinguish different rearing systems

7.3.1 - Difference between two rearing system run under similar management

The objective of this work was by performing two trials in parallel inside a farm of two partners (AFSSA and INRA).

A) Material and methods

In order to limit the individual variability, the similarity of pools of 6 animals within and between housings was studied by CE-SSCP method. Molecular gut patterns of animals coming from the same hatchery and reared under the same conditions in parallel were compared (AFSSA and INRA experiments). Though the feed, the animal origin and the environment (temperature, light) were strictly controlled, the weights of AFSSA animals were higher. At 26 days of age, animals were killed by intravenous injection, ileal and ceacal contents were collected. The numeration of specific flora (Total Aerobic Bacteria, Coliforms, Lactic bacteria) was performed by using conventional cultural methods at INRA. The total culturable and unculturable flora was analysed by performing CE-SSCP. Dendrograms were made from fingerprints with the Bionumerics software by using the UPGMA statistical method and the Pearson coefficient (Protocol in Annex 2).

B) Results and discussion

The efficiency of feed conversion was higher for the animals from INRA. Indeed, more coliforms were detected in caeca and cloacal samples of animals from INRA compared to AFSSA. Clusters from CE-SSCP within husbandries showed a higher similarity with respect to the types of samples than between the two husbandries. The pools of caeca showed highest similarity (90%) compared to the pools of ileum and cloaca, respectively pointing out a specific flora in the caeca. The ileum patterns were more similar to those of cloaca and caeca.

The results showed that some major bacterial taxa are present in the broilers from different houses but at different relative abundances. However, the presence of specific bacterial populations of the husbandry could not be detected in the ileum and cloaca. Moreover, when comparing the samples from the two husbandries, caeca samples are more similar than ileum or droppings. The CE-SSCP method is reproducible enough to cluster pooled samples from husbandry and sensitive enough to discriminate samples from two different husbandries.

7.3.2 - Characterization of intestinal bacterial flora of an organic free-range rearing system

The objectives were to characterize the intestinal bacterial flora of an organic free-range rearing system by conventional and by genotypical methods and to evaluate the molecular methods T-RFLP, SSCP and FISH. Organic free-range rearing systems are expected to include strong differences in the intestinal microflora compared to conventional conditions related to differences in the breeds of birds, feed and bird environment. Few investigations of organic broilers were performed and suitable techniques still are searched for. Farms with bird sizes of 8000-10000 and 4000 were investigated for conventional and organic systems, respectively with sampling in relation to age of birds and season of year.

A) Material and methods

Experimental trial and sampling

The analyses were performed into 8 poultry Italian farms: 4 organic (2 organic and 2 organic-plus) and 4 conventional farms. Organic-plus (OP) system has with the same rules of organic but uses slow-growing poultry strains and with higher grass availability (10 m^2/outdoor area/bird). For each experiment 24 chicken intestines were collected. The caecum and the ileum were removed aseptically from each bird. Six samples of intestine were pooled to obtain 4 samples of each intestinal region (ileum and caecum). In conventional farms, intestinal samples were collected at 40 days whereas samples of the organic groups were collected at both 40 and 80 days of age (end of cycle).

In experiment 1, different farming systems and different seasons were collected (Table 51).

Table 51: Experimental design of experiment 1

Farming system	Number of farms	Day of age	Season	Number of Intestinal samples
Conventional	4	40	Autumn and winter	96
Organic	2	40-80	Autumn, winter, spring, summer	194
Organic plus	2	40-80	Autumn, winter, spring	192

The experiment 2 was executed to verify the effect of age on the bacterial count of Italian and Austrian conventional and organic broilers (Table 52). Intestinal samples were ileum and caecum.

Table 52 : Experimental design of experiment 2

Farming system	Day of age	20	30	40	60	80
Conventional	**Number of farms**	10	10	20		
Organic		10	10	20	20	20

Bacterial counts

One gram of intestinal content was transferred to a sterile measuring tube together with 2 ml of 0.9% sterile saline solution. The stool was pressed and mixed in this solution and the volume was completed to 10 ml with 0.9% sterile saline solution. Each pool (0.1 ml) was diluted serially via 10-fold dilutions (from 10^{-1} to 10^{-10}). MacConkey agar, Violet red bile agar and Kenner Fecal (KF) agar were respectively used for the enumeration of *Enterobacteriaceae*, *Streptococcus* and *Enterococcacea*e. Baird Parker agar and Mannitol salt agar were used for enumeration of Staphylococcaceae. All the plates were incubated at 37 ℃, aerobically, for 24h - 48h and the number of grown colonies was determined. For the enumeration of anaerobic bacteria Schaedler agar, enriched with 5% sheep blood and 1 mg/ml K1 vitamin, was used as anaerobe blood agar. The anaerobe blood agar supplemented with 7.5 mg/ml vancomycin and 100 mg/ml kamamycin was used as kanamycin vancomycin blood agar. Anerobic bacteria were estimated by Reinforced Clostridial agar. Incubation was made in anaerobic jars for 48 h. Anaerobic conditions were obtained with Anaerogen (Oxoid) and were controlled by methyl blue strips as oxidation reduction indicator. For the enumeration of lactobacilli Rogosa agar was used. The plates were incubated for 3 days at 35 ℃ under microaerop hilic condition. All the data were expressed as CFU x Log/g.

Molecular methods

Gel-SSCP, T-RFLP and FISH methods were performed according to protocols described in Annex 2.

B) Results and discussion

Significant differences among bacteria (Enterobacteria, *Staphylococci*, *Enterococci,* Anaerobia and *Lactobacilli*) of ileum and caecum were found with respect to **bacterial counts** even though the variability between farms rendered it difficult to clearly discriminate between rearing systems.

The total aerobic bacteria were higher in the caecum than in the ileum for both organic and conventional systems mainly due to the enterococci and lactobacilli which showed the same tendency. On the contrary, organic chicks at 80 days of age had an opposite development (higher counts in ileum than in caecum) for *Enterococci, Lactobacilli* and total aerobic bacteria. *Staphylococci* were always very low with a non apparent outline related to intestinal section and age. These results indicated a decrease of enterococci counts related to age of birds in organic production. Results from the experiment 2 confirmed the previous findings regarding the farm variability anyway the total anaerobic counts were higher in the caecum than in the ileum and these values were higher in conventional compared to organic farms. Counts increased with age in specific groups. The Total Aerobic Bacterial count was highest in the caecum of organic chickens compared to conventional ones, however, this value decreased with the age. The Member of *Enterobacteriacae* were more frequent in conventional compared to organic production both in ileum and caecum, however, enterobacterial counts increased for organic chickens at 80 days of age with respect to ileum samples. Counts of *Lactobacillus* sp. populations were higher in organic than in conventional chickens in caecum and ileum, respectively. Enterococci were better represented in caecum of organic chickens than in caecum of conventional chickens. Further investigations are necessary to explain the importance of such changes in the physiology of digestive tracts in free-range animals.

The results of the **T-RFLP** showed as expected a more diverse caecal microflora as compared to ileal flora. The ileal samples were very fluctuating and it was not possible to distinguish organic from conventional farms with ileal samples. Also T-RFLP data found a clustering of the caecal samples according to farm. The profiles from the different farms (and sampling date) clustered out together. There was a 75-85% similarity between the samples from the same conventional farm and only around 50% similarity between different conventional farms. Thus, each conventional farm seems to have a specific caecal microflora. The same observation was made regarding organic farms, when samples from 40 days of age were compared. The within-farm-similarity was here around 60-75% and the similarity between farms was only 40-53%.

The **SSCP** analysis of ileum and caecum samples of 40 days old organic chickens showed remarkable differences between these intestinal contents with below 50% similarity whereas the similarity was around 70% within clusters of each section related to high variation between farms. It was remarkable that a region of light fragments of ileum samples was not present in the samples from caecum and a region of medium to heavy fragments in caecum samples lacked from the ileum samples. In addition high variability between farms were recorded. Intestinal samples from conventional farms were not analysed with this method. Comparison of profiles of intestinal samples was made to reference strains of bacteria (see Annex 2) found in the intestine. However, the light ileum fragments did not correspond to any of the reference bacteria. One explanation could be that these light fragments represented yeast in correspondence with observation from FISH.

FISH counts performed on 40 days old organic chickens showed relative higher counts of the Bacteroides group (Bacto1080) (46.4.± 46.9 10^7cells/g) whereas counts of lactobacilli

(LGC354A) (4.1±2.7 0^7cells/g) were lower than those reported for conventional production systems in other investigations. These counts are mean of duplicate samples of 4 farms each with a pool of 6 birds analysed with SD in parenthesis. The variability (SD) between samples is too high to provide quantitative data. Unfortunately, counts were not available for the conventional chickens in the present investigation. The intra-assay coefficient of variation (CV) between triplicate FISH determinations of the same luminal sample was 33% as a mean. This variation seems to be related to the small sample size analysed on each filter. However, the variation was less than the 90% CV determined between organic farms. The relative lower count in organic broilers compared to conventional was explained by lower feed intake by organic chickens and corresponding lower nutrients available for the intestinal microflora. In addition, high numbers of yeast were counted in the organic chickens. These cells were detected without staining by epifluorescence microscopy using UV-region excitation (data not shown) and an unknown yeast component might have reduced counts of lactobacilli by competition for a common nutrient resource.

C) Conclusion

By culture-based methods differences between conventional and organic production were difficult to observe both related to high farm variability and a strong effect related to bird age. A multivariate factor analysis of cultured based counts for mostly allowed discrimination of caecal samples belonging to different housing systems. Molecular methods (SSCP, T-RFLP, FISH) documented high variation between farms including the organic ones and T-RFLP confirmed the age differences observed by culture. In line with culture and T-RFLP, SSCP also showed high variation between ileum and caecum samples. Contrary to the high counts of *Lactobacilli* in organic chickens determined by culture based technique, low counts were found by FISH. The high numbers of yeast cells observed in the lumen of the ileum of the organic reared chicks requires further investigation. All methods were suitable for investigation of the microbial ecology of organic reared broilers. A combination of culture based counting/identification, DNA fingerprinting (SSCP or T-RFLP) and culture independent counting/identification (FISH) will provide detailed information of organic reared broilers for further investigation. In further investigations it is important to account for a high variability between farms when experiments are planned.

7.4 - Possible use of fingerprint methods to monitor the effect of infection by pathogen(s) on the intestinal microflora

7.4.1 - Monitoring of gut microflora from broilers naturally infected with *Salmonella* or *Campylobacter*

The aim of this study was to analyse intestinal and faecal content of broilers naturally and experimentally infected using gel-SSCP analysis for finding specific patterns to identify *Salmonella* and /or *Campylobacter* infected flocks. A 16S rRNA gene specific primer was used (Schwieger *et al.*, 1998; Ott *et al.*, 2004).

A) Material and methods

Experimental trial and sampling

At four different seasons (spring, autumn, winter and summer) four to six flocks at each period were included in the study. In total 15 different poultry flocks from ten different farms were tracked. Caecal and ileal contents from the beginning of the fattening period until slaughter were sampled each week. The first faecal samples were drawn before stabling by analysing transport diapers, followed by the analysis of four pooled faecal samples and 4 individual birds from each flock each week. Organic flocks (N=4) were fattened for eight to nine weeks and conventional flocks (N=11) for four to six weeks. Fractionated slaughter (the flock is slaughtered in two parts; interval: a week) was used for 9 flocks.

Bacterial counts

For identification of *Salmonella and Campylobacter* infected broiler flocks pooled faecal samples were analysed according to the ISO regulations (657-1998 and 10272-2002 respectively).

Molecular method: SSCP

Protocol is decribed in Annex 2.

B) Results

By analysing 15 Austrian flocks for the occurrence of *Salmonella* from farms with intensive production and organic production we were not really lucky in the acquisition of naturally with *Salmonella* infected flocks. Only three flocks became *Salmonella* contaminated during the fattening time. The *Salmonella* serotypes that were identified were Senftenberg and Montevideo. No difference between *Salmonella* infected and *Salmonella* free flocks and individual samples were seen regarding the conventional microbiology results.

Regarding **SSCP** profiles, in *Salmonella* naturally infected and not infected samples, cluster analysis revealed that flocks cluster together independent of their *Salmonella* status. In opposite faecal samples from *Campylobacter* naturally infected flocks cluster together and not infected cluster together.

C) Conclusion

No significant differences between *Salmonellla* or *Campylobacter* infected pooled faecal samples and not infected flocks or individual birds (ileal or caecal samples) were found. Thus, influences of flock, feed and production system may have overlaying specific patterns. In opposite clusters were identified for pooled faecal samples in naturally *Campylobacter*

infected birds. By gel-SSCP cluster analysis differences could be detected at flock level. As are different samples of one flock clustered together. Thus, the individual microbial community of a flock could clearly be detected. Furthermore the method was sensitive enough to distinguish between organically and conventional reared flocks. In addition, the method was suitable to discriminate between different intestinal compartments and pooled faecal samples. These strong specific of differences in profiles between microbial communities of production systems might have masked less strong components of the microbial community e.g. related to an infectious source like *Salmonella* and this explain why *Salmonella* were only been detected in low amounts in the pooled faecal samples as well as the intestinal content.

7.4.2 - Possible use of molecular-based methods to monitor the effect of AGP on the intestinal microflora and on the course of an infection with pathogens by the molecular methods

Salinomycin is an ionophoric coccidiostat, which is widely used as a supplement in poultry feed to control infection with *Coccidia*. In addition, it is known that Salinomycin, has an inhibitory effect on *C. perfringens.* Thus, the use of Salinomycin leads to a decrease in the incidence of necrotic enteritis in broiler chickens (Elwinger *et al*, 1992; Vissiennon *et al*., 2000). It has also been reported that Salinomycin can reduce the occurrence of *Salmonella* in chickens. However, Scalzo *et al*., 2004 found no reduction in the level of *Salmonella* shedding by the use of Salinomycin. In fact, they observed an increase of one Log unit on *Salmonella* CFU between control and Salinomycin treated chickens.

By 2006, the use of all AGP was prohibited in the European Union. Also the use of ionophore coccidiostats is expected to be banned in the near future (Van Immersel *et al.*, 2004). The consequences of such a ban on the incidences of *C. perfringens* related diseases or on the occurrences of *Salmonella* and *Campylobacter* are essentially unknown. Likewise, the action and selective pressure of Salinomycin on the normal intestinal microflora of broiler chickens is still not clearly elucidated.

The purpose of this study was to examine the effect of Salinomycin on *C. jejuni* infection, a *Salmonella* Typhimurium infection and on the composition of the microflora of the ileum and caecum in broiler chickens. This was completed using experimental infections and bacterial culturing. The composition of the intestinal microflora was studied using the DNA fingerprinting techniques SSCP, T-RFLP (for both experimental infections) and DGGE (the *Campylobacter* infection). Cloning and sequencing of 16s rDNA was likewise carried out on samples from the *Campylobacter* experiment.

A) Material and methods

Experimental design, sampling and bacterial counts

The chickens used in this study were conventional broiler chickens (Ross) of mixed sex, purchased as day-old from a local hatchery (DanHatch A/S, Randers, Denmark). The chickens were transferred directly from the hatchery to the experimental unit, where they were housed in isolators (Montair Andersen B.V. HM 1500, The Netherlands). Initially, transport-boxes, feed and water samples from each isolator, and cloacal swabs from 5 randomly selected chickens from each isolator, were analysed for the presence of *Campylobacter* (experiment 1) or *Salmonella* (experiment 2) by cultivation (ISO 657-1998).

Experiment 1: *Campylobacter*

The chickens were divided into 6 experimental groups (Table 53). The 6 groups, each with an initial size of 37 day-old chickens, were kept in separate isolators. The chickens had access to feed and water *ad libitum*. They were fed a conventional wheat based broiler feed without antimicrobial additives (Faculty of Agricultural Sciences, University of Aarhus, Denmark). However, the diet of group 1 and 2 was supplemented with Salinomycin (75 mg/kg) from day 8 and onwards. At day 14, all chickens from group 1 to 4, were orally inoculated with approximately 1.10^9 CFU of *C. jejuni* strain DVI-sc181. The control groups (group 5 and 6) were given a 0.9% NaCl solution. The inoculation was carried out inside the isolators by individual oral gavage of 500 µl of a bacterial suspension or a NaCl solution, using a 1 ml syringe with an attached flexible tube.

Experiment 2: *Salmonella*

The second experiment was conducted in the same way as experiment 1, except that the birds were inoculated with 10^9 CFU rifampicin resistant *Salmonella* Typhimurium at day 15

instead of *Campylobacter*. Samples were taken at day 5, 12, 19, 26 and 35 following the same procedure as exp 1. *Salmonella* were quantified on Rambach agar (Merck 07500) containing 50 µg/ml Rifampicin, incubated aerobically at 37°C for 24 h. CFU counts were performed according to protocols described in section 7.4.1.

Table 53 : Experimental design

	Experiment 1		Experiment 2	
Isolator/Group	Salinomycin – From day 8	*C. jejuni* – at day 14	Salinomycin – from day 8	S. Typhimurium – from day 15
1	+	+	+	+
2	+	+	+	+
3	-	+	-	+
4	-	+	-	+
5	-	-	-	-
6	-	-	-	-

Molecular methods

SSCP, T-RFLP: see Annex 2

DGGE and cloning analysis: see Annex 2

Phylogenetic analysis: The clones were analysed in order to visualize their similarities to known bacterial species. Multiple sequence alignments of the nucleotide sequences were performed using the CLUSTAL W program (Pedersen *et al*., 2003) and the alignment editor GENEDOC. The phylogenetic analysis was made using the software PHYLIP and TREEVIEW. A neighbour joining tree was generated on the basis of 280 base pair sequences and the robustness of the tree was evaluated by the bootstrapping-resembling method with 1000 replicates.

FISH: see Annex 2

B) Results and discussion

Stable infections were obtained by the inoculation with *Salmonella* and *Campylobacter*, respectively, according to previously developed infection protocols. Chickens being supplemented with Salinomycin gained significantly more weight than control birds. *Lactobacilli* were found in high counts in all samples but no effect of Salinomycin on *Lactobacillus* counts was detected. Likewise, no effect of Salinomycin on the course of a *C. jejuni* or a *Salmonella* infection could be demonstrated.

Analysis of **T-RFLP** results using cluster analysis software (BioNumerics) demonstrated no clustering of *Campylobacter* or *Salmonella*-infected versus non infected broilers. Cage groups, caecal and ileal samples cluster together.

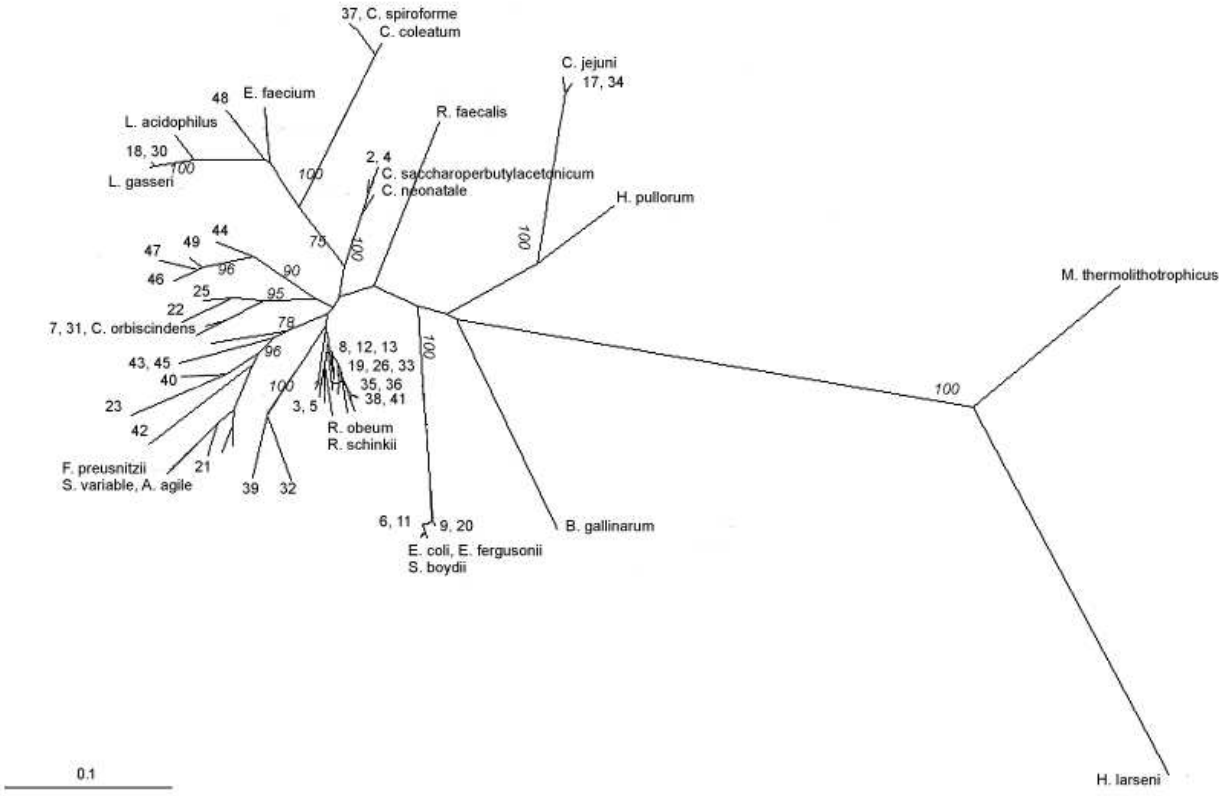
Regarding gel-**SSCP** profiles in *Campylobacter* experimentally infected and not infected flocks, no conclusive cluster was seen for infected and not infected samples. Nevertheless, samples from *Campylobacter* naturally infected flocks cluster together and not infected cluster together (see section 7.4.1). The experimental *Campylobacter* infection seemed to have no effect on the gut microflora. However, a clustering was seen of birds fed Salinomycin versus birds given no coccidiostats in the feed.

DGGE was run on samples from *Campylobacter* / Salinomycin (experiment 1). DGGE profiles of the caecal bacterial community of 7, 13, 16, 23 and 30-day-old chickens were analysed by using BioNumerics software. Already from day 13 and onwards Salinomycin treated chickens and control chickens clustered into two different groups, indicating an effect of Salinomycin on the normal intestinal microflora.

Clone libraries:

Forty-nine clones were chosen for sequencing on basis of their different migrations in the DGGE gel. Blasting the sequences resulted in two clones being most closely related to *C. jejuni*, 2 to *Clostridium* neonatale, 1 to *Lactobacillus* sp. and 3 were most closely related to *Shigella boydii* and *E. coli*. Forty sequences were most closely related to an uncultured bacterium. A neighbor joining distance tree (Figure 14) showed that 12 clones clustered with *Ruminococci.* Seventy-five per cent of these originated from non-treated caecal samples. Another group of 7 clones clustered with *Faecalibacterium prausnitzii* and related organisms. The remaining clones clustered among *E. coli, C. jejuni* and different clostridia or *Lactobacilli*. Two of the clones originating from Salinomycin treated chickens were related to *Clostridium* or *Lactobacillus* sp. respectively.

Figure 14: Clustering of 40 clones and 22 well-known bacteria



Bacteria	Acc. no.
Campylobacter jejuni	DQ174141
Rhodopseudomonas faecalis	AF123085
Clostridium neonatale	AF275949
Clostridium saccharoperbutylacetonicum	U16122
Clostridium spiroforme	X73441
Clostridium coleatum	AF028350
Enterococcus faecium	Y18294
Lactobacillus gasseri	AY730721
Lactobacillus acidophilus	M58802
Clostridium orbiscindens	AY730665
Faecalibacterium prausnitzii	X85022

Bacteria	Acc. no.
Subdoligranulum variable	AJ518869
Anaerofilum agile	X98011
Ruminococcus schinkii	X94964
Ruminococcus obeum	X85101
Shigella boydii	AY696681
Escherichia fergusonii	AF530475
Escherichia coli	DQ182324
Bacteroides gallinarum	AB253733
Methanococcus thermolithotrophicus	M59128
Haloferax larseni	AY838278

In the neighbour joining distance tree, branch lengths reflects genetic distances and bootstrap values, obtained from 1000 resampled datasets, are shown in *italics*.

FISH was performed on the ileal samples from the infection experiments. Different probes were used to quantify: total amount of bacteria, *Lactobacilli*, *Bacillus*, *Enterococci* and *Streptococci*, *E. coli*, *Streptococcus*, Bacteroides and *Clostridia*. The results showed total counts of up to 10^9 cells/g in the ileal content, with the highest total counts found at day 36 in the group given Salinomycin in the feed. *Lactobacillus* counts were found highest at day 23 in all groups and ended up at around 10^8 cells/g at the end of the experiment (day 36). To our surprise the highest counts of *Clostridia* were found in the group fed Salinomycin.

C) Conclusion

In this study no effect of Salinomycin on a *C. jejuni* or a S. Typhimurium infection in chickens was found. This is in agreement with Bolder *et al* 2003 who found that Salinomycin was unable to affect both the incidence and the degree of *Campylobacter* shedding. However, by using the fingerprinting technique DGGE, no effect of Salinomycin was found on the intestinal microflora of the caecum after 5 days of treatment (Day 13) in control chickens and after 15 days of treatment (Day 23) in *C. jejuni* infected chickens. Knarreborg *et al*. found a similar effect of antimicrobial supplementation on the composition of the microflora in the ileum of broilers. The T-RFLP technique did not result in the same clear trend from either the *Campylobacter* nor the *Salmonella* experiment. The clones originating from Salinomycin treated chickens were widely distributed among the phylogenetic groups. Thus, cloning and sequencing gave no unambiguous result concerning specific groups being affected by Salinomycin. Future studies, with larger clone libraries are a necessity to elucidate this result.

7.5 - Possible use of molecular-based methods to detect the impact of feed additive

The main objective of the study was to evaluate the feasibility of the SSCP, CE-SSCP, FISH and RFLP methods to demonstrate perturbations (of high or low extent) in the chicken intestinal flora, induced by the use of AGP and different potential alternatives to AGP (including some feed additives and the change of the feed structure).

The potential additives alternatives to in-feed antibiotics tested were prebiotics (Fructo-olisaccharides, FOS), probiotics (Bactocell ®), botanicals (essential oils and saponin) and organic acids (calcium formate, sodium butyrate, lactic acid, organic acids blend). Diet structure was modified by the use of wheat given as whole grain with a protein concentrate instead of ground in the complete diet. To compare the results obtained with these potential alternatives to those obtained with antibiotics, an AGP (Avilamycin or Salinomycin) was included in all experiments.

Four experiments were performed and the effect of the different alternatives to in-feed antibiotics was studied on animal performance, and the digestive tract particularly on digestive microflora of broiler chickens. Microflora was studied by classical bacterial counts (*E. coli*, *Lactobacillus* sp. and aerobic mesophilic bacteria for INRA and AFSSA experiments; *E. coli*, *Clostridium perfringens*, and *Lactobacillus* sp. for IRTA experiments) and molecular methods as FISH and fingerprint methods. The fingerprint methods used in this work were fingerprint method on gel (SSCP, TTGE) allowing identification of specific bacteria of dietary treatment, and two high-throughput fingerprint methods (CE-SSCP, RFLP).

Moreover the effect of the prebiotic and whole wheat on functionality of the digestive tract was assessed by measuring the gut morphology (villus and crypt size) and the intestinal enzymatic activities of the mucosa. The effect of the probiotic on the morphology of intestinal tract was also studied by gut morphology. The effect of botanicals and organic acids on physical characteristics of the digestive content was assessed by viscosity measurement.

7.5.1 - Effect of botanicals

The study of the effect of **botanicals was** performed**.** This study involved to test the efficacy of **essential oil**s and **Saponin** (Yucca extract) on performance of broilers chickens and on intestinal viscosity, and to elucidate the effect of these compounds on the intestinal microbiota composition by molecular techniques (CE-SSCP, RFLP, FISH).

A) Material and methods

The animal experiment was conducted in the experimental farm of IRTA (Spain) during 36 days. Birds, reared in cage, were fed with a mash diet based on wheat, barley and soybean meal. Six dietary treatments were tested as follow: 1) a negative control; 2) positive control 10 ppm Avilamycin; 3) Thymol at 225 g/ton, 4) Carvacrol at 75 g/ton; 5) Cinnamaldehyde at 500 g/ton and 6) Yucca extract at 750 g/ton. Dietary treatments were replicated six times each and allocated at random by blocks. Body weight, average daily gain, average feed consumption, feed to gain ratio and mortality were determined at 10, 24 and 36 days. At day 22, six chickens per cage were sacrificed and samples from the distal part of the small intestine were taken, pooled and sent for microbiota analysis: conventional counts (CReSA), fingerprint by RFLP (CReSA) and CE-SSCP (AFSSA), and bacterial count by FISH (UOC) (see Annex 2).

The samples for bacterial analysis were successively diluted at 1/10 in 9.5 g/l Maximum Recovery Diluent (Merck) and analysed for *Escherichia coli*, *Lactobacillus* sp and *C. perfringens*. *E. coli* were counted by plating serial dilution on McConkey Agar (Difco) and

incubated aerobically for 24 hours at 37ºC. The Lactic acid bacteria were counted after being plated onto MRS agar (*Lactobacilli* MRS Broth, Difco, supplemented with 15g/l of Bacto Agar, Difco) and incubated anaerobically for 48 hours at 37ºC, and *C. perfringens* were counted by plating on Perfringens Agar (Oxoid) supplemented with Perfringens Supplement A and B (Oxoid) and incubated anaerobically for 24 hours at 37ºC. The results were expressed as Log_{10} CFU/g of digestive contents.

The effect of botanicals on physical characteristics of the digestive content was assessed by viscosity measurement.

B) Results and discussion

From 0 to 24 days, dietary treatments affected significantly ($p<0.05$) body weight, weight gain and feeds conversion ratio (FCR). Birds fed yucca extract grew faster than birds given cinnamaldehyde, thymol or control diets and chickens fed Carvacrol and Avilamycin also grew faster than control birds ($p<0.05$). Birds fed yucca extract showed better feed to gain ratio than those birds fed Avilamycin or control diets. The use of carvacrol or cinnamaldehyde also improved feed to gain ratio compared to control ($p<0.05$). In overall, no differences in feed intake were observed. From 24 to 36 days and in overall experiment (from 0 to 36 days) dietary treatments did not affect significantly performance parameters. The mortality rate in overall experiment was 2.2% and no significant effect of treatments was detected. Supernatant jejunum digesta viscosities ranged from 7.6 centipoises (cps) for the control to 8.7 cps for Yucca extract group, and no effect of dietary treatment was observed.

No effect of botanicals was observed on bacterial counts. The effects of Avilamycin and some botanicals, Yucca extract, Thymol, Carvacrol and Cinnamaldehyde were studied on different bacteria groups (*Lactobacillus, Bacillus, Enterococcus and Streptococcus, Streptococcus, Enterobacteria*) **by FISH counts.** High variability between samples was observed. With Avilamycin, numerically lower counts of each bacteria group were observed (except similar value for *Streptococcus*) compared with negative control. The use of Thymol and Carvacrol also tended to reduce the number of cells of each bacteria group, compared with the negative control, and the effect was similar to that of Avilamycin. With Cinnamaldehyde numerically lower counts of each bacteria group were observed compared with all the other treatments including Avilamycin. Yucca extract resulted in higher counts of Bacteroides, compared with botanicals and Avilamycin, and also compared with negative control.

First analysis of samples by **CE-SSCP** with universal primers revealed no significant differences between the dietary treatments. No high modification of the bacterial microflora was observed whatever the botanical used. In consequence, the samples were not joined together in clusters in function of the treatment. No significant band was detected when the fingerprint with 6-Fam labelling was observed. On the contrary, one band was present in two samples from animals treated with Yucca extract when the fingerprint with hex labelling was studied. This band was absent, in all the other samples. Only Yucca extract may cause a very slight specific modification of the microflora detectable by CE-SSCP with universal primers.

Analysis of samples by **RFLP** showed no significant differences between treatments but a significant effect of block (cage of the same block were located nearest than cages of other blocks). These results could represent a cross-contamination between the microbiota of different treatments located in the proximity of other treatments.

7.5.2 - Effect of a probiotic

The objective was to use two fingerprint methods (CE-SSCP and TTGE) to study the effect of a probiotic (Bactocell®) on digestive microflora, as well as the effect of an AGP

(Avilamycin). This AGP was used as a positive control to study if fingerprint technique were able to detect difference in digestive microflora. Bactocell contained *Pediococcus acidilactici*, a Lactic acid bacterium that produces a bacteriocin.

A) Material and methods

The animal experiment was conducted in the experimental farm of AFSSA (France) during 42 days. The birds were allocated to three dietary treatments: 1) a negative control with no additives, 2) a positive control containing 0.01 g/kg Avilamycin , 3) a treatment containing 1 g/kg (10^{10} CFU / kg) of probiotic Bactocell®.

The feeding program consisted of three different diets for each treatment: a starter diet (from 1 to 28 days of age), a grower diet (from 28 to 35 days of age), a finisher diet (from 35 to 47 days of age). The basal diets (the negative control diets) contained mainly maize, wheat and soyabean. For the treatments Avilamycin and probiotic, the Avilamycin and Bactocell® were incorporated at the expense of the same amount of maize. The feed and the water were supplied *ad libitum*.
At arrival, the birds were randomly distributed with 60 birds in each pen with 5 pens per dietary tretament with a similar weight per pen. The birds were individually weighed on day 14, 25 and 42. The feed intake in each pen was measured at the same age and the FCR calculated. The mortality was checked daily. Feed intake was expressed as animal present each day (i.e. dead birds were not included).
At day 25, fresh droppings were collected on the floor. At day 26, 6 chickens representative of their pens were selected (according to their weight) from each pen and were killed to sample ileal, cloacal and caecal contents. These samples were divided into aliquots for microflora analysis by conventional bacterial counts, molecular approaches and histological analysis (see section 7.5.4). Bacterial counts were performed to determine counts of coliform, Lactic acid bacteria and Aerobic Mesophilic bacteria as described previously. Molecular approaches were fingerprint technics (CE-SSCP, TTGE) and search for the probiotic was made. *Pediococcus acidilactici* was numerated in feed (meal before pelleting, pellet at the beginning and the end of the experiment) onto MRS-NaCl-TTC-vancomycine. It was also quantified by PCR. *Pediococcus acidilactici* was also numerated in dropping, at the beginning (5 days) and at the end (35 days) of the experiment.
The detection of the strain *Pediococcus acidilactici* present in the probiotic Bactocell®, was performed by PCR on pools of samples from negative control, Avilamycin and probiotic (5 pools / treatment). PCR was performed with the primers *pu* and *ppe* specific of the strain *Pediococcus acidilactici* as described by Mora *et al*.,1997.

B) Results and discussion

The **animal performance** results in this experiment showed an increase in feed intake and a negative effect on FCR with antibiotics (Avilamycin) during the whole period (1-42 days) compared to the negative control. With the probiotic a positive effect was observed on FCR during the whole period (1-42 days) although a negative effect was observed from 14 to 25 days.
No difference in surface of villi of ileum was observed between dietary treatments, but a lower crypt area was observed with antibiotic compared to control diet. Moreover a lower heterogeneity of surface of villi was observed with antibiotic compared to control diet.
Classical bacterial counts showed no effect of dietary treatment in ileal, cloacal and caecal content. However, in the fresh droppings, Avilamycin as well as the probiotic increased the number of coliform.

The **fingerprint method (CE-SSCP),** did not reveal high modifications of microflora after treatment with **Avilamycin** or **probiotic** neither with universal primers, nor with specific primers of Lactic acid bacteria. However, the absence or the weak intensity of two peaks in the ileum and the cloaca of only the animals treated with the antibiotic may be related to this treatment. Moreover, the presence of one minor peak in different gut compartment that did

not correspond to lactic flora may be linked to the treatment with the probiotic. Another one was mostly detected in the fingerprints of the caecal lactic microflora of some animals treated with probiotic.

The analysis of samples (5 pools of 6 birds per dietary treatment) with universal primers by the **fingerprint method TTGE** did not lead to cluster according to dietary treatment whatever the digestive content (ileum, cloaca, caeca). However when dietary treatments were compared by using one representative pool of digestive content per dietary treatment (30 birds per pool), some differences were observed. In the ileal content, probiotic diet led to a high intensity for a band corresponding to a *Lactobacillus johnsonii* which was also detected with negative and positive control diets but with low intensity. In the cloacal content, Avilamycin diet lead to the appearance of one band compared to control diet. Probiotic diet lead to the appearance of a band (c) migrating at the same level than the band (b) appearing with Avilamycin diet (Table 54). These bands (b, c) corresponded to a bacteria belonging to the order Clostridiales (Table 55).

Table 54 : Specific bands of dietary treatments observed with CE-SSCP and TTGE

Digestive segment	Dietary treatment	CE-SSCP [2]	TTGE (Univ) [2]
Ileum	Avilamycin	- Presence band 210.5 (LAB, 6-Fam) (Av 2, 4, 5) - Absence band 211.8 (Univ, 6-Fam) (Av 2, 4, 5; Very low intensity Av 1, 3) - Absence band 213 (Univ, 6-Fam) (Av 3, 4, 5 ; Very low intensity Av 1, 2) - Absence band 224 (Univ, 6-Fam)	
	Probiotic	- Presence band 206 (Univ, Hex) (P3, 4, 5)	- Presence band a (high intensity P2,4,5)
Cloaca	Avilamycin	- Presence band 220 (Univ, 6-Fam) (Av 1, 4, 5) - Presence band 210.5 (LAB, 6-Fam) (Av 2, 4, 5) - Absence band 211.8 (Univ, 6-Fam) (Av 2, 3, 4, 5) - Absence band 213 (Univ, 6-Fam) (Av 2, 3, 5; Very low intensity Av 1, 4)	- Presence band b (moderate intensity Av 2,3,4,5)
	Probiotic	- Presence band 206 (Univ Hex) (P2, 4, 5) - Presence band 197 (LAB, 6-Fam) (P1, 2, 4) - Absence band 221 (Univ, 6-Fam)	- Presence band c (moderate intensity P 2,3,4,5)
Caeca	Avilamycin	- Presence band 210.5 (LAB, 6-Fam) (Av 2, 3, 4, 5) - Absence band 185 (Univ, 6-Fam) - Absence band 221 (Univ, 6-Fam)	
	Probiotic	- Presence band 207 (LAB, Hex) (P1, 2, 5) - Presence band 208 (LAB, Hex) (P1, 2, 5) - Absence band 185 (Univ, 6-Fam)	
Fresh dropping	Avilamycin	- Presence band 184.5 (Univ, 6-Fam) (Av 2, 4, 5) - Presence band 204 (Univ, 6-Fam) (Av 3, 4, 5) - Absence band 211.5 (Univ, 6-Fam)	ND
	Probiotic	- Presence band 195 (Univ, Hex) (P1, 2, 4, 5) - Presence band 214.5 (LAB, 6-Fam) (P3, 5)	ND

ND : not determined

[1] Specific bands: absent or present bands with the dietary treatments (Avilamycin, probiotic) compared to negative control diet.

[2] Univ : universal primers ; LAB : Lactic Acid Bacteria primers ; Hex : Hex labelling ; 6-Fam : 6-Fam labelling

Table 55 : Characterization of bands observed by TTGE affected by dietary treatments

Digestive segment	Dietary treatment	Band	Bacteria identified	Accession number	Similarity (%)
Ileum	Probiotic	a	*Lactobacillus johnsonii*	AB295648.1	100%
Cloaca	Avilamycin	b	Order Clostridiales	AF376252.1	100%
	Probiotic	c	Order Clostridiales	AF376252.1	100%

7.5.3 - Effect of organic acids

The objective of the study was to evaluate the feasibility of the CE-SSCP, FISH and RFLP methods to demonstrate perturbations (of high or low extent) in the chicken intestinal flora, induced by organic acids, as alternative to in-feed antibiotics.

A) Material and methods

The animal experiment was conducted in the experimental farm of IRTA (Spain) during five weeks. Birds fed with the same experimental diet were placed in contiguous cages, separated physically from the other dietary treatments. Six dietary treatments replicate six times each with 18 birds per replicate, were studied.

The arrangements of treatments were: 1) negative control, 2) positive control (Salinomycin, 75 mg/kg feed), 3) Calcium formate, 4) Sodium butyrate, 5) Lactic acid, 6) combination of Formic and Propionic acids and Ammonium formate. The organic acids were added on top of the diet at the following doses: Calcium formate (68% formic acid) at 7.4 kg/ton, Sodium butyrate coated (30% butyric acid) at 1 kg/ton from 0-24 d and 0.5 kg/ ton from 24-35 d, Lactic acid (63%) was added at 5 kg/ton, blend of Formic and Propionic acids and Ammonium formate (43% expressed as Formic acid and Ammonium formate) at 4 kg/ton. Chicks were weighed in bulk on arrival, and per cage at 10, 24 and 35 days. Feed consumption per cage was recorded at days 10, 24 and 35. Average daily gain, average daily feed consumption and feed to gain ratio were calculated for the periods 0 to 10 days, 10 to 24 days, 24 to 35 days and for the overall experiment. At day 21, six chickens per cage were sacrificed and samples from the distal part of the small intestine were taken, pooled and sent for microbiota analysis: conventional counts and fingerprint by RFLP by CReSA and CE-SSCP by AFSSA, and bacterial counts by FISH (UOC) (see Annex 2).

B) Results and discussion

In this experiment, with organic acids and during the first 24 days, the blend of Formic and Propionic acids with Ammonium formate, as well as Calcium formate, improved substantially weight gain and increased feed intake, without affecting the FCR. The improvement was similar to those obtained with Salinomycin. In overall experiment (from 0 to 35 days), the organic acid blend tended to increase weight gain and feed consumption compared to the negative control and the Lactic acid group. The use of Calcium formate, Sodium butyrate and the organic acid blend impaired feed to gain ratio compared to negative control and Salinomycin groups. In overall growing period, positive effects of the use of organic acids in terms of weight gain could be detected. The lowest digesta viscosity was observed with calcium formate and the highest with sodium butyrate.

No cluster could be related to a treatment. No detectable high modification of the gut microflora was noticed by **CE-SSCP** with universal primers (to cluster according to dietary treatments). However, some modifications related to the use of Salinomycin were highlighted by using CE-SSCP. Thus, it was observed a disappearance of some bands or bands with lower intensity compared to the other diets.

The effect of **organic acids** analysed by **RFLP** showed significant differences between treatments**.** RFLP profiles corresponding to negative control as well feed supplemented with Sodium butyrate were clearly different to the other treatments. RFLP profiles of feed supplemented with Salinomycin and feed supplemented with Calcium formate were not clearly different. RFLP profiles of feed supplemented with Lactic acid and the blend of Formic and Propionic acids and Ammonium formate have some kind of association. Moreover, differences between treatments were detected in microbial biodiversity, as the number of electrophoretic bands in the RFLP profiles. For the negative control, the bacterial diversity was significantly lower ($p<0.05$), compared to the treatments with Salinomycin, sodium butyrate or organic acid blend. Furthermore, but without statistical significance, feed supplemented with Calcium formate or Lactic acid tended to increase the biodiversity in reference to negative control.

The effect of organic acids on counts of different bacteria groups were analysed by **FISH**. Higher number of bacteria belonging to Domain Bacteria (EUB338 probe) was detected in samples from birds fed sodium butyrate, compared with Lactic acid, organic acid blend or negative control. Lower counts with the LGC354C probe (*Enterococcus, Streptococcus, Lactococcus*) were observed with Salinomycin compared with negative control or organic acids diets. Some organic acids affected members of Enterobacteria, Bacteroides and *Clostridium* significantly. Calcium formate decreased Enterobacteria compared with negative control and Sodium butyrate diets. Lactic acid decreased Bacteroides compared with negative control, Salinomycin and Sodium butyrate diets. Calcium formate, Lactic acid and organic acid blend diet decreased *Clostridium* compared with Salinomycin diet.

7.5.4 - Effect of prebiotic

The objective of this experiment was to study the effect of a prebiotic that could potentially act on the gut microflora, the structure and/or the function of the digestive tract and thereby improve the performance of broilers. The feasibility of the SSCP, CE-SSCP and TTGE methods to demonstrate perturbations (of high or low extent) in the chicken intestinal flora was studied.

A) Material and methods

The animal experiment was conducted in the experimental farm of INRA (France) during six weeks. The birds were allocated to four dietary treatments: 1) a negative control (wheat based) with no additives, 2) a positive control containing 0.01 g/kg Avilamycin, 3) a treatment containing 0.6 g/kg of short chain fructo-oligosaccharides (FOS) and 4) a treatment with the same composition as treatment 1 but in which a part or all (400 g/kg) of the wheat was given as whole wheat and a concentrate complement. After 6 hours of fasting, the birds were individually weighed on day 11, 25, 36 and 42. The feed intake in each pen was measured at the same age and the FCR calculated.

For histological analysis, at 3 weeks of age, 6 chickens representative of their pens were selected (according to their weight). They were killed by intravenous injection of sodium pentobarbital. The digestive tract was removed from the beginning to the end of the intestine. The small intestine was divided into three segments: the duodenum (from gizzard to pancreo-biliary ducts), the jejunum (from pancreo-biliary ducts to Meckel's diverticulum) and the ileum (from Meckel's diverticulum to ileo-caecal junction). The middle part (1.5 cm long) of the duodenum and ileum was taken, from 3 of the 6 sampled animals per pen. The samples were opened longitudinally, rinsed with cold saline (NaCl 9 g/l) and fixed in a buffered formaline solution overnight. They were then rinsed and stored in ethanol/water (70/30, v/v) and stored at 4°C until further analysis.

At 3 and 6 weeks of age, 6 chickens representative of each pen were selected, and the digestive content (cloacal, ileal and caecal) were collected. The cloacal content was obtained by abdominal pressure on the birds before they were slaughtered. The content from the

ileum and caeca was collected by gentle pressure. These digestive content samples were pooled from the 6 animals per pen and stored at -70℃ until further microbial analysis.

For the determination of intestinal enzymatic activities, samples were taken from the 3 animals per pen used for histological analysis. The middle section (one third) of each intestinal segment (duodenum, jejunum and ileum) was split longitudinally, rinsed with cold saline, wiped on a paper towel and the mucosa scrapped off before freezing in liquid nitrogen and stored at -70ºC. The intestinal enzymatic activities that were studied were Alkaline Phosphatase (AP) (EC 3.1.3.1) and of the digestive enzymes maltase (EC 3.2.1.20) and leucine aminopeptidase (LAP) (EC 3.4.11.2).

The intestinal microflora was analysed by conventional bacterial counts and fingerprint techniques (SSCP, CE-SSCP and TTGE) described in Annex 2.

The conventional bacterial counts (*E.coli,* lactic acid bacteria and aerobic mesophilic bacteria) was described in section 7.4.1.

B) Results and discussion

In this experiment, Avilamycin showed a positive effect on performance (FCR and WG) during the whole period and an increase in leucine amino peptidase (LAP) enzyme activity was observed in duodenum..
For the treatment FOS, the feed intake and the DLWG were significantly reduced from day 1 to 25 and for the whole period. However, the FCR was significantly improved for the treatment FOS compared to the control from day 26 to 36 and throughout the experiment, which may be partly explained by the higher LAP activity observed in the duodenum.
At 3 weeks of age, the dietary treatments did not affect the gut morphology.
Avilamycin resulted in a decrease of aerobic mesophil bacteria counts in the caeca at 3 weeks, but with the prebiotic, no effect on bacterial count was observed.
The analyses of ileal samples **by SSCP showed that** six of the 24 samples were not PCR amplified. The eighteen samples that were amplified showed on SSCP gels , fragments at around 12 positions. For all samples two light fragments were obtained and a group of around 4 fragments of medium size. Two heavy fragments were obtained mainly with the **prebiotic** treatment. However, it was difficult to recognize consistent patterns in relation to nutrient treatment.
The analysis of samples (6 pools of 6 birds per dietary treatment) with universal primers by **CE-SSCP** showed no cluster in relation with a specific treatment whatever the digestive content (ceca, ileum, cloaca contents and fresh droppings). However some differences between diets were observed, with bands appearing only in some pools of some of the studied dietary treatments.
Avilamycin appeared to have a regulatory effect on digestive microflora as showed by the higher similarity between microflora TTGE profiles of caecal samples of birds fed Avilamycin compared to profiles of birds fed the control diet (Table 56). A regulatory effect of FOS on digestive microflora was also detected as showed by the higher similarity between microflora TTGE profiles of ileal and caecal samples of birds fed this prebiotic compared to profiles of birds fed the control diet (Table 56).

Table 56: **Similarity coefficient (SC) (Pearson correlations, %) obtained with TTGE for each alternative to antibiotic and each digestive segment [1]**

Intestinal segment	Dietary treatments		SC	Proba
	A: Control	B: Avilamycin		
Ileum	46	39	8.35	NS
Cloaca	34	47	7.72	NS
Caeca	92 b	94 a	0.71	0.024
	A: Control	C : FOS		
Ileum	45 b	67 a	6.97	0.030
Cloaca	47	52	6	NS
Caeca	93 b	98 a	0.53	<0.001
	A: Control	D : Whole wheat		
Ileum	43 b	62 a	6.13	0.038
Cloaca	44 b	59 a	4.93	0.041
Caeca	91 b	94 a	0.64	0.003

(a, b) : Means in the same row with no common letter differ significantly (p<0.05). NS : not significant.
[1] Six profiles of microflora of pools of 6 birds were compared per dietary treatment

As observed by CE-SSCP, the treatment with Avilamycin lead to disappearance of several bands in the three digestive contents compared to control diet. In ileum, a band (position 186, 6-Fam labelling) that was observed in four pools of the control diet was observed only in one pool and with low intensity with Avilamycin (Figure 15). Moreover, two bands that were observed in all the diets, were not observed with Avilamycin (Position 182.5 and 191.5, Hex labelling). In the cloaca, this diet led to the disappearance of one band (position 201.2, Hex labelling), and one bands that was observed in all the diets, it was not observed with Avilamycin (position 224.2, Hex labelling). In the caeca, two band disappeared (position 195.5 and 221.5, 6-Fam labelling). Thus Avilamycin led to the disappearance of some bacterial species. This is in agreement with the significantly lower number of bacteria observed in the caeca of birds fed on the Avilamycin treatment. This could be expected as AGP reduce the number of bacteria in the digestive tract (Thomke and Elwinger, 1998; Engberg *et al.*, 2000). The position of bands was not the same according to the gut compartment. In consequence, it should be not the same bacterial populations.

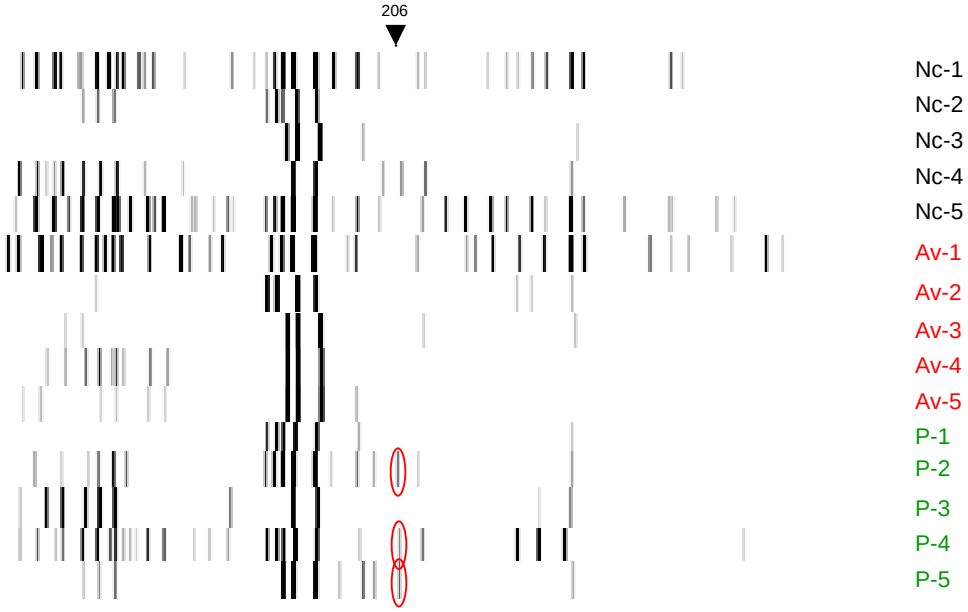


Figure 15: CE-SSCP fingerprints with universal primers (Hex labelling) obtained from cloacal pools (6 individuals). Nc : negative control; Av : Avilamycin; P: probiotic. Arrows indicated specific bands.

However, appearance of some bands was also observed with this AGP compared to control diet, as well as with CE-SSCP and TTGE. Thus, with CE-SSCP, in the caeca, a band absent in the control diet, was observed with Avilamycin (position 210.2, Hex labelling). With TTGE, in cloaca, a modification of profile was observed, as previously reported by Pedroso *et al* (2006) with DGGE. A band identified as long segmented filamentous bacterium was detected (Figure 15, Table 57).

Table 57: **Characterization of bands observed by TTGE affected by dietary treatments**

Digestive segment	Dietary treatment	Band	Bacteria identified	Accession number	Similarity (%)
Ileum	Prebiotic	a	Segmented filamentous bacterium	X87244.1	98%
	Whole wheat	b	Segmented filamentous bacterium	X87244.1	98%
		c	Lactobacillus salivarius	EF519868.1	99%
Cloaca	Control	e	Escherichia coli	EU014689.1	99%
	Avilamycin	d	Long segmented filamentous organism	X80834.1	99%
		f	Escherichia coli	EU014689.1	99%
		h	Order Clostridiales	EF071188.1	98%
	Prebiotic	g	Escherichia coli	EU014689.1	99%
		i	Order Clostridiales	EF071188.1	99%
	Whole wheat	j	Order Clostridiales	EF071188.1	98%

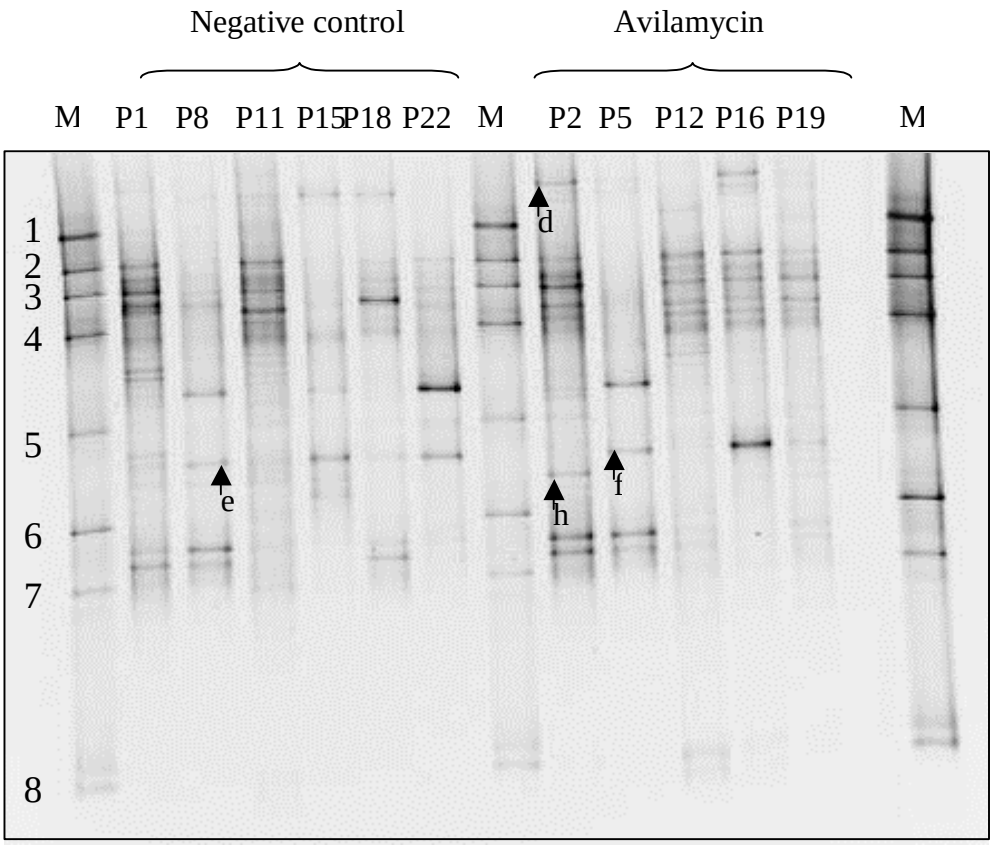


Figure 16 : Fingerprints of TTGE with universal primers obtained from cloacal pools

Pools (6 individuals) from negative control and Avilamycin fed birds. Arrows indicated specific bands. M: markers (1 to 8) of TTGE ladder.

Prebiotics led to the appearance of specific bands compared to control diet, as well as with CE-SSCP and TTGE. In ileum, compared to control diet, a band corresponding to segmented filamentous bacterium was detected with FOS by TTGE. In the cloaca, one band (position 230, 6-Fam labelling) was detected by CE-SSCP. TTGE also detected a band with this diet. In the caeca content, 3 bands were detected by CE-SSCP (position 205.2 and 209.5 with 6-Fam labelling, position 206.5 with Hex labelling). As previously observed with Avilamycin, the position of the bands was not the same according to the gut compartment. The effect of this prebiotic on microflora profiles is in accordance with previous study performed with DGGE (Massias *et al.* (2006)). The detection of a new band with FOS was in accordance previous reported stimulation of lactobacilli and bifidobacteria (Xu *et al.,* 2003; Griggs and Jacob, 2005).

7.5.5 - Effect of whole wheat

The objective of this experiment was to study the effect of a feeding method, which could potentially act on the gut microflora, the structure and/or the function of the digestive tract and thereby improve the performance of broilers. The feasibility of gel-SSCP, CE-SSCP and TTGE methods to demonstrate perturbations (of high or low extent) in the chicken intestinal flora was studied.

A) Material and methods

The same materials and methods that described in the previous section were used.

Moreover for the study of the gastro-intestinal tract, the gizzards were emptied, trimmed for excess fat and weighed, and the pancreases were collected and weighed. The weights were expressed as percentage of live weight.

B) Results and discussion

Whole wheat treatment resulted in a numerically lower feed intake and a significant lower Weigt Gain than in control. The FCR was not affected that maybe due to both positive and negative effects on digestive tract (higher gizzard and pancreas development, Leucine Aminopeptidase (LAP) activity and larger crypts in the ileum).
Although with whole wheat, no effect on bacterial counts was observed, differences were observed with fingerprint techniques.
With prebiotics, the gel-SSCP method was not able to show effect of whole wheat, but CE-SSCPand TTGE were able to show effect of whole wheat.
Regarding the two other dietary treatments studied, a higher similarity between microflora TTGE profiles of samples from birds fed whole wheat was observed compared to profiles of birds fed the control diet, showing a regulatory effect of this mode of feeding on digestive microflora (Table 57). This regulatory effect was previously observed in the microflora of the caeca in another study (Gabriel *et al*, 2007). These last results showed that this effect was also observed in ileum and cloaca.
The use of whole wheat in diet led to modifications of digestive microflora, as well as appearance or disappearance of bands compared to control diet. Resolution of fragments by the TTGE fingerprint technique with subsequent identification of these bands by sequencing showed that it was a segmented filamentous bacterium and a *Lactobacillus salivarius*. In the cloaca, contrary to control diet, a band was detected by TTGE, and in the caeca, a band (position 210.2 with Hex labelling) was observed by CE-SSCP. A fragment corresponding to *E. coli* disappeared in the cloaca of whole wheat fed birds compared to control. This is in accordance with the decrease of *E. coli* counts previously reported with this mode of feeding (Glünder et al, 2002; Gabriel *et al,* 2003).

7.6 - Conclusion

Comparison of results obtained by the molecular methods showed that each method provides unique information depending on target and way of working. All methods tested were able to show differences between production systems as well as between intestinal compartment on chicken intestinal bacteria (Table 58).

Neither effects of Salinomycin on chicken infection by *C. jejuni* or *S.* Typhimurium were found by using T-RFLP, however, cage group and each intestinal compartment clustered together. Nevertheless, by using gel-SSCP, the samples from *Campylobacter* naturally infected flocks clustered together, although no such clustering was found with samples from the experimental *Campylobacter* infection. The effect of Salinomycin was documented by DGGE.

Effects of feed additives were weakly reflected in the CE-SSCP generated fingerprints as well as results generated by the other methods except for RFLP and FISH. However CE-SSCP and TTGE were able to show some specific bands of dietary treatments, which will have to be identified. With chicken GIT samples, RFLP showed significant differences for organic acid treatments of feed. The negative control as well as feed supplemented with Sodium butyrate were clearly different to the other treatments. RFLP profiles of feed supplemented with Lactic acid and feed supplemented with Formic and Propionic acids and Ammonium formate had some kind of association. Moreover, differences between treatments were detected in microbial biodiversity related to the number of electrophoretic bands in the RFLP profiles. In the 2000es, T-RFLP has been more frequently used for analysis of microbial communities than RFLP. In a recent investigation of the effect of non-strach polysaccharides, T-RFLP combined with multivariate data analysis was used to document effects on microflora in ileum and caeca and related these changes with bird performance (Torok *et al.*, 2008).

Results obtained by quantitative FISH method showed that Salinomycin in feed reduced counts of *Lactobacillus* -, *Enterobacteriaceae* - and *Clostridium*-like bacteria in lumen of ileum compared to the conventional control. Increased or decreased bacterial counts were registered by Salinomycin in the ceca compared to the control. Further design of probes including hybridization conditions will be needed to use the FISH technique for routine quantification of bacterial phylotypes in the chicken intestinal tract. Resolution at the species level of bacteria is probably more limited with this method compared to the molecular DNA fingerprinting methods mentioned. The detection threshold of FISH technique limits its use for examining subdominant population.

Neither can one outstanding superior method be chosen nor can methods be excluded for further use due to serious limitations. If a methodology should be chosen to investigate treatment effects on the chicken GIT, the optimal choices seem to be CE-SSCP or T-RFLP together with high throughput DGGE using a multilane system. Both methods are needed since CE-SSCP enables a high processing rate by automation and SSCP or DGGE enable characterization of individual fragments by DNA sequencing. If specific groups of bacteria are identified by DNA sequencing to respond to treatments, further investigation is best carried out by real time PCR with these groups as specific targets.

Regarding the limitations, firstly, the procedures involved extraction and isolation of DNA are still a crucial step of the fingerprint analysis. Secondly, the main problems with these PCR-based techniques are that single fragments observed are not always caused by single PCR fragments. This is both related to divergent sequences migrating to the same length and to heteroduplex molecules formed as artefacts after PCR (Pukal 2006). Moreover, slight differences were noticed between different series of runs by using these fingerprints methods. The change of polymer may influence the profile even when the protocol is strictly followed. According to the sensitivity of the methods, the variability between individuals could

mask the effect of tested treatments. By using CE-SSCP a “pool strategy” consisting to mix samples from individuals coming from the same treatment was tested in order to reduce the variability between individuals.

The interpretation of profiles and dendrograms still is a real challenge because of the lack of reproducibility, the co-migration of fragments and the individual variability. Furthermore, the impact of the feed or the infection on the animal gut microflora is complex and multifactorial and only drastic changes could be showed by analysis of clusters. Other statistical methods such as Principal Component Analysis can be of use for the interpretation of profiles.

Clone libraries offer the highest degree of phylogenetic resolution available for culture-independent methodologies but can be cumbersome for analysis of the large numbers of samples that may be produced by studies of temporal or spatial variability of microbial communities. In addition, comparison of community composition between samples by using clone libraries can be problematic if libraries offer incomplete coverage of a community. The current challenge of these techniques is to compile standardized patterns in a database for interlaboratory use and future reference.

Table 58: Evaluation of molecular methods used to determine treatment effects on chicken intestinal bacteria

Factor	CE-SSCP	gel-SSCP	TGGE	DGGE	T-RFLP	RFLP	FISH
Discriminatory power	+	+	+	+	+	+	-
Reproducibility in the same laboratory	+	+	+	+	+	+	+
Standardization for comparison between laboratories	+	-	-	-	+	-	+
Ease of performance	+	-	-	-	+	+	-
Low analysis time	+	-	-	-	+	-	-
Interpretation of data	+	-	+	+	+	+	+
Low expences	-	+	+	+	-	+	-
Effect of GIT compartment	+	+	+	+	+	+	+
Effect of feed and feed additives	(+)	(+)	(+)	ND	(+)	+	+
Effect of production system	ND	+	ND	ND	+	ND	+
Effect of farm	+	+	ND	ND	+	ND	-
Effect of animal age	ND	ND	ND	ND	+	ND	ND
Effect of zoonotic bacteria	ND	-	ND	-	-	ND	-

+, capability; (+) single fragment difference only;-, inability or weak results
ND, not done or limited information

Section 8: Socio-economics aspects in poultry production in European countries

8.1 - Impact of the managerial models in poultry industry on food safety

8.1.1 - Identifying the current managerial models

A) Introduction

The literature does not explicitly provide a comprehensive view about the managerial approach in the poultry sector with respect to food safety issues. The main observable trends in the European scenarios are the relevant role of large-scale companies, the stable trend toward vertical integration and the attention for quality and safety issues, including organic production. Managerial approaches are thus shaped by these trends, within a competitive environment characterized by oligopolies. Food safety strategies have been elaborated and implemented by the most of companies and tend to affect all the relationships along supply chains. It is noteworthy that the most recent literature is dealing with the study of the factors influencing managerial choices in the field of food safety.

Internal devices (monitoring, Hazard Analysis Critical Control Point (HACCP), etc. are usually recognized as the major tools managing the food safety issues. However, since economic activities are not completely vertically integrated, the route chosen to coordinate agents may influence the final degree of safety.

In the economic analysis of food safety, scholars have increasingly taken into account the relationship between business organizations and management and the quality and safety outcomes in Agri-Food sector. Following diversified approaches, researchers have largely recognized the advantages of the neo-institutional theoretical approach in analysing quality and safety issues in the agro-food system and have pointed out the role of information in supporting viable exchange flows. Solutions for policy intervention have been explored, for example, when problems of moral hazard arise and the efficiency of regulatory approaches have been similarly analysed. Scholars have also examined the relationship between informative flows and signalling strategies providing and increasing amount of outcomes and insights. On the other hand, many natural and behavioural factors underlie the accidents and flaws, which influence the degree of safety of the products supplied to final consumers. Thus

attention is also paid to the relationship among agents: the possibility of reacting in a coordinated manner to exogenous shocks and flexibility of adaptation play an important role in the organization of the supply of safe food. Conversely, agents invest growing amounts of resources in order to improve the safety standards of the goods supplied. Human capital, brands, labels and other usually adopted solutions: thus a theoretical finding suggests that these investments generate different types of asset specificity, the consequences of which could influence the degree of safety of food delivered to consumers. There is small amount of evidences about the factors affecting the strategic managerial choices in field of food safety, whereas both mandatory and voluntary behaviours may be integrated in order to enhance the safety of food supplied. In this perspective there is a need of analysing the managerial choices in terms of both internal organization and of coordinating solutions. The Neo-institutional economics provides the theoretical basis of the study planned, even though attention is paid to different theoretical perspectives.

The main objective of this study was to describe the current managerial models adopted in selected poultry production systems and their classification with respect to quality and safety. This case study using 3 questionnaires was conducted in the field of Italian poultry system and also a French company.

B) Methodology

Questionnaires

According to the total-chain approach of the project, the specific goal of the research are: i) to assess the influence of the managerial factors upon the care over safety (focusing behaviours); ii) to analyse of the resources and competencies of the companies (focusing competencies); and iii) to assess managerial models.

Table 59: Research design elements

Database	**A and C**	**B and D**
Populations	Farmers	Processors
Concepts	Managerial characteristics, Food safety, preferences	Managerial characteristics, Food safety, resources (Physical, Human, Technological, Financial)
Tools	Questionnaire n.1 (A-C)	Questionnaire n°2 (B. 1-D) Questionnaire n°3 (B.2)
Strategy of submitting	Direct	Direct

The steps were: i) to elaborate the questionnaires; ii) to establish agreement and contact with companies and farmers: and iii) to collect data. The planned collected data are described in table 59.

Table 60: Planned collected data

Database	Number of planned collected questionnaires	Candidate area	Goals of analysis
A	100	Italy	To assess the influence of the managerial factors upon the safety
B	10	Italy	Analysis of the resources and competencies of the companies
C	30-50	France	To assess the influence of the managerial factor upon the safety
D	3-5	France	Analysis of the resources and competencies of the companies

Due to the lack of collaboration from companies and associations contacted, the data collecting carried out are described in table 60. A new national level sample as been selected, including units of producers and traders specialized in poultry sector and the questionnaire n°3 was derived from an existing one. The specific objective is to substitute the case study with new data from a postal survey (Database B.2).

Table 61: Data collected

DATABASE		Number of collected questionnaires	Area of collecting data
A		162	Italy
B	**B.1**	5	Italy
	B.2	47	Italy
C		0	France
D		1	France

Questionnaire n°1 (A and C)

This questionnaire is focused on investigating: a) the preferences of growers for contract attributes, where the contract has to be thought of as the main coordinating arrangement in poultry sector; and b) the views of growers about food safety. The basic idea is to analyse the relationship between characteristics of management and of rearing and the food safety concern. The questionnaire also includes general information about the farms and the growers and a specific set of information concerning the skills of growers.

Questionnaire n°2 (B.1 and D)

This questionnaire is focused on analysing the resources and the competences of companies in selected case studies. The approach chosen follows a standard analytical framework derived from the strategic analysis of the firm.

A direct contact was established with the following companies in Central Italy: a) Broiler producers: Pollo Del Campo; Amadori; Gruppo Martini; Fileni; Veronesi; Arena; (Italy), Centre de Management Champagne Ardenne (France); b) Eggs producers: Novelli, Eurovo (Italy).

Questionnaire n°3 (B.2)

The questionnaire n°3 was elaborated in order to ca rry out the postal survey as a corrective action needed due the lack of collaboration from companies and associations. The items of the questionnaire focus on three aspect: a) general information of the firm; b) information about the organizational arrangements established by the firm with other parties along the chains; c) investments made in the field of food safety and quality and their motivations.

Databases

Database A: a total number of 161 farm questionnaires were collected (geographic areas: Central Italy-Emilia Romagna and North of Italy-Veneto).

Database B.1: 5 questionnaires were collected from poultry companies (cases study)

Database B.2: It includes 47 cases of Italian companies involved in poultry production and trade.

Database D: the data collected concern the unique case study elaborated in France

8.1.2 - Results and discussion

The research scientific outcomes achieved by through the analysis of the preferences and the skills of the growers can be summarised as follows (Database A):

- **Proposition 1:** the growers tend to prefer collaboration with the processor and appear to be risk adverse.
- **Proposition 2**: the growers are able to assess the technological tasks both in terms of transformation and transaction costs. These assessments provide opportunities for changing the technology in use.
- **Proposition 3**: the education degree abilities of the growers in executing the productive tasks identified by the processor, while a controversial influence is due to the choices in specific technological field (disinfection, hygienic practices).

The research scientific outcomes achieved by through the case studies can be summarised by the following propositions (Database B.1):

- **Proposition 4**: the food safety strategy appears to be antecedents of differentiating strategies in both meat and eggs productions. There are two reasons for that: a) the search for satisfying the consumer's expectations; b) the compliance with the laws.
- **Proposition 5**: the companies examined are engaged in developing food safety strategy in the context of complex chains relationships. The efforts and investments made in this field provide a basis for productive, quality and marketing strategies.
- **Proposition 6**: each company looks for achieving its own competitive advantage on given market segments by a differentiation strategy. Nonetheless, each company tend also to rely on the possibilities of reducing the costs of the activities. Therefore, the food safety objectives have to be thought of as framed in this strategical context.
- **Proposition 7**: the differentiation strategies adopted tend to rely on tight coordination nexus with downstream agents in the chains, i.e. with large retailers. The safety strategies elaborated at retailer level tend to influence the choices and the behaviours of the companies interviewed by through three basic tools: a) production standards (e.g. production protocols); b) inspections and controls; c) certification. This results in a tight coordination along the last stage of the chains.
- **Proposition 8**: the embeddedness of food safety in the whole strategy of the companies ensures a systematic effort in identifying technological solutions to food safety issues.

The research scientific outcomes achieved by through the analysis of the postal survey can be summarised by the following propositions (Database B.2):

- **Proposition 9**: the investments made in order to ensure the degree of safety of the products, tend to be specific, in the sense of transaction costs economics, to the partners of the transactions
- **Proposition 10**: the specificity of the food safety investments induce the formalization of the contracts among the parties to a transaction along the chains
- **Proposition 11**: the explicit request of the partner promote investments in food safety and influences the adoption of formal contracts
- **Proposition 12**: according to theory predictions, complex organizational arrangements, intermediate between the spot market and the firm, are adopted by the agents as a consequence of food safety strategies

8.1.3 - Analysis of contractual arrangements

A) Introduction

The analysis of contracts in poultry sector was mainly taken into account risk sharing and incentive effects. Koeber, 1989 emphasizes the effects of contractual arrangements on technical changes. Knoeber and Thurman, 1995 found that price risk and common production risk are both shifted to owners of integrator companies, while price risk determine the main component of total risk. Levy and Vukina, 2004 explore differences between contractual arrangements with respect to growers' heterogeneity and production shocks. Goodhue, 2000 examines the implicit long-term contract between growers and processors in broiler production and explains the contractual control of input in terms of heterogeneity of individual ability, risk adversion of growers and systemic uncertainty. Under a complementary view, Mènard, 1996 points out the key role of growers, the variety of agreements and the nature of the contract as a general framework. The analytical framework of the study examines contractual choices expanding on a recent outcome which emphasizes the diffusion of hybrid governance structures as a consequence of food safety oriented strategies. The objective of this study was to identify the role of contracts in poultry sector, with respect to: a) changes in technology, framed within contractual relationship; b) comparison among possible different European contract types. The characteristics of the contract are considered mainly on the basis of existing literature. The aim of the analysis is to identify how the contract contribute to food safety strategy, namely in the field of technological choices. Collection and analysis of three types of contract (Danish and French Cases, based on literature and Italian case based on data collected at company level) were carried out. A questionnaire (n°4) including general information about the potential respondents and request of information on contract types was elaborated. This study was completed by the analysis of literature and brief interviews.

B) Methodology

Questionnaire n°4

The questionnaire n°4 includes: a) general information about the potential respondents; b) request of information on contract types in use). Analysis of literature and brief interviews. The main objectives were to identify the role of contractual arrangements in poultry sector, with respect to: a) changes in technology, framed within contractual relationship; b) comparison among possible different European contract types. No structured information from industry were returned. Literature remains the unique sources of information. The task achievement is an interpretation of how technology is framed in the contracts and what the possibilities are for contract to support food safety strategies, namely in the field of technological choices. Comparison of European contracts was made between Danish and French cases (literature) and Italian case (data).

C) Results and discussion

The research scientific outcomes achieved by through the analysis can be summarised by the following propositions:

- **Proposition 13**: according to theory predictions, the most diffused poultry contract allows agent to cope with food safety issues as it basically represents a hybrid governance form.
- **Proposition 14**: the most diffused poultry contract includes enforcement procedures which secure the food safety objective against the potential opportunistic behaviours
- **Proposition 15**: given the propositions 3-7-10 and 11 the contract in poultry sector can be thought of a reliable organizational arrangements with respect to food safety objectives and to the related technical changes.

The analysis shows that the two basic dimensions of poultry contract supporting food safety strategies relate to the contract as a governance structure and to enforcement procedures. The governance structure allows the processor also to manage the technological change needed in front of change in the competitive strategies and in the expectations of consumers or of the society as a whole (as in the case of EU AGPs ban).

8.2 - Broiler production, trade, consumption and health in the EU25

The objective of this study was to carry out a hazard assessment and an industry/trade analysis for the broiler industry in the EU25 following the integration of 10 New Member States (NMS) into the EU in 2004.

The research was split into 2 categories, industry/trade analysis and hazard identification, covering 4 main topics:

(1) Production (2) Trade (3) Consumption	Industry/trade analysis
(4) Health indicators	Hazard identification

Data for the analysis were extracted from the published literature, Internet and Government sources.

The expansion of the EU into 25 member states has brought about some changes in trade and a more varied broiler farming practice.

In 2005, Spain was the largest broiler producer, producing 15.2% of the total EU25 production by weight with the United Kingdom (UK) and France producing 15.1% and 12.9%, respectively. The fourth largest producer as a NMS, Poland, produced an estimated 0.93 million metric tonnes, amounting to 10.7% of the total EU25. The NMS ranked mainly at the lower end of the scale with Slovenia, Slovakia, Lithuania, Cyprus, Estonia, Latvia, and Malta producing only 2.5% of the EU25 when combined. The distribution of the EU farm sizes can be split into appreciatively three groups: 1) those countries where only small farms exist (0-1,000 birds per farm); 2) countries dominated by a large percentage of small farms (90% in the 0-1,000 birds per farm size) but larger farms do exist; and 3) countries with a wide distribution of farm sizes. NMS are found in each of these categories.

Increases in trade potentially increase the risk of poultry diseases being spread through the EU. However, as the majority of the trade is in meat products rather than live birds, for both EU15 and NMS, this is mitigated. Important broiler commodities are whole fresh/frozen birds, fresh and frozen cuts and offal and processed/prepared chicken products. Between 2003 and 2005, during which accession of the NMS occurred, there was an overall increase in trade of broiler meat and products in the EU25 with an estimated 22.8% increase in imports by weight and a smaller estimated 5% increase in exports.

For certain NMS there was a large increase in imports of chicken products with an estimated 481% increase into the Czech Republic, 471% increase into Malta, and 473% increase into Cyprus. Poland and Slovakia have also been cited as having experienced large increases; however, there is no recorded import trade for the year 2003. Increases for the EU15 and NMS Lithuania, Estonia and Latvia were more modest. In terms of exportation, there has been a mixed experience for NMS. Cyprus and Estonia have all experienced a drop in their exports, and Malta and Hungary have seen little change. There has been a large increase in exports from Latvia (337%), Lithuania (271%) and the Czech Republic (178%). This disparity may be due to the fact that certain NMS were more advanced in implementing the EU food safety and environment regulations and, therefore, entering the EU market than other NMS.

Poultry consumption in the EU15 seems to have stabilised in recent years. It is, however, increasing in the NMS (FAS, 2005). The annual tonnage of chicken and chicken products consumed by each of the EU25 countries were estimated; the estimates are associated with a large amount of uncertainty. From these estimates, the largest national consumers of chicken meat are UK, Spain and Germany. The lowest amounts of chicken are consumed within the NMS: Malta, Cyprus, Latvia and Estonia.

Regarding the status of EU25 countries in regard to former list A and B diseases, classified by OIE and the prevalence's of food borne pathogens, all such diseases and zoonoses are present in both EU15 and NMS. However, there is little information available on strains, virulence and prevalence to provide further analysis. In addition, the surveillance systems and the use of different diagnostics and sampling preclude an overall comparison of such data.

8.3 - Costs and benefits of the removal of AGPs from broiler production in four EU Member States

8.3.1 - Introduction

The marketing and use of AGPs in animal feed was prohibited in the EU from 1 January 2006. The AGP ban was progressive with Avoparcin and Ardacin being withdrawn in 1997 and Bacitracin zinc, Spiramycin, Tylosin and Virginiamycin in 1999. The remaining AGPs Avilamycin, Flavophospholipol, Monensin and Salinomycin were withdrawn in 2006.

During the phasing out of AGPs, there was concern about the potential adverse effects of the ban on broiler production in the EU. Various strategies were proposed to mitigate the effects of the withdrawal of AGPs on broiler performance and health. These included the use of alternative feed additives such as enzymes, organic acids, CE products, probiotics and prebiotics, and changes in management practices including enhanced biosecurity measures.

The main objective of this study was to collect data to estimate the economic costs and benefits of the removal of AGPs and their replacement with alternative products and strategies in broiler chickens in four EU Member States.

To determine the data required for the economic analysis, a cost-benefit analysis framework for the conduct of the study was developed. Knowledge of, and data on, the following topics were required:

- Structure of the broiler industry in the UK, Spain, the Czech Republic and Lithuania
- Broiler production parameters affected by the AGP ban
- Use of AGPs on broiler farms in the four countries before the ban
- Effect of AGPs and their removal on broiler production and health
- Alternative products to AGPs and their effect on broiler production and health
- Management strategies to mitigate the effects of not using AGPs
- Effect of AGP ban on human health
- Effect of the AGP ban on trade, industry structure and the environment

8.3.2 - Methodology

The four case study countries were the United Kingdom (UK), Spain, the Czech Republic and Lithuania. They represented Member States before and after EU enlargement in 2004, broiler industry diversity across the EU and were participants in POULTRYFLORGUT project.

Social cost-benefit analysis was chosen as the economic analysis technique. A framework for the conduct of the study was developed. The chapters of the report mirror the cost-benefit analysis framework.

The approach was to compare the net benefit of broiler production strategies without the use of AGPs (alternative options) with the net benefit of broiler production with AGPs (baseline option).

Data for the cost-benefit analysis were extracted from the published literature, Internet and Government sources.

A questionnaire was developed to obtain qualitative data on the impact of the AGP ban on broiler production and on the use of alternative strategies to AGPs.

The economic analysis proved complex and difficult to achieve within the resources requested. Therefore, effort was concentrated on presenting the science underlying an economic analysis and identifying data gaps.

8.3.3 - Results and discussion

A) Structure of the broiler industry

In 2005, the percent of broilers that were reared on farms with 10,000 or more birds were UK - 99%, Spain - 99%, the Czech Republic - 95% and Lithuania - 82%. Although Spain and Lithuania had many smallholdings, these accounted for only a small proportion of broiler production.

Most commercial holdings were of the conventional production type although around 15% of UK farms were free-range.

Annual data on the distribution of broiler holdings by production type would be a useful addition to the Eurostat database.

Knowledge of the broiler industry in each EU Member State is required for policy analyses of the impact of regulatory changes on the industry. The information should be updated regularly and include projections of trends.

B) Broiler production parameters affected by AGP ban

The primary measures of broiler performance are growth rate, feed conversion ratio, liveability and processing yield.

Many factors affect broiler performance including genetics, stocking density, environment, feeding and nutrition, litter management, lighting and growth management, disease, AGPs, alternative feed additives and therapeutics.

A production (input-output) model that takes into account all factors affecting broiler performance would be useful to estimate the impact of the AGP ban and the use of alternative strategies, including management changes, on broiler performance.

Knowledge about commercial broiler performance and practice in each study country is required to develop appropriate production models to underpin the economic analysis.

It is important to incorporate changes in broiler performance and practice over time into the analysis, especially improvements in genetic performance that may mask the effects of no longer using AGPs.

C) Use of AGPs on broiler farms

The baseline option for the cost-benefit analysis was defined as broiler production with the use of AGPs.

AGP use up to their withdrawal in 2006 has changed over time with the progressive withdrawal of various AGPs between 1997 and 2006 and different regulatory regimes and industry policies in the study countries. It is suggested that the baseline option should be broiler production with AGPs as used in each study country in 1996.

Data are required on the AGPs that were commonly used in commercial broiler holdings in each study country in 1996 as well as levels of AGP usage on broiler farms.

D) Effect of AGPs and their removal on broiler production

There is a large body of clinical trial data on the effects of various AGPs on broiler performance.

Increased growth rates of between 0-26% were reported in broilers given AGPs. The average increase in growth rate is between 3-10%.

Average feed efficiency gains of 3-5% have been observed in broilers given AGPs.

Data from experimental studies on the effects of AGPs on broiler performance may not be representative of the gains likely to be seen from AGP use in commercial broiler production. More studies based on field trials are required.

When measuring the effect of AGPs on broiler performance, the many factors affecting broiler performance should be taken into account in the analysis. Holo-analytical or multiple regression models may be used to analyse such data.

Field trials should include the collection of data on all the relevant factors affecting broiler performance.

Only one comprehensive study on the effect of the removal of AGPs on broiler productivity was found. In Denmark, Emborg *et al*., 2001 used data from the Danish Poultry Council's productivity database to compare broiler performance before and after the AGP ban in Denmark. The withdrawal of AGPs did not affect the weight of broilers produced per square metre nor the per cent total mortality. However, a small increase of less than 1% in the feed conversion ratio was observed over the 18-month period following the withdrawal of AGPs.

Some AGPs may play a beneficial role in preventing enteric diseases such as necrotic enteritis and dysbacteriosis in broilers. The impact of their removal on broiler health and welfare was mitigated to some extent by the use of ionophore coccidiostats to control coccidiosis in broiler flocks.

It is recommended that Member States work with industry to establish broiler productivity databases that could be used to monitor the impact of regulatory changes in the EU on broiler performance, health and welfare. Ways to ensure commercial confidentiality would need to be built into the system.

AGPs may increase or decrease the level of shedding of enteric bacteria that cause no harm to the bird but which are pathogenic for humans, for example, *Salmonella* spp. and thermophilic *Campylobacter*. The effects vary with the type of AGP used and the pathogen studied. For some AGP-pathogen combinations, the results from research studies are inconclusive.

In assessing the potential benefits of the AGP ban on salmonellosis and campylobacteriosis in humans, each AGP-pathogen combination should be considered separately.

E) Alternatives to AGPs and their effect on broiler production: Enzymes

Enzymes are added to poultry feed to improve the digestibility and nutrient utilisation of cereal grains, especially wheat, barley and rye, which are significant components of broiler diets in Europe.

Xylanases break down soluble Non-Starch Polysaccharides (NSPs) in wheat and rye while β-glucanases break down soluble NSPs in barley and oats.

Most clinical trials show that the use of enzymes does increase broiler performance. However, the magnitude of the response depends on the enzyme formulation and the composition of the feed to which it is added. The response also diminishes with increasing age of the bird.

Increased growth rates of around 2-5% were reported in broilers given either β-glucanase or xylanase alone or in combination. However, the differences in growth rates between treated and control groups often were not statistically significant.

The effect of exogenous enzymes on feed conversion ratio is considered more significant with feed efficiency gains of 1.6-3.2% reported.

Exogenous enzymes generally have had little effect on liveability. However, improved litter condition was noted following the addition of enzymes to the diet.

F) Alternatives to AGPs and their effect on broiler production: Organic acids

Organic acids are added to poultry feed to enhance the digestibility and palatability of feedstuffs and to reduce the levels of spoilage organisms and pathogenic bacteria in feed. More recently, they were used to decrease harmful bacteria in the gastrointestinal tract of poultry.

When added to feed, mixtures of formic acid and propionic acid are effective at decreasing levels of *Salmonella* in the feed. They may also reduce other enteric bacteria in feed.

Organic acids are absorbed before they reach the lower gut of birds. Therefore, their addition to poultry feed is not likely to have a significant effect on enteric bacteria in the bird. Recently, coated acid products were developed to enable acids to reach the lower gut.

Coated propionic and butyric acid products in feed were shown to decrease *Salmonella* colonisation and shedding in chickens. Therefore, they may have a beneficial effect on human health.

The effect of organic acids on broiler performance and health is uncertain.

G) Alternatives to AGPs and their effect on broiler production: Competitive exclusion products

CE products are undefined cultures of live bacteria originating from the intestines of healthy adult birds. CE treatment is usually administered once to day-old chicks to replace the enteric flora that chicks reared in modern broiler production no longer obtain from their mothers.

CE products are used primarily to prevent colonisation of the broiler gut by pathogens such as *Salmonella*, *Campylobacter*, *Clostridium* and *E. coli*. Therefore, they may have a beneficial effect on broiler and human health.

CE products may decrease mortality from necrotic enteritis (*C. perfringens*) in broiler chickens.

The effect of CE products on broiler performance is mixed but generally not significant.

H) Alternatives to AGPs and their effect on broiler production: Probiotics

Probiotics are live cultures of defined single bacteria or mixtures of bacteria that are administered to broilers in feed or water over their lifetime to improve the intestinal microbial balance.

Probiotics are used to enhance broiler performance.

Significant increases in growth rates of around 2-8% have been reported in broilers given probiotics.

Probiotics generally have not resulted in significant improvements in feed conversion ratios although feed efficiency gains of 0.6-10.8% have been reported.

The effect of probiotics on colonisation and shedding of *Salmonella*, *Campylobacter*, *Clostridium* and *E. coli* is uncertain with most studies reporting no significant effect.

I) Alternatives to AGPs and their effect on broiler production: Management practices

Many factors influence broiler performance and the risk of enteric diseases in broiler flocks. These factors include biosecurity, housing, stocking density, feed and feeding practices, litter management, AGPs (before 2006), alternative feed additives and therapeutics.

Management practices may be manipulated to mitigate the effects of the withdrawal of AGPs on broiler health and consequently, broiler performance.

The diseases of greatest concern to broiler producers following the withdrawal of AGPs are necrotic enteritis, dysbacteriosis (diarrhoea) and contact dermatitis (pododermatitis, hock burn and breast blisters).

Good biosecurity is essential to prevent the spread of enteric pathogens between flocks. Effective terminal cleaning and disinfection of broiler houses between flocks contributes to coccidiosis control, which is a major predisposing factor for necrotic enteritis.

Improving broiler house ventilation may reduce the incidence of enteric diseases.

Reducing stocking density may lower the risk of necrotic enteritis. However, a lower stocking density may have a significant impact on total meat output and farmer income.

The risk of necrotic enteritis may be lessened by reducing the protein level in broiler diets.

High levels of soluble NSPs in wheat- or barley-based diets may increase the risk of necrotic enteritis. Adding enzymes to the feed may mitigate their effect.

The physical form of feed may affect the level of enteric diseases in broilers. Coursely ground feed and pellets appear to have a protective effect.

Wet litter may be both caused by, and contribute to, enteric diseases in broiler flocks. Therefore, good litter management is important.

Ionophore coccidiostats are used to control coccidiosis in broiler flocks. They have an antibacterial effect and have been shown to suppress the growth of *C. perfringens*. Their continued use may have mitigated an increase in necrotic enteric in broilers, which was expected as a result of the AGP ban.

Ionophore coccidiostats have been shown to increase broiler performance with improvements in growth rates of around 7-11.5% and in feed conversion ratios of around 1.0-6.8%.

A potential negative impact of the AGP ban is increased use of therapeutic antibiotics to treat and control those diseases previously suppressed by AGPs. Increased use of therapeutic antibiotics was noted in Norway after AGPs were withdrawn but the increase was short-lived. There is some evidence that the use of therapeutic antibiotics in food animals increased in the UK and Denmark after AGPs were withdrawn. However, when use in Denmark was calculated in Animal Defined Doses, therapeutic antimicrobial use in broiler chickens did not appear to increase.

Control of immunosuppressive diseases such as infectious bursal disease through vaccination and good cleaning and disinfection contribute to the control of enteric diseases in broilers.

J) Specification of alternative options to AGP use for economic analysis

The alternative options to the use of AGPs in broiler production suggested for the cost-benefit analysis were:

(a) no alternative feed additive

(b) use of a probiotic

(c) use of a mannanoligosaccharides (MOS) prebiotic.

The selection of alternative options was based on the potential of the feed additive as an equivalent replacement for AGPs; that is, the alternative additive had to improve broiler performance and health.

Feed additives that had been in common use before the withdrawal of AGPs were not considered as replacements for AGPs, for example, enzymes and organic acids.

A range of management practices may be manipulated to improve broiler health and performance in the absence of AGPs. However, without detailed knowledge of management practices on broiler farms before the AGP ban, it was difficult to specify an alternative option to AGP use based on a "new" or improved disease prevention measure.

For the economic analysis, it was proposed that management should be assumed to be adequate to achieve average expected broiler performance and health in the study country.

Each alternative option should be analysed across a range of acceptable stocking densities.

Different feed formulations, with and without enzymes as appropriate, should be included in the analysis.

All options should be evaluated with and without the use of ionophore coccidiostats.

To estimate the costs of the replacement of AGPs with alternative strategies, data are required on the uptake of alternative products and management practices by the broiler industries in each study country.

K) Effect of AGP ban on human health and welfare

AGPs were withdrawn from use in food-producing animals in the EU because they add to the development of antibiotic-resistant bacteria that might be passed on to humans with serious implications for health.

The human health benefit of the EU ban on AGP use in broiler production is the value of those losses due to antibiotic-resistant bacteria that are prevented by no longer using AGPs in broilers.

The human losses caused by antibiotic resistance comprise the cost of illness and death from (a) losing an antibiotic to treat a particular infection through antibiotic resistance, and (b) the delay in the effective treatment of a particular infection because of initial treatment with an antibiotic to which the infectious agent was resistant. The cost of the losses will also be increased if human treatment requires a switch to a more expensive antibiotic because of resistance to a cheaper antibiotic.

Each AGP or AGP class has been linked to the emergence of different types of resistance in specific bacterial species.

To quantify the human health losses due to a particular AGP, the following knowledge is required:

(a) link between an AGP and its related antibiotics in human medicine

(b) human infections treated with the AGP-related antibiotics

(c) human diseases affected by the development of antimicrobial resistant bacteria that might have arisen from food animals given the AGP

(d) prevalence of human cases due to the antimicrobial-resistant bacteria

(e) consequences of human infections with the antimicrobial-resistant bacteria in terms of treatment and outcome.

The most difficult methodological issue associated with estimating the benefit from the AGP ban in broiler production is determining how much of the human health loss from antimicrobial resistance is due to the use of AGPs in broilers (attributable risk).

An example of the process to estimate the benefit of the withdrawal of AGPs from broiler production is illustrated for the AGPs spiramycin and tylosin and the rise in macrolide-resistant *Campylobacter* in humans.

L) Costs and benefits of withdrawal of AGPs in EU

Previous economic studies in Sweden and Denmark have indicated that the effect on broiler production from not using AGPs is likely to be small to negligible.

Costs refer to the impact of the withdrawal of AGPs from broiler production on resource use in a study country. They are estimated by comparing broiler production inputs or costs with and without AGPs.

The complete withdrawal of AGPs from broiler production in the EU has resulted in a cost saving for those producers who previously used AGPs.

Additional costs are incurred if alternative feed additives are used to mitigate the effects of the AGP ban on broiler performance and health.

The removal of AGPs from broiler production will have increased the amount of feed required to raise broilers to market weight. As feed is the single biggest input cost in broiler production at around two-thirds of total cost, this is an important component of any economic assessment of the AGP ban.

Other inputs that may be affected as a result of the AGP ban include stcking density, feed composition, use of coccidiostats and therapeutic antibiotics, type of litter used and

frequency of topping up or changing litter. Their inclusion in the economic analysis depends on the degree of detail built into the broiler production model and the data available.

Benefits refer to the effects or outputs generated by the removal of AGPs from food animal production. They are the losses prevented by, or occurring as a result of, the AGP ban.

In broad terms, the AGP ban may affect:

(a) chicken meat output

(b) broiler health and performance

(c) human health and welfare

(d) domestic trade in chicken meat

(e) international trade in chicken meat

(f) broiler industry structure

(g) feed additives industry

(h) environment.

To quantify the effect of the withdrawal of AGPs on broiler meat output, data are required on chicken meat output in each study country before and after the AGP ban. However, production trends must be interpreted in the light of other factors that may influence broiler meat supply.

The AGP ban may have increased the incidence of diseases such as necrotic enteritis, dysbacteriosis, pododermatitis, hock burn and breast blisters in broiler flocks. To quantify these effects, data are required on disease incidence and effects with and without the use of AGPs and with the use of alternative control strategies.

The human health benefit of the withdrawal of AGPs in the EU is the difference in human health and welfare losses with and without the AGP ban. It is very difficult to measure because of the uncertainties about the degree to which AGP use in broilers affected human health.

Quantitative risk assessment models may be needed to estimate the risk and impact of AGP use and their alternatives on human health.

Another potential benefit of the AGP ban is increased consumer confidence in the safety of chicken meat. This should result in increased consumer demand for chicken meat resulting in higher prices for producers.

Data on retail sales of chicken meat before and after the AGP ban are required to quantify this benefit. However, many factors affect consumer demand for chicken meat, which would have to be taken into account in the economic analysis.

One possible effect of the AGP ban is to raise broiler production costs in the EU making the industry less competitive in what is a global market. The benefits (negative or positive) from increased imports and fewer domestic producers of chicken meat are complex.

A potential benefit of the AGP ban is the generation and growth of a new industry that is producing alternative feed additives, which may have a higher value than the AGPs they are replacing.

8.4 - Conclusion

The managerial models have been identified and examined with respect to the issues caused by the European Union ban of AGPs. The analytical perspective adopted has emphasized the costs of the achievement of a new technological base, provided the importance of the AGPs within the Normal Design Configuration of the poultry sector.

To the purpose of the research, the managerial models are thought of as emerging from two pillars: the relationships between the food safety and the search for competitive advantage and the possibility of promoting technological change through the production contract.

The evidences collected showed that food safety act as antecedents of the differentiating strategies elaborated and implemented by the companies within complex chains relationships. Tight coordination nexus exists between the companies and the large retailers, which affect the strategic role of food safety.

Regarding the possibility of promoting technological change through the production contract, the evidences suggests that there are some opportunities to meet the request of technological change caused by the EU ban. Actually the contract, as usual tool of innovation, allows the companies to rely on collaborative approach of the growers who, in turns, appear able to assess the efficiency of specific innovation possibilities, provided that they are framed within the standard contractual approach to technology implementation.

The research activities have also allowed to elaborate on some analytical elements. The technological issues addressed have been conceptualised in terms of Normal Design Configuration and a contribution to the analytical framework of the adaptation costs in the context of hybrid governance structures has been proposed.

The key economic cost associated with the removal of AGPs from broiler production in four case study countries (United Kingdom, Spain, the Czech Republic and Lithuania) has been the increased amount of feed required to raise broilers to market weight. As feed is the single biggest input cost in broiler production, this is the largest cost to producers associated with the AGP ban. Other changes in production, as a result of the ban, include changes in stocking density, feed composition, increased use of coccidiostats and therapeutic antibiotics, type of litter used and increased frequency of topping up or changing litter. Such costs have been borne by the producers. The AGP ban may also have increased the incidence of diseases such as necrotic enteritis, dysbacteriosis, pododermatitis, hock burn and breast blisters in broiler flocks. The benefits of the AGP ban are less tangible - being more difficult to quantify. In broad terms, the AGP ban may have benefited human health and welfare through the food chain, the feed additives industry, and gained greater public trust in the domestic market and international trade for European produce.

Section 9: Conclusion and Recommendations

The strategic sub-objectives of this proposal can be summarized as follows:

1- Improve the characterisation of the digestive troubles occurring in the field since the banning of the in-feed antibiotics and specification of their prevalence, of the incidence and of some causal factors.

2- Associate the occurrence of food-borne pathogens in poultry farms, in different European countries, with different production systems (in-doors, out-doors…) and to determine how to improve management in order to decrease the occurrence of food-borne pathogens.

3- Quantify the food-borne pathogens at the different steps of processing in slaughterhouses in different European countries and to determine possible improvements.

4- Create an European molecular data-base on traditional and (re)emerging food-borne pathogenic strains/species in European poultry production.

5- Assess novel techniques allowing the study of the intestinal flora and of the relation-ship between the whole flora and the food-borne pathogens in poultry.

6- Estimate the socio-economic consequences of the changes occurring in poultry production in response to the new European regulations and to the (public) European citizens concerns.

This project allowed to provide data regarding the occurrences of the main food-borne pathogens (*Salmonella, Clostridium, Listeria, Campylobacter, Helicobacter*) at the farm level from various rearing systems (conventional and alternative) and at the slaughterhouse levels in different EU countries. Moreover, protocols for isolation and typing of new pathogens (*Clostridium perfringens, Listeria monocytogenes, Helicobacter*) were developed; these protocols should be useful for future EU recommendations.

Regarding the comparison between alternative production systems, the data collected in Italy within farms applying different rearing technologies seem to suggest that the occurrence of food-borne pathogens might be slightly lower in free range farms in comparison to organic and conventional farms.

No relationship was found between the *Listeria monocytogenes* strains isolated from the flocks and those from the slaughterhouses. Regarding the analysis of sanitary risks, other

studies of typing of other food-borne pathogens should be interesting to make comparison betweens strains found at the farm and slaughterhouse levels.

At the slaughterhouse level, *C. perfingens* was the only pathogen isolated on surfaces after cleaning. *Salmonella* was not found on the studied surfaces, neither before nor after cleaning. This result is relevant for the future development of cleaning and disinfecting procedures.

Regarding the effect of tested alternatives to AGP (organic acids, prebiotics, probiotics, botanical…), some organic acids increase the biodiversity of the intestinal microflora. They increase the number of bacteroides and decrease the number *Clostridium* of the intestinal flora. The organic acids seem to increase animal performances and to reduce the level of contamination by *Salmonella* but they are not effective against *Campylobacter* infection. Prebiotics as antibiotics decrease the variability of the intestinal flora, this could have a regulatory effect on the digestive flora. Antibiotics decrease the diversity and the number of intestinal bacteria whereas prebiotics increase the biodiversity of the intestinal microflora. Probiotics and antibiotics increase the number of intestinal coliforms. The positive effect of probiotics on zootechnical performances depend on the age of animals, when the product is added along the rearing period, from 1 to 42 days a positive effect was observed whereas a negative effect was observed between 14 to 25 days. Under field conditions, Competitive Exclusion cultures were a useful tool for reducing the incidence of *Salmonella* and *Clostridium perfringens* infection in commercial broiler production. Moreover, this treatment does not interfere negatively with immune response. Under experimental conditions, CE treatment can contribue in controlling *Salmonella* Java, but cannot eradicate the infection. The effect of probiotics depends on the dose, the level of infection, the type of pathogen strains, the time and the date of administration.

Regarding the evaluation of molecular methods to characterise the balanced poultry microflora and the effect of additive on this ecosystem. If one methodology had to be selected to start up investigation or to screen treatment effects on the chicken GIT, the optimal choices seem to be CE-SSCP or T-RFLP, completed by DGGE or SSCP. Indeed, CE-SSCP and T-RFLP methods allow a high processing rate through automation and SSCP or DGGE allow a subsequent characterization of individual fragments by DNA sequencing. When specific groups of bacteria are identified by DNA sequencing in response to treatments, further investigation should be carried out by PCR with bacterial groups as specific targets. Further work beyond the project will be required to exploit results, for example, to improve the knowledge between host pathogen interactions and to develop or study the effect of new additives.

The economic costs and benefits of the removal of AGPs and their replacement with alternative products and strategies in broiler chickens have been estimated in four EU Member States (United Kingdom, Spain, the Czech Republic and Lithuania). Nevertheless, recommendations on where future EU policy spending were focused with regard to best practice on the use of alternatives to AGPs because of the lack of data from national survey in different EU countries. To support "best practice" initiatives on the use of alternative feed additives to AGPs, it is recommended that the EU support independent research on their effectiveness in commercial field situations. Field trials should include the collection of data on all relevant factors affecting broiler performance.

There are many new feed additives on the market or in the process of being approved for sale in the EU. It is recommended that a regularly updated review of feed additives and their properties is produced and placed in the public domain in order that broiler producers and their advisors can make informed decisions about their use. The review should include referenced information on their efficacy as appropriate to their recommended use.

If they have not done so already, it is recommended that EU countries review their advice to broiler producers on "best practice" to control and prevent disease to ensure that it is appropriate to production without AGPs.

To facilitate the sharing of information on "best practice" among EU countries, it would be useful to have a central EU repository of published material on "best practice" issued by each Member State.

It is recommended that when a major regulatory change is proposed, which impacts on primary producers, steps should be taken to set up systems to monitor the impact of the change on those affected.

As many regulatory changes in the food safety and animal health areas impact on food production, systems should be set up to provide on-going data on food animal production. In the case of broiler production, the productivity database run by the Danish Poultry Council is an example of the type of system required to supply the data to measure the actual impacts of a policy change on broiler production and health. As such data are commercially sensitive, safeguards would be needed to protect the confidentiality of individual producer's data.

It is recommended that governments consider making systems to monitor the use of antibiotics in different animal species mandatory. A harmonised system for expressing antimicrobial consumption data in different food animal species across the EU should be useful.

Annual data on the distribution of broiler holdings by production type for each EU Member Country would be a useful addition to the Eurostat database.

Knowledge of the broiler industry in each Member State is required for policy analyses of the impact of regulatory changes on the industry. The information should be updated regularly and include projections of trends.

A production (input-output) model that takes into account all factors affecting broiler performance would be useful to estimate the impact of the AGP ban and the use of alternative strategies, including management changes, on broiler performance. Holistic production models are available but not in the public domain, thus limiting their use for public policy analysis.

Knowledge about commercial broiler performance and practice in Member States is required to develop appropriate production models to underpin economic analysis.

To assess the impact of the AGP ban on EU broiler production, data are required on the AGPs that were commonly used in commercial broiler holdings in Member States before the AGP ban together with details of their use.

A range of management practices may be manipulated to improve broiler health and performance in the absence of AGPs. However, without detailed knowledge of management practices on broiler farms before the AGP ban, it is difficult to specify an alternative option to AGP use based on a "new" or improved disease prevention measure.

To estimate the costs of the replacement of AGPs with alternative strategies, data are required on the uptake of alternative products and management practices by the broiler industries in Member States since the AGP ban.

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Annex 1: Molecular database

Seee excel file

Annex 2: Protocols of molecular methods

10.1.1 - Fingerprint methods

A) SSCP

B) CE-SSCP

C) DGGE

D) RFLP

E) T-RFLP

F) TTGE

10.1.2 - Methods of quantification

A) FISH

USE AND DISSEMINATION

REPORT ON SOCIETAL IMPLICATIONS