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ATTEMPT TO DEVELOP TREATMENTS BASED ON BACTERIA-ENZYME COMBINATION TO REDUCE BROILER CONTAMINATION BY TWO MAIN HUMAN BACTERIAL FOOD-BORN ENTERIC PATHOGENS

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Summary

Broiler flocks become frequently asymptomatically contaminated by the enteric bacteria *Salmonella* sp. and *Campylobacter* sp. which are human pathogens. Among the strategies developed at farm level to reduce the incidence of these pathogens, some lactic acid bacteria have been shown to be interesting because of their antimicrobial activity and their stimulatory properties on the immune system of poultry. The aim of this thesis was to select bacteria with antagonistic activity against *Salmonella* or *Campylobacter*, and to improve their inhibitory effect by the combination with enzymes of polysaccharidase type. The first step of the thesis was an epidemiological study carried out in the Walloon region in order to determine the contamination way of broilers by *Campylobacter* in free range production. Results showed that the major way of contamination is the open-air range to which the animals have access during the rearing period. A preventive treatment of the open-air range and the straw litter with an antagonistic strain in combination with an enzyme seems thus to be suitable in this case. The second step of the work aimed at the selection of a xylanase for using as a dietary additive in combination with an antagonistic bacterial strain against *Salmonella*. Four xylanases were studied *in vivo* for their effect on growth performances of broiler chickens. Diet supplementation with enzyme led to an increased final body weight and daily weight gain ($P < 0.05$), without difference according to the bacterial or fungal origin of the xylanase. The Belfeed B1100MP xylanase, which is commercialized in he Walloon region, was selected in order to develop a probiotic-xylanase feed additive. The purpose of the third part was to select a bacterial strain with antagonistic activity against *Campylobacter* for applying on open-air range and broiler litter. An *in vitro* screening of 12 lactic acid bacteria was realised using a co-culture assay with a growth medium based on straw and dehydrated poultry excreta, supplemented with different cellulase concentrations. *Lactobacillus pentosus* and *Enterococcus faecium* showed inhibitory effect against *Campylobacter* without enzyme which was intensified by cellulose from 200 ppm. Finally, the effect of dietary supplementation with a *L. plantarum* strain combined with the Belfeed B1100MP (PE treatment) on growth performance, microflora, and faecal *Salmonella* Typhimurium concentrations, was studied with experimentally infected broiler chickens. The PE diet allowed to partially overcome the negative effects associated with the infection on growth performance and microflora, and to significantly reduce faecal *Salmonella* concentration.

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Résumé

Les élevages de poulets de chair sont fréquemment contaminés de manière asymptomatique par *Salmonella* sp. et *Campylobacter* sp., des bactéries entériques pathogènes pour l'homme. Parmi les stratégies développées au niveau de la production primaire pour réduire l'incidence de ces pathogènes, certaines bactéries lactiques se sont montrées intéressantes par leur activité antimicrobienne et leur effet stimulateur sur le système immunitaire des volailles. L'objectif de cette thèse était de sélectionner des bactéries antagonistes vis-à-vis de *Salmonella* ou *Campylobacter* et d'améliorer leur effet inhibiteur par la combinaison avec une enzyme de type polysaccharidase. La première étape a été une étude épidémiologique réalisée en région wallonne afin de déterminer les voies de contamination par *Campylobacter* des productions de poulets de chair de type extensif. Les résultats ont permis de déterminer que la source principale de contamination est le parcours extérieur auquel ont accès les animaux durant la période d'élevage. Un traitement préventif du parcours et de la litière paillée au moyen d'une souche antagoniste combinée à une enzyme semble donc approprié dans ce cas de figure. La deuxième étape du travail avait pour but la sélection d'une xylanase pour combiner dans un additif alimentaire avec une souche antagoniste à *Salmonella*. Quatre xylanases ont été étudiées *in vivo* pour leur effet sur les performances zootechniques de poulets de chair. L'ajout d'enzyme à la ration a amélioré le poids final et le GQM ($P < 0,05$), sans différence entre les enzymes en fonction de leur origine bactérienne ou fongique. La Belfeed B1100MP commercialisée en région wallonne a ainsi été retenue afin d'élaborer un additif alimentaire probiotique-xylanase. La troisième partie avait pour objectif de sélectionner une souche antagoniste à *Campylobacter* à appliquer sur parcours et litière. Un criblage *in vitro* de 12 bactéries lactiques a été réalisé en co-culture en milieu à base de paille et de fientes, supplémenté en cellulase à différentes concentrations. *Lactobacillus pentosus* et *Enterococcus faecium* ont montré un effet inhibiteur vis-à-vis de *Campylobacter* sans enzyme, et celui-ci est renforcé en présence de cellulase à partir de 200 ppm. Enfin, l'effet de la supplémentation alimentaire avec un *L. plantarum* combiné à la Belfeed B1100MP (traitement PE) sur les performances zootechniques, la microflore, et la charge en *Salmonella* Typhimurium dans les fientes, a été étudié chez des poulets de chair infectés expérimentalement. Le régime PE a permis de surmonter en partie les effets négatifs associés à l'infection sur les performances de croissance et la microflore, et de réduire significativement la concentration fécale en *Salmonella*.

This dissertation is dedicated to
Emmanuel Van Olmen and Quirin Renard.
Both of them, using their own means, teached me how to use knowledge and passion to face adversity.
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Abbreviations

AFSCA:
Agence Fédérale pour la Sécurité de la Chaîne alimentaire

AFSSA:
Agence Française de Sécurité Sanitaire des Aliments

***C.*:**
Campylobacter

CE:
Competitive Exclusion

CFU:
Colony-Forming Unit

EFSA:
European Food Safety Authority

EU:
European Union

GHP:
Good Hygiene/Farming Practices

GIT:
Gastrointestinal Tract

ICGFI:
International Consultative Group on Food Irradiation

***L.*:**
Lactobacillus

mCCDA:
modified Cefoperazone Charcoal Deoxycholate Agar

MIC:
Minimum Inhibitory Concentration

PCR:
Polymerase chain reaction

PFU:
Plaque-Forming Unit

***S.*:**
Salmonella

VNC:
Viable but Non Culturable

WHO:
World Health Organization

Fast-growing broiler chicken

Low-growing broiler chicken

General Introduction

The primary objective of the research presented in this manuscript was to develop control measures able to reduce broiler contamination by the human enteric pathogens *Salmonella* spp. and *Campylobacter* spp. It was initiated in the framework of two successive projects subsidized by the « Direction générale des Technologies de la Recherche et de l'Energie » (Research project n° 001/4545 « *Recherche d'un probiotique travaillant en synergie avec une hémicellulase en alimentation aviaire* », 2001-2005) and by the « Direction générale de l'Agriculture » (Research project n° D31-1093 « *Sélection d'un complexe enzymo-bactérien antagoniste de Campylobacter ssp. pour l'élevage, sur parcours extérieur, de poulets de chair* », 2004-2010) of the Walloon Region Ministry. These projects come within the scope of the European Union (EU) target, specified especially by the regulation (EC) n° 2160/2003, intending the reduction of poultry and poultry products contamination by food-borne pathogens. Modern controlled rearing systems are frequently confronted with flock infection by enteropathogens such as *Salmonella* and *Campylobacter* especially because broiler chickens have been genetically selected for growth, feed efficiency, and meat production rather than infection resistance (Cheema *et al.*, 2003). Moreover, these bacteria present some etiological specificity, meaning that broiler flocks may be differently affected according to the production type. For intensively raised broilers, the short breeding time and the high stocking density may especially favour the flock infection by *Salmonella* (Tuyttens *et al.*, 2008). On the other hand, the access of the birds to the outer environment and the far longer rearing period in extensive free-range production are major risk factor for *Campylobacter* contamination (Van Overbeke *et al.*, 2006; Tuyttens *et al.*, 2008). Consumption of carcasses or transformed meat products contaminated by both pathogens is recognized as the major cause of food-borne gastroenteritis worldwide, with an estimated 160,000 cases reported annually in the EU alone.

The overall objective of this PhD thesis was to study the antagonistic potential against *Salmonella* or *Campylobacter* of bacterial strains when combined with enzymes of polysaccharidase type. The research was based on the hypothesis that hydrolysates produced from the polysaccharidase activity should be specifically metabolised by the antagonistic strain to the detriment of pathogen bacteria. After the determination of the type of treatment the most adapted for both pathogens according to their aetiology, different enzymes were screened *in vivo* and lactic acid bacteria with interesting antimicrobial potential were screened *in vitro*. The last step of the work was to develop a diet additive composed of a *L. plantarum* strain combined with a bacterial xylanase and to test its effect on fast-growing broiler chicken.

This manuscript is a compilation of published and submitted papers and is structured as follows: Chapter I presents a review of the literature that is divided in two parts, dealing with current and developing strategies to reduce the prevalence in poultry flocks of *Campylobacter* and *Salmonella* respectively (Articles I and II). In the subsequent chapter, the research strategy that was adopted in this PhD study is developed, before to describe the epidemiological study on *Campylobacter* realised in the Walloon free-range broiler productions in Chapter III (article III). Screening of xylanases *in vivo* (Article IV) and of bacterial strains with potential antagonistic activities against *Campylobacter in vitro* (Article V) are then assessed. Results are reported in Chapter IV. Chapter V presents a study on the effect of the dietary supplementation of a broiler feed with a *Lactobacillus plantarum*-Xylanase combination on growth performance, nutrient

digestibilities, microflora populations and *Salmonella* colonization (Article VI). Finally, a general conclusion and future prospects are drawn in Chapter VI.

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Van Overbeke I., Duchateau L., De Zutter L., Albers G., Ducatelle R. (2006). A comparison survey of organic and conventional broiler chickens for infectious agents affecting health and food safety. *Avian Dis.* **50**: 196-200.

Chapter I. Review of the literature

Campylobacter **– Review of the literature**

Article I. Contamination of poultry flocks by the human pathogen *Campylobacter* spp. and strategies to reduce its prevalence at the farm level

Salmonella **– Review of the literature**

Article II. *Salmonella* in chicken: current and developing strategies to reduce contamination at farm level

Campylobacter – Review of the literature

Campylobacter contamination of poultry flocks is frequent in free-range broiler production. The main factor incriminated in this high prevalence is the access of the birds to the outer environment. Moreover, many studies have shown that *Campylobacter* colonization is a relatively late event, occurring generally after 3 weeks of age, so that the long rearing period of such poultry production system may increase contamination risk. Although several risk factors for infection of broilers have been identified, knowledge about the various routes by which flocks become infected and their relative importance is still incomplete. The development of an efficient control treatment against *Campylobacter* requires a better understanding of these transmission ways.

Thus, the following review, reported in part I of this Chapter, summarizes firstly our current knowledge about poultry colonization by this pathogen, with an exhaustive description of the transmission vectors and risk factors at farm level. It appears from the collected informations that horizontal transmission from the outer environment, especially from the open-air range and from wild and domesticated animals, is the main factor responsible for flock contamination. It can be concluded that an environmental treatment could be an efficient measure to reduce *Campylobacter* prevalence in free-range broiler production.

The second part of the review describes usual and complementary prevention method developed in order to meet the requirements of the European legislation about the establisment of effective measures to control zoonotic agents like *Campylobacter* at the level of primary production. Besides usual hygiene measures and the study of antibiotics use, other strategies based on environmental acidification, on immunity stimulation and on microbiological competition by acidifying bacteria, have been reported. Concerning antagonistic bacterial strains, combination with specific substrates like prebiotics showed interesting stimulation of bacterial activity against pathogens. As acidficiation seems to be an efficient way to reduce *Campylobacter*, and as guidelines of extensive broiler production only accept the use of biological treatments, environmental measure based on antagonistic acidifying strains, combined with enzymes supplying specific substrates, was hypothesized to be a powerful option in *Campylobacter* control.

Contamination of poultry flocks by the human pathogen Campylobacter spp. and strategies to reduce its prevalence at the farm level

Article I — Vandeplas *et al.* (2008)
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Running head: Strategies to reduce Campylobacter prevalence in poultry

Abbreviations: AFSCA, Agence Fédérale pour la Sécurité de la Chaîne alimentaire; AFSSA, Agence Française de Sécurité Sanitaire des Aliments; *C.*, *Campylobacter*; CE, Competitive Exclusion; cfu, Colony Forming Unit; EFSA, European Food Safety Authority; EU, European Union; GHP, Good Hygiene/Farming Practices; ICGFI, International Consultative Group on Food Irradiation; *L.*, *Lactobacillus*; MIC, Minimum Inhibitory Concentration; VNC, Viable but Non Culturable; WHO, World Health Organization

Abstract

Enteric *Campylobacter* spp. bacteria are human pathogens that frequently contaminate poultry flocks. Consumption of products from poultry origin may then lead to acute bacterial enteritis called campylobacteriosis of which prevalence is increasing for about ten years in Europe. This review summarizes *Campylobacter* epidemiological data, risk factors for contamination in poultry flocks and conceivable strategies to control this pathogen.

Keywords: Campylobacter, epidemiology, poultry, prevalence reduction, prevention

Résumé

Campylobacter spp. est une bactérie entérique pathogène pour l'homme qui contamine fréquemment les élevages de volailles. La consommation de produits d'origine aviaire peut ainsi entraîner une gastro-entérite bactérienne aiguë appelée campylobactériose, dont la prévalence augmente depuis une dizaine d'année en Europe. Cette synthèse bibliographique résume les données épidémiologiques sur *Campylobacter*, les facteurs de risque de contamination dans les élevages de volailles et les stratégies envisagées pour lutter contre ce pathogène.

Mots-clés : Campylobacter, épidémiologie, prévention, réduction de la prévalence, volaille

I. Introduction

Even though pig meat, with a worldwide share of about 50 %, is by far the most preferred meat by European Union (EU) consumers, the poultry meat production has showed the more favourable progression, with a mean annual increase rate of 2.5 % from 1992 to 2002 (European Commission, 2005); it recorded a worldwide share of around 26 % in 2005 (i.e. 70 million tons). Moreover, world poultry production and consumption are predicted to still increase over the next seven years by more than 20 %, i.e. an average annual growth of approximately 2.5 %. This expansion is mainly driven by low poultry meat production costs (relative to beef and pork), strong consumer preference, increased use in food preparations and a high demand for low price proteins on the worldwide market. Furthermore, poultry meat has generally benefited from the Bovine Spongiform Encephalopathy and Foot-and-Mouth Disease outbreaks, in the past few years.

Nevertheless, the avian sector has also faced several sanitary problems to which media coverage was given since a few years. In June 1999, the dioxin crisis in Belgium was caused by dioxin-contaminated food components. The widespread Avian influenza epidemic since 2003 has completely disrupted production and trade in many areas of the world, notably South East Asia but also the US and Canada. Beside these time-limited outbreaks, poultry production is confronted with a major permanent problem which is much less known. Poultry remains an important vehicle of bacterial human pathogens, leading to foodborne diseases by contaminated poultry products consumption and incriminated by epidemiological reports all over the world. The most reported pathogen agent is *Salmonella* spp. but, over the last three decades, *Campylobacter* spp. has emerged as an increasing concern all over the world. It is a major cause of a human acute bacterial enteritis called campylobacteriosis (van Vliet and Ketley, 2001). Unlike *Salmonella*, *Campylobacter* is mainly a problem in extensive poultry production, with up to 100 % of organic farms being contaminated (Engvall, 2001). This review will focus on prophylactic measures and curative treatments developed to reduce the incidence of *Campylobacter* infections in broiler flocks, at the primary production level.

II. Campylobacteriosis

Campylobacter spp. have been recognised as a cause of diarrhoeal illness in human since 1972. The *Campylobacter* species associated with food poisoning include *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari* and *Campylobacter upsaliensis*. *C. jejuni* is predominant while *C. coli* accounts for most of the remainder (Hariharan *et al.*, 2004). According to a French study, *C. jejuni* was found in ca. 68 % of the isolates from human campylobacteriosis cases (Dachet *et al.*, 2004).

Dose-response studies have shown that ingestion of about 10 (Ridley and Newell, 2004) to 500 cells (Rosenquist *et al.*, 2003) could already be sufficient to infect the human host. In human, pathogens invade epithelial cells in the ileum and large intestine thanks to chemotaxis and high motility, which causes inflammatory diarrhoea with usually moderate uncharacteristic symptoms (van Vliet and Ketley, 2001).

Complication following *Campylobacter* infection are uncommon, but an association with certain neurological disorders is noteworthy (Butzler, 2004). It is estimated that one in 1000

Campylobacter infections lead to the Guillain-Barré syndrome, an acute demyelinating disease characterized by muscular paralysis and leading to a 2 to 3 % mortality, (Allos, 1997). This syndrome is usually confined to very young or elderly patients or the immuno-compromised suffering (Altekruse *et al.*, 1999).

II.1. Public health impact

In most industrialised countries, the reported incidence of campylobacteriosis has increased during the last decade. In 2004, a total of 183,961 cases of laboratory confirmed campylobacteriosis were recorded in the EU-25, compared to 120,462 cases in 1999. The overall incidence was 47.6 per 100,000 population, which is slightly higher than for *Salmonella* (42.2). This makes *Campylobacter* the most commonly reported gastrointestinal bacterial pathogen in humans in the EU (EFSA, 2006).

On the other hand, in Belgium, *Campylobacter* infections represent the first cause of foodborne illness, just before *Salmonella* (CSH, 2005), with an estimated annual number of cases of about 48 per 100,000 population (Ducoffre, 2008). In 1984, the sentinel laboratories network recorded only just 1703 cases of infection. During the nineties, campylobacteriosis incidence has continually increased to reach 7473 cases in 2000, although the increase in the number of *Campylobacter* infections cases until 1996 could mainly be attributed to problems at the surveillance level (van Dessel, 2005). From 2000 to 2003, the illness incidence was reduced. However, it tends to increase again since 2004, without reaching the levels observed in 2000. It is usually estimated that 90 % of *Campylobacter* contamination are due to meat consumption and 80 % specifically come from poultry meat. Nevertheless, the rise of *Campylobacter* incidence observed for more than 20 years may also be partly due to increase of the poultry meat consumption during this period, rather than only an increase in the proportion of contaminated poultry (ICGFI, 1999).

The high incidence of *Campylobacter* spp. diarrhoea, its duration and possible sequelae, make campylobacteriosis important from a socio-economic perspective.

II.2. Economic and social importance

Campylobacteriosis affects each year a significant proportion of humans worldwide. Foodborne gastrointestinal diseases are major burdens on society causing considerable suffering and loss of productivity. Besides the discomfort felt by sick people, these infections have major economic repercussions by direct illness costs (laboratory diagnosis, consultations, medical cares, hospitalization, etc.) and indirect costs (work inefficacy, days lost work, etc.) (ICGFI, 1999; Bogaardt *et al.*, 2004). In the Netherlands, the economic costs of campylobacteriosis are estimated at 21 million euros per year for a population of 16 million (Mangen *et al.*, 2005a). Costs for campylobacteriosis are difficult to estimate because of differences in the simulation models used. Differences in one case cost according to two recent studies, i.e. 465 € in the United Kingdom (Roberts *et al.*, 2003) and 77 € in The Netherlands (van den Brandhof *et al.*, 2004) show the complexity of estimating these costs.

III. *Campylobacter* and the animal hosts

III.1. Characteristics of Campylobacter species

Campylobacters are Gram-negative, non-sporing, slender, helical or curved rods. In culture exposed to environmental stresses such as oxygen, the cells can change to spherical or coccal forms. Their polar flagellum conferred them a characteristic darting, and corkscrew-like motility. They are unable to oxidize or ferment carbohydrates but they reduce nitrate and nitrite. *Campylobacter jejuni* is the most frequent of the four thermophilic *Campylobacter* species that is isolated from human, and is one of 20 species and subspecies within the genus *Campylobacter* and family *Campylobacteraceae*. The other thermophilic species include *C. coli*, *C. upsaliensis* and *C. lari*. The thermophilic species are characterizeded by their ability to grow best between 37 and 42°C and their inability to grow at 25°C. For the most part, campylobacters require a microaerobic atmosphere for growth and can be very difficult to work with in laboratory settings, due to their fragile nature. However, isolates are extremely diverse, compared to some other enteropathogens. There are more than 60 different heat-stable serotypes, more than 100 heat-labile serotypes, differences in adherence properties, invasive properties, toxin production, serum resistance, colonization potential, aerotolerance and temperature tolerance. This diversity may be partly explained by the genomic plasticity of *Campylobacter*. The high levels of multiple-strain colonization and high frequency of incidence in mammals and birds mean there is substantial opportunity for exchange of genetic material and explain the ability of bacteria to survive in extreme conditions.

III.2. Transmission vectors

Campylobacter, as *Salmonella*, may be carried asymptomatically, as commensal organism, in the alimentary tract of all warm-blood animals. Because this pathogen can be transferred from animals to man, *Campylobacter* is considered as a zoonotic bacteria (WHO, 2000). Human infection may be caused by direct contact with contaminated animals or animal carcasses. In the case of domesticated animals as cattle, sheep, goats, pigs and especially poultry, pathogens can spread via the slaughter process to raw and finished products. *Campylobacter* may also be transferred to humans by consumption of undercooked or recontaminated meat, or the handling of raw products (Bryan and Doyle, 1995). It is noteworthy that, despite the meat importance, this does not represent the only food vehicle for *Campylobacter* and large campylobacteriosis outbreaks are usually associated with contaminated drinking water or raw or contaminated milk (Friedman *et al.*, 2004). According to Mead *et al.* (1999), food contamination could originate for 80 % of *Campylobacter* infection cases. Regarding inter-humans transmission, it is considered to be relatively exceptional (Adak *et al.*, 1995; Studahl and Andersson, 2000; Winquist *et al.*, 2001).

As mentioned above, the most important *Campylobacter* species known to cause human illness are the thermophilic species: *C. jejuni, C. coli and C. lari*. Birds, especially breeding poultry, appear to be the main reservoir for these pathogens, their internal temperature of 41-42°C being favourable for thermophilic *Campylobacter* proliferation (Hariharan *et al.*, 2004). Therefore, foods of poultry origin have been identified as significant sources of human campylobacteriosis. In Belgium, more than 40 % of campylobacteriosis cases would be associated to poultry meat consumption (Vellinga and Van Loock, 2002). In 1999, the finding of dioxin-contaminated feeding stuffs caused the Belgian authorities to withdraw all poultry meat and eggs from the

market. The estimated reduction in campylobacteriosis cases during the following crisis months was 40 % without any other explicative event that happened in this period. Furthermore, the belgian poultry reintroduction 4 weeks later on the market lead back to the previous campylobacteriosis incidence situation.

Another factor that could link together chicken consumption and human pathogen acquisition is the important similarity between human and poultry serotypes (Petersen *et al.*, 2001). Nevertheless, it is advisable to relativize this affirmation. Several studies have shown that some *Campylobacter* strains colonizing chicken are not human pathogens while some human isolated strains are unable to colonize poultry (Corry and Atabay, 2001).

III.3. Poultry colonisation

Colonized chickens usually show no observable clinical symptoms of infection even when young animals are exposed to high doses under experimental conditions (Newell and Fearnley, 2003). Corry and Atabay (2001) reported furthermore possible observation of enteritis and hepatitis symptoms or excessive mortality of very young chicks.

Experimentally, the dose of viable *C. jejuni* required to colonize chicks and chickens can be as low as 40 cfu even if numbers from 10^4 to 10^7 cfu can be frequently found in literature (Udayamputhoor *et al.*, 2003; Bjerrum, 2005). Furthermore, a strain variability concerning the ability to colonize the chicken digestive tract is also reported (Stas *et al.*, 1999). Infection pattern in poultry is also age-dependent. Actually, *Campylobacter* is not detected in chicks less than 2 to 3 weeks of age under commercial broiler production conditions, and that may be related to high levels of circulating *Campylobacter*-specific maternal antibodies in young chickens, which gradually decrease to undetectable levels at 2 to 3 weeks of age (Sahin *et al.*, 2001).

In chickens, *C. jejuni* colonizes the mucus overlying the epithelial cells primarily in the ceca and the small intestine but may also be recovered from elsewhere in the gut and from spleen and liver (Beery *et al.*, 1988; Achen *et al.*, 1998). The microorganism remains in the intestinal lumen at the crypts level, without adhesion. Once colonization is established, campylobacters can rapidly reach extremely high numbers in the cecal contents, from 10^5 to 10^9 cfu/g of content (Schoeni and Doyle, 1992; Achen *et al.*, 1998; Woodall *et al.*, 2005).

III.4. Poultry flock prevalence

The reported proportion of *Campylobacter*-positive broiler chickens flocks (the flock prevalence) varies between countries, ranging from 5 % to more than 90 % (EFSA, 2005), as summarized in Table 1. This apparent variation in the flock prevalence may reflect significant differences between countries, but is affected by sampling time, during the breeding period, and age and type of birds (conventional, free-range, organic). The method of detection (direct plating *vs* enrichment), and type of sample (cecal contents, fresh droppings, litter) also influence the detection of *Campylobacter* spp. (Jørgensen *et al.*, 2002; Oyarzabal *et al.*, 2005).

Table 1. Review of *Campylobacter* contamination prevalence in broiler chickens

Country	Number of analysed flocks	Contamination rate	Additional informations	Reference
Denmark	4286 (standard)	46 %	One year study ; cloacal swabs	Wedderkopp *et al.*, 2001
Great Britain	100 (standard)	4 weeks old broilers: 40 % 7 weeks old broilers: >90 %		Evans et Sayers, 2000
Denmark	10 (8 standard poultry farms)	50 %	Cloacal swabs just before slaughtering	Hald *et al.*, 2001
France	24 (standard)	79.2 %	Fresh droppings from 35 to 40 days of age	Denis *et al.*, 2001
Denmark	*160 (39 farms):* standard: 79 organic: 22 extensive indoor:59	 36.7 % 100 % 49.2 %	Study leading from 1998 to 1999	Heuer *et al.*, 2001
France	*620 of which:* standard: 403 *"Label Rouge": 62 export: 155	 56,6 % 80 % 51.3 %	Monitoring program leading in 1999	Avrain *et al.*, 2001
United States	3 farms with open-air range	32 to 68 %		McCrea *et al.*, 2006

* "Label Rouge": French extensive rearing broiler production with access to an open-air range

Table 2 shows more frequently contaminated broilers flocks in extensive rearing systems, especially those allowing access of the birds to an open-air range (organic, "Label Rouge", etc.). According to Heuer *et al.* (2001), the higher contamination rate of free-range broiler production could be explained by an unimpeded access to soil and water in the open-air range, a longer rearing period and differences in chicken host lineages.

Table 2. *Campylobacter* species distribution according to the poultry production system (from Heuer *et al.*, 2001).

Poultry production system	*C. jejuni*	*C. coli*
Standard	86.2 %	10.3 %
Organic	91.0 %	4.5 %

Distribution of *Campylobacter* species is also dependent on the rearing system, as shown in Table 2 and on the country. *C. jejuni* is the most frequently isolated species in poultry farms, whatever the production system. *C. coli* is less common although it is predominant in some EU Member Stats as Slovenia (Zorman *et al.*, 2006). Moreover, this species tends to become more frequent from a few years (Desmonts *et al.*, 2004). Finally, although relatively scarce, *C. lari* can also be isolated from poultry samples (Denis *et al.*, 2001; Hald *et al.*, 2001).

III.5. Risk factors for contamination at farm level

Although several risk factors for infection of broilers with *Campylobacter* spp. have been identified, knowledge about the various routes by which flocks become infected and their relative importance is still incomplete. The risk factors that have repeatedly been identified are summarized below.

Vertical Transmission

Campylobacter can be present in the poultry reproductive system. Nevertheless, several authors dismiss the assumption that vertical transmission is a major source of pathogen transmission (van de Giessen *et al.*, 1992; Jacobs-Reitsma, 1995; Chuma *et al.*, 1997; Sahin *et al.*, 2003). The main reasons proposed are a poor campylobacters survival on egg shells and inability to penetrate, to survive and to multiply into eggs in natural conditions. Meanwhile, some evidence could be found for vertical transmission of *Campylobacter* (Callicott *et al.*, 2006).

Horizontal transmission from the outer environment

Flock-to-flock transmission and litter role

Campylobacter transmission from a contaminated flock to the following flock seems to be not very important. Campylobacters are actually sensitive to detergents and disinfectants as well as dry conditions found in the poultry house during the empty period, although a little number of bacteria could survive during flocks intervals (Evans and Sayers, 2000 ; Petersen *et al.*, 2001).

Dry and aerobic conditions of clean fresh litter are considered harmful to *C. jejuni* as reported by Newell and Fearnley (2003) and Hutchinson *et al*. (2005). On the other hand, litter can be contaminated by broiler fecal droppings and then favours pathogen transmission through the flock. Nevertheless, in Belgium, the problem of litter as contamination vector is not recognized because houses are generally cleaned and disinfected and the litter is replaced between two subsequent flocks.

Dirty contaminated litter spread over the land can scatter the microorganism in the environment. Contaminated sewage are attractive for wild birds and insects that can be infected and then become *Campylobacter* vectors (Jones, 2001; Stanley and Jones, 2003).

Environment and open-air range

Campylobacter is able to survive in the house surroundings soil (Bull *et al.*, 2006) and the farmer can therefore act as a pathogen vector for campylobacters entrance in the broiler house, for instance via farmer's boots (Newell and Fearnley, 2003). The open-air range to which broilers have access in free-range poultry production could also be a major environmental source for flock contamination. When campylobacters are isolated from the

open-air range soil or from stagnant water, before the birds go out, the precedent flock may be responsible for the contamination. Furthermore, even if the open-air range seems to be *Campylobacter*-free, it is possible that campylobacters are present under a Viable but Non Culturable (VNC)-form. Induced through cell stress, particularly in drastic soil conditions (Rivoal *et al.*, 2005), VNC represents a resting or dormant stage extremely difficult to detect which could return to virulent form under appropriate conditions (Moore, 2001).

This transmission route seems yet not negligible as shown by Rivoal *et al.* (2005). Among seven poultry farms sampled from 1996 to 1999, four had get informations about the respect of strict biosecurity measures aimed at preventing the introduction of *Campylobacter* into flocks. In these farms, flock contamination appeared from six weeks of age, at the time of outdoor rearing period. In the three farms for which no biosecurity measures were applied, broiler contamination appeared from two weeks of age, then before the access to the open-air range. The influence of the open-air range on the contamination is yet not fully understood. According to a recent study of the Agence Française de Sécurité Sanitaire des Aliments (AFSSA), access to an open-air range is not the main *Campylobacter* contamination route of free-range broiler production. Among 73 farms, close to three quarter of flocks were contaminated before access to the open-air range. At the end of the rearing period, all the flocks were *Campylobacter*-positive, and concerned mainly *C. jejuni* (Huneau-Salaün *et al.*, 2005).

Feed and drinking water

Campylobacter can not survive in poultry feed because of a too low moisture rate (Altekruse *et al.*, 1999; Newell and Fearnley, 2003) although feed, as drinking water, can be contaminated by fecal droppings during the rearing period and can serve as transmission route (Bull *et al.*, 2006). On the other hand, water can be a real contamination vector for broiler chickens, as shown by Shanker *et al.* (1990) who succeeded to infect broilers with artificially contaminated water.

Air

Campylobacters can be isolated from air, both in the broiler house and from the house surroundings (Bull *et al.*, 2006). Pathogens are entrapped in aerosols or dust (Berndtson *et al.*, 1996) which could then be considered as pathogen transmission vector (Berrang *et al.*, 2003). Nevertheless, there is an assumption that *C. jejuni* can not survive for long period within the dehydrating conditions of dust. Saleha (2004) failed to isolate *Campylobacter* from 114 swabs samples of the walls, floors and dust from a total of 19 Malaysian chicken houses. According to Newell and Fearnley (2003), the location of ventilation fans can affect the risk of flock positivity, and the use of air conditioning increased this risk.

Wild and domesticated animal

Because of the pathogen unability to multiply outside warm-blooded animals, farm animals like poultry, cattle, pigs, sheep and goats (Oporto *et al.*, 2007), pets like cats and dogs, and wild animals like birds and rodents, are often considered as important *Campylobacter* reservoir. Although the broiler contamination by wild and domesticated animals does not seem to be direct, except for the free-range broiler productions, animal bearing and fecal shedding of the bacteria have been actually pointed out in several studies (Stanley and Jones, 2003; Hutchinson *et al.*, 2005) as a potential origin of environmental contamination (Nicholson *et al.*, 2005).

Because of their microaerophilic metabolism and their inability to growth at temperatures below 31°C, the presence of campylobacters in streams, rivers and ponds can then be taken as a sign of recent faecal contamination by livestock or wild animal (Friedman *et al.*, 2000) but can last up to four months (Rollins and Colwell, 1986; Hazeleger *et al.*, 1998). *Campylobacter* serotypes and genotypes are not systematically corresponding, and the wild animals role should be relatively limited after all (Petersen *et al.*, 2001).

Insects

Some authors have made the assumption that insects like flies could play a part in the *Campylobacter* epidemiology (Skov *et al.*, 2004). They could act as mechanical vectors, transmitting pathogens from reservoir environment or animals to broiler flocks (Ekdahl *et al.*, 2005; Nichols, 2005). Nevertheless, insects seem to be contaminated by the broilers and may act as pathogen vector only afterwards.

IV. European legislation

Following several different sanitary crisis, the Community legislation on food hygiene has been progressively restructured and strengthened in order to establish a coherent and consistent network of hygiene rules based on an integrated approach covering the whole food chain “from stable to table”. The new legal instrument on food hygiene ensures that the Member States comply with the Good Hygiene/Farming Practices (GHP) in livestock production, as applied in Belgium. The reflection of the Commission on the new approach to food safety, covering the entire production chain of all foodstuffs, both of animal and of plant origin, resulted in the adoption of the White Paper on food safety in January 2000.

The main principles depicted in the White Paper are : the assurance of a high standard of food safety ; the responsibility for food safety primarily upon food businesses, including feed manufacturers and farmers ; the assurance of a “farm to table” policy ; the possibilities for traceability and transparency and the possibilities to take into account the precautionary principle and other legitimate factors, where appropriate.

These rules would be essential to prevent contamination and spread of zoonotic agents in farms and are the basis of the European legislation concerned with the monitoring and control of zoonoses and zoonotic agents at the primary production, transformation and distribution levels. With the aim of decreasing the incidence of zoonoses in humans, of improving the control of zoonoses in the primary production and of strengthening the collection of relevant data to support risk assessment activities and risk management decisions, the European Union has decided more recently to integrate and to standardize the different national monitoring and survey plans by the establishment of the Directive 2003/99/CE and the Regulations (EC) n° 2160/2003 and n° 1003/2005.

The specific purpose of these Regulations is “to ensure that proper and effective measures are taken to detect and to control *Salmonella* and other zoonotic agents, particularly at the level of primary production, in order to reduce their prevalence and the risk they pose to public health”. *Salmonella* is the primary zoonotic agent targeted at primary production as it represents an important burden to public health. From 2010, poultry meat containing *Salmonella* in 25 g shall not be placed on the market without any industrial treatment able to eliminate *Salmonella*.

Such measures are not yet implemented for *Campylobacter* at this time but are actually examined by The Community Economic and Social Committee, a small number of Member States and at a preliminary stage the European Parliament (Kremer, 2005). It is within this context that the European Food Safety Authority (EFSA) has formulated several recommendations in its Scientific Report in 2005 (EFSA, 2005). They concern particularly the intensification of epidemiological studies about *Campylobacter* and the reduction of the proportion of *Campylobacter*-infected poultry farms, by the application of strict biosecurity measures.

Since 1996 in Belgium, the « Institut d'Expertise vétérinaire » that became included in the « Agence Fédérale pour la Sécurité de la Chaîne alimentaire » (AFSCA), with the help of Universities and Community Reference Laboratories, has setting up an annual monitoring program of zoonotic agents in humans and animal products. Since 1998, the survey program, intended for all foodborne pathogens including *Campylobacter*, is coupled with a hygiene plan based on biosecurity measures at primary production level which aims to reduce contamination from live animals. Such interventions measures can lead to additional production costs that are at the moment difficult to estimate. The Dutch CARMA Project has tried to evaluate these costs by means of an economic model. According to Mangen *et al.* (2005b), the annual income of broiler farmers could not bear increased production costs without any additional bonus, and this situation is all the more actual for extensive small-sized poultry farms.

V. Integrated approach to reduce flock contamination

Given the public health and economic problem represented by *Campylobacter*, and the strengthening of the European legislation relating to animal products contamination by zoonotic agents, it is important to take measures in order to reduce *Campylobacter* prevalence throughout the poultry production chain leading to a reduced incidence of the human illness. In a recent risk evaluation, the CSH (2005) showed that the risk to contract illness decreases significatively if the proportion of contaminated meat-based preparations may be limited or eliminated in the food distribution chain. Moreover, it is not just presence or absence of pathogenic bacteria that is important, but also the amounts in which they are present. Dutch (Nauta *et al.*, 2007) and Danish (Rosenquist *et al.*, 2003) studies have particularly developed quantitative microbiological risk assessment models based on mathematical dose-response model to estimate the relationship between ingested dose and the probability of developing campylobacteriosis.

Many broiler flocks can become infected with *Campylobacter* spp. at many stages of the poultry production chain. Therefore, the only intervention strategy to reduce the exposure of humans to *Campylobacter* spp. seems to be an integrated approach (Snijders and van Knapen, 2002), with multiple control measures along the poultry production chain, for instance at farm level, during transport, at the slaughterhouse and/or at the product transformation step (Line, 2002; Hariharan *et al.*, 2004; Whitaker, 2006).

Risk factors and sanitary measures for contamination during catching and transportation have been presented by Ramabu *et al.* (2004) and Rasschaert *et al.* (2007). The risk factors associated with the slaughter operations on the contamination of carcasses have been studied by Rosenquist *et al* (2006) and EFSA (2005) have reviewed the risk management options available at this level. Furthermore, techniques of preventing contamination or decontaminating raw meat and poultry meat products in the food processing industry have been discussed by several

authors (Huffman, 2002; Woteki and Kineman, 2003; Dinçer and Baysal, 2004). Woteki and Kineman (2003) have also presented in details necessary strategies at the consumer level.

VI. Usual prevention methods

VI.1. Hygiene measures

Practical biosecurity measures at the farm level have been determined as the primary strategy to prevent colonisation of housed broiler flocks with campylobacters entering the processing plant and hence the food chain (van de Giessen *et al.*, 1992; ICGFI, 1999; Gibbens *et al.*, 2001; Rivoal *et al.*, 2005). Nevertheless, many authors have shown that biosecurity measures are only partly effective in controlling *Campylobacter* contamination (Pattison, 2001; Sahin *et al.*, 2003; Van Gerwe *et al.*, 2005).

Measures that are important to protect the flock include the washing of hands, the wearing of protective clothing and dedicated footwear, the respect of house cleaning and disinfection protocoles, provision of *Campylobacter*-free water, feed and the removal of spent litter between two flocks. Details about biosecurity measures designed to control *Campylobacter* have been reported by Allen and Newell (2005).

The limited action of hygiene procedures is based on the fact that in conditions where broilers are confronted with environmental factors which are scarcely controllable (open-air range, wild birds, domesticated animals faeces, etc.), i.e. organic and free-range flocks, biosecurity is difficult to apply. In these production systems, Rivoal *et al.* (2005) have shown that, even if strict hygiene measures allow broiler flocks to be *Campylobacter*-negative during the first weeks of age (the indoor period), birds are almost always colonised at slaughter, after the access of birds to the open-air range.

Nevertheless, even if high levels of environmental exposure to campylobacters may overwhelm best practice biosecurity measures and that these practices can not guarantee infection prevention, they can help to delay the onset of *Campylobacter* colonisation and are consequently essential.

VI.2. Antibiotics use

The use of antibiotics in modern intensive animal production as growth-promoters and for therapy and prevention of diseases could not be a rational solution to reduce *Campylobacter* incidence. Several studies have actually pointed out the partial association between the veterinary use of antibiotics and the emergence of resistant strains of campylobacters related to human enteritis (Pezotti *et al.*, 2003; Desmonts *et al.*, 2004; Luangtongkum *et al.*, 2006). Nevertheless, Bywater (2004) assessed the sum total contribution of antibiotic use in animal production to human bacterial resistance as $< 4\,\%$. Moreover, variation is seen in antibioresistance in different countries, reflecting various veterinary practices in antimicrobial usage. Whatever the opinion we have in this debate, these antibiotics have been banned in the EU since January 2006, according to the “Precautionary Principle”.

VI.3. Acidification

It is generally acknowledged that the campylobacters are sensitive to acid conditions (AFSCA, 2006). Several strategies developed to reduce *Campylobacter* populations are based on the acidification of the pathogen environment.

Drinking water and feed acidification

The *in vitro* studies realized by Chaveerach *et al.* (2002) have pointed out the bactericidal activity of organic acids used individually or in combination. The four studied acids (formic, acetic, propionic and hydrochloric), alone or in combination at different formulation ratios, were mixed with a commercial broiler feed into bottles containing 250 ml of tap water. The acid combinations have shown an interesting bactericidal activity at pH 4.0 with *Campylobacter* numbers declining below 1 log cfu/ml within 1 h, and the reduction was higher than the decreasing effect observed with the different acids used individually.

Water being an efficient *Campylobacter* vector, Chaveerach *et al.* (2004) studied *in vivo* the drinking water acidification by the same four organic acids as a prophylactic measure. During all the experiment, no *Campylobacter* was found in acidified drinking water. Although acidification seems to be an effective measure to control water as a prominent contamination vector, most chickens were infected at the end of the experiment, demonstrating the impact of other contamination ways. Byrd *et al.* (2001) have also studied drinking water acidification during pre-slaughter feed withdrawal. The addition of 0.5 % lactic acid in drinking water significantly reduced crop contamination with *Campylobacter* as compared with the controls (62.3 % *vs* 85,1 %).

Another study by Heres *et al.* (2004) has tested fermented feed containing high concentrations of organic acids (5.7 % lactic and 0.7 % acetic) on susceptibility of chickens to *Campylobacter* and *Salmonella*. Broilers fed with fermented feed until 21 days of age needed a ten times higher dose of *Campylobacter* to achieve the same proportion of infected chickens as the control population. Nevertheless, the protective effects seem relatively limited and dependent on the infection dose according to the pathogen inoculated.

Litter acidification

Acidification of poultry litter has also been suggested as a method to limit pathogen proliferation in breeding flocks. Line (2002) assessed two commercially available litter treatments (aluminium sulfate and sodium bisulfate) on *Campylobacter* prevalence and cecal colonization of broilers. For example, treatment of pine shavings litter with the lowest level of aluminium sulfate, i.e. 3.63 kg per 4.6 m^2 litter significantly reduced cecal *Campylobacter* colonization frequency by 65 % and effected a 3.4 log reduction in cecal pathogen populations. Nevertheless, it is noteworthy that, even at the lowest treatment level, such high concentrations are difficult to include in an environmental-respectful rearing system.

VII. Complementary developing strategies

VII.1. Non antagonism-based studies

Active and passive immunity

Vaccination of poultry against *Campylobacter* has been considered to be a more effective measure than strict hygiene practices by some studies (de Zoete *et al.*, 2007), because of the observation of a *Campylobacter*-specific immune response in chickens (Rice *et al.*, 1997).

So, the study of Wyszynska *et al.* (2004) has shown that chicken immunization with avirulent *Salmonella* vaccine strain carrying *C. jejuni* cjaA gene, encoding highly immunogenic proteins, may be an attractive and efficient approach for bird vaccination.

About the passive immunization, Sahin *et al.* (2003) have observed that *C. jejuni*-specific maternal antibodies have a role in protection against colonization in young *Campylobacter*-negative chicks. Furthermore, Tsubokura *et al.* (1997) showed prophylactic and therapeutic effects against *C. jejuni*, for at least 5 days post-infection, by oral administration of bovine and chicken immunoglobulin preparations to 22-day-old chickens. Nevertheless, the use of maternal antibodies could be hindered by their short protection period, unable to cover the whole rearing period. Wilkie (2006) purified and concentrated egg yolk antibodies from *C. jejuni* vaccinated hens. Three hours after experimentally infecting day-of-hatch broiler chicks with $5 \cdot 10^7$ cfu *C. jejuni*, yolk antibodies were administered via oral gavage or in the feed at a final concentration of 0.5 % (w/w) until day 11 post-challenge. Despite measurable antibody activity *in vitro*, no significant reduction in the intestinal colonisation by *C. jejuni* could be demonstrated.

Bacteriophage therapy

The use of *Campylobacter*-specific bacteriophages has been attempted by several authors to face pathogens in poultry farms (Goode *et al.*, 2003; Carrillo *et al.*, 2005; Wagenaar *et al.*, 2005). Atterbury *et al.* (2005) demonstrated a correlation between the presence of natural environmental phage and a reduction in the *Campylobacter* population colonizing broiler chicken caeca. Although it is a relatively new developing technique, it has already given some interesting results. However, Goode *et al.* (2003) emphasize the limitation of phage use at farm level i.e. the potential for fast selection of resistant campylobacters following the simultaneous pathogen and bacteriophage release. These authors would limit consequently the bacteriophages use at the slaughter stage. On the other side, Wagenaar *et al.* (2005) consider the release of phage-infected campylobacters in the environment to be acceptable, since phages have been shown to reside in *Campylobacter* populations present on naturally infected poultry.

Diet modification

Heres *et al.* (2003) have studied the effect of feed fermentation on the *Campylobacter* contamination of broiler chickens. They used a moistened commercial standard broiler feed (feed: water ration = 1 : 1.4) supplemented with a *Lactobacillus plantarum* strain to ferment the mixture. The resulting product, named FLF (fermented liquid feed), lead to a significant reduction of *Campylobacter* susceptibility in chickens. This reported effect was particularly due to the high organic acids concentrations and the resulting pH decrease in the feed. FLF had also an effect on the chicken intestinal microflora (Heres, 2004).

Cereal-based broiler diets contain anti-nutritive Non-Starch Polysaccharides (NSP) that increase intestinal viscosity, impairing digestion and reducing broiler performances (Bedford, 2001). Addition of exogenous enzymes, in particular xylanases and glucanases, reduces anti-nutritive effects of NSP and improves zootechnical poultry performance. Moreover, growth-promoting enzymes have also shown interesting antagonistic effect against *Campylobacter*. By reducing viscosity of the intestinal contents, xylanases can induce modifications of the chicken flora (Vahjen *et al.*, 1998) and reduce *C. jejuni* contamination when these enzymes are added to the broiler diet, as shown by Fernandez *et al.* (2000). These authors have found significant reductions of the *C. jejuni* cecal colonization (from 0.3 to 0.5 log cfu/g cecal content on average) by 0.1 % xylanases supplementation of the diet. This reduction can be due to a lower intestinal viscosity as well as to the reduction of the digesta transit time, leading to a too short time for the pathogen establishment. Viscosity reduction could stimulate mucin production in the small and large intestines and in the caeca, as well as changes in the mucin composition. Some mucin glycoproteins are responsible for the protective properties of the mucus gels in the gastrointestinal tract.

It is however important to point out that the use of feed additives is subjected to strict European legislations. Regulation (EC) n° 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, including enzymes, lays down rules governing the Community authorisation of the additives and, in particular, defines the conditions that a substance or a product should meet to be granted authorisation, and the labelling conditions for these additives. Authorisation of the additive needs to pass the risk assessment by EFSA. To be legally placed on the market and used, feed additives must be proved to have a favourable effect on the characteristics of the feed to which it is added or on animal production, to have no harmful effect on animal health, human health or the environment and that the presentation of the additive or alteration of the features of the products to which it is added does not harm or mislead the consumer. All these procedures are expensive and time-consuming so that enzymes approach may only be attractive if the purpose of pathogen prevention is combined with performance improvement.

VII.2. Microbiological competition

Competitive exclusion flora

Competitive exclusion (CE) is a concept taking advantage of bacterial antagonism to reduce animal intestinal colonization by pathogenic microorganisms. The study of defined or undefined flora acting by competitive exclusion mechanisms was first initiated in the 70s by Nurmi and Rantala (1973). They observed that introduction of gut contents originating from adult cocks to 1-2 d old chicks can protect young birds against *Salmonella infantis* infection. Figure 1, adapted from van der Wielen (2002), summarizes possible interactions between competitive exclusion flora and potential pathogens in broiler caeca. A twofold competition may operate in the gastrointestinal tract, i.e. competition for nutriments and for adhesion sites. Moreover, CE bacterial formulations may have a direct antimicrobial effect by the production of lactic acid, volatile fatty acid, hydrogen peroxide or bacteriocins.

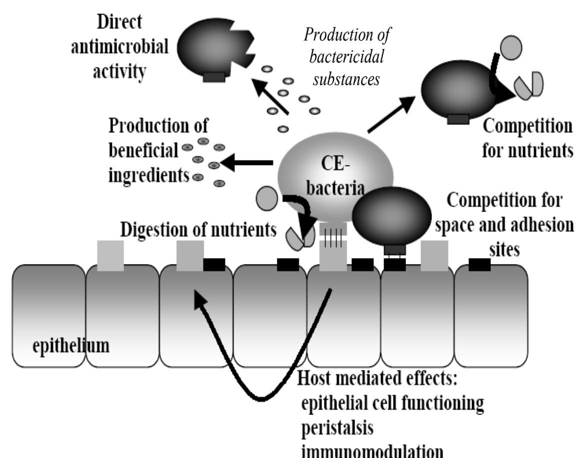

Figure 1. Interactions between CE bacteria and potentially pathogen bacteria in the caeca and with epithelial caecal cells of broiler chickens. Bactericidal substances: volatile fatty acids, organic acids, bacteriocins, hydrogen peroxide (adapted from van der Wielen, 2002)

Afterwards, such CE flora has displayed variable results according to the experiments, generally because of their undefined composition. Oral treatment of newly-hatched chicks, challenged at day 24 with $5.7 \cdot 10^4$ cfu, $5.4 \cdot 10^4$ cfu or 7.3 10^3 cfu *C. jejuni*, with the commercial CE Broilact® reduced both the proportion of positive chicks from 100 % to 0-62 % and the numbers of the challenge organism in the caeca by 10^8 to 10^9-fold according to the infection dose (Hakkinen and Schneitz, 1999). Aho *et al.* (1992) also observed a reduction in *Campylobacter* cecal population with Broilact-treated chicks. Stern *et al.* (2001) showed a *Campylobacter* average reduction of 0.38 log cfu/g and 2.01 log cfu/g cecal material in 6-days chicks treated with CE and a mucosal CE cultures respectively. The average incidence colonization reduction observed in CE- and MCE-treated birds was 2.2 % and 15.6 % respectively. On the other hand, Laisney *et al.* (2003) failed to show beneficial effect of cecal CE flora on broiler infection with 10^2-10^3 cfu *C jejuni* at 15 days of age. Because of the limited advantage for the poultry producers, the practical application of CE has only a great success in Finland.

Furthermore, it is difficult to ensure the absence of potentially pathogen organisms in the bacterial compositions. It is noteworthy that Chen and Stern (2001) aimed to prevent *Campylobacter* colonization of the chicken intestinal tract by early inoculation, in these chickens, of non-pathogenic *C. jejuni* strains used as defined CE preparation. Nevertheless, some authors predict a promising future for CE (Schneitz, 2005), among others owing to the ban of growth-promoting antibiotics in animal production and sanitary requirements that become more and more strict.

Acidifying bacteria

Because of the CE disadvantages, the current trend is now the development of defined flora although the work is made complicated by lack of knowledge of the mechanism of CE and of the type of bacteria involved in the process (Chaveerach *et al.*, 2004; Bjerrum, 2005). Acidifying bacteria, particularly lactic acid bacteria (LAB), contribute since several thousand years to preserve food. Nevertheless, their antimicrobial properties are not limited to the food

industry field. Several *in vitro* and *in vivo* studies, summarized in Tables 3 and 4, have investigated the bacterial antagonistic activities against *Campylobacter*.

Table 3. *In vitro* studies assessing the antagonism of microorganism against *Campylobacter*

Antagonistic microorganisms	Principal tests	Observed or assumed antagonistic effects	References
Mixture of *lactobacilli* *L. acidophilus* *L. fermentum* *L. crispatus* *L. brevis*	*C. jejuni* and *lactobacilli* mixed with sterile poultry feed followed by successive incubations at 41.1°C in *in vitro* tests simulating the poultry digestive tract	*C. jejuni* and *lactobacilli* enumeration: absence of *C. jejuni* for the last incubation	Chang and Chen, 2000
L. plantarum *Bifidobacterium bifidum*	Agar diffusion Co-culture with *C. jejuni*	Significant inhibition of *C. jejuni* growth ; increased number of probiotic after 24h ; lactate and acetate production	Fooks and Gibson, 2002
Lactobacillus spp.	Agar diffusion Co-culture with *C. jejuni*	Production of formic and acetic acids; production of an antimicrobial peptide	Chaveerach *et al.*, 2004
Bacillus circulans *Paenibacillus polymyxa*	Spot test	Production of bacteriocins inhibiting *Campylobacter*	Svetoch *et al.*, 2005

Lactobacilli are frequently used in these *in vitro* studies. Chaveerach *et al.* (2004) have assessed the inhibitory activity of a *Lactobacillus fermentum* (P93) strain isolated from the chicken gut on ten C. *jejuni*/*coli* strains by diffusion agar assay and co-culture in anaerobic conditions. The experiment revealed an antagonistic effect of the *L. fermentum* strain against all the ten *Campylobacter* tested strains, which decreased of 4.10 ± 2.15 log cfu/ml during 24 h of co-culture incubation. The authors have suggested that the inhibitory effect of *Lactobacillus* (P93) on *Campylobacter* growth could be explained mainly by organic acids production, resulting in pH reduction. Furthermore, the inhibitory effect was enhanced when the pH level in the culture media was low. Levels and types of organic acids produced depend on bacterial species or strains, culture composition and growth conditions (Ammor *et al.*, 2006). According to van der Wielen *et al.* (2000) and Chaveerach *et al.* (2004), the acid dissociation stage is an essential factor for antagonism effect. Van der Wielen *et al.* (2000) stated that the undissociated form of these short-chain acids can diffuse freely across the bacterial membrane and dissociates inside the cell, thereby reducing the internal pH and causing internal pathogen cell damage. Some authors mention also the damage caused by the anion itself as well, and in particular the inhibition of fundamental metabolic functions (van der Wielen *et al.*, 2000; Chaveerach *et al.*, 2002).

Table 4. *In vivo* studies of probiotics showing antagonism on *Campylobacter*

Antagonistic microorganisms	Observed effects	References
Combination of *Citrobacter diversus, Klebsiella pneumoniae, Escherichia coli*, with mannose	Flock colonization rate: - 62 %	Schoeni and Wong, 1994
L. acidophilus + *Streptococcus faecium*	Frequency of *C. jejuni* shedding: - 70 % Jejunal colonization: - 27 %	Morishita *et al.*, 1997
Enterococcus faecium	No significant difference	Netherwood *et al.*, 1999
Purified bacteriocin of *Paenibacillus polymyxa*	Significant reduction of intestinal contamination rate and frequency	Stern *et al.*, 2005

The *in vitro* study realised by Fooks and Gibson (2002) aimed to investigate antagonistic effects of lactobacilli (*L. plantarum*, *L. pentosus*, *L. acidophilus*, *L. reuteri*). *Lactobacillus plantarum* 0407 showed the most promising inhibitory activity on *Campylobacter* growth, both using plate assays and co-culture. This antimicrobial activity appeared to depend on the carbohydrate source supplied *in vitro*, suggesting that a suitable carbohydrate substrate supplementation may enhance competitive exclusion by lactobacilli. The experiment of Chang and Chen (2000) tried to get closer to *in vivo* conditions, by investigating the impact of a selected lactobacilli mixed culture (*L. acidophilus*, *L. fermentum*, *L. crispatus*, *L. brevis*) on *C. jejuni* in simulated chicken digestive tract. The *C. jejuni* and lactobacilli were mixed with sterile poultry feed and incubated at 41.1°C for various lenghts of time and pH values, simulating five segments of the digestive tract. All the tested lactobacilli spp. showed an antagonistic effect on *Campylobacter* in individual sections and the whole simulated digestive tract models.

Then, several studies have pointed out the bactericidal activity of hydrogen peroxide (H_2O_2) produced by LAB in the presence of oxygen (Felten *et al.*, 1999; Strus *et al.*, 2006). Hydrogen peroxide may inhibit growth of bacteria which do not possess protective mechanisms like catalase or peroxidase. Its antimicrobial effect may result mainly from oxidation phenomenons causing denaturing of a number of enzymes and from the peroxidation of membrane lipids and proteins leading to increased membrane permeability (Edens, 2003; Ammor *et al.*, 2006). Zhao and Doyle (2006) showed that incubation of 7.0 log cfu/ml *C. jejuni* with 0.1 and 0.2 % H_2O_2 in suspension reduced *C. jejuni* populations by ca. 2.0 and 4.5 log cfu/ml, respectively. Furthermore, some authors studied the efficacy of broiler carcasses decontamination with H_2O_2 during the slaughter processing. Although Wagenaar and Snijders (2004) observed that immersion of carcasses in 1, 2, 3 and 4 % H_2O_2 solutions containing glycerol resulted in average reductions of 0.3 up to 1.4 log cfu for the mesophilic aerobic counts, they did not measure *Campylobacter* loads on carcasses. Moreover, Dickens and Whittemore (1997) demonstrated that addition of up to 1.5 % H_2O_2 to sprays waters during defeathering had no effect on total aerobic plate counts of picked uneviscerated carcasses when compared to the water control.

Besides organic acids and H_2O_2, bacteriocins are the third kind of compounds that may help to inhibit *Campylobacter* growth, as shown by Stern *et al.* (2006) for a bacteriocin produced by a *Lactobacillus salivarius* strain. Bacteriocins are peptidic compounds with antimicrobial

properties produced by some bacteria. Their target is mainly the cytoplasmic membrane, forming pores that allow the unregulated outflow of essential ions, leading to bacteria death (Papagianni, 2003). The bacteriocins have often a relatively restricted spectrum of activity against bacteria strains closely related to the producing strain. Particularly, the genus *Paenibacillus* have be pointed out by Russian and American researchers. Svetoch *et al.* (2005) have revealed the production, by three *Paenibacillus polymyxa* strains, of bacteriocins effective against *Campylobacter*. One of these bacteriocins, secreted by *P. polymyxa* NRRL-B-30509, was purified and microencapsulated to evaluate a bacteriocin-based treatment to reduce *C. jejuni* colonization in poultry (Stern *et al.*, 2005). The purified preparation was incorporated in chicken feed at the rate of 0.25 g/kg. One-day-old chicks were orally infected with 10^8 cfu *C. jejuni* and were provided from day seven to chicken feed containing or not (control) bacteriocin. Ten days after *C. jejuni* challenge, comparison of cecal contamination rate between control and treated chickens showed that bacteriocin treatment reduced levels of intestinal colonization by *C. jejuni* from 4.6 to 6.3 log cfu/g of feces ($P \leq 0.05$).

Probiotics

The probiotic notion derives directly from the competitive exclusion concept. Unlike the CE treatments, probiotics are compositions containing one or several well-defined strains. Several descriptions have been proposed for probiotics (Jin *et al.*, 1997) but they may globally be defined as living microorganisms which, once ingested, beneficially affects the host animal by improving its microbial balance (Fuller, 1989). The main expected characteristics and functions for an efficient probiotic strain in poultry production, presented in Table 5, include maintaining normal intestinal microflora by competitive exclusion and antagonism, altering metabolism by increasing digestive enzyme activity, improving feed intake and digestion and neutralizing enterotoxins and stimulating the immune system (Ghadban, 2002). The use of probiotic microorganisms in animal production is well controlled and is considered, as enzymes and feed additives, by Regulation (EC) n° 1831/2003 of the European Parliament and of the Council of 22 September 2003.

Table 5. Expected characteristics and functions of probiotics in animal production (adapted from Edens, 2003)

Characteristics	Functions
Host adapted by creation of a beneficial microecology	Exclusion (colonization prevention) or bactericidal effect against pathogens
Non pathogenic	Production of inhibitory substances against other bacteria
Resistances to gastric and biliary acids	Alteration of microbial metabolism
Rapidity to colonize intestinal epithelium and mucus	Active competition for adhesion sites
Viability in the gastrointestinal tract	Competition for essential nutriments
Tolerance with industrial manufacturing and storage	Stimulation of the immune system
	Improvement of nutriments absorption
	Improvement of animal performances
	Reduction of pathogen excretion in feces

About the *in vivo* studies, Morishita *et al.* (1997) have assessed the antagonistic effect of probiotic containing a *L. acidophilus* strain combined with a *Streptococcus faecium*. This avian-specific probiotic was given to chicks from day one to day three; moreover, birds were challenged with *C. jejuni* 6 h after the first oral administration of probiotic. At 40 days of age, the probiotic-treated group had a 70 % ($P = 0.0001$) decreased number of birds shedding *C. jejuni* when compared with the control group given distilled water instead of probiotic. They also found a 27 % ($P = 0.0001$) reduction in the number of chickens that were colonized in the jejunum at slaughter in comparison with the control birds.

VII.3. Prebiotics and synbiotics

Prebiotics are defined as poorly digestible food ingredients that beneficially affect the hosts by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995). Among the mostly reported prebiotics are polyols (xylitol, etc.), or di-, oligo- and polysaccharides (lactilol, fructo-oligosaccharides, inulin, etc.) (Šušković *et al.*, 2001).

Some specific carbohydrates used as prebiotics, like mannanoligosaccharides (Spring *et al.*, 2000) and isomaltooligosaccharides (Chung and Day, 2004), have been shown to reduce *Salmonella* colonization in the caeca of poultry. Such carbohydrate substrates are fermented in the latter intestinal segments and give rise to a mixture of carbon dioxide, hydrogen and short-chain fatty acids (Grizard and Barthomeuf, 1999; MacFarlane *et al.*, 2006) that lead to intestinal pH reduction and may partially explain the pathogen antagonism.

Combinations of prebiotics and probiotics, for example *Lactobacillus* and lactitol, are known as synbiotics, and may have antimicrobial activity (Klewicki and Klewicka, 2004). Then, the survival and the development of the probiotic organism could be improved, because its specific substrate is readily available (Collins and Gibson, 1999). Fooks and Gibson (2002) have yet recorded a *C. jejuni* inhibition *in vitro*, with a population reduction below detectable level after 24 h culture, with a *L. plantarum* or *Bifidobacterium bifidum*, when combined with oligofructose or an oligosaccharide : xylo-oligosaccharide mixture (50 : 50, w/w) at 10 $g \cdot l^{-1}$. The observed antagonistic effect was related to a pH decrease of the cell culture.

VIII. Conclusion

Zoonose, particularly food pathogen transmission from animals to man, is a major concern of food safety. Consequently, the European Union has recently established the Directive 2003/99/CE and the Regulations (EC) n° 2160/2003 and n° 1003/2005, in the way to decrease the incidence of zoonoses in humans, to improve their control in the food chain and to strengthen the collection of relevant data to support risk management decisions. *Salmonella* is the primary zoonotic agent targeted at primary animal but similar measures and recommendations are actually examined for *Campylobacter* by the European authorities. *Campylobacter* is one of the main recognized causes of human acute enterocolitis called “campylobacteriosis”. Foods of poultry origin appear to be the main source of this pathogen. In order to reduce the exposure of humans to *Campylobacter* spp., an integrated approach including control measures implemented throughout the poultry production chain (chicken meat and eggs) appears to be the only effective intervention strategy. At the primary production

level, biosecurity measures are only partly effective and subtherapeutic antibiotics, which were used as growth-promoting but also helped to prevent pathogen contamination, are baned in the EU since January 2006. Many alternative procedures have been investigated. They are based on active/passive immunity, on bacteriophage, NSP-hydrolysing enzymes or bacteriocins incorporated in chicken feed, or on diet modification. Nevertheless, direct and indirect acidification- and antagonism-based measures seem to be the more promising strategies. Beside competitive exclusion flora, defined bacterial strains like probiotics and acidifying bacteria have shown interesting *in vitro* and *in vivo* antagonistic effects against *Campylobacter* spp., especially by organic acids production and pH reduction. Several studies have showed that synbiotics, i.e. combinations of probiotics and prebiotics which can be used specifically as substrate by probiotics, may also have antimicrobial activity. Feed additives, i.e. components other than feedstuffs like probiotics, synbiotics, bacteriophage or exogenous enzymes, are yet subjected to strict European legislations. With the cost inherent to these authorisation procedures, application of monitoring plans and developed measures to control *Campylobacter* contamination in poultry farms will be expensive for the producer. Only the strategies that combine low cost and efficacy to prevent or reduce *Campylobacter* contamination in broiler flocks, in order to fit the EU Directives and Regulations, would be applicable in practice.

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Salmonella – Review of the literature

Unlike *Campylobacter*, bacteria of the species *Salmonella enterica* can infect young chicks from the day of hatch. The probability for broiler chickens to become infected at the slaughter age is consequently very high. The following section describes the characteristics of the bacteria and the mechanisms of poultry colonization. Transmission vectors of *Salmonella* at farm level, also reported in this section, are relatively well known and need to be controlled in order to prevent flock contamination and subsequent human disease. Problems linked to salmonellosis and public health impact, which has led to the establisment of a specific European legislation to reduce its prevalence, are also especially pointed out.

Knowledge of infection mechanisms in the gastro-intestinal tract of poultry host and mechanisms of immunity against the pathogen has first led to the development of vaccination programs. Nevertheless, vaccination strategy cannot be applied in broilers due to the short life span of the animal. The use of feed additives is then more and more accepted as a valuable way to combat *Salmonella* infection in broilers standard production. Among the studied strategies are organic acids, prebiotics, probiotics, synbiotics, competitive exclusion products, passive immunity and bacteriophages. All the current and developing strategies to reduce *Salmonella* contamination at farm level are detailed in the Article II presented in the second part of this Chapter.

An attractive developing approach is the combination of different treatments allowing potential addition, complementarity, or even synergism, of the protagonist effects. Especially, supplements based on microbial strains (competitive exclusion products, probiotics, etc.) may be easily combined with several kinds of molecules, like fermentable oligosaccharides which can be used by the bacteria for their metabolism. As dietary modulation by xylanase supplementation shows additionnal interest by improving the nutritionnal value of the broiler diet, it was hypothesized that enzyme activity could also produce C-5 oligosaccharides specifically metabolizable by the antagonistic bacteria in order to efficiently compete with *Salmonella*.

I. Introduction

Salmonella is a major cause of gastro-enteritis in human worldwide. It has been shown that the human infection comes from consumption of a variety of foodstuffs covering both food of animal and plant origin. The food that is most often incriminated is raw or undercooked poultry products (EFSA, 2007a). Facing the public health concern associated with salmonellosis, the EU has progressively established a European regulation ordering its Member States (MS) to settle up survey and control programmes focused on *Salmonella* and other pathogen agents in poultry. Consequently, in Belgium, like in overall European Union (EU), the salmonellosis incidence tends to decrease since 2003. This reduction may be explained by the prevalence reduction of the *S*. Enteritidis serovar, mostly associated with the consumption of egg, following recent vaccination programmes carried out in breeder and laying hens flocks. Nevertheless, vaccination appears to be only partly effective in controlling *Salmonella* in poultry, and the need of complementary strategies has arisen. As poultry carcass contamination can not be avoided when chickens are contaminated at the arrival in the slaughter house, even with optimal slaughter and process (Corry *et al.*, 2002), implemented prophylactic measures target mostly the poultry flocks at the primary production level. This review will focus on preventive and curative developed strategies aiming to reduce the incidence of *Salmonella* infections in broiler chickens.

II. *Salmonella* characteristics

Salmonella sp. are Gram-negative and oxidase negative bacteria belonging to the *Enterobacteriaceae* family. The small rod-shaped, straight-sided, and non-sporeforming cells are mostly motile with peritrichous flagellae. *Salmonellae* can reduce nitrate to nitrite, fermentate glucose, and grow under aerobic or anaerobic conditions. They are mesophilic organisms with an optimum for growth between 35°C and 43°C, close to the body temperature of warm-blooded animals, while they are able to grow over a wide temperature range (5-46°C). Values of water activity (a_w) lower than 0.94 and pH greater than 9.5 or lower than 3.8 also inhibit the growth of *Salmonellae*.

The genus *Salmonella* contains two species (*Salmonella enterica* and *S. bongori*) based on phenotypic criteria. Members of *Salmonella enterica* subspecies *enterica* are mainly associated with warm-blooded vertebrates, while strains of species *bongori* and the other subspecies of species *enterica* are usually isolated from cold-blooded animals (Chan *et al.*, 2003). The species *S. enterica* is further divided into 6 subspecies (*arizonae, diarizonae, enterica, houtanae, indica and salamae*). The serology, based on the characterisation of the somatic (O), flagellar (H), and envelope (Vi) antigens, allows classification into serotypes. Of a total of 2541 serotypes officially recognized, 1504 were of *S. enterica* subspecies *enterica* (National Reference Center for *Salmonella* and *Shigella*, 2006).

III. Salmonellosis

III.1. Human disease

A clinical cases due to human *Salmonella* infection is called salmonellosis. Three clinical forms of salmonellosis are generally recognized, mainly dependent on the degree of host adaptation of the *Salmonella* serotypes. Serotypes adapted to humans, such as *S.* Typhi and *S.* Paratyphi, usually cause enteric fever, including typhoid fever. These serotypes are not usually pathogenic to animals. Secondly, non-typhoid serotypes which are highly adapted to an animal host such as S. Abortusovis (sheep), S. Gallinarum (poultry), S. Choleraesuis (pigs), and S. Dublin (cattle) may cause more or less serious clinical signs like abortion or septicaemia. The third form of salmonellosis which is most common in human, is a self-limited, febrile gastroenteritis characterized by diarrhea, abdominal cramps, vomiting, nausea and fever. The non-typhoid serotypes *S*. Typhimurium and *S*. Enteritidis are the most frequent causes of human gastroenteritis in many EU countries, but other serotypes like *S*. Hadar, *S*. Virchow and *S*. Infantis have been also often reported (EFSA, 2007a). In approximatively 5 % of cases, human infections can develop outside the intestine, progressing to systemic infections and resulting in various complications such as reactive arthritis, and osteomyelitis (Hohmann, 2001), and even death in about 1.4 % of salmonellosis cases (Voetsch *et al.*, 2004; Hohmann, 2001). The severity of the infection and whether it remains localized in the intestine or disseminates to the bloodstream may depend on the resistance of the patient and the virulence of the *Salmonella* isolate. Moreover, the infective dose of salmonellae varies according to the serotype, the strain, the immuno-competence of individuals, and the type of food involved. Experimental studies reported that ingestion of 10-45 cells, for outbreaks, to 10^5-10^9 *Salmonella* bacteria was required to establish an infection (European Commission, 2003).

The recent increase inthe relative proportion of *Salmonella* strains with reduced sensitivity to antibiotics, like the multi-resistant *Salmonella* DT104 (Hohmann, 2001), as well as the extension of their spectrum of antibiotic resistance, lately including fluoroquinolone resistance, may impede the antibiotic therapy efficiency.

III.2. Public health impact

BSN (Basic Surveillance Network) data showed that the number of human salmonellosis cases in the EU has decreased by 12.6 % from 2005 to 2007 with a reached incidence of 31.1 cases/100,000 populations (EFSA, 2009). In Belgium, a strong increase in *Salmonella* isolates in humans has been observed since 1987 that reached 15774 cases in 1999 (National Reference Center for *Salmonella* and *Shigella*, 2006). From 2003 to 2006, the total number of salmonellosis cases in Belgium decreased from 12,894 to 3693. This reduction was mainly explained by the reduction of the Enteritidis serovar, by 53 % and 83 % compared to 2005 and 2004 respectively. As observed for overall EU data, the serovars most frequently involved in human infection in 2006 in Belgium were *S*. Typhimurium and *S*. Enteritidis, with relative frequency of 49,44 % and 28,49 % respectively (National Reference Center for *Salmonella* and *Shigella*, 2006). Trend analyses has also revealed that, as a percentage of overall isolates, the number of multi-resistant *S*. Typhimurium Definitive Type (DT) 104 isolates recovered from human patients tend to increase.

Food-borne gastrointestinal diseases like salmonellosis are major burdens on society. Besides the discomfort felt by sick people, these infections have major economic repercussions by direct

illness costs (consultations, medical cares, hospitalization, etc.) and indirect costs (loss of productivity, days lost work, etc.) (ICGFI, 1999). Costs for human *Salmonella* infections are difficult to estimate because of differences in the simulation models used. Differences in estimated salmonellosis costs in England and Wales according to Sockett (1991) and Sockett (1995), i.e. £18 million and £231-331 million respectively, illustrates this complexity. In the US, recent reports from the department of agriculture (USDA) have estimated that salmonellosis costs about 2.4 billion dollars per year, for more than 4 million individuals infected with this pathogen (Voetsch *et al.*, 2004). Furthermore, based on the assumption that 95 % of salmonellosis are from food origin, the annual costs in the EU are estimated from 560 million to 2.8 billion euros (Korsak *et al.*, 2004).

IV. Poultry as a source of human *Salmonella* infection

IV.1. Reservoirs and transmission vectors

Several reservoirs and sources for *Salmonella* infection have been identified and were summarized by the European Commission (2003). These include the environment, fruits and vegetables, milk and milk products, chocolate, water or even spices. Nevertheless, the principal reservoir of the common *Salmonella* sp. is the gastrointestinal tract of animals, especially mammals and birds, while *Salmonella* sp. may also be isolated from cold-blooded animals. This involves that human infection can be be acquired through direct contact with animals or by person-to-person contacts, whereas the most frequent transmission route is the ingestion of contaminated products of animal origin (Hohmann, 2001).

Furthermore, the European Food Safety Authority has specifically showed, from data on food-borne outbreaks in all MS since 2004, that poultry products were the most frequently implicated vehicles of salmonellosis outbreaks (EFSA, 2007a). For example, in Spain, there was recently a *Salmonella* outbreak with more than 2000 cases due to consumption of pre-cooked chicken of a particular brand (Lenglet, 2005). Part of the reason is, as explained by Mead (2004), that *salmonellae* may be carried asymptomatically in the alimentary tract of the live bird and then spread via the slaughter process to raw, finish products. Subsequent consumption of undercooked or cross-contaminated poultry meat, or the handling of raw products are major risk factors in human infections. In Belgium, Uyttendaele *et al.* (1998) reported that turkey and chicken carcasses were frequently contaminated with *salmonellae* from the intestinal tract or from faecal material on feet and feathers. The true proportion of human cases associated with poultry is difficult to estimate because many incidents are sporadic, which cannot be identified with the consumption of a particular food item. Previous estimates from the literature showed that poultry was likely to be responsible for 20-35 % of all reported cases (Mead, 2004). *S.* Typhimurium and *S.* Enteritidis, which are the serovars most frequently associated with human illness in the EU (EFSA, 2007a), are also the serotypes most frequently associated with poultry products, especially broiler meat and eggs respectively.

IV.2. Poultry colonisation

The intestines, especially the ceca have been reported to be the primary sites of colonization for *Salmonella* in the live chickens (Desmidt *et al.*, 1997). Ingested organisms easily overcome the

acidic conditions of the proventriculus, because of the rapid adaptation of the bacteria to lower pH (Kwon and Ricke, 1998). *Salmonella* is then able to colonize the small intestine but also the underlying lymph tissue where it can proliferate. As the number of organisms increases, *Salmonella* can further infect essentially all tissues of the body, including the crop, spleen, liver, gall bladder, heart, ovaries and oviducts (Desmidt *et al.*, 1997).

The pathogenicity of *Salmonella* depends on the serotype, the strain, the susceptibility and the age of the bird. Chickens infected with the non-host adapted *Salmonella* strains usually shows no observable clinical signs of infection, whereas they may sometimes exhibit clinical signs of low or moderate severity like pericarditis or necrotic foci (O'Brien, 1988). Young birds are more susceptible to *Salmonella* infections that can lead to increased early mortality. On the other hand, adult birds can harbour *Salmonella* in their intestinal tract without showing clinical signs. Infective dose is consequently generally lower for adult than for chicks (Desmidt *et al.*, 1997; Milner and Shaffer, 1952). Milner and Shaffer (1952) infected chicks experimentally with *S.* Typhimurium and showed that 50 % of day-old birds became infected with an oral challenge of only 10 cells. By day 14, however, even a challenge dose of a million cells infected only 10 % of birds, indicating an increased resistance due to the establishment of an adult type intestinal microflora.

IV.3. Poultry flock prevalence

In 2006, data from national *Salmonella* survey programmes showed that a total of 3.4 % of tested broiler flocks were found infected in the EU (EFSA, 2007a). This represented a decrease by 1.5 % in comparison with 2004, while the occurrence of *Salmonella* in broiler flocks has remained at the same level or decreased since 2004 for most MS reporting, (EFSA, 2007a). Only Norway reported no positive broiler flocks. Among the other reporting MS, the proportion of positive flocks ranged from less than 0.1 % to 66.0 %. Belgium was, with Austria, and The Netherlands, the only MS where there appeared to be decreasing trends in *Salmonella* spp. prevalence in poultry flocks, from 7.2 % in 2004 to 2.4 % in 2006, whereas prevalence increased in Germany and Poland. A similar reduction was also observed in parent-breeding flocks for laying hen production (EFSA, 2007a).

A European-wide baseline survey was also carried out in commercial flocks of broilers with at least 5,000 birds, between October 2005 and September 2006 (EFSA, 2007b), and reported prevalence of *Salmonella*-positive flocks of 23.7 % with wide variety amongst the MS, from 0 % to 68.2 %. In laying hen flocks, 0.1 % to 13 % flocks were found infected with *Salmonella* in 2005. In flocks of turkeys, ducks and geese, 0 % to 17 % of the flocks were also reported infected (EFSA, 2007b). Differences between results from national routine monitoring and data from the EU-wide, fully harmonised *Salmonella* baseline study, may reflect the different sensitivities of sampling scheme and sample types used and demonstrates that a harmonised protocol should be used when comparing the situation in one MS with another.

Finally, the five most frequently isolated *Salmonella* serovars from broiler flocks in the EU were respectively in decreasing order *S.* Enteritidis, *S.* Infantis, *S.* Mbandaka, *S.* Typhimurium and *S.* Hadar. A total of 11.0 % of the broiler flocks was estimated to be positive for *S.* Enteritidis and/or *S.* Typhimurium, the two most common serovars found in *Salmonella* infections cases in human.

V. European legislation

Salmonellosis is an infection transmissible directly or indirectly between animals and humans, so-called "zoonoses". Following the incidence of zoonotic agents like *Salmonella* and their public health impact, protecting human against zoonoses has became of capital importance for the EU authorities. This public health concern has led to the establishment of the Directive 2003/99/CE and the Regulations (EC) n° 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of *Salmonella* and other specified food-borne zoonotic agents. The main aim of these Regulations is to reduce the prevalence of certain zoonoses in animal populations at the level of primary production and, where necessary, at other appropriate stages of the food chain. To meet this purpose, the EU have especially established Community rules concerning the setting up of specific national control programmes and the adoption of rules on certain specific control methods. Control measures are planned for poultry, i.e. breeding flocks of *Gallus gallus*, laying hens, broilers, turkeys, and pigs (slaughter and breeding herds). *Salmonella* is the primary zoonotic agent targeted at primary production. The Community Regulation 2073/2005 applied from 1 January 2006 prescribes additional specific rules for sampling and testing of *Salmonella*, and set limits for its presence in specific food categories. From 2010, samples of 25 g raw or 10 g cooked meat products must be *Salmonella*-free or shall not be placed on the market without any industrial treatment able to eliminate *Salmonella* (EFSA, 2007a).

In order to meet the Community targets concerning the establishment of national control programmes in Belgium, the « Agence Fédérale pour la Sécurité de la Chaîne alimentaire » (AFSCA), has designated national reference laboratories for the analysis and testing of zoonoses and has set up an annual monitoring program of zoonotic agents in humans and animal products. Since 1998, the survey program intended for all food-borne pathogens including *Salmonella*, is coupled with a hygiene plan based on biosecurity measures at primary production level which aims to reduce contamination from live animals.

VI. Contamination at farm level

In order to implement effective measures for *Salmonella* control in primary production, it is first necessary to clearly identify the sources of flock infection, the means of transmission and the risk factors at the poultry house level. These parameters, determined by different epidemiological studies, are summarized below.

VI.1. Vertical transmission

Vertical transmission can originate from an infection of the reproductive organs of the breeder hen via systemic infection, or from an ascending infection from the cloaca to the vagina and lower regions of the oviduct (Rasschaert, 2007). Via vertical transmission, the yolk, the yolk membrane or the albumen surrounding it, are directly contaminated as a result of *Salmonella* infection of the reproductive organs before the eggs are covered by the shell. *Salmonella* may also penetrate through the egg shell after oviposition via for example faecal contamination on the shell (Messens *et al.*, 2005). However, it is difficult to distinguish between contamination during formation of the egg and contamination after oviposition. According to Heyndrickx *et*

al. (2002), vertical transmission is nowadays of less importance mainly due to the vaccination of breeder flocks, so that horizontal transmission is the main determinative factor for colonization of broiler flocks.

VI.2. Horizontal transmission from the outer environment

The houses used for rearing broilers can largely be considered as closed environments. However, *Salmonella* sp. are able to survive and, under certain conditions, maybe even multiply in the external environment (Winfield and Groisman, 2003), which represents a risk of propagation into the house. In the following paragraphs; each potential source is reviewed. However, it must be keep in mind that for some factors inside the house, as rodents, insects, feed, or litter, it is generally difficult to determine if the contamination follows or precedes the flock contamination.

Feed and drinking water

Several analyses of commercially manufactured feeds have linked the occurrence of Salmonella in poultry and its presence in feed (Heyndrickx *et al.*, 2002; Davies and Wray, 1996a). Davies and Wray (1996a) actually showed that Salmonella can survive for at least 26 months in artificially contaminated poultry food, and Heyndrickx *et al.* (2002) demonstrated that 3.5 % of fresh feed samples tested positive for *Salmonella*.

Although the waterline and the water cup, as well as the drinking water, in poultry houses with Salmonella-positive flocks, has been shown to be contaminated with Salmonella (Bailey *et al.*, 2001), this water contamination usually follows flock colonization rather than preceding it (Heyndrickx *et al.*, 2002). The drinking water source is actually considered as a low risk factor for flock colonization (Rose *et al.*, 1999).

Broiler house cleaning and disinfection

Salmonella infection may also be associated with poor hygiene standards at poultry sites (Wray *et al.*, 1999). Indeed, *Salmonellae* are frequently isolated in poultry houses after the cleaning and disinfection process (Davies and Wray, 1996b; Rose *et al.*, 2000), mainly due to over-dilution or inconsistent application of disinfectants. *Salmonella* may then persist in dry livestock buildings for many months, because of the failure of disinfection routines, but also because of the presence of wildlife carriers or vectors like rodents and insects (Davies and Wray, 1996a; Rose *et al.*, 2000). Moreover, Wales *et al.* (2006) stated that wet cleaning may cause increased mobilisation and activation of *Salmonella*, and that bacteria in residual wet matter may benefit from increased protection against penetration by disinfectants

Human traffic and activities

The main human traffic in and out of a broiler house is the farm staff for the purpose of routine animal husbandry. Many authors have reported that *Salmonella* can be carried into the house via boots, clothes and equipment (Bailey *et al.*, 2001; Heyndrickx *et al.*, 2002). In an epidemiological study, Bailey *et al.* (2001) showed that boots from farm workers were contaminated by *Salmonella* for 12 % of 32 integrated broiler houses followed in all the year 1998.

Wild animals

Though conventionally reared poultry flocks are kept in closed poultry houses, some animals such as rodents and insects may have free access to the house. The significance of rodents as vectors and reservoirs of *Salmonella* has been shown by several studies (Davies and Wray, 1996b; Bailey *et al.*, 2001; Rose *et al.*, 2000). *Salmonella* contamination in the environment may consequently be amplified by mice defecating into feed hoppers and on egg-collection belts and may be spread further throughout the house by automated feeding systems (Wray *et al.*, 1999). Risk of transmission by rodents is however considered as low as most farms apply efficient rodent control programs.

More difficult to control are insects. Flies and beetles in and around poultry houses have often been reported to carry *Salmonella* (Bailey *et al.*, 2001; Wray *et al.*, 1999; Rasschaert, 2007). In the study of Hazeleger *et al.* (2008), individually housed chickens fed with several groups of beetles or larvae that were inoculated with *S.* Java, showed colonization to levels of 50-100 %.

Wild birds are also frequently colonized with *Salmonella* (Bailey *et al.*, 2001; Wray *et al.*, 1999; Pennycott *et al.*, 2006). Though they have usually no access to the broiler houses under good management practices, contaminated droppings can be brought into the house by footwear, clothing or material. However, Pennycott *et al.* (2006) observed that many of the genotypes of the isolates from wild birds and from broilers are seldom the same. This suggests that the importance of wild birds as a reservoir of infections is limited.

Pet animals and livestock

The presence of livestock such as pigs, cattle and sheep around the broiler house has also been sometimes associated with higher *Salmonella* prevalence in broiler flocks. As wild animals, they may excrete *Salmonella* in the environment surrounding the broiler house, which can result in the contamination of boots, clothes or equipment taken into the house. Liebana *et al.* (2002) found that the cattle located in the proximity of a broiler house were colonized with the same *Salmonella* strain as the broilers. However, this hypothesis was not confirmed by epidemiogical studies. Other broiler houses on the farm site also represent a risk, since an increasing number of poultry houses may be associated with a higher risk of colonization with *Salmonella*, as demonstrated by Skov *et al.* (1999).

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Salmonella in chicken: current and developing strategies to reduce contamination at farm level

Article II — Adapted from Vandeplas *et al.* (2010)
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Running head: Strategies to reduce *Salmonella* prevalence in chicken

Abbreviations: AFSCA, Agence Fédérale pour la Sécurité de la Chaîne alimentaire; CE, Competitive Exclusion; CFU, Colony Forming Unit; EFSA, European Food Safety Authority; EU, European Union; GIT, Gastrointestinal Tract; ICGFI, International Consultative Group on Food Irradiation; *L.*, *Lactobacillus*; PFU, Plaque-Forming Unit; *S.*: *Salmonella*

Abstract

Salmonella sp. is a human pathogen that frequently infects poultry flocks. Consuming raw or undercooked contaminated poultry products can induce acute gastro-enteritis in human. Faced with the public health concern associated with salmonellosis, the European Union (EU) has established a European regulation forcing Member States (MS) to implement control programs aimed at reducing *Salmonella* prevalence in poultry production, especially at the primary production level. The purpose of the present review article is to summarise the current research and to suggest future developments in the area of *Salmonella* control in poultry, which may be of value to the industry in the coming years. The review will focus especially on preventive strategies that have been developed and that aim at reducing the incidence of *Salmonella* colonization in broiler chickens at farm level. Besides the usual preventive hygienic measures, different strategies have been investigated, like feed and drinking water acidification by organic acids and immune strategies based on passive and active immunity. Modulating the diet in terms of ingredient and nutrient composition with the intent of reducing the bird's susceptibility to *Salmonella* has also been examined. Because in-ovo feeding has been shown to accelerate small intestinal development and to enhance the epithelial cell function, it could also be an efficient tool to control enteric pathogens. Microflora-modulating feed additives, like antibiotics, prebiotics, probiotics, and synbiotics, represent another field of investigation, whose success depends on the additive used. Finally, recent control methods, such as chlorate products and bacteriophages, have also been studied.

Keywords: contamination reduction, poultry, prevention, primary production, *Salmonella*

I. Introduction

Salmonella is a major cause of gastro-enteritis in human worldwide. It has been shown that consuming raw or undercooked poultry products can cause a human infection, known as salmonellosis (*35*). Faced with the public health concern due to salmonellosis, the EU has progressively established a European regulation mandating its MS to set up surveys and control programs focused on *Salmonella* and other pathogenic agents in poultry. Applying decontaminating treatments on the meat or eggs as final products is the easier way of controlling *Salmonella*. However, carcass disinfectants are prohibited in the EU and decontaminating fresh table eggs is difficult. Therefore, the European control programs have to be applied as an integrated approach along the poultry production chain (*112*), and to focus on prevention and on monitoring/eradication during the living phase. Simultaneously, the products need to be controlled during transport, at the slaughterhouse and/or at the transformation stage. In Belgium, like in most of the EU, the salmonellosis incidence has decreased since 2003, after national plans were set up. This reduction may be explained by the reduced prevalence of *Salmonella* Enteritidis serovar, which is mostly associated with the consumption of egg, due to recent vaccination programs of breeder and laying hen flocks. Nevertheless, vaccination appears to be only partly effective in controlling *Salmonella* in poultry, and the need for complementary strategies has arisen, especially for chicken production. Fries (*42*), Dinçer and Baysal (*28*) and Rasschaert (*102*) discussed management practices to prevent contamination during catching, transportation, and slaughter operations, as well as techniques to decontaminate raw meat and poultry meat products in the food processing industry. However, poultry carcass contamination can not be avoided if chickens are contaminated when arriving at the slaughterhouse, even with optimal slaughtering and processing (*26*), and implemented prophylactic measures need to target mostly poultry flocks at the primary production level.

The purpose of the present review article is to summarise the current research and to suggest future developments in the area of *Salmonella* control in poultry, which may be of value to the industry in the coming years. The review will focus especially on preventive and curative strategies aiming at reducing the incidence of *Salmonella* colonization in broiler chickens at farm level. The most widely used control measures against *Salmonella* are preventive hygienic measures and vaccination with dead and live *Salmonella* strains, which will be briefly summarized below. Another immune strategy is passive immunity of the birds that were fed specific antibodies produced from eggs of hyper-immunized hens. Supplementing feed with cationic antimicrobial peptides like avian gallinacins, known to play an important role in chicken's innate host defence, has also been suggested to reduce *Salmonella* incidence in poultry because of their antimicrobial activity (*10*). Other strategies include the use of genetically more resistant chicken lines (*30*), and the acidification of feed and drinking water with short- and medium-chain fatty acids (*130*). Modulating the ingredient and nutrient composition of the diet could also be useful to reduce the bird's susceptibility to infectious pathogens, as stated by Klasing (*71*). In this field, the most promising strategies seem to consist in incorporating high-fiber dietary sources and non-starch polysaccharides hydrolysing enzymes in the diet, which may modify the microfloral and physico-chemical balance in the gastro-intestinal tract. Amino acids, dietary antioxidants, and essential minerals levels are also important to maintain the gastrointestinal integrity (*1*). Moreover, in-ovo feeding has been shown to accelerate small intestinal development and to enhance function of the epithelial cells (*111*). These effects may improve the bird's resistance to colonization by *Salmonella* and other pathogens. Microflora-modulating feed additives, like antibiotics, prebiotics, probiotics, and synbiotics, represent another field of investigation, whose success varies with the additive used.

Finally, recent control methods, in particular chlorate products and bacteriophages, have also been investigated (*2, 17*).

II. Sanitary barrier

Biosecurity is an action plan designed to minimize the risk of introducing diseases or zoonotic agents into a flock. Practically the term "biosecurity" refers to measures taken to prevent or control the introduction and spread of infectious agents to flocks. As everything introduced into the house is potentially contaminated, an effective control program has to encompass several biosecurity measures. In effect, the starting point is to ensure that poultry breeding flocks are kept *Salmonella* free, as well as the house, feed and litter. Water should also be properly treated (*30*). The European Food Safety Authority (*34*) and Helm (*55*) have reported specific hygiene measures to be followed for equipment, animals, and people. However, such hygiene strategies have been shown to be only partly effective in controlling pathogen colonization of the poultry house and thus complementary measures are necessary to control *Salmonella* efficiently (*80*).

III. Dietary modulation

As stated by Klasing (*71*), dietary characteristics can modulate the bird's susceptibility to infectious pathogens and the level or type of ingredients may be of critical importance. Diverse dietary strategies have been developed or investigated to prevent or reduce the establishment of *Salmonella* in the gastro-intestinal tract. The feed structure has been shown to further change the poultry susceptibility to *Salmonella* infection. Whole wheat feeding was shown to lower pH in gizzard contents significantly, which in turn decreased the *Salmonella* population (*11*). Huang *et al.* (*62*) investigated the effects of feed particle size and feed form on *Salmonella* colonization, and they observed higher cecal *Salmonella* Enteritidis concentrations in broilers fed a pelleted diet than in those fed a mashed diet, while cecal volatile fatty acids (VFA) concentrations were significantly increased in broilers receiving the pelleted diet. Moreover, evidence can be found that various raw feed materials have useful characteristics to inhibit the growth of pathogens in animal's gastrointestinal tract (GIT). The level of protein content, as well as the nature of the protein source may help to protect the intestine from colonization. Plant-protein based feeds especially contain various non-starch polysaccharides (NSP) that are fermented in the cecum into organic acids, and that are detrimental to pathogens, as shown for *Campylobacter* (*121*). High-fiber dietary approaches were also proposed. They are based on the assumption that dietary fiber is preferentially utilized by *Lactobacillus* and *Bifidobacteria* species, to produce lactic acid and VFA, which results in normal microbial population maintenance and low pH (*31*). For example, alfalfa is one of the most extensively studied high-fiber dietary sources that increase lactic acid production and show potential inhibitory effect on *Salmonella* (*29*). Immunomodulation by the diet is another important part of nutrition strategies in order to maintain animal health. Large molecular weight compounds such as β-glucans (*77*), and antioxidants (*23*), have been identified as featuring immunomodulatory properties. Blount *et al.* (*12*) pointed out an immune response to the lutein, a yellow carotenoid, in avian species. β-Glucans from yeast cell walls are used as feed additive and have also been shown to up-regulate the innate immune response in immature chickens against *Salmonella* Enteritidis (*77*).

Damage to the GIT structure may lead to increased risk of invasion of the body by pathogens. Therefore it is very important to develop nutritional strategies that maintain gastrointestinal integrity. Several amino acids such as glutamine, serine, threonine, and arginine are known to play an important role in maintaining the GIT integrity, as well as being indicated as *Salmonella* growth-limiting nutrients. They are fermented into lactic acid and VFA (*64*) which have antagonistic activity against different pathogens. Formulating an optimal amino acid balance in the chicken diet is thus necessary to control the proliferation of enteric pathogens in the gut. Other micronutrients, like iron, may impair the animal mechanisms of natural resistance to colonization (*14*). These authors also showed that free dietary Fe increases bacterial virulence. It could be interesting to investigate whether the incorporation of chelators like phytic acid (*84*) and organic acids could improve the natural resistance to *Salmonella* infection in birds. It should be noted that phosphorus in phytic acid however is not bioavailable to non-ruminant animals because they lack the digestive enzyme phytase. The unabsorbed phytic acid consequently passes unabsorbed through the gastrointestinal tract, elevating the amount of phosphorus in the manure, which can lead to environmental problems such as eutrophication.

Rapid digestion and absorption of nutrients is certainly an important aspect as this reduces the amount of substrate remaining in the GIT which could be used by pathogenic microorganisms. The use of NSP-hydrolysing enzymes to improve the nutritional value of feed cereal like wheat and barley is common practice but their effect on pathogen colonization in chickens has been studied to a lesser extent (*41, 53*). Exogenous enzymes can alter microbial populations indirectly within the GIT through their action on the substrates that bacteria use as carbon source. Santos (*104*) demonstrated that supplementing a corn-based diet with an enzyme product containing endo-xylanase and alpha-amylase activities significantly reduced *Salmonella* prevalence and cecal population. It also improved growth performance in turkeys at 15 weeks of age.

It was previously demonstrated that chicks that were provided a hydrated nutritional supplement during the first 2 to 3 days of post-hatch life, initiated early immune development in comparison with chicks that had fasted and were given no water over the same period, and those birds had improved resistance to disease challenge with coccidial oocysts (*27*). The early feeding concept was further expanded to *in-ovo* feeding, which is the intra-amniotic administration of nutrients. During late embryogenesis, the injected solutions are consumed by the embryo, and these are digested and absorbed by the embryonic intestine prior to pipping. As the immediate post-hatch period is characterized by a transition from absorbing lipid-rich yolk to consuming exogenous feed that is rich in carbohydrates and proteins, solutions containing carbohydrates have been administered *in ovo* (*111, 115*). These were shown to accelerate the small intestine development and to enhance the functioning of enterocytes. Increased digestive capacity may improve growth performance in poultry, however other effects linked to *in-ovo* feeding were described. Mousavi *et al.* (*85*) demonstrated that injecting *in-ovo* a solution containing 25g/l maltose, 25g/l sucrose, 200g/l dextrin and 1 % threonine in 0.5 % saline into the amniotic fluid of eggs on day 17.5 of incubation led to a significant increase in the body weight gain and FCR of post-hatch broilers. Threonine was added because it is recognized as one of the most important amino acids that have a maintenance function in the intestine, and it is one of the main precursors for intestinal mucin production. However, Smirnov *et al.* (*111*) injected a similar carbohydrate treatment solution, without threonine, and still observed that goblet cells containing acidic mucin had increased by 50 % and that the mucin mRNA expression had been enhanced in comparison with the control group. Early and *in-ovo* feeding could therefore be useful complementary methods to prevent chicken's intestinal colonization by bacterial pathogens because they improve the immune development and the mucin production which is essential to prevent bacteria from adhering to the gut epithelium and from invading the body

subsequently. Nevertheless, the effects of treatments with different carbohydrate and amino acid compositions should be further investigated and compared in order to determine the relative importance of each component for growth and mucin production.

IV. Acidification of the chicken environment

IV.1. Short- and medium-chain fatty acids

VFA, or short-chain fatty acids (SCFA), which are mainly acetic, propionic and butyric acids, are produced by the normal anaerobic intestinal flora as end products of their metabolism (*79*). As demonstrated *in vitro* (*118*), these acids exert bacteriostatic or bactericidal effects against gram-negative bacteria, by entering the bacterial cell in undissociated form. Once inside the bacterial cell, the acid dissociates, so that the intracellular pH decreases and anion accumulates (*129*). Within practical limits, the acid concentration in undissociated form increases as pH decreases, so that the antibacterial effect is more efficient at low pH. Durant *et al.* (*32*) evidenced this *in vitro* as they observed a reduced growth rate of *Salmonella* Typhimurium at decreasing pH levels in the presence of VFA.

Consequently, many studies have investigated the potential acidification of feed or water by these SCFA to reduce the *Salmonella* colonization of broilers. These studies, reviewed by Doyle and Erickson (30) and Van Immerseel *et al.* (129), yield conflicting results. It appears that the administration mode, the type of acid and its concentration are very important. Furthermore, the colonization is not always affected by the treatment if the infection pressure is high or when the chickens are highly stressed. It has been proved that several products containing VFA mixtures decrease *Salmonella* shedding (18, 60, 118) and these appeared to be most effective in the gizzard and the crop (118, 130). Researchers have also developed microencapsulated organic acids in order to transport them further down in the GIT, which should prevent the absorption of acids in the upper tract and ensure their release further down in the GIT. Most commercial products that are actually applied in the field consist of propionic and formic acids, either in powder form or encapsulated in silica beads. Byrd *et al.* (18) evaluated the addition of 0.5 % acetic, lactic, or formic acids in drinking water during an 8-h pretransport feed withdrawal and showed reduced numbers of *Salmonella* Typhimurium recovered in the crop (log 0.79 *vs* log 1.45) in the case of lactic and formic acid, in comparison with control birds. Coated butyric acid was also tested in the feed for its potential effect on *Salmonella* colonization (128). While the number of chickens shedding *Salmonella* was significantly lower in the group receiving butyric acid, cecal colonization at slaughter age was equal to the control group of chickens. Moreover, Van Immerseel *et al.* (126) used chickens challenged at 5 and 6 days post-hatch with 5×10^3 CFU *Salmonella* Enteritidis in order to test the effect of feed supplementation with microencapsulated SCFA on *Salmonella* invasion, which is measured in internal organs at 8 days post-hatch. While butyric acid-impregnated microbeads resulted in a significantly decreased colonization by *Salmonella* Enteritidis in the ceca, feed supplementation with acetic acid, and to a lesser extent formic acid, led to an increased colonization of the ceca and internal organs.

Some *in vitro* studies suggested that VFA might also have an undesirable effect by promoting the invasiveness of *Salmonella* (*124*), or enhancing *Salmonella*'s resistance to acid upon exposure to VFA (*73*). While *Salmonella* bacteria normally do not survive at pH 3.0 in culture,

Kwon and Ricke (*73*) showed that a high number of bacteria survived for some hours after exposure to any VFA, suggesting that bacteria could be protected against the gastric acidity when passing through the gastro-intestinal tract. This protection is possible thanks to the induction of the genes that are involved in an acid-tolerance response and the synthesis of a series of acid shock proteins that are protective against extreme acidic conditions (*73*). Nevertheless, this resistance might only be effective if the pathogen is already in contact with the VFA before ingestion, in the feed, which is unlikely to occur, and this is not observed for all VFA (*124*). SCFA may also modulate the expression of the *Salmonella* genes that are involved in virulence (*32, 129*). An acetate-induced increasing invasion was actually detected for some serovars *in vitro*, whereas propionate or butyrate resulted in decreasing epithelial cell invasion (*125*). Butyrate was shown to down-regulate the expression of the Salmonella Pathogenicity Island 1 gene, which is crucial for bacterial invasion of the epithelial cells and virulence (*45*).

Medium-Chain Fatty Acids (MCFA), from 6 to 12 carbons (caproic, caprylic, capric and lauric acid), appear to be much more effective against *Salmonella* than the SCFA. As little as 25 mM C6 to C10 acids were bacteriostatic to a *Salmonella* Enteritidis strain, while the same strain tolerated 100 mM of SCFA (*125, 127*). When *Salmonella* Enteritidis and Typhimurium were incubated with 5 mM monocaprin combined with an emulsifier, the bacteria did not survive (*119*). However, large-scale studies are needed to confirm the antibacterial activity of MCFA against *Salmonella*.

In conclusion, in order to reduce *Salmonella* bacteria in broilers and the infection pressure on the farm, SCFA and MCFA can actually be effective products since these acids act fast and can also be active in young chickens, in contrast to many other types of feed additives. However, selecting the type of organic acids used in commercial products has been rather empirical until now, and little information is available concerning the best combination of acids in these products. Complementary studies are needed in order to analyse the effects of the different single VFA on *Salmonella* virulence.

IV.2. Fermented liquid feed

Another type of acidified poultry diet is Fermented Liquid Feed (FLF). The fermentation may happen spontaneously, by inoculating the moistened feed at a certain temperature before feeding the birds, or it may be induced. The induced fermentation can be achieved either by backslopping or inoculating with a Lactic Acid Bacteria (LAB) strain. Consequently FLF is characterized by high numbers of LAB, high concentration of lactic acid, and low pH of 3.5 to 4.5 (*98*). Feeding fermented feed to pigs has been shown to influence the bacterial ecology of the GIT and to reduce *Enterobacteriaceae* all along the GIT (*98*). In the case of broiler chickens, Heres and co-workers tested the effect of FLF on the susceptibility to *Salmonella* (*56, 57*). Heres *et al.* (*56*) infected chickens orally with 10^2 to 10^5 CFU *Salmonella* Enteritidis at 8 days of age and observed that the proportion of *Salmonella*-shedding chickens was significantly decreased in FLF-fed birds, regardless of the infection dose used. In another experiment, a fermented feed containing high concentration of lactic and acetic acid induced a *Salmonella* decrease in the anterior parts of the GIT that was more important in chickens inoculated with *Salmonella* Enteritidis at 8 or 22 days of age than in chickens fed dry feed (*57*). Similar results were obtained with *Salmonella* Typhimurium by Savvidou *et al.* (*105*) who showed moreover that FLF controlled *Salmonella* more effectively than liquid feed acidified with 30.3 ml/kg lactic acid. However, several variables in the fermentation process, like the composition of the feed varying with the production stage, may affect the advantages of such FLF and should be further studied.

IV.3. Litter treatment

Feed additives, in other words, components other than feedstuffs, such as the organic acids discussed above, are subjected to strict European legislations. With the cost inherent to these authorization procedures, strategies like litter treatment, inducing lower costs, would be more applicable in the practice for the producer to prevent or reduce *Salmonella* contamination in broiler flocks. Litter treatments with different acidifying compounds were documented in the past years. Their first aim was to reduce the emission of ammonia by modifying the litter pH. The effect of organic acids, formalin, sodium bisulfate, aluminium sulphate, or sulphuric acid on *Salmonella* and other pathogens has also been described (*65, 74, 99, 133*). Poultry Guard® litter amendment is a product based on 40-50 % sulphuric acid, used to control ammonia volatilization by converting litter ammonium to ammonium sulphate, thereby decreasing litter pH and resulting in pathogen reduction in the litter (*132*). Such treatment might lead to decreasing *Salmonella* concentration in chick ceca, as observed by Vicente *et al.* (*132*). Otherwise, Line (*74*) did not show that two commercially available acidifying litter treatments, aluminium sulphate and sodium bisulphate, affected the *Salmonella* colonization frequency in chickens raised on pine shavings.

V. Immune strategies

V.1. Passive immunity by specific antibodies

As maternal antibodies transferred from the yolk to the chicks were shown to prevent the chick's colonization by *Salmonella* (*54*), it was hypothesized that feeding these antibodies might provide passive immune protection to the hatchling. Several studies, reviewed by Schade *et al.* (*106*), have investigated how hyper-immunizing hens could produce pathogen specific antibodies in large quantities from eggs. In recent years, the efficacy of such antibodies against *Salmonella* has been the focus of *in vitro* (*21*) as well as *in vivo* (*52, 101*) experiments and has yielded promising results. In the study by Rahimi *et al.* (*101*), 3-day old chickens that were challenged with 1×10^6 CFU/ml *Salmonella* Enteritidis and received purified yolk IgY in drinking water showed significantly lower fecal shedding (0 % *vs* 14 %) and lower concentration of *Salmonella* Enteritidis from the ceca (0.27 $\log_{10}$*Salmonella*/g *vs* 3.98 $\log_{10}$ *Salmonella*/g) at 28 days of age. This study also investigated the effect of IgY combined with Primalac, a probiotic mixture of *Lactobacillus acidophilus, Lactobacillus casei, Enterococcus durans, Bifidobacterium thermophilus*, and showed similar results as with the antibody alone. Tellez *et al.* (*117*) also showed inhibitory effect of a similar association, between Avian Pas Plus, combining *Lactobacillus Acidophilus* and *Streptococcus faecium*, and egg-source antibodies for *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Heidelberg. Producting IgY that are simultaneously directed against several *Salmonella* serovars was shown to be effective (*21*). Furthermore, it has been found that non-immunized egg yolk powder was also effective in eliminating and preventing *Salmonella* colonization in poultry (*70*). Egg yolk contains anti-infectious/anti-adhesive factors besides IgY, like ovotransferrin, and the antimicrobial activity may involve the pathogens' agglutination to the egg yolk, generating a competition for adhesion sites, or the stimulation of the immune system by egg yolk components. Finally, lymphokines may be used for passive immunity, as tested by McGruder *et al.* (*78*). These authors administered *Salmonella* Enteritidis-immune lymphokines *in ovo*, and

after challenging the day-of-hatch chicks with 5×10^4 CFU *Salmonella* Enteritidis, they showed that organ invasion was significantly decreased in 2-days old chicks with lymphokine treatment. Readers can find more information about passive immunity in the review by Chalghoumi *et al.* (*20*).

V.2. Antimicrobial peptides

Antimicrobial peptides are small molecules with a molecular mass of 1 to 5 kDa (*68*). Their structure usually contains elements that facilitate the interaction with the negatively charged membrane of target pathogen organisms. In chickens, the cationic antimicrobial peptides, including avian β-defensins called gallinacins, are natural endogenous antibiotics and constitute bioactive molecules of the innate immune system (*10*). The antimicrobial activity of different cationic antimicrobial peptides against *Salmonella* has been describedin some *in vitro* studies (*36, 82, 123*). Using a time-kill assay Milona *et al.* (*82*) demonstrated the antimicrobial activities of gallinacins 4, 7, and 9 against strains of *Salmonella* Enteritidis and Typhimurium and provided evidence for the synergistic interaction of gallinacins 7 and 9 against *Salmonella* Enteritidis. Chicken and turkey heterophil peptides were shown to reduce by over 90 % the survival of *Salmonella* Enteritidis and Typhimurium strains at concentrations from 2 to 16 µg/ml in 0.01 % sterile acetic acid (*36*). These antagonistic properties and the recent advances in cationic antimicrobial peptide technologies may lead to develop new feed additives in order to prevent or limit poultry contamination by pathogens like *Salmonella*. The production of small antimicrobial peptides occurs in all living organisms, from bacteria to mammals. Currently chemical synthesis appears too costly for large-scale production of peptides, and biological production ought to be attempted with microorganisms, tissue cultures, or transgenic plants and animals. Peptide-containing plant material could also be added to animal feed (*68*). However, the potential use of peptides as feed additives faces some important economic and ecological issues that need to be resolved. For example, chemical modifications or encapsulation methods should be developed to make them more resistant to proteolysis in animals. Some of the peptides such as defensins or bactencin exhibit toxic effects (*69, 100*). Further aspects about properties and applications of antimicrobial peptides have been reviewed by van 't Hof *et al.* (*131*).

V.3. Vaccination

Developing effective vaccines and disease control strategies requires that host immunological mechanisms against the pathogen are understood. Little is known about the poultry's immune response to virulent and attenuated *Salmonella* strains. The importance of cell-mediated immunity for tissue clearance (*9, 87*) and systemic clearance (*38*) of virulent *Salmonella* strains is generally recognized. Vaccines have been designed with killed and live attenuated *Salmonella* strains, as well as live attenuated vaccine strains developed by non reverting mutations of genes involved in metabolic functions or in virulence factors.

Killed vaccines exert a poorer protective immunity than live vaccine because they stimulate mainly the antibody production, they are rapidly destroyedd and eliminated from the host system, and relevant antigens are destroyed during vaccine preparation (*8*). Also killed vaccines generally fail to induce cytotoxic T cells (*87*) and secretory IgA responses, which are potentially important to protect mucosal surfaces (*9*). Although a number of different live *Salmonella* strains have been tested for their efficacy, only a few are registered and commercially available for use in poultry in Europe. One example is the rough strain *Salmonella* Gallinarum 9R (*40*),

which is registered for prophylactic use against *Salmonella* Enteritidis. The reader is invited to learn more about the inhibitory effect of the different types of vaccines against *Salmonella* in the detailed reviews by Barrow (*8*). Finally, subunit vaccines, like outer-membrane protein (OMP) (*81*) or toxoids (*83*), have also been used in poultry.

VI. Microflora-modulating feed additives

VI.1. Antibiotics

In modern poultry production, therapeutic antibiotics are used to control economically important infections such as coccidiosis and bacterial enteritis caused by *Salmonella*. Several types of antibiotic agents are available with different modes of action based on inhibition or alteration of bacteria metabolic functions (*46*). Next to their curative use, antibiotics have also been given to poultry in feed for their growth promoting properties and their preventive effect against pathogen colonization. Fluoroquinolones, salinomycin sodium, trimethoprim and polymyxin B were tested as feed additive before *Salmonella* challenge and were shown to eliminate *Salmonella* Enteritidis from poultry flocks, or at least reduce *Salmonella* incidence (*13, 50, 109*). Most growth promoters act by reducing mainly gram-positive bacteria, which are known to depress animal growth, either directly or indirectly, through their metabolic activities (*46*).

In recent years, concerns arose about using antibiotics in livestock. Antibiotic feed additives were linked to the emergence of antibiotic resistant bacteria (*134*), the presence of undesired antibiotic residues in meat and environmental contamination. Consequently all prophylactic and growth-promoting antibiotics used in animal rearing have been banned in the EU since January 2006 according to the "Precautionary Principle". These assumptions have however led to conflicting conclusions and are still debated (*19, 97*). Casewell *et al*. (*19*) stated however that the suppression of growth-promoting antibiotics led to deteriorating animal welfare and increasing infections, which in turn substantially increased the use of therapeutic antibiotics. Anyway, antibiotic use does not seem to be interesting in view of the burden of antibiotic resistance in the food chain and the harmful consequences for the public health, as pointed out by the European Commission about fluoroquinolones (*135*). The European Commission consequently specified prudent-use guidelines and recommended the development of alternative measures. Other strategies to cope with pathogens have thus become popular. These include probiotics, prebiotics, and synbiotics, which, unlike antibiotics, have been designed partly for promoting the growth of some microflora communities.

VI.2. Probiotics

A probiotic is defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (*44*). The administration of probiotic strains actually induces changes in the bacteria community structure (*89*) and stimulates the immune system as well as the indigenous enzymes, which may lead to a beneficial nutritional and growth-promoting effect (*96*) as Jin *et al.* (*66*) observed. Probiotic bacteria may be especially useful in poultry under stress conditions (feed withdrawal, heat stress, colonization by an enteric pathogen like *Salmonella*), when sensitive bacterial populations of the microflora

with main antimicrobial activities, like lactobacilli and bifidobacteria, tend to decrease and are no more able to provide sufficient resistance to enteric pathogens (*96*). Many studies have actually shown that inoculating cultures of one or several probiotic strains to broiler chickens may inhibit the bird's contamination by *Salmonella* (*3, 59, 122*). Several potential mechanisms allowing probiotic cultures to exclude enteric pathogens have been proposed, including competition for receptor sites, competition for limiting nutrients, and production of antimicrobial metabolites (bacteriocins, VFA, hydrogen peroxide), which may inhibit certain enteric pathogens (*92, 110*).

Microorganisms used as probiotic in animal feeding are mainly bacterial strains belonging to the genera *Lactobacillus*, *Enterococcus*, *Pediococcus* or *Bacillus* although microscopic fungi including *Saccharomyces* yeasts have been also used. However, much *in vitro* selection research has focused on the *Lactobacillus* species isolated from various sources (*67, 120, 122*) and was based on their various modes of action against pathogens. Jin *et al.* (*67*) showed that *Lactobacillus fermentum* and *Lactobacillus acidophilus* reduced significantly the attachment to the ileal epithelial cells of respectively *Salmonella* Typhimurium and *Salmonella* Pullorum, through exclusion (lactobacilli prior to addition of salmonellae) and competition (lactobacilli and salmonellae added together). Two other strains of *Lactobacillus acidophilus* and *Lactobacillus fermentum* were also selected *in vitro* for their ability to adhere to intestinal epithelium cells from poultry and for their inhibition effect against *Salmonella* Typhimurium due to their lactic acid production (*120*). In the study by Van Coillie *et al.* (*122*), two strains - *Lactobacillus reuteri* R-17485 and *Lactobacillus johnsonii* R-17504 - inoculated into the proventriculus at 2×10^8 CFU significantly decreased the pathogen colonization of 6-day-old chicks infected at 2 days of age with 10^4 CFU *Salmonella* Enteritidis in ceca, liver and spleen. Furthermore, the inoculation of *Enterococcus* spp. was also shown to protect chickens against *Salmonella* challenge, due to the combined effects of lactic acid production and bacteriocins (*3*). In all the *in vivo* trials described here, the probiotic was inoculated orally in one or few steps although the probiotic could also be incorporated into the feed, as described by Jin *et al.* (*66*) and Line *et al.* (*75*). Line *et al.* (*75*) observed that continuous supplementation of live yeast cells of *Saccharomyces boulardii* in a standard feed reduced cecal colonization from log 1.64 to log 0.15 in broiler chickens that had been co-challenged with *Salmonella* Typhimurium and *Campylobacter jejuni* at 4 days of age.

VI.3. Prebiotics

Prebiotics are feed ingredients that are not digested by the host, not or little used and/or metabolized as they pass through the upper portion of the intestinal tract, so they can interact with the flora of the large intestine. In the lower intestine, they have to be able to serve selectively as a substrate to stimulate the growth and/or activity of bacteria species that are present in the GIT and beneficial to the health (*51*). These characteristics are summarized in the *definition* of prebiotics by Gibson and Roberfroid (*47*). In principle, non-digestible, fermentable feed carbohydrates but also sugar alcohols may be considered as prebiotics. Diverse types of prebiotic were listed by Šušković *et al.* (*114*). The non-digestible carbohydrates are divided according to their molecular weight and the most important prebiotics are the di- and oligo-saccharides (*86*). It is generally recognized that prebiotics exert beneficial health effect on animal and human, especially by inhibiting intestinal colonization by enteric pathogens like *Salmonella* (*51*). This effect can be direct by binding to pathogens in the intestinal lumen and therefore blocking the adhesion of the bacteria to the epithelial cells (*113*). Their effect may also be indirect, as fermentable prebiotics may constitute a substrate for the metabolism and the

growth of intestinal flora. The multiplication of normal flora may inhibit the colonization with pathogenic bacteria by competitive exclusion and may stimulate production of antibacterial metabolites, such as lactic acid, VFA and bacteriocins (*114*). However, the capacity of prebiotics to control the colonization of enteropathogens showed inconsistent results, which are detailed in the review by Rehman *et al.* (*103*).

Lactose, lactulose and lactosucrose are natural disaccharides, or isomerization products, which reportedly have shown prebiotic effects in chickens (*22, 116*). Tellez *et al.* (*116*) observed a significant reduction in the total number of positive *Salmonella* Enteritidis organ invasions 14 days and 19 days after infection in chicks fed with lactose. On the other hand, Barnhart *et al.* (*7*) observed that adding 2.5 % lactose in the drinking water of broilers during the last 5 to 11 days of growth period prior to slaughter failed to reduce *Salmonella* population from crops and ceca. Fructo-oligosaccharides (FOS) and mannan-oligosaccharides (MOS) are the most extensively studiedoligosaccharides in chickens with respect to their prebiotic effect and their activity against *Salmonella*. The short-chain polymers of β 1-2-linked fructose units, FOS, produced commercially by hydrolysis of inulin or by enzymatic synthesis from sucrose or lactose (*86*), have been actually shown to reduce chicken intestinal colonization by *Salmonella,* in a way that varied with the mode of preparation, and when they were added to the experimental diet (*22*). Mannose-based carbohydrates, or MOS, occur naturally in many products such as yeast cell walls and gums (*113*). Spring *et al.* (*113*) showed that the number of birds infected at 3 days of age with 1×10^4 CFU of *Salmonella* Dublin which were positive in their ceca at 10 days of age was significantly reduced by 34 % when MOS was incorporated at 4,000 ppm in the diet. More recently, other oligomers, like bacterial isomaltooligosaccharides produced from *Leuconostoc mesenteroides* fermentation (*24*) or xylo-oligosaccharides produced by hydrolysis of arabinoxylans from cereal (*33*), have also been studied for their potential in *Salmonella* inhibition. Finally, while being not considered as a prebiotic since it can be metabolized by the host, mannose has been shown to block *Salmonella* Typhimurium adherence to chicken intestinal epithelial cells *in vitro* (*94*), and to decrease cecal colonization of 3-days old *chickens in vivo (93).*

VI.4. Synbiotics

Since prebiotics are able to stimulate some beneficial microflora populations, like bifidobacteria or lactobacilli (*114*), a further step consisted in developing synbiotics in which probiotic strains and prebiotic substrate were combined (*25, 108*). According to Schrezenmeir and de Vrese (108), this term is reserved for products in which the prebiotic compound selectively favors the probiotic strain, as observed *in vitro* by Kontula *et al.* (*72*) who found that β-gluco-oligosaccharides and xylo-oligosaccharides may be fermented by three specific lactic acid bacteria. This results in advantages to the host offered by the antimicrobial activity of the probiotic supplemented by the prebiotic effect. For example, the combination of fructose with *Bifidobacterium* strain is a potentially effective synbiotic, as is the use of lactilol or lactulose in conjunction with lactobacilli (*25*). The results of much research (*48, 76, 114*) have pointed out that combining probiotics and prebiotics has a synergistic effect on the fecal microflora of experimental animals. This effect was demonstrated by the increased total anaerobes, aerobes, lactobacilli, and bifidobacteria counts as well as by the reduction of bacteria considered as negative, such as clostridia, *E. coli*, and *Enterobacteriaceae* comprising *Salmonella*. Nevertheless, these results are based on experiments carried out on humans and rats. Most data available for poultry reported the effect of different synbiotics on growth performance and intestinal metabolism (*4, 15*). When Nisbet *et al.* (*90*) used a combination of dietary lactose and

competitive exclusion flora to reduce *Salmonella* colonization in chickens, they reported actually that the combination was more effective in reducing *Salmonella* colonization than lactose or competitive flora alone. On the other hand, Fasina *et al.* (*39*) failed to show any beneficial effect from the combination of pectin and a commercially available probiotic on cecal contamination of chickens infected at 4 days of age with 10^6 CFU of *Salmonella* Typhimurium.

Table 1 summarizes the studies that have reported efficiency of feed additives based on pre-, pro- and synbiotics on the intestinal colonization of chickens by *Salmonella* sp. Concerning synbiotic use in poultry, no studies are currently available with probiotic strains, but some were carried out with competitive exclusion culture of bacteria (see next paragraph) and these are reported in table 1. Nevertheless, some research is being carried out in our laboratories in order to study another kind of synbiotic that combine a probiotic with an enzyme in a feed additive. By specifically metabolizing the prebiotics supplied by the enzyme from the hydrolysis of the broiler diet, the probiotic could efficiently compete in the GIT and exert its antagonistic activity more efficiently.

VI.5. Competitive exclusion

In intensive production system, newly hatched chicks are very sensitive to enteric infections since they have little opportunity to develop a normal intestinal microflora quickly due to the clean housing conditions in which the chicks are reared. Nurmi and Rantala, (*91*) then stated that a suspension of alimentary tract contents from adult birds administrated orally to newly hatched chicks may establish an adult-type microflora early in the chicks, which protects from *Salmonella* colonization. The process through which normal intestinal microflora protects the host against invading pathogens is called competitive exclusion (CE). This treatment is generally used as a prophylactic measure aiming at increasing the resistance of young chicks to *Salmonella* (*79*), but it can also be used after a curative antibiotic therapy applied to eliminate an existing infection and, in this case, the CE flora restores the normal microbiota (*109*).

CE treatment is normally given to newly hatched chicks or turkey poults as soon as possible after hatching. It is either added to the first drinking water or sprayed in the hatchery or on the farm (*6, 95, 107*). The latter method has been designed to overcome the problems linked to the reduced viability of anaerobic strains in water and to the variable drinking water uptake in the first 24h after hatching (*79*). At present, different CE products are commercially available, for example AviFree, Aviguard, Broilact, MSC, Preempt, and CF-3. They are all mixed cultures derived from the cecal contents and/or gut wall of domestic fowl (*107*). CE aimed originally at controlling *Salmonella* infections, however they were shown to protect chicks against other pathogens, like *E. coli*, *Yersinia enterocolitica* and *Campylobacter jejuni* (*107*). Nakamura *et al.* (*88*), Bailey *et al.* (*6*) and Palmu and Camelin (*95*) actually showed the protective effect of Aviguard, MSC, and Broilact, respectively, against overwhelming colonization by salmonellae.

Very little is known about the action mechanism of CE flora. The effect on *Salmonella* in the ceca is mainly bacteriostatic rather than bactericidal (*79*) and was partly explained by the VFA production by the bacteria (*107*). Hume *et al.* (*63*) observed a significant increase in the cecal propionic acid concentrations in chicks challenged with 10^4 CFU *Salmonella* Typhimurium 4 h after CF-3 treatment by oral gavage. Other proposed mechanisms referred to competition for receptor sites in the intestinal tract and competition between pathogens and native microflora for nutrients (*79*).

Table 1. *In vivo* studies reporting inhibitory effect of pre-, pro- and synbiotics against *Salmonella* sp. in chickens

Feed additive	*Salmonella* challenge	Observed effects	Reference
Probiotic			
Saccharomyces boulardii[a] at 100g/kg feed	3.2×10^8 CFU *S.* Typhimurium at 4-d-old	Reduction of colonized birds number by 65% Reduction of *Salmonella* counts in ceca from log 1.64 to log 0.15/g ceca	Line *et al*. (*75*)
Lactobacillus reuteri R-17485 and *Lactobacillus johnsonii* R-17504 at 2×10^8 CFU (oral gavage)	10^4 CFU *S.* Enteritidis at 2-d-old	Significant reduction of *Salmonella* counts in ceca, liver and spleen	Van Coillie *et al*. (*122*)
Enterococcus feacium J96 at 10^9 CFU (oral gavage)	0.2×10^5 CFU *S.* Pullorum at 4-d-old	Increased resistance to *Salmonella* infection (25% mortality vs 50% for the control)	Audisio *et al*. (*3*)
Eleven lactic acid bacteria isolates[b] at ~ 7.5×10^5 CFU (oral gavage)	10^4 CFU *S.* Typhimurium or *S.* Enteritidis at 1-d-old	Reduction of the incidence of *S.* Enteritidis by 60-70% and of *S.* Typhimurium by 89-95% in the cecal tonsils 25h post-challenge Reduction by log 2.9 of total cecal *S.* Enteritidis 25h post-challenge	Higgins *et al*. (*59*)
Prebiotic			
Arabinoxylooligosaccharides 0.4%	2.5×10^9 CFU *S.* Enteritidis at 14-d-old	Significant reduction of frequency of positive cloaca swabs from 1 to 11 days post-infection Significant reduction of log *Salmonella* CFU/g ceca	Eeckhaut *et al. (33)*
Fructooligosaccharides 5% or lactulose 5% or lactosucrose 5%	10^7 CFU *S.* Typhimurium at 1-d-old	Reduction of *Salmonella* scores[c] at 6 wk, from 1.13 to 0.61, 1.08 and 1.11 for FOS, lactosucrose and lactulose	Chambers *et al*. (*22*)
Mannanoligosaccharides 0.4%	10^4 CFU *S.* Typhimurium at 3-d-old	Reduction of cecal *Salmonella* concentrations from log 5.40 to 4.01 CFU/g)	Spring *et al*. (*113*)
Mannanoligosaccharides 0.4%	10^4 CFU *S.* Dublin at 3-d-old	Reduction of the number of positive bird in the ceca by 34% at 10-d-old	Spring *et al*. (*113*)
Fructooligosaccharide 0.75%	10^9 CFU *S.* Typhimurium at 7-d-old	Reduction by 42% of colonized chickens number	Bailey *et al*. (*5*)
Lactose 10%	10^8 CFU *S.* Enteritidis at 13- or 18-d-old	Significant reduction in the total number of positive organ invasions one day after infection	Tellez *et al*. (*116*)
Synbiotic			
Anaerobic culture of cecal bacteria encapsulated + lactose 2%	10^4 CFU *S.* Typhimurium at 3-d-old	Reduction of *Salmonella* counts in ceca by 3.4 to 5.3 log at 10 days of age	Hollister *et al*. (*61*)
Competitive exclusion culture 0.02 ml (oral gavage) + fructooligosaccharides 0.1%	10^8 CFU *S.* Enteritidis at 7-d-old or 21-d-old	Significant reduction of the mean numbers of *Salmonella* in chicks at 1 and 7 days post-inoculation	Fukata *et al*. (*43*)
Continuous-flow (CF) derived bacterial culture + lactose 2%	10^4 CFU *S.* Typhimurium at 3-d-old	Significant reduction of *Salmonella* counts in ceca at 10 days of age Protection factor[d] = 9.26 *vs* 2.49 for control	Nisbet *et al*. (*90*)

S. : *Salmonella*

[a] Levucell™SB20, Lallemand, Inc., Rexdale, ON, Canada, M9W 4Z51

[b] FM-B11, IVS-Wynco LLC, Springdale, AR

[c] *Salmonella* score: 0= 0 CFU, 1= 1 to 100 CFU, 2= > 100 CFU of *Salmonella* per cecal swab

[d] Protection factor = $\log_{10}$ Salmonella control diet divided by $\log_{10}$ *Salmonella* treatment group

VII. Recently developed strategies

Byrd and co-workers have recently investigated an experimental chlorate product on broiler contamination by *Salmonella* Typhimurium (*16, 17*). *Salmonella* respires under anaerobic conditions by converting nitrate into nitrite while using nitrate reductase. As nitrate reductase does not distinguish between nitrate and chlorate, chlorate is reduced to chlorite that builds up to toxic levels, which are able to eliminate susceptible bacteria. In their articles Byrd *et al.* (*16, 17*) actually showed that a chlorate product added to control feed (5 to 18.5 %) or to drinking water (7.5 to 30 m*M* chlorate ion) led to significantly lower incidence and concentrations of *Salmonella* Typhimurium in the crop and ceca. Sodium chlorate has low toxicity for animals but such a nutritional strategy would probably face serious regulatory obstacles.

Bacteriophages were also tested for their inhibitory effect against *Salmonella*. They represent a group of viruses that specifically infect and replicate in bacteria. Bacteriophage treatments were first applied to decontaminate poultry carcasses and products (*49, 58)*. Higgins *et al.* (*58*) observed that applying 5.5 ml of 10^8 or 10^{10} PFU/ml of a single bacteriophage significantly reduced the frequency of *Salmonella* recovery in carcasses inoculated with *Salmonella* Enteritidis. Subsequently Andreatti Filho *et al.* (*2*) studied the effect of such treatment on *Salmonella* contamination *in vivo*. By treating day-of-hatch chicks via oral gavage with cocktails of 4 or 45 bacteriophages at 10^8 PFU/chick 1 h after oral challenge with 9×10^3 CFU *Salmonella* Enteritidis, these authors showed significantly reduced *Salmonella* Enteritidis recovery from cecal tonsils 24 h post-treatment. However, no significant difference was reported at 48 h. These results suggest that further investigation is necessary in order to determine the actual efficiency of bacteriophages in reducing *Salmonella* in poultry for long period.

VIII. Conclusion

Human gastroenteritis caused by *Salmonella* is a major food safety concern. As foods of poultry origin appear to be an important source of this pathogen, recent European regulations were established with the aim of forcing Member States to implement programs to control *Salmonella* in poultry efficiently. These are based on an integrated approach and include control measures applied throughout the poultry production chain. At the primary production level, biosecurity measures are only partly effective and subtherapeutic antibiotics, which were used as growth-promoters but also helped to prevent pathogen contamination, have been banned in the EU since January 2006. Other strategies have therefore been investigated. They are mostly based on diet modulation and feed supplementation with different types of additives other than feedstuffs. Vaccination was shown to be efficient only in laying hens and does not seem applicable in meat-producing birds, whereas passive immunity is still under development and should offer broader applications. Acidification- and microbial-based measures have been studied for about 10 years but give conflicting results. Beside competitive exclusion flora, defined bacterial strains like probiotics have shown interesting *in vitro* and *in vivo* antagonistic effects against *Salmonella* spp., especially by producing organic acids and antimicrobial metabolites. Prebiotics, which are non-digestible carbohydrates being fermented by specific microflora bacteria, are also potentially efficient additives for *Salmonella* control, as are synbiotics, in which prebiotics can be used specifically as substrate by probiotics, as many

studies have demonstrated in the last decade. None of these treatments are actually effective to eliminate *Salmonella* contamination from poultry flocks completely. Combining several measures seems therefore the only efficient strategy to reduce the prevalence of this pathogen at farm level. The most important criterion to select appropriate control measures is their efficacy, which depends on many breeding parameters, but economical costs and phenomena of *Salmonella* resistance, as observed with some organic acids, have also to be taken into account.

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Chapter II. Research strategy

The overall objective of this PhD thesis was to study the potential efficiency of combinations between antagonistic bacteria and polysaccharidases to prevent or reduce *Salmonella* and *Campylobacter* contamination of broiler chickens.

As expressed previously, *Campylobacter* and *Salmonella* prevalences in broiler flocks may be dependent on the rearing system (intensive *vs* free-range). Some parameters like the access to the surrounding environement and the length of the rearing period can particularly be important risk factors. Determining the most appropriate treatment method for each production type was consequently the first step of the present work. The second step was the selection of bacterial strains with antagonistic activities against both pathogens, and of enzymes able to supply hydrolysis products metabolizable by the bacteria. Finally, an *in vivo* study was carried out in order to validate a bacteria-enzyme formulation used as feed additive to reduce pathogen colonization of experimentally infected birds.

Concerning *Salmonella* in intensive broiler productions, treatments based on stimulation of the immune system by microbial products have showed promising effects (Patterson and Burkholder, 2003; Dunkley *et al.*, 2009). Such methods could especially be efficient when applied as feed additive (Van Immerseel, 2007). In this way, active ingredients may directly interact with the Gut-Associated Lymphoid Tissue (GALT) which is responsible for inducing immune responses against viral, parasitic, and bacterial enteral antigens like *Salmonella* (Bar-Shira *et al.*, 2003). Moreover, defined bacterial strains like probiotics have shown interesting *in vitro* and *in vivo* antagonistic effects against *Salmonella* spp., especially by production of organic acids and antimicrobial metabolites (Ouwehand *et al.*, 1999). In order to improve the antagonistic activity of probiotics, substrates which can be specifically metabolizable by the strain have been added to the bacterial feed additive. Such product, called synbiotics, have been investigated and showed promising effects on *Campylobacter* and *Salmonella* (Hollister *et al.*, 1994; Fukata *et al*., 1999; Klewicki et Klewicka, 2004). Fermentable carbohydrates are usually used as substrate for improving the probiotic metabolism. However, such substrate could also be supplied by enzymes that are able to hydrolyse some carbohydrate fraction of the chicken diet to produce fermentable oligosaccharides. In many Northern European countries, poultry diet formulations often use wheat as source of energy because culture conditions are little suited to maize grain production. However, wheat presents a high content in arabinoxylans (AX), which are major anti-nutritive factors in cereals (Choct and Annison, 1990). These antinutritionnal effects can be partially suppressed by supplementing wheat-based diet with xylanases able to hydrolyze AX into fermentable C-5 mono- and oligo-saccharides. Addition of xylanase to the probiotic formulation may then exert two beneficial effect, by increasing the nutrient digestibility and the energy content of the diet for the chicken, and by supplying specific substrate to the probiotic strain in order to favour its competition potential. A feed additive combining a xylanase and a probiotic strain that is able to metabolize C-5 oligosaccharides was consequently selected. The epidemiology and transmission factors of *Campylobacter* in free-range broiler houses are less known, while some hypothetical ways of transmission into the broiler houses have been pointed out in reviews (Newell and Fearnley, 2003; Bull *et al.*, 2006). So, we have first decided to carry out an epidemiological study in free-range broiler farms of the southern part of Belgium to assess the main sources of flock infection in such rearing system. Underscoring these sources aimed to determine an efficient treatment to reduce *Campylobacter* prevalence in broiler flocks. The results of this study are presented in Chapter III and are published in *Biotechnology, Agronomy, Society, and Environment* (Article III).

AX are the principal endosperm and aleurone cell wall components in wheat. The antinutritionnal effects of AX could especially be related to the content of water-soluble AX in

the endosperm, which are able to increase digesta viscosity and consequently reduce the absorption of nutrients (Steenfeldt *et al.*, 1998). For the last decade, many studies have shown that xylanase supplementation has been able to increase the nutritive value of wheat-base diets by reducing the antinutritive effect of AX, and to consequently improve nutrient digestibilities and growth performance of broiler chickens. For the development of a xylanase-probiotic formulation against *Salmonella*, different xylanase preparations from bacterial or fungal origin were supplemented to a wheat-based diet in order to study their effects on broiler chicken performance and on nutrients digestibilities of the diet. The aim was to select the most efficient product for incorporating in the feed additive. The results, which are presented in Article IV, are published in the *Canadian Journal of Animal Science*.

In the same way, screening of Lactic Acid Bacteria (LAB) with potential antibacterial activity against *Campylobacter* was carried out. LAB are characterized by their ability to prevent the adherence, the establishment, the replication and/or the virulence of specific enteropathogens (Reuter, 2001). Different antagonistic mechanisms have been proposed: pH decrease via organic acid production, nutrient competition with pathogens, and/or production of specific inhibitory compounds such as bacteriocins (Salminen *et al.*, 2004). The antagonistic effect of LAB was assessed using *in vitro* experiments. Especially, the co-culture assay was selected because this method allows bringing directly into contact the LAB strain and *Campylobacter* in broth culture. Bacteria were grown in culture medium containing compound similar to those encountered in the environment of broiler house. Such medium being rich in cellulose, an enzyme preparation with cellulase activity was added to the broth medium in order to supply glucose and C-6 oligossacharides to the antagonistic LAB strain. These hydrolysis products were hypothesized to selectively favour growth and metabolism of LAB to the detriment of *Campylobacter* which is unable to utilize glucose as a carbon source (Velayudhan and Kelly, 2002). The results of these co-culture assays are reported in Article V and are accepted with minor revisions in the *Journal of Microbiology and Biotechnology*. Article IV and V are grouped in Chapter IV.

Finally, two polysaccharidase-antagonistic strain combinations were developed:

- a feed additive composed of the xylanase selected *in vivo* and a *Lactobacillus plantarum* strain selected *in vitro* by the Bio-industry Unit of the GxABT, for the reduction of *Salmonella* colonization of fast-growing standard broiler chickens;
- an environmental treatment based on a cellulase and a *Lactobacillus pentosus* strain selected *in vitro*, for pulverisation on litter and open-air range to reduce *Campylobacter* colonization of slow-growing broiler chickens from free-range production.

The *in vitro* experiments can provide subtantial information on the antagonistic potential of these formulations. However, they do not take into account the effect of the complex environment of the gut, namely that of the gastrointestinal acidity, or the effect of the environmental microflora present in litter or soil, which might influence the activity and the survival of the antagonistic strain and of the pathogen. Therefore, it is of prime importance to evaluate the interest of these enzyme-bacterial strain formulations *in vivo*. Thus, three experiments were further conducted to study the efficiency of the feed additive, combining the xylanase and the *L. plantarum*, on *Salmonella* colonization of standard broiler chickens fed a wheat-based diet, and experimentally infected with *S.* Typhimurium. Fecal *Salmonella* spp. were monitored by plate counting and by quantitative real-time polymerase chain reaction (PCR). The effect of feed supplementation on growth performance, nutrient digestibilities and microfloral populations was also assessed. The results observed with the xylanase-*L. plantarum*

combination were compared with those of *Salmonella*-challenged chickens fed or not with the *L. plantarum* and the xylanase alone. The results of this late study are presented in Chapter V and are published in *Poultry Science*.

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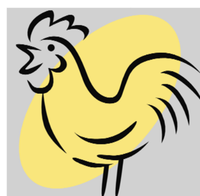
Chapter III. Determination of risk factors of Campylobacter infection infree-range broiler productions

Article III. Prevalence and sources of *Campylobacter* spp. contamination in free-range broiler production in the southern part of Belgium

Determination of risk factors of Campylobacter infection in free-range broiler productions

It is generally assumed that vertical transmission is not a major source of *Campylobacter* contamination of broiler flocks, while horizontal transmission from the outer environment is frequently incriminated. Some environmental factors, like litter, air, and feed, were not identified as important transmission routes. On the other hand, the broiler house surroundings, the open-air range in free-range productions, and wild and domesticated animals, were hypothesized to play an important part in *Campylobacter* contamination of broiler flocks. Free-range broiler production is consequently highly susceptible to contamination by this pathogen because of the bird's access to an open-air range during the rearing period. Nevertheless, knowledge about the various routes by which flocks become infected, the risk factors, and their relative importance, is still incomplete.

If chickens are mainly infected via the outer environment, reduction of the environmental contamination, with the help of an antibacterial treatment, should reduce *Campylobacter* colonization in the birds. Before establishing the applicability of such treatment, the first step was to determine the exact environmental factors responsible of flock contamination in Walloon free-range broiler farms. The following chapter describes the methodology used in this epidemiological study, and the representative results.



Prevalence and sources of Campylobacter spp. contamination in free-range broiler production in the southern part of Belgium

Article III — Adapted from Vandeplas *et al.* (2010)
Biotechnology, Agronomy, Society and Environment **14**(2), 279-288

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Running head: *Campylobacter* in free-range broiler production in Belgium

Abbreviations: *C.*, *Campylobacter*; EFSA, European Food Safety Authority; mCCDA, modified Cefoperazone Charcoal Deoxycholate Agar; PCR, Polymerase Chain Reaction

Abstract

A one year epidemiological study was carried out between February 2005 and January 2006 in the southern part of Belgium to assess the *Campylobacter* prevalence in free-range broiler production. Three successive broiler flocks from six Belgian farms were investigated for the presence of *Campylobacter* spp. during the rearing period. Each flock was visited four times, before and after the outdoor rearing period. During each visit, samples were taken in the broiler house (litter, cecal droppings, water-lines, feed, anteroom) as well as from the outer rearing environment (open-air range). The *Campylobacter* detection in all samples was carried out according to the ISO 10272 standard. Identification was based on colonial morphology, microscopic examination, and biochemical tests. PCR multiplex was used for genetic confirmation. *Campylobacter jejuni* was the main species isolated from all contaminated samples. Overall, mixed infections C. jejuni/C. *coli* represented 40.6 %, while C. *jejuni* and C. *coli* represented 46.9 % and 12.5 % of chicken samples respectively. A 100 % flock contamination was observed in the 6 farms during the summer/autumn period, whereas only 66.7 % and 33.3 % of the flocks became Campylobacter-positive in spring and winter respectively, at the end of the rearing period. Half of contaminated flocks were infected before chickens have access to the open-air range. Environmental samples, especially the open-air range soil, were found to be Campylobacter-positive before flock infection. The other potential sources of contamination were delivery tray, anteroom floor and water-lines. Other animal productions like cattle on the farm, no applied rodent control, no cleaning and disinfection of water-lines between flocks, no detergent used for cleansing and thinning were recorded as risk factors. In conclusion, the contact with the environment, particularly the access to an open-air range, appeared to be the major way of *Campylobacter* contamination of chickens in free-range broiler production.

Keywords: *Campylobacter*, epidemiology, free-range broiler flocks, open-air range, prevalence, risk factors

Résumé

Une étude épidémiologique d'un an a été menée de février 2005 à janvier 2006 en Région Wallonne de Belgique afin d'évaluer la prévalence de *Campylobacter* dans les productions de poulets de chair élevés en plein air. Trois lots successifs dans six exploitations belges ont été investigués pour la présence de *Campylobacter* spp. pendant la période d'élevage. A chaque visite, des échantillons ont été prélevés dans le poulailler (litière, matières caecales, lignes d'eau, aliment, sas d'entrée) ainsi que de l'environnement extérieur (parcours). La détection de *Campylobacter* dans les échantillons a été réalisée selon le standard ISO 10272. L'identification était basée sur la morphologie des colonies, l'examen microscopique et des tests biochimiques. La PCR multiplex a été utilisée pour confirmation génétique. *Campylobacter jejuni* était la principale espèce isolée de tous les échantillons contaminés. Globalement, les infections mixtes *C. jejuni*/*C. coli* représentaient 40,6 %, tandis que *C. jejuni* et *C. coli* représentaient 46,9 % et 12,5 % des isolats de poulets respectivement. Tous les lots (100 %) étaient contaminés dans les 6 exploitations pendant la période été/automne, alors que seulement 66,7 % et 33,3 % des lots étaient positifs à *Campylobacter*, à la fin de la période d'élevage, au printemps et en hiver respectivement. La moitié des lots contaminés étaient infectés avant que les poulets n'aient accès au parcours extérieur. Différents échantillons environnementaux, plus particulièrement le sol du parcours, ont été détectés positifs à *Campylobacter* avant l'infection du lot. Les autres sources potentielles de contamination étaient le véhicule de livraison, le sol du sas et les lignes d'eau. La présence d'autres productions animales comme des bovins dans l'exploitation, l'absence de contrôle des rongeurs, l'absence de nettoyage et de désinfection des lignes d'eau entre les lots, le nettoyage sans détergent et la séparation des lots pour l'abattage ont été déterminés comme facteurs de risque. En conclusion, le contact avec l'environnement, plus particulièrement l'accès à un parcours extérieur, apparaît comme une source majeure de contamination des poulets par *Campylobacter* en production de poulets de chair élevés en plein air.

Mots-clés : *Campylobacter*, épidémiologie, facteurs de risque, lots de poulets élevés en plein air, parcours extérieur, prévalence

I. Introduction

According to several reports from countries all over the world, *Campylobacter* is now recognized as the most common cause of human bacterial enteritis in developed countries. In 2006, the overall incidence of campylobacteriosis was 46.1 per 100 000 population in the EU-25 (European Food Safety Authority, 2007). More than 95 % of registered *Campylobacter* enteritis is caused by thermotolerant species, i.e. *Campylobacter jejuni* and *Campylobacter coli* (Butzler, 2004). *C. jejuni* and *C. coli* have been traditionally differenciated by the hippurate hydrolysis test, for which only *C. jejuni* gives a positive reaction.

Case control studies have identified consumption of contaminated raw or insufficiently cooked poultry products as the major vehicle for campylobacteriosis (Moore *et al.*, 2005 ; Zorman *et al.*, 2006), for a variable percentage of cases ranging from 10 % in Denmark to more than 70 % at a US university (Friedman *et al.*, 2000). Moreover, a quantitative risk assessment carried out by Rosenquist *et al.* (2003) showed a linear relationship between the poultry flock prevalence of *Campylobacter* and the incidence of human campylobacteriosis. Therefore, the European Union and governmental agencies are focused on eradicating *Campylobacter* in live bird and at the processing plant, particularly by improving the control of pathogen in the primary production and by the intensification of epidemiological studies about *Campylobacter* at national level.

The development of cost-effective control strategies requires a more precise knowledge of the mechanisms of transmission and epidemiology of campylobacters in poultry. The transmission routes, the risk factors and sources for flock colonization in poultry production have been identified and quantified in several studies. Horizontal transmission is generally considered the most significant cause of broiler flock colonization (Newell and Fearnley, 2003), with the environment of the poultry house being the major reservoir of pathogen. The factors showed to be associated with an increased risk of contamination are the lack of hygiene measures (Kapperud *et al.*, 1993 ; Evans and Sayers, 2000), the presence of other farm animals on the farm (Kapperud *et al.*, 1993 ; Van de Giessen *et al.*, 1996 ; Bouwknegt *et al.*, 2004), several poultry houses on the same farm site (Refrégier-Petton *et al.*, 2001 ; Bouwknegt *et al.*, 2004), the first disinfection being performed by the farmer instead of a hygiene specialist (Huneau-Salaün *et al.*, 2007), drinking unchlorinated water (Arsenault *et al.*, 2007), as well as rodents, insects and wild birds (Berndtson *et al.*, 1996; Stern *et al.*, 2001; Hansson *et al.*, 2007).

The potential of the environment as source of *Campylobacter* has lead to the assumption that characteristics of extensive organic broiler productions, including the access to an open-air range, could be associated with a higher prevalence of *Campylobacter* than conventional standard production, as mentioned by Huneau-Salaün *et al.* (2007) and Newell and Fearnley (2003), and confirmed by several studies. Data on the prevalence of *Campylobacter* in conventional in comparison with non-conventional broiler production actually gave values from 36.7 % to 66 % and from 89 % to 100 % respectively (Heuer *et al.*, 2001; Luangtongkum *et al.*, 2006) Furthermore, a seasonal variation in the prevalence of *Campylobacter*-positive broiler-flocks has also been reported from Norway (Kapperud *et al.*, 1993) as well as from France (Refrégier-Petton *et al.*, 2001) and United Kingdom (Wallace *et al.*, 1997).

In this context, the aim of the present study was to determine the flock prevalence of *Campylobacter* from free-range broiler production, a fast-expanding poultry rearing system in the southern part of Belgium. Furthermore, the study aimed to evaluate potential contamination sources at the farm level, including among others food, litter, drinking water or free-open range. In accordance with the request related to *Campylobacter* national surveillance recorded in the EFSA scientific report, the collected data will help to develop an effective control program to reduce the broiler flocks prevalence of *Campylobacter*, and than the campylobacteriosis incidence at national level.

II. Materials and methods

II.1. Farm characteristics

This study was conducted from February 2005 to January 2006 in the southern French-speaking part of Belgium. Six farms, designated farms “A” through “F”, were selected as a convenient sample representing approximatively 10 % of Belgian free-range broiler production farms. They were affiliated with three different chicken meat production companies (I, II, III), with two farms/production company. The companies were chosen for inclusion in this study due to their large size and their readiness to collaborate in the project. They supplied the farm locations and subsequent farm selection was based on homogenous geographical distribution, and on diversity regarding broiler house and rearing managements. All contacted farmers accepted free cooperation to the study.

Two companies were of type organic farmer production system, while the third was of type free-range production system. Characteristics of both broilers production systems are based on the references to free-range chickens according to Commission Regulation (EEC) n°1538/91 introducing detailed rules for implementing Regulation (EEC) n°1906/90 on certain marketing standards for poultry meat.

Only one of the separate broiler houses on each farm was chosen for the epidemiological study. Three successive broiler flocks coded “a” through “c” were sampled on each broiler house, for the presence of *Campylobacter* ssp. during the rearing period. Flocks a were reared from February to June, flocks b from July to September, and flocks c from October to January. The flock size ranged from 1200 to 4800 (mean 3582) birds at the day of placement. All housed were closed, isolated and had regulated temperature and ventilation. Chickens were raised on floor, with either straw or wood shavings as litter. Slow-growing broiler strains had free access to an open-air range from six weeks of age and were slaughtered at minimum 82 days of age. The chicken densities for organic production in the broiler house (10 birds/m^2) and on the open-air range (4 m^2/bird) are however different than for free-range production (11 birds/m^2 for the broiler house, and 2 m^2/bird for the open-air range). Cereals in the feed accounted for at least 70 % in weight. Between each successive flock, there was a two to four weeks period where the house is cleaned, disinfected and left empty before input of new chickens. The thinning system, i.e. partial flock depopulation at slaughter age, was used for most flocks.

II.2. Sample collection

Each flock was visited four times, before (1 and 27 days of age) and after (54 and 81 days of age) the outdoor rearing period. The first visit was carried out just before the setting up of the chicks, and samples were taken aseptically in the disinfected broiler house (clean straw litter, water-lines, drinking water, feed, exit trap doors, floor from the anteroom), in the transportation truck (delivery tray liners, floor) as well as in the outer rearing environment (open-air range soil).

One composite sample of 25 g litter from four areas of the broiler house, one composite sample of 1 l drinking water from one to four drinkers, two composite samples of 250-500 g from fifteen areas of the open-air range and one composite sample of about 130 g feed from the feeders were collected. About 1 l of tap water was sampled in the anteroom to exclude the risk of contamination from the chickens. Samples from the anteroom floor, exit trap doors, paper liners from the chick delivery trays, water drinkers and transport truck floor, were obtained by rubbing a sterile cotton gauze moistened with sterile distilled water over about 10×0.0025 m^2 of the object’s surface. All the samples were placed in sealed sterile bags or containers.

For each of the three following visits, samples consisted of four composite samples of caecal droppings taken from the four quarters of the whole house litter and stored in a sterile plastic bag tightly sealed after excluding the air, as well as of swabs from the anteroom and of surface soil of the open-air range, sampled as described for the first visit. All samples were collected on each occasion within 1 to 2 h and transferred in insulated boxes containing ice packs for transport to the laboratory. They were kept at 4°C less than two weeks prior to the microbiological tests.

II.3. Isolation and identification of Campylobacter

Two reference strains (*Campylobacter jejuni* LMG 8841 and *Campylobacter coli* LMG 6440) were used as controls.

Campylobacter detection and isolation methods were based on the ISO 10272 procedure. Briefly, each swabs or 25g of solid material were inoculated into 100 ml selective enrichment Bolton broth (Oxoid, Belgium) supplemented with 0.5 % lysed defibrinated horse blood. Water samples were first filtered through a sterile 0.45 µm membrane filter (Zetapor, CUNO Benelux, Belgium) prior to add the latter to 100 ml of broth. All samples were then subjected to pre-enrichment step at 37°C for 4 h followed by enrichment cultures at 42°C for 44 h in microaerophilic atmosphere. Microaerophilic conditions were generated by using commercial gas generating kits (Anaerocult C, VWR International, Belgium). After enrichment, the samples were streaked onto selective agar media (Karmali agar, Biokar, Belgium; mCCDA agar, Oxoid, Belgium) and the plates were incubated in jars at 42°C for 48 h in microaerophilic atmosphere. From each positive agar plate, several typical *Campylobacter* colonies was subcultured onto Brucella agar (48 h, 42°C), confirmed as a member of the genus by examination of cellular morphology and motility on a wet mount under phase contrast microscopy and tested for Gram-staining, production of oxidase and catalase, hippurate hydrolysis and antibiotic susceptibility to nalidixic acid and cephalothin.

A multiplex PCR test was used for the final confirmation. After morphological and biochemical confirmation, 3 colonies from the mCCDA confirmation plates were picked up and anaerobically sub-cultured into Brucella broth at 42°C for 48 h. The total DNA was extracted form cell pellets, using the Genomic DNA Wizard kit (Promega, WI, USA) according to the salting-out technique. The multiplex PCR reaction was carried out with *16S rDNA* primers designed to obtain the specific identification of the genus (Denis *et al.*, 1999) and *CeuE* and *MDmapA* primers to discriminate *C. Coli* and *C. jejuni* species (Nayak *et al.*, 2005). PCR products were visualized on a 1 % agarose gel.

II.4. Flock informations

On the first and fourth sampling day, detailed farm and flock management data were collected through the submission of a standardised management questionnaire. The questionnaire was conducted in the form of an in-person interview with the farm owner or manager. A single member of the study team administered the questionnaire on all farms to eliminate inter-observer error. The questionnaire consisted of 111 questions. Thirty-one (28 %) of the questions were open-ended (requiring descriptions), 12 (11 %) were semi-closed, (asking about number of days, animals, or rooms) and 68 (61 %) were closed (with Yes/No or preset options for answers). The questionnaire was previously pilot tested by the French Agency for Food Safety (Ploufragan, France), but repeatability of answers was not tested directly. The questionnaire took about 2 h to complete. Collected data, detailed in Table 1, concerned sanitary practices, litter, conditions of chick placement, dead-bird management, control of wildlife, house and flock characteristics, house surroundings, water supply and others. At each sampling day, a separate questionnaire was filled out by the person taking the samples, in order to give information about the rodents/insects presence and the sanitary status of the broiler house and surroundings. Farms were considered as Campylobacter-positive when the pathogen was detected in cecal droppings at least at slaughter age.

Table 1. Headings and short descriptions of the questions (n=111) in the questionnaire at the first and last visit for the 18 broiler flocks

Staff	Number of farmers/employees, visitors, hygiene barrier for visits, handling of animals
Buildings	Number and description of buildings on the farm site, materials, equipment for ventilation and heating, feeding system, drinking system
Chicken flock	Flock size, age and weight at slaughter, age at which the flock had access to the open-air range
Surroundings	Geographical location, other farm animals, open-air range characteristics and surface, concrete aprons and paths around the house, location according to fields and forest
Flock management	Feed, litter and water origin, clothing routines, use and management of boot dips
Cleansing and disinfection	Washing and disinfection procedures, equipment and products, manure management, empty period
Wildlife control	Management and equipment against wild birds, rodents and insects
Setting up	Hygiene barrier, hatchery and transport time, staff
Chicken health status	Vaccination, diseases, dead-bird management, use of coccidiostatics and antibiotics

II.5. Statistical analysis

Descriptive variables of the flock assessed by questionnaire (in qualitative form) were first selected to eliminate those that generated the same responses for at least 75 % of the 18 flocks (Denis *et al.*, 2008). Association between these remaining descriptive variables and explanatory variables were tested using Fisher's exact test available in SAS software (SAS Institute Inc., NC, USA). Variables generating a response frequency lower than 11 % (corresponding to two flocks) were removed. Finally, associations between the remaining descriptive variables and explanatory variables were tested with Fisher's exact test ($P \leq 0.05$). Although risk factors generally result from the combination of several parameters, a multivariate statistical analysis was not performed because of the low number of data.

III. Results

III.1. Flock contamination by C. jejuni and C. coli

Data for all successive flocks for the six farms are summarized in Table 2. From February to June 2005, i.e. in spring, the results of Table 1 show that four farms (66.7 %) were *Campylobacter*-positive before slaughter age. The extent of contamination increased in summer until October (flock b), to reach a prevalence of 100 %. Furthermore, in winter, only 33.3 % of

the farms were contaminated by *Campylobacter*. The species distribution among the *Campylobacter*-positive flocks shows that *C. jejuni*, alone or mixed with *C. coli*, was predominant in broiler chickens from free-range broiler production in Belgium. Overall, mixed infections by *C. jejuni*/*C. coli* represented 40.6 %, while *C. jejuni* and *C. coli* only represented 46.9 % and 12.5 % of chicken samples respectively, taking into account all days of sampling. At slaughter age, five of the 12 contaminated flocks (41.6 %) were contaminated by mixed population of *C. jejuni*/*C. coli,* four flocks (33.3 %) by *C. jejuni* and three flocks (25 %) by *C. coli.*

Table 2. Free-range broiler flock contamination by *Campylobacter jejuni* (j) and *Campylobacter coli* (c) in Belgium, according to the age of the broilers.

Flock	Farm	Day(s) of age				% Flock contaminated at slaughter
		1 [a]	27 [a]	54 [a]	81 [a]	
a (febr-june)	A	-	-	-	-	66.7
	B	-	+ (j/c)[b]	+ (j/c)	+ (j/c)	
	C	-	-	-	-	
	D	-	-	+ (j)	+ (j)	
	E	-	+ (j)	+ (j)	+ (c)	
	F	-	-	+ (j)	+ (c)	
b (july-sept)	A	-	-	+ (j/c)	+ (j/c)	100
	B	-	-	+ (j/c)	+ (j/c)	
	C	-	-	+ (c)	+ (c)	
	D	-	-	+ (j/c)	+ (j/c)	
	E	-	+ (j)	+ (j)	+ (j)	
	F	-	+ (j)	+ (j)	+ (j)	
c (oct-janv)	A	-	-	-	-	33.3
	B	-	-	-	-	
	C	-	-	-	-	
	D	+ (j/c)	+ (j/c)	+ (j/c)	+ (j/c)	
	E	+ (j)	+ (j)	+ (j)	+ (j)	
	F	-	-	-	-	

[a] 1 day of age : setting up of the chicks; 27 days of age: before the outdoor rearing period; 54 days of age: during the outdoor rearing period; 81 days of age: at slaughter age.
[b] j : *C. jejuni* identified by hippurate test and multiplex PCR; c : *C. coli* identified by hippurate test and multiplex PCR.

For the winter period (flocks c), the two *Campylobacter*-positive flocks were yet contaminated from 1 d of age. In the spring period (flocks a), *Campylobacter* was detected in two flocks before the exit of the chickens on the free-open range (day 27) and two additional flocks were contaminated after the exit. In the summer time, contamination was detected in flocks Eb and Fb at 27 days of age while three more flocks were *Campylobacter*-positive at 54 days of age.

Table 3 gives details of flocks in which environmental samples were found to be *Campylobacter*-positive before or at the same time that flock infection was detected. In 83.3 % of the flocks did positive environmental samples come partly of fully from the open-air range. Moreover, the anteroom floor was detected *Campylobacter*- positive in three of the 13 infected flocks (23 %). Other contaminated environmental samples were litter (flock Da) and exit trap door (flock Eb).

Moreover, results for the third flock, from October to January, are distinctive. Contamination for the two *Campylobacter*-positive farms was revealed just before the setting up of the chicks, only from the free-air range soil, the water-line, and the delivery tray. Broilers fecal droppings were infected later, at four to eight weeks of age (day 54).

Table 3. Environmental samples found to be *Campylobacter*-positive prior or simultaneously to the appearance of flock infection

Flock	Farm	Day of *Campylobacter* detection	Positive samples
a (febr-june)	A	-	-
	B	27	Open-air range
	C	-	-
	D	54	Open-air range, litter
	E	27	Open-air range, anteroom
	F	54	Open-air range
b (july-sept)	A	54	Open-air range
	B	54	Anteroom
	C	81	Open-air range
	D	54	Anteroom
	E	1	Open-air range, exit trap door
	F	27	Nd
c (oct-janv)	A	-	-
	B	-	-
	C	-	-
	D	1	Open-air range, delivery tray
	E	1	Open-air range, water-line
	F	-	-

nd: not determined

III.2. Flock characteristics correlated to Campylobacter infection

Among the 111 items included in the questionnaires submitted to the farmer, seven variables were retained and studied in relation to the *Campylobacter* infection of flocks. These variables concerned poultry house and flock management (Table 4).

The kind of other animal production systems in the farm, the mode of cleansing, the disinfection of aprons surrounding the broiler house, the control of rodents, and the environment around the open-air range had significant effect on flock contamination ($P \leq 0.05$). On the other hand, the season when sampling was performed did not affect the contamination status of the flock ($P > 0.05$), although the *Campylobacter* prevalence decreased from 100 % to 33.3 % between summer and winter.

Table 4. Variables retained from the questionnaires and results of Fisher's exact test ($P \leq 0.05$)

Farm characteristics	Modalities	Number of flocks		Probability (*P*)
		Negative	Positive	
Open-air range surroundings	Fields	3	0	0.037
	Meadows	1	11	
	Meadows and forests	2	1	
Animal productions on the farm site	Cattle	1	8	0.025
	Other	2	1	
	Cattle and other	3	3	
Rodent control	Yes	6	3	0.029
	No	0	9	
Use of detergent for cleaning	Yes	4	1	0.022
	No	2	11	
Cleaning and disinfection of water-lines between flocks	Yes	1	9	0.043
	No	5	3	
Thinning	No	3	2	0.009
	In 2 times	3	1	
	In 3 times	0	9	
Farmer passage on the open-air range before the chicken exit	No	1	8	0.025
	More than once a week	2	1	
	Less than once a week	3	3	
Season	Spring-summer	2	4	0.085
	Summer-autumn	0	6	
	Autumn-winter	4	2	

IV. Discussion

The *Campylobacter* prevalence in free-range broiler production of the southern part of Belgium was found very high with 33.3 to 100 % of flocks being infected from February 2005 to February 2006. Similar observations were reported in Denmark for 22 organic broiler flocks (Heuer *et al.*, 2001), and in France for 73 flocks from extensive outdoor broiler production (Huneau-Salaün *et al.*, 2007). These values are higher than those observed in conventional broiler production by Herman *et al.* (2003) and Rasschaert *et al.* (2006) who reported *Campylobacter* prevalence at broiler flock level from 39 to 72 % in Belgium. Higher *Campylobacter* prevalence in extensive production in comparison with conventional intensive farms was frequently observed in Denmark (Heuer *et al.*, 2001) and the USA (Luangtongkum *et al.*, 2006). However, high variations in the proportion of colonized broiler flocks have been observed between countries, from 3 % in Finland to >90 % in the United Kingdom (Raaschaert, 2007), which could partly reflect different sampling and isolation methods used.

A season effect for *Campylobacter* presence is generally reported in the literature (Refrégier-Petton *et al.*, 2001; Bouwknegt *et al.*, 2004). In our study, the *Campylobacter* prevalence varied from 33.3 % in winter to 100 % in summer, the higher number of *Campylobacter*-positive flock being found from July to October. The statistical analysis showed no significant effect of the season but only a trend ($P = 0.085$). The reason for these seasonal variations is still debated but may indicate a possible relationship between temperature and *Campylobacter* spp. survival and transmission of infection to broilers as stated by Patrick *et al.* (2004). Insects have been frequently implicated in this seasonal effect of *Campylobacter* prevalence. Some researches carried out by Hald and coworkers (Hald *et al.*, 2008) showed that insects may be an important source of *Campylobacter* infection of broiler flocks. Insects that may be present in poultry house are mainly flies identified to the families Muscidae (house fly *Musca domestica*, little house fly *Fannia canicularis*, etc.), but also insects of the families Tenebrionidae (Litter Beetle *Alphitobius diaperinus*), Dermestidae (Hide Beetle *Dermestes maculates*), Calliphoridae (Blue bottle fly *Calliphora vomitoria*), as well as several species of mites, lice and fleas (personal communication). In summer, hundreds of flies passed through the ventilation system into the broiler house and the influx of insects was correlated with the outdoor temperature. The climate impact could be especially important in free-range production for which the birds are in close contact with the environmental conditions. In the future, any epidemiological study should take into account potential correlations between climate factors, like the average temperature or hours of sunlight, and *Campylobacter* prevalence in poultry flocks.

The thermotolerant species *C jejuni* and *C coli* account for most of the human foodborne infections (Zorman *et al.*, 2006). The species distribution among the *Campylobacter*-positive flocks in this study showed that *C. jejuni* is predominant in free-range broiler production in Belgium, with a prevalence varying according to the flock considered, from 42.9 % to 50 % for *C. jejuni* alone, and from 80 % to 100 % when combining *C. jejuni* and mixed *C. jejuni/C. coli* isolates. These results are in agreement with most of epidemiogogical studies where identification to species level has been undertaken (Heuer *et al.*, 2001 ; Siemer *et al.*, 2005 ; Denis *et al.*, 2008), while *C. coli* and even *C. lari* had also been isolated as a common species from chickens in some study (Zorman *et al.*, 2006 ; Kilonzo-Nthenge *et al.*, 2008). Van Looveren *et al.* (2001) found that among 677 *Campylobacter* isolates from broiler carcasses and meat from Belgian slaughterhouses, 79 % was identified as *C. jejuni*. It should be mentioned that poultry carcasses could be cross-contaminated by different *Campylobacter* species during the slaughter processing, leading to different results compared to *Campylobacter* contamination of the broiler flocks.

Identification of the sources of flock colonization would enable control measures to be targeted towards the areas posing the greatest risk. In most infected flocks, the first time *Campylobacter* spp. was found in caecal samples was when broilers were 27-days-old. This is in agreement with other studies where it has been reported that most flocks become infected only two to three weeks after the setting up of chicks into a broiler house (Saleha, 2004; Bull *et al.*, 2006; Hansson *et al.*, 2007). This lag-phase in contamination is a possible protection effect of maternal immunity, as proposed by Sahin *et al.* (2003). This observation may support the hypothesis that campylobacters are not transmitted vertically from parents to chicks, as stated by Saleha (2004).

Moreover, results for the c flocks, from October to January, are distinctive. Contamination for the two *Campylobacter*-positive farms was revealed just before the setting up of the chicks, from the free-air range soil, the water-line, the delivery tray and the anteroom floor swabs, whereas broilers caecal droppings were infected later, at four to eight weeks of age. This may have been due partly to the residual presence of pathogens either from previous *Campylobacter*-

positive flocks or to the environmental contamination of the house surroundings from which the infection could have arisen. Campylobacters are ubiquitous in the environment and around broiler houses and may be easily transported into the house either in utilities, such as feed, litter and water. In this study, *Campylobacter* spp. were not isolated from any samples of litter and feed after the setting up of the chick, as observed by Bull *et al.* (2006) and Hansson *et al.* (2007). In case of flocks Ec and Fc, water-lines were detected positive from the first day. As Newell and Fearnley (2003) pointed that contamination of the water lines usually follows rather than precedes colonization of the flock, it may be hypothesized that poor disinfection of water-line after flocks Eb and Fb may be responsible for the *Campylobacter*-detection in the following flocks. As pointed out by Newell and Fearnley (2003), the analysis of risk factors in this study showed that cleaning and disinfection of water-line between flocks may help to reduce the risk of chicken colonization.

Human activities as entrance of farmers, maintenance staff, veterinarians, and catching crews, may also carry out *Campylobacter* into the house from the external environment via boots, external clothes and equipment (Newell and Fearnley, 2003; Ramabu *et al.*, 2004). The contamination of the anteroom floor may actually suggest the possible infection of flock by the farmer, visitor and equipments. Unsuitable hygiene practices at the farm level, especially poor cleaning and disinfection of the house and not dedicated protective clothing, could then be a major reason of *Campylobacter* contamination persistence in poultry flocks, as summarized by Allen and Newell (2005). Moreover, most samples found to be *Campylobacter*-positive in this study were from the open-air range which appears to be a major source of *Campylobacter* contamination. Such conclusion was also reported by Rivoal *et al.* (2005) who studied genomic diversity and sources of *Campylobacter* contamination in French free-range broiler farms The subsequent flock infection may be related to contamination of farmer equipment by the open-air range soil or through contact in the open-air range with wild birds and other animals and with their faeces (Rodenburg *et al.*, 2004). However, the carrying of *Campylobacter* into the house by human activity and environmental factors has yet to be proven by genotyping confirmation of strains in the environment which subsequently result in flock colonization.

Other risk factors for *Campylobacter* contamination have been identified in this study. Efficiency of the rodent control applied at the farm appeared to be important as wild animal like insects and rodent are recognized vectors of *Campylobacter* (Stern *et al.*, 2001; Saleha, 2004). The presence of other domestic animals on the farm site is main risk factor underlined by several authors (Bouwknegt *et al.*, 2004; Saleha, 2004). The risk of spreading *Campylobacter* from other animals on the farm to the broiler flock, or generally from environmental surroundings, can be reduced by applying strict hygiene measures, like walk-over benches, using boot dips or house dedicated footwear, at least until the access of the birds to the open area.

Thinning also appeared to be a major risk factor for the introduction of *Campylobacter* into the broiler house. Thinning or partial depopulation of the flock is a common procedure in many European countries, including Belgium. This practice enables higher productivity and provides the market of birds of different weight. During thinning, the doors of the poultry house are opened and the catching crew and the catching equipment enter the poultry house without any hygiene measures. Ramabu *et al.* (2004) found that catching equipments like trucks, forklifts, or crates may actually be contaminated by *Campylobacter*, which represent a major risk of contamination for the remaining birds.

In summary, the results of this study provided for the first time information about the *Campylobacter* prevalence in free-range broiler production from the southern part of Belgium

and pointed out potential sources of *Campylobacter* for this kind of rearing system. The high prevalence of enteric *Campylobacter* in free-range broiler production in Belgium reported was similar to results from other European states. The *Campylobacter* prevalence increased in summer-fall. The environment surrounding the broiler house, especially the open-air range, seems to be an important way of contamination of broilers and appears consequently as a burdensome parameter to take into account in further development of *Campylobacter* control programs.

Suitable biosecurity measures to exclude campylobacters from free-range broiler flocks are currently the only intervention available. However, it is generally considered that adequate biosecurity procedures are difficult to sustain in the farm environment (Newell and Fearnley, 2003). The development of supplementary on-farm control strategies may be required to achieve predominantly *Campylobacter*-negative flocks.

V. References

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Chapter IV. Selection of polysaccharidases and bacterial strains with antagonistic activities

Enzymes — Selection of polysaccharidases and bacterial strains

Article IV. Effect of the bacterial or fungal origin of exogenous xylanases supplemented to a wheat-based diet on performances of broiler chickens and nutrient digestibilities of the diet

Bacteria — Selection of polysaccharidases and bacterial strains

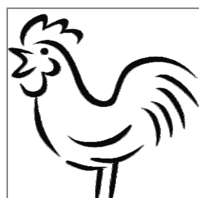
Article V. *In vitro* antagonistic activity evaluation of Lactic Acid Bacteria combined with cellulase enzyme on *Campylobacter jejuni* growth in co-culture

Enzymes – Selection of polysaccharidases and bacterial strains with antagonistic activities

Northern European countries often substitute wheat for maize as source of energy in broiler diets because of its more suitable culture conditions and its lower costs. Supplementation of polysaccharidases to the diets is able to circumvent the anti-nutritional effects of wheat due to its high content in non-starch polyssacharides, especially in arabinoxylans. Such enzymes, like xylanases, are necessary in order to sustain competitive growth performance in comparison with maize-based diets.

Hydrolysis products from xylanase activity in the gastro-intestinal tract are essentially xylooligosaccharides based on C-5 xylose and arabinose. They can not be digested by broilers as they are, because of the lack of endogenous appropriate digestive enzymes, but they can be fermented by specific bacteria of the gut microflora. If these bacterial strains present some antibacterial activity against enteric pathogens like Salmonella, it should be interesting to favour their growth and metabolism by supplying specific xylooligosaccharides based on C-5 xylose and arabinose.

The first part of this Chapter describes a screening *in vivo* of 4 xylanase products. The comparison criterions were the improvement in growth performance of fast-growing broiler chickens, compared with birds fed unsupplemented diet, and the simultaneous increase in nutrient digestibilities. Studies that are carried out with experimental infection of animals have shown that *Salmonella* colonization of broiler chickens induces a significant reduction of growth performance, which is dose-dependent. Therefore, performance can be considered as a relevant measurement parameter for evaluating feed treatment efficiency. As xylanases used in chicken diet formulations are mainly produced from fungi and from bacteria, enzymes from both microbial origins were also compared in the study. The final purpose of the experiments was to select a growth-promoting enzyme for including in an enzyme-bacteria formulation. The bacterial strain will present a potential antagonistic activity against Salmonella while being able to metabolize xylooligosaccharides.



Effect of the bacterial or fungal origin of exogenous xylanases supplemented to a wheat-based diet on performances of broiler chickens and nutrient digestibilities of the diet

Article IV — Adapted from Vandeplas *et al.* (2010)
Canadian Journal of Animal Science **90**(2), 221-228S

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Running head: Exogenous xylanases for broiler chickens

Abbreviations: A, xylanase from *Aspergillus aculeatus*; AME, apparent metabolizable energy; AMEn, nitrogen-corrected apparent metabolizable energy; AX, arabinoxylan; B, xylanase Belfeed B1100MP; DM, dry matter; DWG, daily weight gain; FCR, feed conversion ratio; G, xylanase Grindazyme G; NSP, non-starch polysaccharide; R, Roxazyme G; RSD, residual standard deviation

Abstract

Two identical experiments were carried out to study the effect of four xylanases from bacterial or fungal origin supplemented to a wheat-based diet, on growth performance of broiler chickens and nutrient digestibilities. Experimental treatments consisted of a control basal diet containing 600 g kg^{-1} wheat (C), and the basal diet supplemented with 0.1 g kg^{-1} Grindazyme G from *Aspergillus niger* (G), 0.1 g kg^{-1} Belfeed B1100MP from *Bacillus subtilis* (B), 0.1 g kg^{-1} Roxazyme G from *Trichoderma viride* (R), or 0.0125 g kg^{-1} of a xylanase from *Aspergillus aculeatus* (A). Each experimental diet was given to four groups of six chickens each. Growth performance and feed conversion ratio (FCR) were recorded weekly, from 7 to 21 days of age. In the second experiment, a digestion balance trial was performed from 27 to 31 d of age to evaluate the nitrogen-corrected apparent metabolizable energy (AMEn) and the digestibilities of nitrogen, crude fat, starch and crude fibre. From 7 to 21 d of age, xylanase supplementation led to increased final body weight and daily weight gain by 3.7 % and 4.5 % ($P < 0.05$) respectively, without significant difference according to the xylanase origin. Xylanase supplementation significantly increased the AMEn (+2.6 %), and the digestibilities of crude fibre (+58.9 %) and nitrogen (+1.6 %). Increase in AMEn, as well as in crude fat and starch digestibilities was significantly different according to the xylanase, but was not dependent on with the fungal or bacterial origin. In conclusion, the microbial origin of xylanases supplemented to wheat-based diets influenced neither the performance of broiler chickens nor the nutrient digestibilities improvements.

Keywords: broiler, growth performance, nutrient digestibility, wheat, xylanase

Résumé

Deux expériences identiques ont été menées afin d'étudier l'effet de quatre xylanases d'origine fongique ou bactérienne, additionnées à une ration à base de froment, sur les performances de croissance de poulets de chair et la digestibilité des nutriments. Les traitements expérimentaux consistaient en un contrôle composé d'un aliment à base de 600 g kg^{-1} de froment (C), et l'aliment contrôle supplémenté avec 0,1 g kg^{-1} de Grindazyme G d'*Aspergillus niger* (G), 0,1 g kg^{-1} de Belfeed B1100MP de *Bacillus subtilis* (B), 0,1 g kg^{-1} de Roxazyme G de *Trichoderma viride* (R), ou 0,0125 g kg^{-1} d'une xylanase d'*Aspergillus aculeatus* (A). Chaque aliment expérimental a été donné à quatre groupes de six poulets chacun. Les performances de croissance et l'indice de conversion alimentaire (ICA) ont été mesurés de manière hebdomadaire, de 7 à 21 jours d'âge. Dans la seconde expérience, un bilan digestif a été réalisé de 27 à 31 jours d'âge afin d'évaluer la teneur en énergie métabolisable apparente corrigée pour une rétention d'azote nulle (EMAn) ainsi que les digestibilités de l'azote, des matières grasses, de l'amidon, et de la cellulose brute. De 7 à 21 jours d'âge, l'addition de xylanase a induit une augmentation du poids final et du gain quotidien moyen de 3,7 % et 4,5 % ($P <$ 0,05) respectivement, sans différence significative en fonction de l'origine des xylanases. L'addition de xylanase a augmenté significativement l'EMAn (+2,6 %) et les digestibilités de la cellulose brute (+58,9 %) et de l'azote (+1,6 %). L'augmentation de l'AMEn ainsi que des digestibilités des matières grasses et de l'amidon, était significativement différente en fonction des xylanases, sans être dépendante de l'origine fongique ou bactérienne. En conclusion, l'origine microbienne de xylanases supplémentées à un aliment à base de froment n'a pas influencé l'amélioration des performances des poulets et de la digestibilité des nutriments.

Mots-clés : digestibilité des nutriments, froment, poulet de chair, performance de croissance, xylanase.

I. Introduction

In many Northern European countries, culture conditions are little suited to maize grain production which is generally expensive. Consequently, poultry diet formulations often substitute wheat for maize as source of energy. However, wheat can only be used to a limited extent as feed grain for poultry because of its high content in non-starch polysaccharides (NSP), especially arabinoxylans (AX), which are considered as the major anti-nutritive factors in cereals (Choct and Annison 1990). It is generally recognized that their detrimental effects are based on the decrease in nutrient digestibility or metabolizable energy content of the ration. For the last two decades, many authors have showed that water-soluble AX produce highly viscous conditions in the intestine. Such effect interferes with digestion and absorption of nutrients (Cowieson *et al.* 2006), resulting in depressed growth performance of poultry (Adeola and Bedford 2004; Józefiak *et al.* 2007). Moreover, insoluble AX are constituents of the cell wall and they can entrap nutrients in the cell lumen. This effect is frequently called "cage effect" (Cowieson *et al.* 2006).

Supplementation of NSP-degrading enzymes to poultry diets in order to circumvent the anti-nutritional effects of wheat was already investigated by Jensen and co-workers in the 50s (Jensen *et al.* 1957) and has been sustained up to now (Jia *et al.* 2009). The addition of specific xylanase into wheat-based diet allows the degradation of wheat AX fraction in the gut. The enzyme activity results especially in improved growth performance of broiler chickens, and increased nutrient digestibilities, because of the reduction in intestinal viscosity and in cage-effect. (Choct *et al.* 2004; Meng *et al.* 2005; Gutierrez del Alamo 2008).

In 2009, 99 enzyme products containing NSP-hydrolysing activities were approved as feed additives in the EU (European Commission 2009). Xylanases used in chicken diet formulations are mainly produced from fungi, especially from the genera *Aspergillus* and *Trichoderma*, but also from bacteria, especially from the genus *Bacillus*, and from yeast like *Saccharomyces cerevisae* (Beg *et al.* 2001) However, difference in enzyme efficiency according to the microbial origin is relatively unknown. The main advantage of fungal enzymes is their higher activity levels in comparison with bacterial enzymes (Polizeli *et al.* 2005). Fungi typically produce several accessory xylanolytic enzymes, which are necessary for debranching substituted NSP (Haltrich *et al.* 1996). Furthermore, xylanases from bacteria are generally not subjected to post-translation modifications such as glycosylation (Polizeli *et al.* 2005), which can impair their efficiency. However, according to enzyme manufacturers, bacterial xylanase may present a greater stability at high temperature, because most fungal xylanases used in chicken are synthesized by mesophilic organisms, with relatively low optimum temperature (Polizeli *et al.*, 2005). The characteristic data of xylanases reported in the review from Beg *et al.* (2001) confirm such hypothesis. Higher temperature stability may be useful for conditioning and processing of animal feed, for example for pelleting. Beg *et al.* (2001) showed that the optimum pH of activity is lower for fungal than for bacterial xylanases, with pH range of 2-6 and 5.5-7 respectively. Using broiler chickens fed wheat, Józefiak *et al.* (2007) measured pH values of 6.06-6.10 in the small intestinal chime where hydrolysis of NSP primarily occurs. Therefore, NSP-degrading enzymes from bacteria could show higher activities and induce more pronounced effect in chicken.

To the author's knowledge, no previous study was published concerning the effect of xylanase origin (bacterial *vs* fungal) on growth performance and nutrient utilization of broiler chickens fed a wheat-based diet. The purpose of this study was to determine if the effect of xylanase on broiler chickens could be influenced by the microbial origin of the enzyme. The effect of supplementing fungal or bacterial xylanase preparations to a diet containing 600 g kg^{-1} wheat on growth performance of broiler chickens, as well as on nutrient digestibilities and on AMEn, was compared according to the origin of the producer microorganism.

II. Materials and methods

II.1. Experimental Diet

Five experimental diets were based on wheat ("Corvus" cultivar) and were formulated to meet Cobb broiler nutrient requirements, according to recommandations of DSM Nutritionnal Products n.v. (Deinze, Belgium). Their composition is presented in Table 1 and Table 2. The wheat and soybean meals were milled to pass through a 3 mm screen in a hammer mill, and then mixed with the other ingredients. Minor ingredients (less than 1 %) were first mixed in a small rotary mixer (Tecator 1094 Homogenizer, Tecator AB, Höganäs, Sweden) and were then pooled with major ingredients for thorough mixing in a horizontal mixer. All the ingredients originated from the same batches.

Table 1. Composition of the experimental diets

Ingredient	Experimental diets
	Incorporation level (g kg^{-1} of DM)
Wheat[z]	600.0
Soybean meal	272.4
Soybean oil	83.3
Vitamin-mineral premix[y]	36.6
L-Lysine HCl	3.4
DL-Methionine	1.2
L-threonine	0.8
Sodium chloride	2.3
Xylanase supplementation[x] *(g kg^{-1} of DM)*	
Xylanase from *A. aculeatus*	0.0125
Belfeed B1100MP	0.1
Grindazyme G	0.1
Roxazyme G	0.1
Xylanase activity in the diet (IU kg^{-1})	
Xylanase from *A. aculeatus*	0.0000046
Belfeed B1100MP	0.00001
Grindazyme G	0.0012
Roxazyme G	0.004335

[z] Wheat of cultivar Corvus : specific viscosity : 3.0 mL g^{-1}; real viscosity : 2.7 mL g^{-1} (Piron *et al.*, 2005).

[y] Provides (per kg of diet): vitamin A (retinyl acetate), 3.7 mg; cholecalciferol, 0.061 mg; DL-α-tocopheryl acetate, 18.3 mg; bioflavonoids, 42.6 mg; thiamin, 1.2 mg; riboflavin, 7.3 mg; nicotinic acid, 9.7 mg; pyridoxine hydrochloride, 1.2 mg; cyanocobalamin, 0.024 mg; menaquinone, 1.2 mg; folic acid, 0.62 mg; choline chloride, 622.2 mg; calcium, 8784 mg; phosphorus, 3660 mg; sodium, 366 mg; magnesium 36.6 mg; iodine, 0.59 mg; cobalt, 0.59 mg; copper, 2.42 mg; iron, 45.75 mg; manganese, 97.36 mg; zinc, 85.39 mg; selenium, 0.11 mg; methionine, 1647 mg.

[x] Each of the four enzymes is added separately to the basal diet to formulate 4 experimental diets (B, G, R, A). The fifth experimental diet is the basal diet used as control.

Four xylanase products were tested: Grindazyme G® (G) from *Aspergillus niger* (Danisco Animal Nutrition, Marlborough, UK), Roxazyme® G (R) from *Trichoderma viride* (DSM Nutritional Products Ltd, Basel, Switzerland), Belfeed® B1100MP (B) from *Bacillus subtilis* (Beldem s.a., Andenne, Belgium), and a pure xylanase (A) from *Aspergillus aculeatus* (Beldem s.a., Andenne, Belgium). The fungal multi-enzymatic preparation Grindazyme® G presented endo-1,4-β-xylanase (12 IU kg^{-1}), endo-1,4-β-glucanase (5 IU kg^{-1}) and low pectinase (0.01 IU kg^{-1}) activities with optimum pH of 4.5-5. Roxazyme® G contained not only endo-1,3:1,4-β-glucanase (27.17 IU kg^{-1}) and endo-1,4-β-xylanase (43.35 IU kg^{-1}) but also pectinase and amylase activities, and optimum pH of 4-5 (King and Moughan, 1998). The xylanase from *Aspergillus aculeatus* presented an enzymatic activity of 0.3702 IU kg^{-1} and had an optimum pH of 4-5 (de Vries, 1999). Belfeed B1100MP was an enzymatic preparation with endo-1,4-β-xylanase produced by *Bacillus subtilis* (LMG S-15136) as active compound, with a minimum activity of 0.10 IU kg^{-1} and optimal activity at pH 7-8. Enzyme incorporation levels were recommended by the manufacturer for wheat-based broiler diets, i.e. 0.0125 g kg^{-1} for the

enzyme A and 0.1 g kg^{-1} for enzymes G, R and B. One IU is the amount of enzyme which liberates 1 µmol of xylose from birchwood xylan per minute at pH 4.5 and 30°C. Xylanase activities per kg of diet for each experimental diet are reported in Table 1.

Table 2. Calculated chemical composition, digestible amino acids, and metabolizable energy of the experimental diets[z]

Item	Composition (g kg^{-1} of DM)
Crude protein	202.5
Crude fibre	28.6
Starch	352.0
Crude fat	97.5
Digestible lysine	11.5
Digestible methionine	5.5
Digestible threonine	6.8
Calcium	10.0
Total P	5.0
ME (kcal kg^{-1})	3000.0

[z] DSM Nutritionnal Products n.v., Deinze, Belgium.

II.2. Chicken Management and Experimental Design

The experimental protocols complied with the guidelines of the Animal Care and Use Committee (protocol FUSAGx00/13) of Gembloux Agricultural University.

Growth Performance

Two experiments (Exp.1 and 2) were carried out with exactly the same procedure. For each experiment, a total of 120 seven-day-old male broiler chickens (Cobb) were born in the hatchery of the Animal Science Unit (Gembloux Agricultural University, Belgium) and were randomly allotted to 20 groups of six chickens (4 groups per treatment) in a randomised complete block design using weight at 7 d of age as basis for blocking. Groups of chickens were housed in 39 × 98 × 62 cm (height × length × width) cage with a 5 cm squared wire mesh bottom, in two three-tier batteries. All batteries were placed in the same windowless house provided with forced ventilation. Broiler chickens were kept at 32°C during the first week of age. Thereafter, temperature was decreased by 3°C/week to reach 22°C by 21 days of age. The room was lit 23 h per day during the whole experimental period to ensure frequent feed intake. The 5 diets (control, B, G, R, A) were given *ad libitum* as mash, and water was available all the time. Chickens were weighed per pen at day 7, 14 and 21. Feed intake per pen was recorded at the same time and feed conversion ratio (FCR**,** g g^{-1}) was calculated on a pen weight basis. Chickens were inspected daily and dead birds were removed and weighed. To calculate FCR, the body weight of dead birds was considered.

Dry matter (DM), ash, crude protein, crude fat, gross energy and metabolizable energy of the experimental diets were determined according to the methods of the Association of Official Analytical Chemists (1990) for comparison with the theoretical composition.

Digestibility Trial

This animal trial was performed at the end of the second growth performance experiment (Exp. 2) in the same experimental conditions. To determine nutrient digestibilities and apparent metabolizable energy (AME) corrected to zero nitrogen balance (AMEn) of the experimental diets, the total collection procedure and *ad libitum* feeding were performed according to Bourdillon *et al.* (1990). The digestion balance trial started on d 21 with a six days adaptation period during which the five experimental diets were given to the chicken groups as described for the performance experiments. On d 27, chickens were weighed per pen after a 18-h fast period, and then fed the experimental diets for four days. They were then weighed after a 18-h fast period. Excreta were quantitatively collected once daily and kept at 4°C, pooled per pen for the whole experimental period, and stored at -20°C before being freeze-dried. Diets and excreta samples were later ground through a 1 mm sieve and stored at 4°C until analysis. Apparent digestibilities of nitrogen, crude fat, starch, crude fibre, and the AMEn of the diets were estimated from the analyses of excreta and diets. Nutrient digestibility is defined as follows: [nutrient intake-nutrient output]/nutrient intake. DMof diet was measured after drying diet sample at 105°C for 24 h in an oven. DM of excreta samples was determined by freeze-drying. Fat was measured after Soxhlet extraction with diethyl ether during 8 h. Fecal samples were first hydrolysed with 3 M HCl. Measurements of crude fibre were performed according to Weende (AOAC, 1990). The determination of nitrogen (N) was performed with the Kjeldahl method. Faecal N in the excreta was calculated as total N minus N in the uric acid. Uric acid was analysed with the method of Terpstra and De Hart (1974). AME was calculated from the gross energy values of diets and freeze-dried excreta. Gross energy was measured using an adiabatic bomb calorimeter (Parr 1241EF, Parr Instrument Company, Moline, Illinois). AMEn values were determined as described by Bourdillon *et al.* (1990). Total starch was determined in diets and excreta according to Faisant *et al.* (1995).

II.3. Statistical Analysis

To test the effect of enzyme addition on broiler chickens, the analysis of variance was conducted with the general linear models procedure (GLM) of SAS (SAS Institute Inc., Cary, NC, USA). A randomized complete block design was used, a single cage representing the experimental unit (replicate) for FCR, daily weight gain (DWG), and digestive parameters. Performance data were analysed by three-way ANOVA, including the effect of the experiment (n=2), the block (n=4), enzyme addition (control diet *vs* enzyme diet; n=2), and any interactions, when appropriate. The cage was used as random effect. In order to test the nature of the xylanase products on bird performance, the effect of the microbial origin of xylanase (B, G, R, A; n=4) was substituted for the effect of enzyme addition. The initial weight (d 7) of the broiler chicks was used as covariate in the model. The Type III mean square for enzyme x block(experiment) was used as an error term to test the effect of enzyme addition. The Type III mean square for nature of xylanase x block(experiment) was used as an error term to test the effect of the microbial origin of the xylanase. A two-way ANOVA (diet × block) was applied for results of the balance trial. All the results were presented as means and SEM calculated by standard procedures. Means were compared using the least-significant-difference procedure and considered significant at $P < 0.05$.

III. Results

III.1. Nutritional Characteristics of Experimental Diets

Measured DM, ash, crude protein, crude fat, gross energy, and metabolizable energy values were slightly different from the theoretical chemical composition values presented in Table 2 but were similar for the five dietary treatments (data not shown).

III.2. Impact of Exogenous Enzyme on Broiler Performance

The effect of xylanase supplementation on growth performance in Exp. 1 and 2 are presented in Table 3. Supplementing xylanase to wheat-based diet significantly improved the body weight at 21 d of age and the DWG (g/bird) of broiler chickens by 3.7 % and 4.5 % respectively, in comparison with the control birds. Xylanase supplementation had no significant effect on feed intake and FCR.

Table 3. Effect of xylanase supplementation on growth performances of male broilers fed a diet containing 600 g kg^{-1} wheat

Item	Control diet	Enzyme diet[z]	RSD[y]	*P*-value		
				Enzyme	Exp.	Enzyme x Exp.
Body weight (g/bird)						
7 days	131.4	131.3	1.64	0.910	< 0.001	0.467
14 days	336.1	352.3	9.82	0.135	0.026	0.787
21 days	665.9	690.0	23.02	0.048	0.171	0.464
Daily weight gain (g/bird/d)						
7-14 days	29.3	31.6	1.40	0.134	0.024	0.774
14-21 days	47.1	48.1	2.87	0.573	0.253	0.568
7-21 days	38.2	39.9	1.64	0.049	0.169	0.472
Feed intake (g/bird/d)						
7-14 days	37.5	39.3	1.62	0.252	0.021	0.061
14-21 days	62.2	62.6	2.08	0.544	0.434	0.053
7-21 days	49.8	50.9	1.50	0.365	0.386	0.014
FCRx (g DM feed/g BWG)						
7-14 days	1.280	1.244	0.048	0.162	0.904	0.040
14-21 days	1.321	1.301	0.052	0.346	0.015	0.338
7-21 days	1.304	1.276	0.040	0.203	0.289	0.077

z Enzyme diet = control diet supplemented with 0.1 g kg-1 Belfeed B 1100 MP or 0.1 g kg-1 Grindazyme G or 0.1 g kg-1 Roxazyme G or 0.0125 g kg-1 xylanase from Aspergillus aculeatus.

y RSD = residual standard deviation.

x FCR = feed conversion ratio.

The enzyme × experiment interaction was significant for feed intake from 7 to 21 d and for FCR from 7 to 14 d. Feed intake of chickens fed the xylanase-supplemented diet was significantly improved in Exp. 1 but was not significantly different in Exp. 2, compared with the control chickens. FCR of chickens fed the xylanase-supplemented diet was significantly lower in Exp. 2, but showed no significant difference in Exp. 1, in comparison with the control birds.

The effect of the xylanase microbial origin on growth performance is reported in Table 4. Body weight, DWG, feed intake, and FCR from 14 to 21 d and from 7 to 21 d were not significantly affected by the fungal or the bacterial origin of the enzyme. The effect of enzyme origin was only significant for FCR from 7 to 14 d, and the most important difference was observed between the xylanase from *A. aculeatus* and the Grindazyme G (1.213 g DM feed/g DWG *vs* 1.28 g DM feed/g DWG).

Table 4. Effect of the microbial origin of xylanases on growth performances of male broilers fed a diet containing 600 g kg^{-1} wheat

Item	**Xylanase**[z]				**RSD**	***P*-value**		
	A	**B**	**G**	**R**		**Enzyme**	**Exp**	**Enzyme x Exp**
Body weight (g/bird)								
7 days	131.5	130.5	131.8	131.5	1.73	0.481	<0.001	0.761
14 days	354.9	353.5	348.7	351.9	9.88	0.496	0.044	0.418
21 days	688.3	683.3	687.4	701.0	21.39	0.484	0.066	0.105
Daily weight gain (g/bird/d)								
7-14 days	31.9	31.9	31.0	31.5	1.40	0.489	0.041	0.423
14-21 days	47.6	47.1	48.4	49.9	2.66	0.206	0.124	0.284
7-21 days	39.8	39.5	39.7	40.7	1.52	0.465	0.066	0.103
Feed intake (g/bird/d)								
7-14 days	38.6	39.6	39.8	39.1	1.61	0.509	0.015	0.295
14-21 days	62.5	61.5	62.9	63.6	2.06	0.168	0.713	0.884
7-21 days	50.5	50.5	51.3	51.4	1.56	0.634	0.103	0.476
FCRx (g DM feed/g DWG)								
7-14 days	1.213*a*	1.241*ab*	1.280*b*	1.241*ab*	0.032	0.005	0.559	0.006
14-21 days	1.319	1.315	1.302	1.283	0.048	0.550	0.011	0.086
7-21 days	1.276	1.284	1.293	1.267	0.035	0.489	0.418	0.036

[z] A, B, G and R : Control diet supplemented with 0.0125 g kg^{-1} xylanase from *Aspergillus aculeatus* or 0.1 g kg^{-1} Belfeed B 1100 MP (*Bacillus subtilis*), 0.1 g kg^{-1} Grindazyme G (*Aspergillus niger*) *or* 0.1 g kg^{-1} Roxazyme G (*Trichoderma viride*), respectively.
[y] RSD = residual standard deviation.
[x] FCR = feed conversion ratio.

The enzyme × experiment interaction was significant for FCR from 7 to 14 d and from 7 to 21 d. From 7 to 14 d, significant difference for FCR was only observed in Exp. 1. From 7 to 21 d, no statistical difference between FCR values was measured, but diet classification differed according to the experiment.

III.3. Nutrient digestibilities and AMEn of the experimental diets

Enzyme supplementation induced significant higher apparent N digestibility and crude fibre digestibility, with an average increase by 1.6 % and 58.9 % respectively, compared with the control diet (Table 5). Starch and crude fat digestibilities were not significantly improved with xylanase supplementation by 2.2 % and 1.8 % respectively. At the same time, AMEn values of the wheat-based diets were significantly increased by 2.6 %.

Table 5. Effect of xylanase supplementation on nutrients digestibilities and on AMEn values for male broilers fed a diet containing 600 g kg^{-1} wheat from 27 to 31 days of age

Nutrient	Digestibility		RSD[y]	*P*-value
	Control diet	Enzyme diet[z]		
Crude fibre	0.095	0.151	0.0229	0.047
Crude fat	0.836	0.851	0.0206	0.255
Starch	0.952	0.973	0.0174	0.061
Nitrogen	0.833	0.846	0.0082	0.016
AMEn (MJ/kg DM)	14.85	15.24	0.28	0.047

[z]Enzyme diet = control diet supplemented with 0.1 g kg^{-1} Belfeed B 1100 MP *or* 0.1 g kg^{-1} Grindazyme G *or* 0.1 g kg^{-1} Roxazyme G *or* 0.0125 g kg^{-1} xylanase from *Aspergillus aculeatus*.

[y] RSD =residual standard deviation.

The xylanases that were tested differently influenced nutrients digestibilities but differences were only significant for crude fat and starch (Table 6). AMEn also showed siginificant differences between the xylanases, but the differences did not depend on the fungal or the bacterial origin of the xylanase.

Table 6. Effect of the microbial origin of xylanases on the nutrients digestibilities and on AMEn values for male broilers fed a diet containing 600 g kg^{-1} wheat from 27 to 31 days of age

Digestibility	Xylanase[z]				RSD[y]	*P*-value
	A	B	G	R		
Crude fibre	0.150	0.149	0.150	0.156	0.0262	0.985
Crude fat	0.829*a*	0.866*b*	0.845*ab*	0.863*b*	0.0128	0.009
Starch	0.948*a*	0.985*b*	0.979*b*	0.980*b*	0.0051	<0.001
Nitrogen	0.840	0.849	0.850	0.846	0.0080	0.378
AMEN (MJ/kg DM)	14.93*a*	15.44*b*	15.16*ab*	15.42*b*	0.17	0.006

[z] A, B, G and R : Control diet supplemented with 0.0125 g kg^{-1} xylanase from *Aspergillus aculeatus* or 0.1 g kg^{-1} Belfeed B 1100 MP (*Bacillus subtilis*), 0.1 g kg^{-1} Grindazyme G (*Aspergillus niger*) *or* 0.1 g kg^{-1} Roxazyme G (*Trichoderma viride*), respectively.
[y] RSD =-residual standard deviation.
[a-c] Means within a row lacking a common superscript are significantly different ($P < 0.05$).

IV. Discussion

Over the last decade, many studies have reported that adding exogenous enzymes like xylanase to wheat-based diets may reduce the anti-nutritional effects of AX and consequently improve performance in poultry (Steenfeldt *et al.* 1998; Engberg *et al.* 2004; Meng *et al.* 2005; Yang *et al.* 2008). In this study, broiler chickens fed a wheat-based diet supplemented with exogenous endo-xylanases from 7 to 21 d of age showed significant increase of the final body weight and of the DWG during this period. The increase in growth performance is relatively lower than those reported in previous studies, which may be explained by the relatively lower wheat inclusion level in this study (600 g kg^{-1}) and/or by the AX level in the wheat variety used in the experiment, as stated by Adeola and Bedford (2004). The response of broilers to xylanase supplementation may also be influenced by the wheat hardness (Amerah *et al.*, 2009), and by the susceptibility of exogenous enzymes to inhibition by wheat constituents (Cowieson *et al.*, 2006), which especially depends on the concentration of enzyme inhibitors. These parameters may contribute to the great variability in poultry performance responses described in the literature. Finally, performance parameters are also likely to depend on the enzyme incorporation rate in the diet (Olukosi *et al.* 2007), and consequently on the enzymatic activity of the xylanase. In this study, enzyme activities were quite different according to the concentrations recommended by the manufacturer (Table 1). However, difference in activities did not induce any significant difference in performance improvement. Thus, others factors, like the molecular architecture of the enzyme or the contribution of side activities, could play a part in enzyme efficiency and should be further investigated.

Many studies demonstrated that the beneficial effects of exogenous enzymes on growth parameters are based on increased nutrients digestibilities and AMEn of the cereal-based diet (Gutierrez del Alamo *et al.* 2008; Selle *et al.* 2009). These observations are in agreement with the results of the digestion balance trial described in this study. Xylanases hydrolyse AX and suppress consequently their anti-nutritional effect, which impair the digestion of the diet and the absorption of nutrients. These enzymes reduce viscosity of the intestinal contents and release nutrients entrapped in the endosperm cell wall structure of wheat grain in the gut. The nutrients contained in plant cells are consequently more accessible for digestive enzymes, and this may induce a better diet utilisation by the poultry host and improved growth performance (Meng *et al.* 2005; Cowieson *et al.* 2006).

Especially, absorption improvement of energy-producing nutrients, i.e. fat, protein, and principally starch (Svihus and Gullord 2002; Meng *et al.* 2004), may render more energy for maintenance and growth. This results in increased AMEn of cereal-based diet (Gutierrez del Alamo 2008). Our results showed AMEn improvement similar to values reported by Gutierrez del Alamo (2008) who tested wheat-based diets with a lower inclusion level of wheat (650 g kg^{-1}). Improvement of the AMEn content of cereal-based broiler diet is a main economic advantage in ration formulation, allowing higher inclusion level of cereals like wheat while impeding anti-nutritional effects of AX.

The increase in crude fibre digestibility that was observed with xylanase supplementation could be the result of the degradation of the hemicellulose fraction of crude fibre by exogenous xylanase, as stated by Pettersson and Åman (1989). Nevertheless, the low crude fibre concentrations in our diets and the lack of specificity of the Weende method may lead to inaccurate digestibility values.

Moreover, this study aimed to investigate potential differences in mode of action of four xylanase preparations, according to their bacterial (B) or fungal origin (R, G and A). These enzymes were representative of the main genera of producer micro-organisms, i.e. *Trichoderma* and *Aspergillus* for fungi, and *Bacillus* for bacteria (Beg *et al.* 2001). The two selected *Aspergillus* xylanases had different activity levels and consequently different incorporation rate in broiler diet. They were also selected according to their conformational characteristics, allowing hypothetical different modes of hydrolysis along the AX chain. Bacterial xylanases are not subjected to post-translation modifications such as glycosylation (Polizeli *et al.*, 2005), which can hinder the enzymatic activity. Moreover, Beg *et al.* (2001) showed that the optimum pH of activity is lower for fungal than for bacterial xylanase, with pH range of 2-6 and 5.5-7 respectively. These characteristics could lead to different intestinal site of action and different efficiency between fungal and bacterial xylanases. Most previous studies that investigated xylalase effect on chickens tested fungal enzymes. Few studies compared the enzymatic efficiency according to the nature of the producer microorganism. Choct *et al.* (2004) showed that enzyme preparations from three fungal species (*Thermomyces lanuginosus, Humicola insolens* and *Aspergillus aculeatus*) similarly improved growth performance of broiler chickens fed a low- or a normal-metabolizable energy diet, as showed for R, G and A diets in this study. However, these enzymes showed significant differences in insoluble and soluble NSP digestibilities in jejunal and ileal digesta. Cowieson *et al.* (2005) presented similar observation with a thermostable arabinoxylanase from *Trichoderma reesei*. In this study, no significant difference was reported between the performance parameters observed for the four enzyme products, even if crude fat and starch digestibilities, which were related to the AMEn content of the diet, were significantly different according to the enzyme. These differences were not dependent on the microbial origin of the xylanase, which does not seem consequently to be an important parameter for enzymatic efficiency. In contrast, 0.0125 g kg^{-1} xylanase A showed digestibility coefficients significantly lower, in comparison with the other enzymes which were incorporated at 0.1 g kg^{-1} (Table 6). Significant differences in digestibility improvements could then be related to incorporation level of xylanase in wheat-based diet, as stated by Pourreza *et al.* (2007). These authors observed that higher levels of enzyme may have significant effect on nutrient digestibilities of broiler chicks fed triticale-based diets, without affecting growth performance. Finally, we must remember the complex and often uncontrolled interactions between the nutrient digestibilities and the resident microflora in the large intestine.

The beneficial effects of xylanase preparations to reduce the detrimental effect of wheat AX were previously demonstrated in many studies. This work aimed to determinate if these xylanase effects could be influenced by the microbial origin of the enzyme. Although the tested exogenous enzymes were produced from fungi or bacteria and presented different optimum pH, the present study failed to highlight any difference between them. Xylanase supplementation of a wheat-based diet was shown to similarly improve the growth performance of broiler chicken, as well as the crude fibre and nitrogen digestibilities, and the AMEn. Significant differences for crude fat and starch digestibilites, related to the AMEn, were observed between the xylanase preparations, but these differences were not related to the microbial origin. Therefore, xylanase selection for practical applications should be firstly based on financial and efficiency considerations. The microbial origin does not seem to be an important criterion. Moreover, the mode of action of xylanases according to the microbial origin and to the optimal pH of activity is not fully understood and needs to be further investigated. The average time of digestive transit is relatively short in poultry compared to monogastric animals. Therefore, degradation of NSP in the gut should be more complete with enzymes that are effective in all the intestinal segments. Selecting mixtures of xylanases with complementary enzymatic activities, which

could be dependent on the optimal pH and on the microbial origin, may be beneficial to improve diet utilization.

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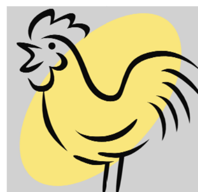
Bacteria – Selection of polysaccharidases and bacterial strains with antagonistic activities

For developing an antagonistic treatment able to reduce *Campylobacter* prevalence in the environment of free-range broiler flocks, the first step was to select a bacterial strain with antibacterial potential against the pathogen. Antagonistic *in vitro* screening is traditionally performed with disc diffusion assays. However, co-cultures experiments can supply more precise responses about the antibacterial activities. In such assay, the antagonistic strain is directly brought together with *Campylobacter* in liquid culture flask, with various concentrations. The culture conditions are defined for favouring the pathogen growth. Production of antibacterial metabolites by antagonistic strains can then be measured in culture broth. As the antagonistic strain has to be able to grow in the chicken environment, the culture medium should moreover mimic environmental conditions, so that a broth based on straw and dehydrated poultry excreta was designed.

LAB strains produce organic acids from carbohydrate fermentation, leading to pH reduction. Selection of a polysaccharidase that is able to act synergistically with the bacterial strain ought to focus on its optimum pH of activity. We decided to supplement the co-culture medium with an enzymatic system presenting optimal pH range from 4.5 to 5. It contains three glycohydrolase activities: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Several enzymatic activities represent an advantage for degrading the complex coculture medium, in order to supply sufficient nutrient amount for bacterial growth.



In vitro antagonistic activity evaluation of Lactic Acid Bacteria (LAB) combined with cellulase enzyme on *Campylobacter jejuni* growth in co-culture

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Abbreviations:

Abstract

The antibacterial effects of nine Lactic Acid Bacteria (LAB) against *Campylobacter jejuni* were investigated by using agar gel diffusion and co-culture assay. Some differences were recorded between the inhibition effects measured with these two methods. Only two LAB, *Lb. pentosus* CWBI B78 and *E. faecium* THT, showed anti-*Campylobacter* activity in co-culture assay with Dehydrated Poultry Excreta mixed with Ground Straw (DPE/GS) as the only substrate source. It was observed that supplementation of such medium with a Cellulase A enzymatic complex (Beldem S.A.) enhanced antimicrobial effect of both LAB strains. The co-culture medium acidification and the *C. jejuni growth* inhibition were positively correlated with the Cellulase A concentration. The antibacterial effect was characterized by the lactic acid production by the homofermentative *E. faecium* THT and by the lactic and acetic acids production by the heterofermentative *Lb. pentosus* CWBI B78. The antagonistic properties of LAB strains and Cellulase A combination could be used in strategies aiming at the reduction of *Campylobacter* prevalence in the poultry production chain, and at the subsequent decrease of the human infection risk.

I. Introduction

During the last decade the alimentary toxi-infection due to *Campylobacter* has considerably increased worldwide (Shane, 2000). At the same time "organic" and "traditional" products have gained popularity among consumers (Winter and Davis, 2006), and the increased production of such products will not solve food safety problems. The principal source and reservoir of *Campylobacter* pathogen is actually poultry and derived products (Wilson *et al.*, 2008), especially when chickens have access to the open-air range (Esteban *et al.*, 2008). A Danish study showed that 100 % of 22 organic broiler flock samples were positive for *Campylobacter* spp. compared with 36.7 % of 79 conventional broiler flock samples (Heuer *et al.*, 2001). A recent epidemiological study carried out in the southern part of Belgium for one year demonstrated that 100 % of sampled free-range broiler flocks were positive for *Campylobacter* during the warm season (Vandeplas *et al.*, 2010). Campylobacters are ubiquitous enteric bacteria in the environment and in domestic animals. So, they are widely disseminated through the farm environment, and they can be concentrated in raw chicken meat. Due to its susceptibility to multiple environmental stresses (e.g., temperature, relative humidity and air composition), it is known that *Campylobacter* does not grow outside its host, but is able to survive in litter (Line, 2006), in sewage (Abulreesh *et al.*, 2006), for a long period in poultry biofilms (Hanning *et al.*, 2008) and for more than two weeks in feces (Sinton *et al.*, 2007). The reduction of *Campylobacter* contamination at different levels of the chicken production chain, especially at the primary production level, should decrease the risk of human infection. The control of *Campylobacter* prevalence in broilers is possible but still remains a real challenge. Many different strategies have been developed with more or less success and have been recently reviewed by Lin (2009) and Vandeplas *et al.* (2008). At present, usual methods that have been implemented to prevent *Campylobacter* colonization and that have been admitted by sanitary authorities are based on strict biosecurity (FAO/WHO, 2004). However, hygiene procedures are limited by uncontrollable environmental factors in organics flocks (eg., open-air range, wild birds, rodents, farm animals, etc.) and by the difficulty to strictly respect biosecurity rules in conventional flocks throughout the rearing stages. In addition, many complementary methods have been developed during the last decade. Acidification of the flock environment (litter, drink water and feed) was based on acid susceptibility of *Campylobacter* (Van Deun *et al.*, 2008). Other investigated strategies include vaccine, phage therapy or the antibiotic use as growth promoters. However, the latter are completely prohibited by the EU authorities since 2006 according to Regulation (EC) n°1831/2003. However, biological controls, especially microbial competition, are the most investigated approaches (Mead, 2002; Zhang *et al.*, 2007; Vandeplas *et al.*, 2008). The most usually studied bacteria belong to the Lactic Acid Bacteria (LAB) group, which have beneficial effects on health and an important role in preserving foods and preventing food poisoning (Ljungh and Wadstrom, 2006). These bacteria are characterized by their potential to prevent the adherence, the establishment, the replication, and/or the virulence of specific enteropathogens (Reuter, 2001). Different antagonistic mechanisms have been proposed: pH decrease via production of organic acids, nutrient competition with pathogens, and/or production of specific inhibitory compounds like bacteriocins (Salminen *et al.*, 2004). So, the ubiquitous LAB use in chicken environment (chicken house, litter, pasture, etc.,) could reduce pathogen populations and their horizontal transmission in farm, which could subsequently promote animal health.

The global aim of this research was therefore to establish the effect of some LAB combined with glycanase enzyme on growth of the enteropathogen *Campylobacter jejuni*. The LAB

survival in the chicken excreta and their antagonistic potential could be enhanced by glycanase enzyme, as stated by Vandeplas *et al.* (2009). These authors showed that a combination between an enzyme of xylanase type and a *Lactobacillus* strain used as feed additive was able to reduce *Salmonella* Typhimurium concentration in caeca of challenged chickens. Enzymes can hydrolyze non-digestible polysaccharides into oligo- and monomers which can be specifically metabolized by LAB. In this study, combinations of LAB strains and a cellulase enzyme were tested in order to further develop anti-*Campylobacter* treatments to be spread on poultry litter and/or open-air range. Thus, the antagonistic activity of different LAB has been tested in agar gel diffusion and co-culture batch techniques. Subsequently, the Cellulase A enzymatic complex was combined with two anti-*Campylobacter* strains, *Lactobacillus pentosus* CWBI B78 and *Enterococcus faecium* THT, in order to assess its ability to improve the antimicrobial activity s of both LAB in co-culture. The molecular mechanism that is involved in the observed antagonistic effect was investigated by monitoring pH changes and organic acids levels recorded throughout the time of co-culture.

II. Materials and methods

II.1. Bacterial strains

Nine lactic acid bacteria (LAB), which were isolated from environmental samples of chicken farms, were tested in this study: *Pediococcus pentosaceus* (CWBI B73 and CWBI B605), *Enterococcus faecium* (THT and CWBI B411), *Paenibacillus* ssp. (CWBI B1070 and CWBI B60), *Weissella confusa* CWBI B902, *Lactobacillus pentosus* CWBI B78, and *Lactobacillus plantarum* CWBI B76. All these strains were phenotypically and genetically characterized. The LAB cultures were preserved in MRS agar (Biokar Diagnostic, Beauvais, France) and in cryopreservation tubes at – 80°C. Two *C. jejuni* strains (LMG 6446 and CWBI B1444) and two *C. coli* strains (LMG 6440 and CWBI B1445) were used as indicator organisms. These strains were kept without antibiotics in Brucella agar, and conserved at – 80°C in broth medium containing 15 % (w/v) glycerol (Merck, Darmstadt, Germany).

II.2. Lactic acid bacteria and C. jejuni culture preparation

All LAB were subcultured two times in 10 mL of MRS broth, overnight at 30°C before used. The cells were harvested at the end of the log phase and prepared for transfer into anaerobic culture flask by centrifuging at 14 000 × *g* for 5 min. The supernatant was removed and cells were washed twice in anaerobic phosphate buffer (1 M, pH 7.4). Cells were re-suspended to a final OD_{560} of approximately 0.1 in Brucella broth, and 0.5 mL of this culture was added to an anaerobic culture flask. *Campylobacter* inoculum was prepared by streaking one loop of stock solution on Campy-Cefex agar, without antibiotics. The plates were kept at 42°C in hermetic jars for 48 h, under microaerophilic conditions (5 % O_2, 10 % CO_2, 85 % N_2) generated with a Anaerocult C Gas-Pak envelope, (Merck, Darmstadt, Germany). Subsequently, the inoculum was prepared by transferring typical *Campylobacter* colonies into Brucella broth kept under microaerophilic atmosphere for 48 h.

II.3. Assessment of inhibitory activity

II.3.1. Agar diffusion test

The antimicrobial activity of LAB was screened with the 'colony-overlay' method (Schillinger and Lucke, 1989). The method was modified as follows: LAB were cultured overnight in MRS and 5 µL were taken for spotting on fresh MRS plate, which were buffered with 0.2 M sodium phosphate (pH 7). Plates were incubated for 16 h at 30°C and then overlaid with 5 mL of Campy-Cefex soft agar (0.5 % agar) which was supplemented with 8 mM of triphenyltetrazolium chloride (Sigma, St. Louis, Mo., USA) and seeded with 0.5 mL of 48 h microaerobic *Campylobacter* culture. Overlaid plates were incubated for 24 h at 42°C under microaerobic conditions. Positive inhibition was recorded as a clear zone of 10 mm or greater that spread laterally in a circle from the LAB spot.

II.3.2. Co-culture experiments

The antagonistic activity of LAB on *Campylobacter* growth was tested into a culture medium that simulated the composition of the chicken environment. Dehydrated Poultry Excreta (DPE) was prepared by hot drying at 60°C for 48 h and finely ground in a cyclone mill with a 2-mm mesh screen (Cyclotec Sample Mill 1093, Höganäs, Sweden), and was used as nitrogen source. Ground Straw (GS), which was ground in a grinder Cyclotec 1093, was used as a carbon source into the co-culture medium.

The co-cultures experiments were performed by inoculating both LAB strain and *C. jejuni* LMG 6446 in 250 mL anaerobic culture flask filled with 150 mL of DPE/GS medium, which contained 1 % (w/v) DPE, 1 % (w/v) GS, 500 µL Tween 80, and 2 g L^{-1} peptone. All ingredients were mixed thoroughly and autoclaved for 20 min at 121°C before inoculation of the bacterial strains. The medium was inoculated with 1 mL of a 48 h *C. jejuni* culture (~ 10^8 – 10^9 CFU mL^{-1}) and 0.5 mL of an overnight LAB culture diluted 100-fold (~10^4 – 10^5 CFU mL^{-1}). Microaerobiosis conditions were obtained by flushing a gas mixture composed of 10 % CO_2, 5 % O_2, and 85 % N_2, through the medium in anaerobic culture flask for 20 min. The co-culture flasks were placed continually on an orbital shaker (Gallenkamp Orbital Incubator, Sanyo, Pocklington, UK) set at 80 rpm at 37°C. To avoid disturbing the flask microaerobic atmosphere, the co-culture medium was sampled every 24h.

Numeration of *C. jejuni* and the LAB strain were performed on co-culture samples. One mL was serially diluted 10-fold in bacterial diluent (8.5 g L^{-1} NaCl, 1.0 g L^{-1} peptone, and 1mL tween 80 [pH 7.0]) and appropriate dilutions were plated. Direct plating onto Campy-Cefex agar supplemented with antibiotics (33 mg L^{-1} cefeperazone and 0.2 g L^{-1} cycloheximide) was used for *Campylobacter* enumeration. Plates were incubated in jars at 42°C for 48 h under microaerobic conditions using an Anaerocult C Gas-Pak envelope (Merck, Darmstadt, Germany). Enrichment of the first dilution was realized to confirm the absence of *Campylobacter* in samples that were detected negative with direct plating.

LAB strains were quantified by plating onto MRS agar supplemented with 0.2 % calcium carbonate, and incubating the plates overnight at 37°C. Each sample dilution was plated in triplicate for *Campylobacter* and LAB.

II.3.3. Co-culture: enzyme effect

The enzymatic complex called Cellulase A (Beldem s.a., Andenne, Belgium) was used to study the enzyme effect on the antagonistic activity of LAB. This enzymatic system from *Trichoderma reesei* contains three glycohydrolase activities: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) and was chosen for its ability to hydrolyze straw/litter cellulose in easily metabolizable compounds (Kumar *et al.*, 2008). Cellulase A was diluted in PBS buffer at pH 6 and sterilized using Pall Acrodisc® filters, 0.2 µm (Pall Life Sciences, St Germain en Laye, France) before being added into the co-culture medium adjusted to pH 6.0, in concentrations ranging from 0 to 500 ppm. After C. *jejuni* and LAB co-inoculation as described above, the co-culture flasks were incubated at 37°C for 72 h under microaerobiosis. *Campylobacter* and LAB were quantified every 24 h as described above.

II.4. Determination of co-culture pH and lactate, acetate, glucose concentrations

Ten mL of co-culture sample were adjusted to pH 10 with NaOH 2 M, treated with 0.2 g of polyvinylpolypyrrolidone (Sigma, St. Louis, MO, USA), shook vigorously for 5 min., and subsequently filtered on Whatman n°1 filter (VWR International, West Chester, PA, USA). The filtrate was diluted two times before lactate and acetate concentrations were quantified by high-performance liquid chromatography (HPLC). The HPLC module consisted of a Waters 2690 Alliance System (Waters, Milford, MA, USA) fitted out with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA), a Waters 717 automatic injector (Waters, Milford, MA, USA), a Waters 600 MS pump and system controller (Waters, Milford, MA, USA), and a column oven at 40 °C. The mobile phase was 0.003 N H_2SO_4 at 5 % (v/v) CH_3CN with a flow rate of 0.6 ml min^{-1}. The internal standard was isocaproic acid (Sigma, St. Louis, MO, USA), and the compounds were detected by a Waters 486 UV-visible detector (Waters, Milford, MA, USA) at 210 nm. The final glucose content in the co-culture supernatant was measured using the enzymatic glucose assay kit (Megazyme K-GLUC, Wicklow, Ireland). The co-culture pH was determined directly on samples with a 3151 pH meter (WTW GMBH, Weilheim, Germany).

II.5. Chemical analysis

The total neutral sugar content in the co-culture medium was quantified with the phenol – sulphuric acid method as described by Dubois *et al.* (1951), whereas the reducing sugar content was determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Ammonia nitrogen and total Kjeldahl nitrogen concentrations in the DPE/GS medium were evaluated with the international standard method (ISO1871:1994) in comparison with Brucella broth supplemented or not with 250 ppm of Cellulase A.

II.6. Statistical analyses

Statistical analysis was conducted with the general linear models procedure (GLM) of SAS (SAS Institute Inc., Cary, NC). The time course of LAB and *C. jejuni* in co-culture was analysed by two-way ANOVA, including the effect of the LAB strain (n = 4), the sample time (n = 5), and any interactions when appropriate. A one-way ANOVA was applied for analysing the effect of enzyme concentration on LAB antagonist activity (enzyme concentration as fixed

factor, n = 5), and metabolic activities of LAB in co-culture (sample time as fixed factor, n = 4). Means were compared using the Newman-Keuls test (Dagnelie, 1996) and considered significant at $P < 0.05$. Correlations between glucose and organic acid concentrations in co-cultures were determined using the CORR procedure of SAS, with a parametric Pearson's test.

III. Results

Estimation of the antimicrobial activity of LAB against *C. jejuni*/*coli,* determined with the agar spotting method, is presented in Table 1.

Table 1. Antimicrobial activities of lactic acid bacteria selected against *Campylobacter* indicator strains[a]

Indicator strains	*C. jejuni*		*C. coli*	
Tested strains	**LMG 6446**	**CWBI B1444**	**LMG 6440**	**CWBI B1445**
E. faecium THT	+ +	+ +	+ +	+ +
E. faecium CWBI B411	-	+	+ +	+ + +
Lb. plantarum CWBI B76	-	-	+ +	+ +
Lb. pentosus CWBI B78	+ +	+	+ + +	+ + + +
P. pentosaceus CWBI B73	+	+	+ + +	+ +
P. pentosaceus CWBI B605	-	+	+ +	+
W. confusa CWBI B902	-	-	+ + +	+ +
Paenibacillus ssp. CWBI B1070	-	-	+	+
Paenibacillus ssp. CWBI B60	-	-	+	-

[a] The different scores try to reflect different degrees of growth inhibition; −, no inhibition; +, 10–12 mm inhibition zone; ++, 13–15 mm inhibition zone; +++, 16–18 mm inhibition zone; ++++, 19–20 mm inhibition zone. All indicator strains were assayed at least twice.

All the tested LAB were active against *C. coli*, even if weak activities were recorded for the two *Paenibacillus* strains (CWBI B1070 and CWBI B60). Five isolates produced antimicrobial substances that were active against at least one of the *C. jejuni* strains used as indicators, and only three isolates (*P. pentosaceus* CWBI B73, *E. faecium* THT and *Lb. pentosus* CWBI B78) were antagonistic against both *C. jejuni* LMG 6446 and CWBI B1444. No clear inhibition zone was observed with *Paenibacillus* ssp. CWBI B1070, *Paenibacillus* ssp. CWBI B60 and *Lb. plantarum* CWBI B76 with both *C. jejuni* strains used as indicators.

P. pentosaceus CWBI B73, *E. faecium* THT, and *Lb. pentosus* CWBI B78, were selected for their antimicrobial activity against *C. coli* as well as against *C. jejuni* and were tested in co-culture with *C. jejuni* LMG 6446. *Paenibacillus* ssp. CWBI B1070 was used as negative reference strain which do not inhibit the *Campylobacter* growth. In comparison with Brucella broth, the analysis of the DPE/GS medium, reported in Table 2, showed a poor availability of 0.364 g L^{-1} reducing sugars in comparison with 8.847 g L^{-1} in Brucella broth. The total sugar content was also 2.2 times less important in DPE/GS medium than in Brucella broth. The sugar composition difference between both culture media led to a decrease of the bacterial growth by

approximately 1.5 to 2.0 Log_{10} CFU mL^{-1} in DPE/GS medium (data not reported). On the other hand, no significant differences were reported in total Kjeldahl nitrogen and ammonia nitrogen between DPE/GS medium and Brucella broth. While the DPE/GS medium supplemented with 250 ppm Cellulase A increased the reducing sugar and total sugar availabilities, no significant effect was recorded on the total Kjeldahl nitrogen and ammonia nitrogen content. Otherwise, it was observed that *C. jejuni* enumeration after 48h incubation at 37°C was not different in DPE/GS medium supplied or not with Cellulase A. *Campylobacter* concentration reached 7.8 and 7.11 Log_{10} CFU mL^{-1} in DPE/GS medium, supplied or not with enzyme, and Brucella broth, respectively.

Table 2. Effect of sugar and nitrogen sources combined or not with Cellulase A on *C. jejuni* growth after 48h incubation

	Reducing Sugar	**Total Sugar**	**Total Kjeldahl Nitrogen**	**Ammonia Nitrogen**	***C. jejuni* growth after 48h**
	Eq. [Glc] g L^{-1}	Eq. [Glc] g L^{-1}	%	%	Log_{10} CFU mL^{-1}
Brucella Broth	8.84 ± 0.47	9.41 ± 0.84	7.73 ± 0.26	0.64 ± 0.05	7.11 ± 0.48
DPE/GS	0.36 ± 0.02	4.25 ± 0.02	7.95 ± 0.11	0.68 ± 0.01	7.81 ± 0.30
DPE/GS + enz.	0.67 ± 0.02	5.15 ± 0.53	8.00 ± 0.15	0.71 ± 0.01	7.82 ± 0.40

DPE/GS: Dehydrated Poultry Excreta (1 %, w/v) mixed with Ground Straw (1 %, w/v)

enz.: Cellulase A (250 ppm)

All the LAB strains were in stationary phase after 24 h in co-culture (Fig. 1A). With initial inocula of approximately 10^3-10^4 CFU mL^{-1}, the concentrations of *Lb. pentosus* CWBI B78, *P. pentosaceus* CWBI B73, and *E. faecium* THT, were maintained approximately at 10^8-10^9 CFU mL^{-1} from 24h of fermentation until the end of experiment, while *Paenibacillus* ssp. CWBI B1070 only reached 10^6-10^7 CFU mL-1. During the first 24 h, pH reduction can be correlated with the log-phase of LAB strains (Fig. 1B). The highest pH reduction was observed in *E. faecium* THT and *Lb. pentosus* CWBI B78 co-cultures, reaching 5.8 at 24h and 6.0 at 48h, respectively. Changes in pH throughout the fermentation did not affect the LAB growth in co-culture. The pH reduction during the first 24h did not induce any decrease of *Campylobacter* concentration during this period (Fig. 1C). It is only after 24h of fermentation that a slow but steady decline of *Campylobacter* population was observed in *E. faecium* THT and *Lb. pentosus* CWBI B78 co-cultures, reaching 4.6 Log_{10} and 2.4 Log_{10} at 96 h, respectively. *C. jejuni* inhibition could not be correlated with the pH decrease in *E. faecium* THT and *Lb. pentosus* CWBI B78 co-cultures. The pH reduction was observed during the first 24 hours of fermentation, while the pathogen concentration only began to decrease from 24 h.

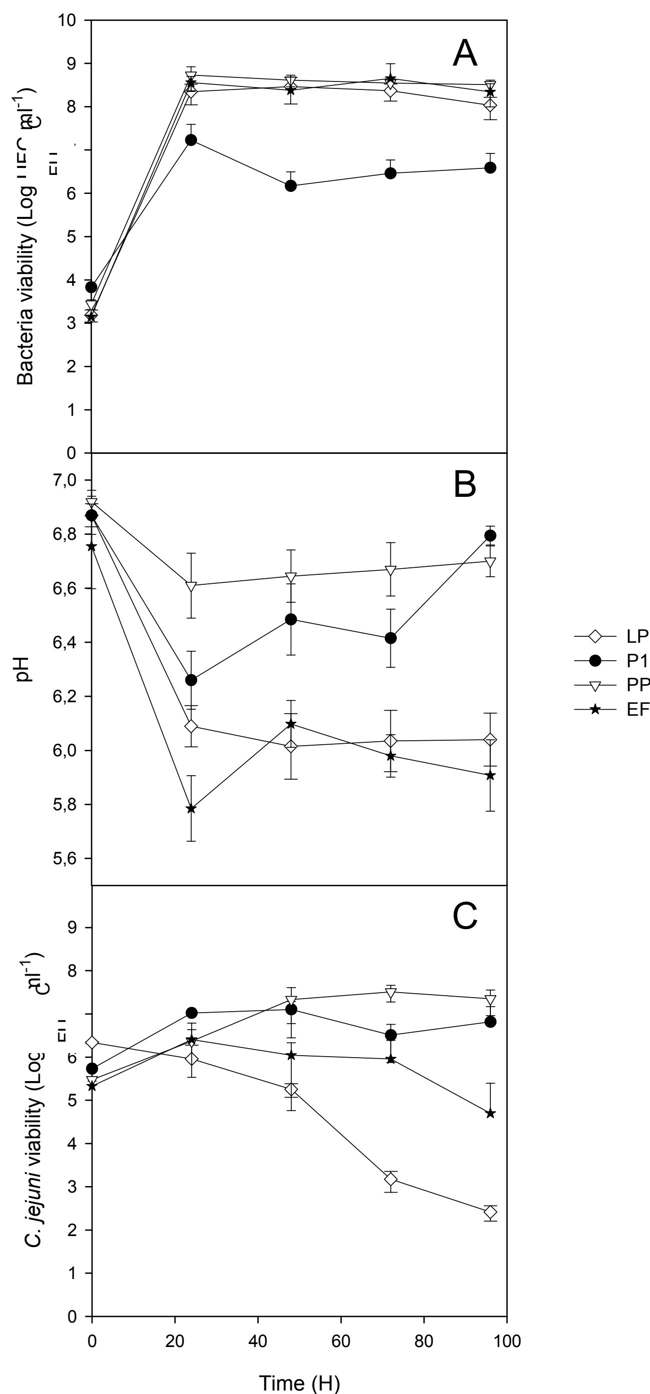

Figure 1. Time course of lactic acid bacteria and *C. jejuni* in co-cultures, representing the viability of LAB (A), the viability of *C. jejuni* LMG 6446 (C) and pH evolution (B). Co-cultures were realized in DPE/GS broth medium under microaerophilic condition at 37°C. Each value is the mean of at least 4 values from two sets of experiments ± SD

Cellulase A was added to the co-cultures in order to evaluate the possible impact of enzyme treatment on the antimicrobial activity of LAB. The effect of Cellulase A concentration on the inhibition potential was tested with *Lb. pentosus* CWBI B78 (Fig. 2A) and *E. faecium* THT (Fig. 2B) in DPE/GS medium. After 72 h of fermentation, enzyme supplementation did not affect LAB growths. However, enzyme amplified the acidification effect of both strains in DPE/GS medium, which was positively correlated to the enzyme concentration. The pH variation between the DPE/GS medium supplemented or not with 500 ppm of enzyme, was statistically significant ($P < 0.0001$) and reached 1.1 and 0.9 of pH unit, into the *Lb. pentosus* CWBI B78 and the *E. faecium* THT co-cultures, respectively. The pH decrease was correlated ($P < 0.0001$) to the reduction of the *Campylobacter* population into both fermentations. *C. jejuni* was reduced to an undetectable level after 72 h in *Lb. pentosus* CWBI B78 co-culture medium supplemented with 500 ppm of Cellulase A. Under the same conditions with *E. faecium* THT (Fig. 2B), a pathogen concentration of about 10^2 CFU mL^{-1} was still measured 72 h after inoculation. The enzymatic complex had not bactericidal activity against LAB and *C jejuni* by itself (data not shown).

Organic acids and glucose concentrations were measured throughout fermentation into *Lb. pentosus* CWBI B78 (Fig. 3) and *E. faecium* THT (Fig. 4) co-cultures supplemented with 250 ppm of Cellulase A. Initial inocula were of 2.4 and 3.1 Log_{10} CFU mL^{-1} for *Lb. pentosus* CWBI B78 and *E. faecium* THT respectively (Fig. 3A & 4A). After 24 h, the stationary phase was reached for both LAB strains, with stable concentrations of about $5\ 10^8$ CFU mL^{-1} until the end of the experiment. From 0 to 24 h, *Campylobacter* concentration in both co-cultures remained stable at ~ 5.8 Log_{10} CFU mL^{-1}. When the antagonistic strains reached the stationary phase at about 24 h, with a mean pH value of ~5.2, *C. jejuni* concentrations decreased in both co-cultures (Fig. 3A & 4A). In the *Lb. pentosus* CWBI B78 co-culture (Fig. 3A), the pH was lowered slightly from ~ 5.2 to ~ 4.7 after 24 h, with a pronounced decrease of *Campylobacter* numbers at the same time, reaching about 2 Log_{10} CFU mL^{-1} at 96 h. In the *E. faecium* THT co-culture (Fig. 4A), the pH value was stabilized at pH ~5.2 after 24 h, while the *C. jejuni* population began to linearly decrease to reach ~ 2.2 Log_{10} CFU mL^{-1} at 96 h.

Glucose in both co-cultures was consumed in less than 24 h (Fig. 3B & 4B). At the same time, lactate concentration increased proportionally ($P < 0.009$) to glucose reduction, reaching concentrations of 33 and 21 mg L^{-1}, in the *Lb. pentosus* CWBI B78 and *E. faecium* THT, co-cultures respectively. From 24 h fermentation, the lactate content continued to increase with *E. faecium* THT to a maximum of 36 mg L^{-1} at 96 h (Fig. 4B). On the other hand, the lactate concentration in the *Lb. pentosus* CWBI B78 culture was decreased from 24 h to 96 h, reaching a final value of 19 mg L^{-1} (Fig. 3B). The lactate concentration was negatively correlated to the acetate content in the co-culture medium. Acetic acid concentration steadily increased to reach 12 mg L^{-1} after 96 h. In the *E. faecium* THT co-culture, only low acetic acid level which did not excess 5 mg L^{-1} was recorded (Fig. 4B).

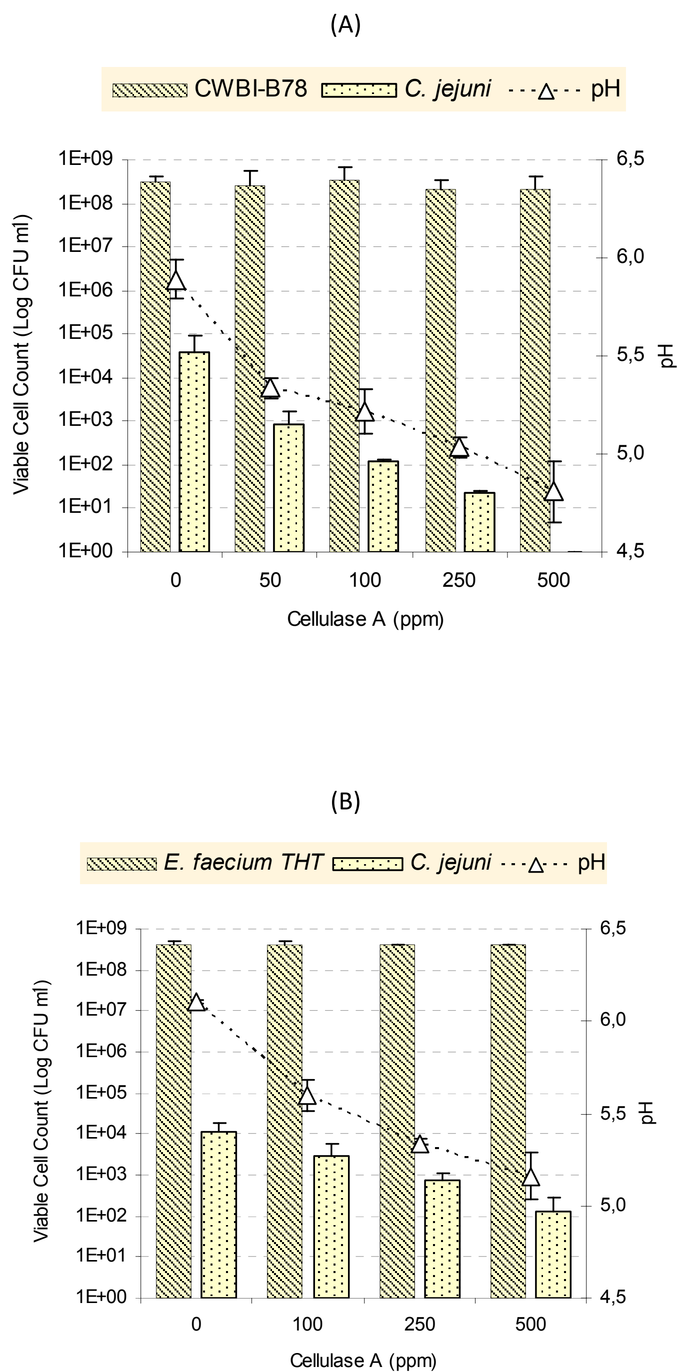

Figure 2. Effect of Cellulase A concentration on pH values and on the antagonistic activity of *Lb. pentosus* CWBI B78 (A) and *E. faecium* THT (B) with *C. jejuni* LMG 6446 in co-culture. Bacteria enumeration and pH in co-culture were determined at 72 h incubation under microaerophilic condition at 37°C. The values are expressed as the mean of at least 4 values from two sets of experiments ± SD

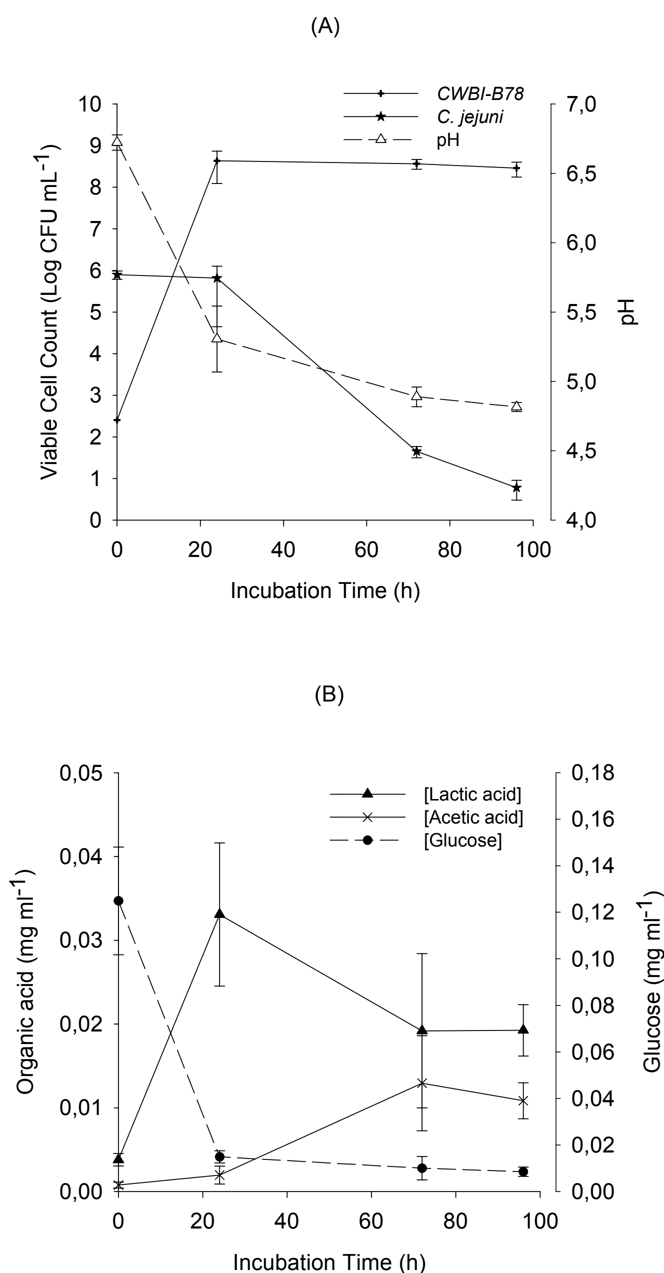

Figure 3. Metabolic activity of *Lb. pentosus* CWBI B78 in co-culture. (A) pH evolution of medium, and growth curves of *Lb. pentosus* CWBI B78 and *C. jejuni* LMG 6446. (B) Evolution of glucose, lactic acid and acetic acid concentrations. The co-culture was realized in DPE/GS medium supplemented with 250 ppm of Cellulase A under microaerophilic condition at 37°C. Each value is the mean of at least 4 values from two sets of experiments ± SD

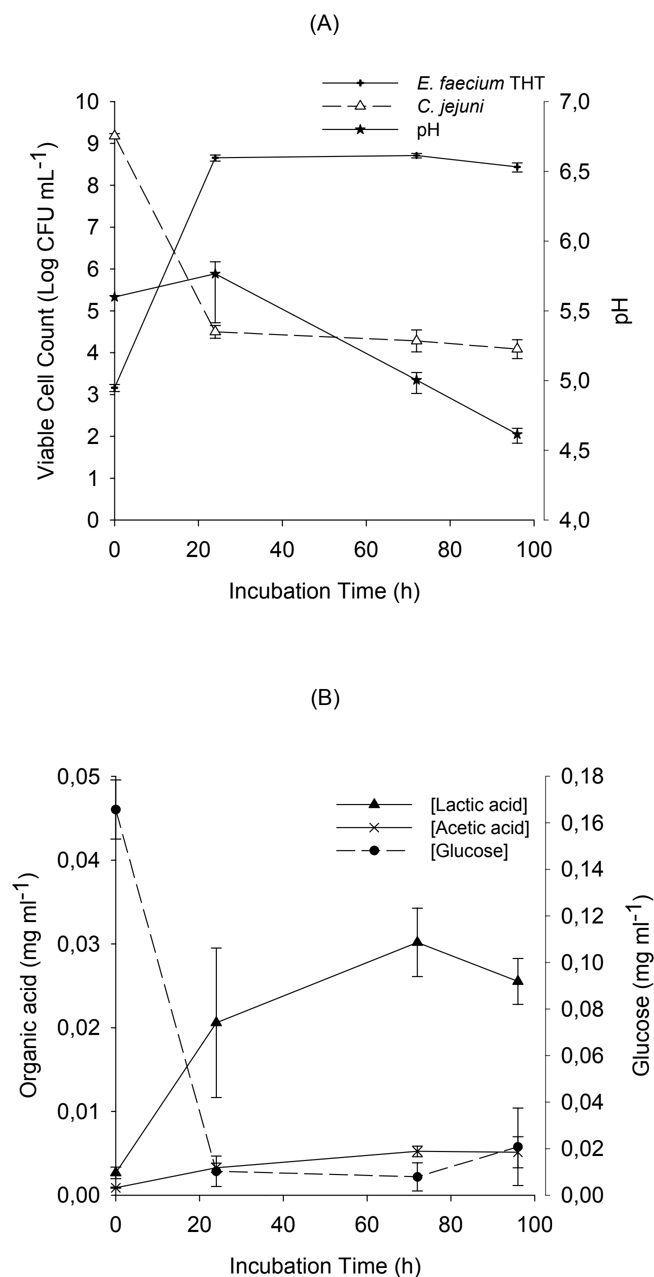

Figure 4. Metabolic activity of *E. faecium* CWBI B78 in co-culture. (A) pH evolution of medium, and growth curves of *Lb. pentosus* CWBI B78 and *C. jejuni* LMG 6446. (B) Evolution of glucose, lactic acid and acetic acid concentrations. The co-culture was realized in DPE/GS medium supplemented with 250 ppm of Cellulase A under microaerophilic condition at 37°C. Each value is the mean of at least 4 values from two sets of experiments ± SD

IV. Discussion

In the first part of this study, results of agar diffusion tests, which were used for estimating the antimicrobial activity of microorganisms from the poultry environment, confirmed that some LAB have an antagonistic effect against *Campylobacter*. Most of the studies carried out with LAB strains belonging to the *Enterococcus* and *Lactobacillus* genera that were shown to inhibit *C. jejuni*/*coli*, were performed *in vitro* using the agar gel diffusion test based on the Mayr-Harting method. This method uses culture supernatant extracted from LAB fermentation performed in optimal growth conditions (Mayr-Harting *et al.*, 1972). In this study, the agar gel diffusion method revealed that culture supernatant from *P. pentosaceus* CWBI B73, *Lb. pentosus* CWBI B78 and *E. faecium* THT can inhibit *C. jejuni* as well as *C. coli*. These bacterial species are actually known to produce antimicrobial substances such as organic acids, fatty acids, ethanol, hydrogen peroxide, and bacteriocins (Ouwehand and Vesterlund, 2004), and were shown to inhibit Gram-positive bacteria (Gilliland and Speck, 1977). Moreover, the inhibition ability of some LAB has also been demonstrated on Gram-negative bacteria, especially on *Campylobacter* growth (Chaveerach *et al.*, 2004; Nazef *et al.*, 2008; Musikasang *et al.*, 2009). In our experimental conditions, *Paenibacillus* ssp. CWBI B1070 did not exert any anti-*Campylobacter* activity, contrary to the results of Svetoch *et al.* (2005), who reported antagonistic activities against *Campylobacter* of one *Bacillus* and three *Paenibacillus* strains isolated from poultry production environment.

A co-culture method with a mixed DPE/GS medium was developed in this study and allowed us to directly observe the anti-*Campylobacter* effect of *Lb. pentosus* CWBI B78 and *E. faceium* THT in liquid medium. In comparison with the synthetic Brucella broth, the DPE/GS medium showed significantly different reducing sugars contents whereas nitrogen contents were statistically similar. Nevertheless, it appears to be a culture medium as efficient as Brucella broth for co-cultures with *Campylobacter*. Gilpin *et al.* (2009) has already reported that animal excreta can support *Campylobacter* growth. The co-culture technique allows to further appreciate the impact of LAB strains on the medium composition, on pH values, and on *Campylobacter* growth for the whole length of the experiment. The results of *P. pentosaceus* CWBI B73 co-culture showed a weak pH decrease throughout the culture, which was related with the absence of *Campylobacter* reduction, contrary to the results from the agar diffusion method. This strain did not seem to be adapted to the less favourable conditions of the DPE/GS medium. This experiment also demonstrated the inability of *Paenibacillus* ssp. CWBI B1070 to be antagonistic against *Campylobacter*.

The use of exogenous enzymes is a common practice in modern biotechnology industry for biomass valorization, environmental sanitation, and food (Wolfgang, 2004). Cellulase and hemicellulase enzymes are largely involved in optimization of several agricultural and waste treatment processes (Angenent *et al.*, 2004) and in improvement of feed efficiency and food fermentation (Bhat, 2000). These enzymes act by converting dominant polysaccharides in plant cell wall to oligo- and monomers. We hypothesized that these monomers could be specifically used by LAB strains for growth and metabolic activities. At this stage, *Lb. pentosus* CWBI B78 and *E. faecium* THT were the strains under investigation because of their strong inhibition activity against *C. jejuni* observed with the agar diffusion tests. The addition of the enzymatic complex called Cellulase A to DPE/GS medium significantly enhanced the anti-*Campylobacter* activity of both strains (Fig. 2). The antagonistic effect increased for both strains as enzyme concentration raised, confirming the potential cooperation between Cellulase A and LAB strains. These results support the hypothesis that this enzyme complex could hydrolyze the cellulosic part of the DPE/GS medium to more easily fermentable carbon sources. The enzyme

use during composting of poultry litter has already been reported by Tiquia *et al.* (2002), who demonstrated that an enzyme consortium (proteases, phosphatases, amino-peptidases and glycosyl-hydrolases) eliminated the fecal coliform population in a mixture of poultry litter and yard trimmings. In the same way, Logan and Bartlett (2000) claimed that enzyme-bacteria combination could act, like competitive exclusion flora (Schoeni and Wong, 1994), by reducing opportunistic microbes in favour of the bacteria that are present in the complex. Exogenous supplementation of hydrolases like Cellulase A may support the LAB antagonism efficiency and the LAB growth, especially during the adaptation or the lag phase. This effect can be explained by LAB metabolization of oligosaccharides at the expense of *Campylobacter*, which exclusively used amino acids or TCA cycle intermediates as carbon sources, and by enhanced microbial adhesion to saccharide substrate, which makes substrate capture easier (Urdaneta *et al.*, 2000).

The antimicrobial activity that was observed in co-culture was investigated in the second part of this study in order to elucidate the underlying molecular mechanism of LAB-enzyme antagonism. During *Lb. pentosus* CWBI B78 and *E. faecium* THT co-cultures, it was observed that the weak *Campylobacter* reduction for the first 24 h of fermentation was not correlated with pH decrease. *Campylobacter* population began to decrease significantly once the culture pH reached its lowest value, after 24 h of culture. It appears that a pH diminution is a prerequisite to trigger the antimicrobial activity of the tested LAB strains. However, results of lactic and acetic acid concentrations, which were measured by HPLC in co-cultures, suggest that production of organic acids may directly induce *Campylobacter* inhibition. Indeed, lactate and acetate are major metabolic end products from LAB strain fermentation (Salminen *et al.*, 2004), and their toxicity at low pH has been explained by transmembrane flux of undissociated SCFA, which ionized in the cell, yielding protons that acidify the alkaline interior of the cell (Russell, 1992). The time course of *Lb. pentosus* CWBI B78 fermentation clearly showed that this strain produced preferentially lactate during the first hours (2 - 24h) until glucose is consumed. From 24 h of incubation time, lactate concentration decreased while acetate was produced (Fig. 3), probably by shifted the metabolic flux of the hexose mono-phosphate to the pentose phosphate (Zaunmuller *et al.*, 2006). The advantage of such facultative heterofermentative strain is to produce acetate which has a more inhibitory potential against *Campylobacter* than lactate because of its higher pKa value (Heres *et al.*, 2004). Moreover, the co-culture supplementation with Cellulase A complex improved the antimicrobial activity of *Lb. pentosus* CWBI B78 which can ferment hexoses as well as pentoses released from the DPE/GS medium. On the other hand, *E. faecium* is an obligated homofermentative strain, which uses hexoses to produce exclusively lactic acid so that its bactericidal effect is more limited (Fig. 2B and 4).

In conclusion, the results of this study demonstrated the antibacterial effect of *Lb. pentosus* CWBI B78 as well as *E. faecium* THT against *C. jejuni*, by the means of co-culture techniques and agar diffusion tests. The antagonism was enhanced in co-culture by supplementation of the medium, which was based on dry poultry excreta and ground straw, with the Cellulase A complex. This apparent synergistic effect is consistent with observations of other authors with other pathogens. The co-culture model seems consequently to be more relevant than the agar gel diffusion technique to evaluate microbial interactions between antagonistic strains and *C. jejuni* in complex ecosystems like poultry litter. It has been pointed out that the inhibitory mechanism underlying this antagonistic effect is the ability of LAB to produce organic acids like lactic and acetic acids. This work suggests that application of LAB in combination with cellulase-type enzymes in the poultry environment could be used as a method for reducing viable *Campylobacter* populations, which could help to lower the risk of contamination in the poultry production industry. This hypothesis needs to be checked by developing an environmental

treatment that could be spread on the poultry litter and on the open-air range and by testing it a in a free-range chicken experiment.

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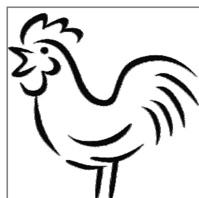
Chapter V. *In vivo* effect of a xylanase-probiotic formulation on performance and *Salmonella* colonization of broiler chickens

Article VI. Efficiency of a *Lactobacillus plantarum*-Xylanase Combination on Growth Performance, Microflora Populations, and Nutrient Digestibilities of Broilers Infected with *Salmonella* Typhimurium

In vivo effect of a xylanase-probiotic formulation on performance and Salmonella colonization of broiler chickens

At this work stage, a formulation was developed as a feed additive to supplement wheat-based diet in order to reduce Salmonella colonization in the gastro-intestinal tract of birds. This additive is composed of a xylanase and a *Lactobacillus plantarum* strain. The enzyme with xylanase activity was selected *in vivo* for its ability to improve growth performance of broiler chickens, in relation with the increase in nutrient digestibilities and AMEn of a diet based on 600 g kg^{-1} wheat. The selection of the bacterial strain for its probiotic properties was realised by *in vitro* tests performed by the Bio-industry Unit of the GxABT. The L. plantarum was shown to be resistant *in vitro* to the drastic conditions encountered in the gut (pH, bile salts, etc.) which is an essential condition to be considered as probiotic. Absorption of the bacteria to the gut epithelium was demonstrated in situ with a molecular cell probes. The strain was also able to ferment xylose and presented a high antagonistic activity against several Salmonella serotypes in agar gel diffusion assay.

However, the effects of the enzyme and the bacterial strain on performance and Salmonella populations were not yet investigated while bringing them together. Specific interactions can develop in the gut because of the complex composition of intestinal chyme, the microflora or the digestive enzymes, and these can influence the antibacterial effect against the pathogen. Being aware of such interactions, it was necessary to extend the individual tests to a global chicken model. The main objective of the succeeding chapter was to assess the effectiveness of in-feed inclusion of the xylanase and the probiotic in combination, to prevent Salmonella intestinal colonization in experimentally challenged broiler chickens.



Efficiency of a Lactobacillus plantarum-Xylanase Combination on Growth Performance, Microflora Populations, and Nutrient Digestibilities of Broilers Infected with Salmonella Typhimurium

Article VI — Adapted from Vandeplas *et al.* (2009)
Poultry Science 88(8), 1643-1654

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Running head: *Lactobacillus* and xylanase to reduce *Salmonella* in broilers

Abbreviations: AX, arabinoxylans; C, control basal diet; C-, noninfected chickens fed the basal diet; C+, infected chickens fed the basal diet; DWG, Daily Weight Gain; E, basal diet supplemented with xylanase; EU, European Union; FCR, Feed Conversion Ratio; FISH, Fluorescent *In Situ* Hybridization; MRS, de Man, Rogosa, and Sharpe agar; P, basal diet supplemented with *L. plantarum*; PE, basal diet supplemented with *L. plantarum* and xylanase

Abstract

Three experiments were performed to assess the ability of a *Lactobacillus plantarum* probiotic combined with a xylanase to reduce the effects of *S.* Typhimurium infection in broiler chickens from 1- to 30- or 42-d-old. Chicks were challenged at 3-d-old with 10^8 or 10^5 cfu *S.* Typhimurium/chick. Four diets were studied: a wheat-based diet (C+) supplemented with 0.1 g/kg xylanase (E), or 10^6 cfu/g *L. plantarum* (P), or both (PE). Uninfected chicks fed the C diet were used as negative control (C-). Six or 8 chicks were housed per cage with 9 cages/treatment. Growth performance and feed conversion ratio (FCR) were recorded weekly. In experiment 1, bacterial enumeration in caeca was achieved using the fluorescent *in situ* hybridization technique. *Salmonella* enumeration was realized in excreta by microbiological cultures (Exp. 2 and 3). Nutrient digestibilities and AMEn were determined in experiment 3 from d 35 to d 39. Infection with *S.* Typhimurium led to a significant decrease in the daily weight gain (DWG) by 23.6 % to 32.8 %, whereas FCR was increased by 1.0 % to 19.7 %. Chickens fed the PE diet showed significantly improved performance in comparison with C+ birds (DWG: +12.5 % in Exp. 1; FCR: -2.1-8.6 %), and in comparison with the P and E treatments (DWG: +6.3-8.3 % in Exp. 1; FCR: -2.7-6.4 %). In experiment 3, the FCR was significantly improved by 3 % with the PE diet in comparison with C- chickens. The PE combination tended to restore a microflora similar to that of uninfected broilers, whereas the P and E diets had less of an effect on the profile of bacterial communities. At slaughter age, *Salmonella* contamination was reduced by 2.00 and 1.85 log cfu for the E and PE treatment, respectively. The PE diet significantly reduced the crude fat digestibility by 9.2 %, in comparison with the C+ chickens. These results suggest that combination between *L. plantarum* and a xylanase as feed additive could be effective for reduction of detrimental effect following *S.* Typhimurium infection of broilers.

Keywords: probiotic, xylanase, caecal microflora, *Salmonella*, broiler

I. Introduction

In industrial countries, contamination of food from animal origin by human enteric pathogens remains a major public health concern. The major pathogen involved in bacterial food-borne infections world-wide is *Salmonella* spp., with an infection incidence of 38.2 per 100,000 population in 2005 in the European Union (**EU**) (EFSA, 2007). As this micro-organism may be carried asymptomatically in the alimentary tract of wild and domesticated birds, consumption of food from poultry origin was identified as the major source of *Salmonella* infection, by spreading via the slaughter process to raw, finished products. In broiler meat, the most commonly reported serovar is *S.* Typhimurium, which represent the predominant serovar associated with salmonellosis in Belgium (National Reference Center for *Salmonella* and *Shigella*, 2007). With the aim of decreasing the incidence of food-borne infections in humans, the EU has progressively established a coherent European legislation (Directive 2003/99/EC and the Regulations (EC) n° 2160/2003 and n° 1003/2005), especially for the monitoring and control of *Salmonella* spp. at the primary production, transformation and distribution levels. From 2010, the European legislation states that poultry meat shall not be placed on the market without any industrial treatment if a 25 g sample is detected *Salmonella*-positive. In order to match the EU directive, interventions procedures will consequently be progressively implemented by poultry producers to reduce the *Salmonella* colonization in the intestinal tract of poultry and the subsequent potential carcass contamination during slaughter.

Therefore, different strategies have been proposed to prevent *Salmonella* intestinal colonisation of chickens at the primary production level, including the use of feed additives (Van Immerseel *et al.*, 2002). The prevention of *Salmonella* infection by competitive exclusion bacteria, which are undefined or partially defined cultures derived from poultry intestinal microflora, have been widely investigated (Schneitz, 2005). In order to counteract the disadvantages linked to the undefined nature of competitive exclusion products, treatments based on one or some well-identified microbial strains as probiotics have also been studied. Species of *Lactobacillus* have been the most common microorganism studied for their probiotic properties in order to control *Salmonella* infection (Pascual *et al.*, 1999; Tsai *et al.*, 2005). The positive effects can result from a health effect, with probiotics acting as bioreactors of the intestinal microflora (Netherwood *et al.*, 1999; Mountzouris *et al.*, 2007). By production of anti-microbial substances, stimulation of the immune system, and competition for nutrients and adhesion sites in the gastro-intestinal tract (Jin *et al.*, 1997), probiotics may also help to exclude or prevent pathogen colonisation in the host.

The use of feed enzymes is also becoming an increasing part of the proposed strategies to mitigate the intestinal colonization of *Salmonella* spp. in poultry. Exogenous enzymes such as xylanase are usually incorporated in poultry wheat-based diets to degrade the anti-nutritional arabinoxylans (**AX**) of wheat which may consequently improve the nutrient digestibilities and the growth performance of poultry (Simon, 2000; García *et al.*, 2008). Several studies have showed that such enzymes have also been found to reduce the bacterial colonization in the gut. By improving the digestibility and absorption of nutrients, AX-hydrolysing enzymes have been hypothesized to leave less residual nutrients to fuel bacterial growth in the small intestine, and more particularly pathogen growth. Vahjen *et al.* (1998) showed that xylanase supplementation to wheat-based diets for broilers significantly lower total presumptive enterobacteria and total gram-positive cocci in intestinal samples. Similar results were observed in wheat or rye-based diets for enterobacteria, gram-positive cocci but also enterococci and total anaerobic microbes (Dänicke *et al.*, 1999; Hübener *et al.*, 2002). According to their effect on enterobacteria,

subsequent studies have demonstrated the inhibitory properties of growth-promoting enzymes against *Salmonella* in chickens (Hruby, 2003).

Nevertheless, combining xylanase and probiotic strains in wheat-based diet for chickens, with expected complementary improvement of growth and inhibitory activities against *Salmonella* spp., has not yet been investigated. Hence, the objective of the present work was to study the effectiveness of a feed additive, composed of a *Lactobacillus* strain as probiotic combined with a xylanase, on *S.* Typhimurium colonization in excreta of chickens experimentally infected and fed a wheat-based diet. Moreover, the effects of this combination on the performance and nutrient digestibilities, in relation to modification of the microflora, in infected chickens were evaluated, in comparison with non-contaminated birds.

II. Materials and methods

II.1. Strains and Cultivation Conditions

The *S.* Typhimurium CWBI-B1199 strain antibiotic penta-resistant (spectinomycin, tetracyclin, ampicillin, chloramphenicol and nalidixic acid) was used for oral infection challenge assays. Pathogen working culture, from bacterial stock stored at -80°C in 15 % glycerol/water, was achieved on Plate Count Agar or Tryptone Soya Broth (Merck, Darmstadt, Germany) in 18 h at 37°C. The *S.* Typhimurium inocula were grown in 15 mL of Tryptone Soya Broth. In order to determine the final concentration, serial dilutions were made on Plate Count Agar plates. The biomass cells were stored at 4°C and subsequently used in the challenge experiments.

The probiotic strain used to supplement diets was the *L. plantarum* CWBI-B659 from the CWBI lactic acid bacteria collection (CWBI, Gembloux, Belgium). The strain was kept frozen at −80°C before being replicated twice for 24 h at 37°C in De Man, Rogosa and Sharpe (**MRS**) agar (Oxoid Ltd., Basingstoke, United Kingdom). The dry powder of the CWBI-B659 culture incorporated in the poultry ration was obtained by bacterial fermentation in 15 L of MRS broth in a 20 L fermentor (Biolafitte & Moritz, Pierre Guerin Technologies, Lyon, France) for 8–12 h at 37°C. The fermentation was performed under controlled pH (i.e. 6.0) and with moderate agitation (120 rpm). The culture was then centrifuged at 8 000 × *g* for 30 min at 4°C in a Beckman Avanti J25 I centrifuge (Beckman Instruments, Fullerton, CA). After adding cryoprotective medium containing glycerol 1.5 % (wt/vol) and skim milk 20 % (wt/vol), the pellet was frozen at -40°C and, then, lyophilized in a Dura-Top freeze-dryer (FTS systems Inc., Stone Ridge, NY). The drying conditions were set at 14 Pa pressure, -25°C, during 18 h. Before use, the freeze-dried culture was analyzed for total viable cell counts (TVC). The TVC was performed by the standard dilution method on MRS agar after incubation at 37°C for 48 h.

II.2. Experimental Diets

Two basal diets, based on wheat at about 600 g/kg from the same batch for all *in vivo* trials, were formulated to meet Ross broiler nutrient requirements for grower (1 to 20 days of age) and finisher (21 to 42 days of age) growth periods (Table 1). The chemical compositions of the experimental diets were determined according to the methods of the Association of Official Analytical Chemists (AOAC, 1990). Dry matter was obtained by drying the diet samples for at least 48 h in an oven at 105°C (AOAC, 1990; method 934.01). Nitrogen was determined with

the Kjeldahl method (AOAC, 1990; method 988.05). Total protein was obtained by multiplying nitrogen value by 6.25. Fat was measured by Soxhlet extraction with diethyl ether (AOAC, 1990; method 920.39). Measurements of crude fibre were performed according to Weende (AOAC, 1990; method 978.10). The AME was calculated from the gross energy values measured using an adiabatic bomb calorimeter (Parr 1241EF, Parr Instrument Company, Moline, IL).

Table 1. Composition of the basal diets

Item	**Grower (0-14 d)**		**Finisher (15-42 d)**	
Experiment	***Exp. 1***	***Exp. 2 / Exp. 3***	***Exp. 1***	***Exp. 2 / Exp. 3***
Ingredient		(g/kg)		
Wheat[1]	592.8	600.0	580.8	600.0
Soybean meal	296.4	283.0	290.4	265.3
Soybean oil	64.5	68.8	87.5	94.0
Vitamin-mineral premix[2]	21.4	20.0	23.0	20.0
L-Lysine HCl	2.8	3.5	1.2	1.6
DL-Methionine	1.4	1.7	0.8	1.3
L-threonine	0.3	0.8	-	0.3
Sodium chloride	1.6	1.3	1.7	0.9
Limestone	-	4.7	-	3.5
Dicalcium phosphate	18.8	16.2	14.5	13.1
Analysed composition		*(g/kg diet)*		
Dry matter	882.9	885.9	882.9	884.6
		(g/kg of DM)		
Crude protein (N × 6.25)	26.8	22.1	26.2	22.7
Crude fat	10.6	9.0	11.5	11.8
Crude fibre	4.5	2.7	3.8	2.8
AME (kcal/kg DM)[4]	3,503	3,546	3,595	3,728
Calculated composition[3]		(g/kg of DM)		
Starch	310.8	311.8	310.8	311.4
Met	5.3	5.3	4.4	4.4
Thr	6.2	6.2	6.2	5.3
Lys	10.6	10.6	9.7	8.9

[1] cultivar Meunier, specific viscosity: 6.2 mL/g.

[2] Premix Leg 2 % (Provimi b.v., Rotterdam, The Netherlands), provides (per kg of diet): vitamin A (retinyl acetate), 12 188 IU; cholecalciferol, 2438 IU; vitamin E (DL-α-tocopheryl acetate), 18.3 IU; pantothenic acid, 42.6 mg; vitamin B_1, 1.2 mg; vitamin B_2, 7.3 mg; vitamin B_3, 9.7 mg; vitamin B_6, 1.2 mg; vitamin B_{12}, 0.024 mg; vitamin K_2, 1.2 mg; folic acid, 0.62 mg; choline chloride, 622.2 mg; calcium, 8784 mg; phosphorus, 3660 mg; sodium, 366 mg; magnesium 36.6 mg; iodine, 0.59 mg; cobalt, 0.59 mg; copper, 2.42 mg; iron, 45.75 mg; manganese, 97.36 mg; zinc, 85.39 mg; selenium, 0.11 mg; methionine, 1647 mg.

3 DSM, Deinze, Belgium

4 AME values were determined on the basis of gross energy, measured using an adiabatic bomb calorimeter (Parr 1241EF, Parr Instrument Company, Moline, IL).

The enzyme preparation is Belfeed B1100MP (Beldem s.a., Andenne, Belgium), which the producing strain is *Bacillus subtilis* (LMG S-15136). It is a specific pentosanase of xylanase type (105 IU/g) with optimal activity at pH 7-8 and added as a dry powder to experimental diets. In the International Union of Biochemistry system, the activity is classified as endo-1.4-β-xylanase (No. EC 3.2.1.8). It hydrolyses AX into oligosaccharides and some mono-, di- and trisaccharides. One IU is the amount of enzyme which liberates 1 µmol of xylose from birchwood xylan per minute at pH 4.5 and 30°C. The feed additive probiotic was the lactic acid strain *Lactobacillus plantarum* CWBI-B659, producing acid lactic, able to resist and to hydrolyze bile acids, and able to adhere to gut epithelial tissue (Roblain *et al.* 2002). Four dietary treatments were produced by addition of the probiotic strain and the xylanase alone or together to the basal diets. The survival of the supplemented bacteria in the diet was assessed following preparation and during storage at room temperature every week. The four experimental diets, fed as mash, contained no additional coccidiostats or antibiotics as growth promoters. A preliminary investigation of *Salmonella* presence was performed by culture technique.

II.3. Chicken Management and Experimental Design

This study consisted of three experiments. Chicks, hatched in the hatchery of the Animal Science Unit (Gembloux Agricultural University, Gembloux, Belgium), and receiving the P and PE diets, were sprayed at hatching with the cellular suspension of *L. plantarum* at 10^{10} cfu/mL by a manual vaporizer every hour during 8h in the hatchery environment. The chicks were wing-sexed, wing-banded and housed in 2 three-tier batteries, with each of the batteries containing a total of 18 cages. The combination of the two tiers at the same level on each battery represented one block. Chickens were placed in 39 × 98 × 62 cm (height × length × width) cages fitted out with a 5 cm square wire mesh bottom and plates underneath to collect excreta. All batteries were placed in a windowless house provided with forced ventilation. The temperature was controlled and gradually reduced from 33°C to 20°C until 44 d of age. Chickens had 23 h light per day during the whole experimental period to ensure frequent feed intake evenly distributed over 24 h. Water and experimental diets were offered for ad libitum consumption. Nine similar cages located in a separated room housed the non-infected chickens fed the control basal diets C (= negative control, C-) in identical rearing conditions. Detection of *Salmonella* according to the ISO 6579 standard (Anonymous, 2002) was performed in the house before the start of the experiment. The three *in vivo* trials were performed successively.

The experimental protocols complied with the guidelines of the Animal Care and Use Committee (protocol FUSAGx03/03) of Gembloux Agricultural University.

Experiment 1

A total of 270 one-day-old male broiler chickens (Ross) were used. The experiment was carried out from 7 to 28 d of age. From 1 to 6 d of age, chicks were fed the starter basal diet. Confirmation of *Salmonella* absence according to the ISO 6579 standard (Anonymous, 2002) was performed in excreta at 2 d of age. Chicks were infected at 3-d-old by oral gavage with 10^8 cfu of *Salmonella* Typhimurium CWBI-B1199 per chick. A heat stress was used to intensify stress conditions for the birds and favour the intestinal tract colonisation by the pathogen. So, the rearing room temperature was reduced by 5°C during the day and restored during the night. This temperature program was applied the day before infection, the day of infection and the day post-infection. At 7 days of age, 36 groups of 6 chicks with homogeneous weight were obtained by picking up a chick in each of 5 g weight classes, and the groups were randomly

housed in batteries. The C, P, E and PE experimental diets were randomly assigned to three cages per block (9 cages per dietary treatment), each cage representing one replicate. The C basal diet was distributed to 54 *Salmonella*-free chicks housed in nine cages of the separated house with 6 chicks/cage. The chickens were weighed individually at 7- and 28-day-old. Feed intake per cage was recorded at the same time and feed conversion ratio (**FCR**) was calculated on a cage weight basis. To measure bacterial populations from the gut, one cage per block was randomly selected from each dietary treatment. At 19 d of age, two chickens per cage were euthanized by an intracardiac dose of Nembutal (Abbott Laboratories, North Chicago, IL) 2 mL/kg live weight to collect aseptically one caecum per chicken.

Experiment 2

Experiment 2 was similar to experiment 1 with regard to the sex and number of chickens, the experimental design, the infection protocol with *S.* Typhimurium and the growth performance recording. In order to quantify *Salmonella* in excreta by conventional microbiological methods, excreta were collected aseptically from 4 cages selected randomly per treatment at 4, 9, 16, 23 and 30 d of age.

Experiment 3

Two hundred and eighty-eight one-day-old broiler chickens (Ross) were fed the starter basal diet from 1 to 6 d of age. At 7 d of age, they were randomly divided into 36 groups of 8 chicks each (four males + four females) with homogeneous weight. Each group was assigned to a cage of the three-tier batteries. The C+, P, E and PE experimental diets were randomly assigned to three cages per block (9 cages per treatment), each cage representing one replicate. The C basal diet was distributed to 72 *Salmonella*-free chicks housed in nine cages of the separated house with 8 chicks (four males + four females) per cage. The experiment was carried out from 7 to 42 d of age. Confirmation of *Salmonella* absence according to the ISO 6579 standard (Anonymous, 2002) was performed in excreta at 2 d of age. At 3 d of age, all the chicks were orally inoculated with 1 mL of *Salmonella* Typhimurium CWBI-B1199 at 10^5 cfu/mL, with a heat stress as described above. The chickens were weighed individually at the beginning (7 d) and then weekly until the end (42 d) of the experiment. Feed intake by cage was recorded at the same time and FCR was calculated on a cage weight basis. *Salmonella* quantification was performed by conventional microbiological methods in excreta collected aseptically from all the 45 cages (9 cages × 5 diets) at 4, 11, 18, 25, 32 and 44 d of age. From 35 to 38 d of age, 6 of the 9 cages per treatment (2 cages per block, 6 × 8 chicks per treatment) were used to perform a balance trial. To determine the nutrient digestibilities and the AME of experimental diets, total collection procedure and ad libitum feeding were performed according to Bourdillon *et al.* (1990) with some modifications. The digestion balance trial started on d 29 with a 6 d adaptation period. During this period, birds were fed their respective experimental diets as during the performance trial. On d 35, after an 18 h fast, chicks were fed the experimental diets for 4 d, followed by an 18 h fast. Fresh diets and non-ingested food were dried for minimum 24 h in an oven at 105°C. Excreta were quantitatively collected twice daily, pooled per cage for the whole experimental period and stored at -20°C, before being freeze-dried.

II.4. Measurements

Analysis of Caecal Microbial Communities by Fluorescent In Situ Hybridization (FISH)

Samples of the fresh caecal contents were homogenized and diluted 10-fold by weight in PBS (pH 7.5). To remove particulate material, the samples were centrifuged (700 × *g* for 3 min) and 1mL of supernatant was treated with freshly prepared 4 % paraformaldehyde to fix the cells. The samples were incubated overnight at 4°C, transported to the University of Groningen on dry ice, and then stored at -80C until they were analyzed.

For FISH analysis, samples from the caeca were assessed with general and group specific 16S ribosomal RNA targeted oligonucleotide probes. Seven probes were used to enumerate bacterial groups: Eub338 for total bacteria (Amann *et al.*, 1990), Chis150/Clit135 for the *Clostridium histolyticum* group and the *Clostridium lituseburense* group, which comprise *C. perfringens* and C. *difficile,* respectively (Franks *et al.*, 1998), Lab158 for lactobacilli/enterococcci (Harmsen *et al.* 1999), Enfl3/Enfm2 for *E. excretais/E. faecium* (Waar *et al.*, 2005), and Bif164 for *Bifidobacterium* spp. (Langendijk *et al.*, 1995). The FISH method used was described previously by Harmsen *et al.* (2002). Briefly, 10 µL of diluted cell suspensions were applied to gelatin coated slides and hybridized with 10 µL of each oligonucleotide probe (50 µg/mL stock solution) in 110 µL of hybridization buffer overnight. The total number of cells was determined by staining with 4',6-diamidino-2-phenylindole. Fluorescent cells were counted with an epifluorescence microscope (Leica DMRXA, Leica Camera AG, Solms, Germany) (Jansen *et al.* 1999). Twenty-five fields were counted in duplicate for each sample.

Excreta Quantification of S. Typhimurium

The counting method (cfu) of *Salmonella* in excreta of the chickens was based on direct plating on Brilliant Green Agar (Biokar Diagnostics, Beauvais, France) without an enrichment step. Ten g of fresh excreta sample were diluted 10 fold with buffered peptone water and mixed thoroughly. The samples were serially diluted, and plated on Brilliant Green Agar. The plates were incubated aerobically at 37°C for 18-22 h.

Balance Trial

Diets and excreta samples were ground through a 1 mm sieve and stored at 4°C until analysis. Apparent digestibilities of nitrogen, crude fat, crude fibre, dry matter and the AMEn of the diets were estimated from the analyses of excreta and of distributed and non-ingested feeds, according to the methods of the Association of Official Analytical Chemists (AOAC, 1990) as described above. For excreta, dry matter was obtained by freeze-drying the samples. Nitrogen in the excreta was calculated as total nitrogen minus nitrogen in the uric acid. Uric acid was analysed by the method of Terpstra and De Hart (1974). For fat analysis by Soxhlet extraction, excreta samples were first hydrolysed with HCl 3 M. The AMEn values were determined as described by Bourdillon *et al.* (1990).

II.5. Statistical Analysis

To test the effect of the experimental diets on the bird performance, the analysis of variance was conducted with the general linear models procedure (GLM) of SAS (SAS Institute Inc., Cary, NC). A randomized complete block design was used, a single cage representing the experimental unit (replicate) for FCR, daily weight gain (**DWG**), and digestive parameters. Experimental performance data were analysed by three-way ANOVA, including the effect of the experimental diet (n = 5), the block (n = 3), and any interactions when appropriate. The initial weight (d 7) of the broiler chicks in the performance experiments was used as covariate in the model. A two-way ANOVA (diet × block) was applied for results of the balance trial. The results were presented as means and SEM calculated by standard procedures. Means were compared using the Newman-Keuls test (Dagnelie, 1996) and considered significant at $P < 0.05$. Microbiological data for *S.* Typhimurium concentration and FISH analysis were performed using the MIXED procedure (SAS Institute Inc., Cary, NC). For data collected over time (*S.* Typhimurium concentration), a repeated-measures-in-time analysis was conducted and the smallest value of the Akaike's information criteria was used to select the most appropriate covariance structure. Effects in the model included time and treatment as fixed factors, cage as a random factor, and treatment × time interaction. When treatment× time interactions were significant, variables were analyzed within time periods. Differences among treatments were considered significant when $P < 0.05$.

III. Results

The C, E, P and PE experimental diets were negative for *Salmonella* for the whole period of the three experiments (data not shown). The un-inoculated 2-d-old chicks were also *Salmonella*-free before the start of the three *in vivo* trials. No clinical signs were recorded in any of the birds in Experiment 1, 2 or 3.

III.1. Experiment 1: Growth Performance and Microflora Measurements

The effects of block and of the diet × block interaction on growth performance were not significant and only *P* values for diet effect were reported in Table 2. The infection pattern with *S.* Typhimurium CWBI-B1199 led to a significant decrease of the DWG of control infected birds by 32.8 % (43.2 *vs* 64.3 g/bird/d), in comparison with uninfected birds. In the same way, the FCR of infected chickens fed the control basal diet was increased by 19.7 % (1.40 *vs* 1.17) following infection.

The *L. plantarum* strain and the xylanase preparation, added separately to the C+ basal diet, significantly increased the DWG of infected birds from 43.2 g/bird/d to 45.9 and 46.8 g/bird/d, with similar mean improvement for the 2 additives. The FCR was significantly improved by probiotic or xylanase supplementation, with a mean reduction of FCR values by 6.4 % (1.31 *vs* 1.40) in comparison with infected birds fed the C+ control diet. Combination of *L. plantarum* CWBI-B659 strain and xylanase Belfeed B1100MP significantly increased growth rate by 12.5 % (48.6 *vs* 43.2 g/bird/d) and decreasing FCR by 8.6 % (1.28 *vs* 1.40), in comparison with non-treated infected birds.

Table 2. Growth performance from 7 to 28 d of age of male broiler chickens infected with 10^8 cfu *S.* Typhimurium CWBI-B1199 according to the dietary treatment in experiment 1

Item	Dietary treatment[1]					SEM	*P* value
	C-	C+	E	P	PE		
Body weight[2]							
Initial weight (g/bird) (7 days)	134.7[b]	138.7[a]	143.3[a]	130.3[b]	133.6[b]	2.08	0.0009
Final body weight (g/bird) (28 days)	1437[a]	986[c]	1065[b]	1083[b]	1145[b]	27.3	< 0.0001
DWG[3] (g/bird/d)	64.3[a]	43.2[d]	46.8[bc]	45.9[c]	48.6[b]	1.62	< 0.0001
Feed consumption[2]							
Feed intake (g DM/bird/d)	78.9[a]	60.9[b]	60.7[b]	61.1[b]	65.8[b]	1.55	< 0.0001
FCR[3] (Feed intake/DWG)	1.17[c]	1.40[a]	1.31[b]	1.31[b]	1.28[b]	0.017	< 0.0001

[1] C-: control wheat-based diet fed to non-infected chickens; C+: control wheat-based diet fed to infected chickens; E: control wheat-based diet supplemented with 0.1g/kg of the xylanase Belfeed B1100MP (Beldem s.a., Andenne, Belgium); P: control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *L. plantarum* CWBI-B659; PE: control wheat-based diet supplemented with the combination of xylanase and probiotic. Composition of the control wheat-based diet is given in Table 1. From 1 to 6 days of age, chickens were fed the starter control diet.
[2] Each mean represents 9 cages with 6 animals each.
[3] DWG: Daily Weight Gain; FCR: Feed Conversion Ratio.
[a-d] Means within a row lacking a common superscript are significantly different ($P < 0.05$).

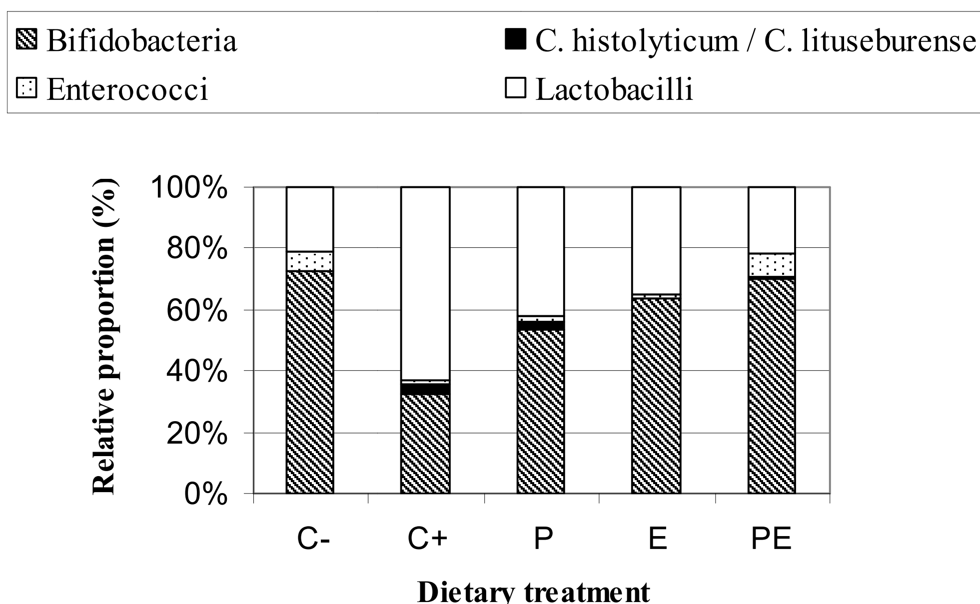

Figure 1. Relative proportions regard to total hybridized cells of microbial communities from caeca of 19 d-old chicken, according to the dietary treatment (n = 4). C-: control wheat-based diet fed to non-infected chickens; C+: control wheat-based diet fed to infected chickens; E: control wheat-based diet supplemented with 0.1 g/kg of the xylanase Belfeed B1100MP (Beldem s.a., Andenne, Belgium); P: control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *L. plantarum* CWBI-B659; PE: control wheat-based diet supplemented with the combination of xylanase and probiotic. *P* values for the treatment effect were as followed: bifidobacteria, $P = 0.1034$; *C. histolyticum / C. lituseburense*, $P < 0.0001$; enterococci, $P = 0.0004$; lactobacilli, $P = 0.0221$

From 4 to 19 days of age, FISH enumeration in caecal samples, reported as relative proportion of the total hybridized cells in Figure 1, showed differences between the treatments for clostridia ($P < 0.0001$), lactobacilli ($P = 0.0221$), enterococci ($P = 0.0004$) and bifidobacteria ($P = 0.1034$). Until 19 days old, *Salmonella* infection, in comparison with uninfected chickens, increased the relative proportion of lactobacilli and clostridia genera by 43.8 % ($P = 0.0412$) and 2.31 % ($P = 0.0529$) respectively, in relation to total hybridized cells. In contrast, the relative proportion of bifidobacteria population decreased by 37.2 % ($P = 0.0785$) following *S.* Typhimurium infection. Otherwise, *Salmonella* reduced the relative proportion of *E. faecium*/*E. faecalis* by 0.07 % ($P = 0.0003$) only, in comparison with uninfected broilers. The probiotic and enzyme treatments, alone or combined, reversed nearly completely the effect of contamination. Bacteria relative proportions observed for the PE treatment were not significantly different from the uninfected birds. In contrast, the P and E treatments showed significant differences in comparison with C- birds while being not significantly different from C+ birds. The combination of xylanase and *L. plantarum* increased the relative proportion of bifidobacteria by 36.7 % and decreased the relative proportion of lactobacilli by 42.9 % in comparison with C+ treatment. Furthermore, the relative proportions of clostridia and *E. faecium*/*E. faecalis* were also similar to those in the healthy broilers, with an increase of 6.1 % at 19 days old of relative proportion of enterococci in relation to total hybridized cell by xylanase-*L. plantarum* supplementation, as compared to infected C+ chickens.

III.2. Experiment 2: Growth Performance and Mean Concentration of S. Typhimurium in Excreta

The effects of block and of the diet × block interaction on growth performance were not significant and only *P* values for diet effect were reported in Table 3. Infection of 3-d-old chicks with *S.* Typhimurium CWBI-B1199 had a significant detrimental effect on growth performance of infected birds, with a 23.6 % decrease (46.4 *vs* 60.7 g/bird/d) of DWG and a 2.4 % increase (1.45 *vs* 1.36) of FCR in comparison with unifected animals.

The *L. plantarum* strain and the xylanase preparation, added separately to the C+ basal diet, had no significant effect on DWG of infected birds. The FCR was significantly improved from 1.45 to 1.42 with xylanase or xylanase combined with probiotic supplementation, in comparison with infected birds fed the C+ control diet. *L. plantarum* CWBI-B659 strain had no significant effect on the FCR, in comparison with non-treated infected birds.

The concentrations results of *Salmonella* in excreta of chickens challenged with *S.* Typhimurium CWBI-B1199 (Figure 2) showed that the combination of xylanase and *L. plantarum* CWBI-B659 was the most effective to significantly reduce the concentrations of *Salmonella*. At 30 days old, the PE treatment reduce ($P = 0.0116$) the *Salmonella* concentration from 7.39 log cfu/g to 6.90 log cfu/g in comparison with the control C+.

Table 3. Growth performance from 7 to 28 d of age of male broiler chickens infected with 10^8 cfu *S.* Typhimurium CWBI-B1199 according to the dietary treatment in experiment 2

Item	Dietary treatment[1]					SEM	*P* value
	C-	C+	E	P	PE		
Body weight[2]							
Initial weight (g/bird) (7 days)	145.5[b]	156.8[a]	157.3[a]	143.5[b]	144[b]	1.2	< 0.0001
Final body weight (g/bird) (28 days)	1417[a]	1103[b]	1085[b]	1046[b]	1092[b]	25.9	< 0.0001
DWG[3] (g/bird/d)	60.7[a]	46.4[b]	45.7[b]	44.6[b]	46.3[b]	1.04	< 0.0001
Feed consumption[2]							
Feed intake (g DM/bird/d))	84.6[a]	66.8[b]	63.9[b]	64.2[b]	64.5[b]	1.76	< 0.0001
FCR[3] (Feed intake/DWG)	1.36[c]	1.45[a]	1.42[b]	1.46[a]	1.42[b]	0.008	< 0.0001

[1] C-: control wheat-based diet fed to non-infected chickens; C+: control wheat-based diet fed to infected chickens; E: control wheat-based diet supplemented with 0.1g/kg of the xylanase Belfeed B1100MP (Beldem s.a., Andenne, Belgium); P: control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *L. plantarum* CWBI-B659; PE: control wheat-based diet supplemented with the combination of xylanase and probiotic. Composition of the control wheat-based diet is given in Table 1. From 1 to 6 days of age, chickens were fed the starter control diet.
[2] Each mean represents 9 cages with 6 animals each.
[3] DWG: Daily Weight Gain; FCR: Feed Conversion Ratio.
[a-b] Means within a row lacking a common superscript are significantly different ($P < 0.05$).

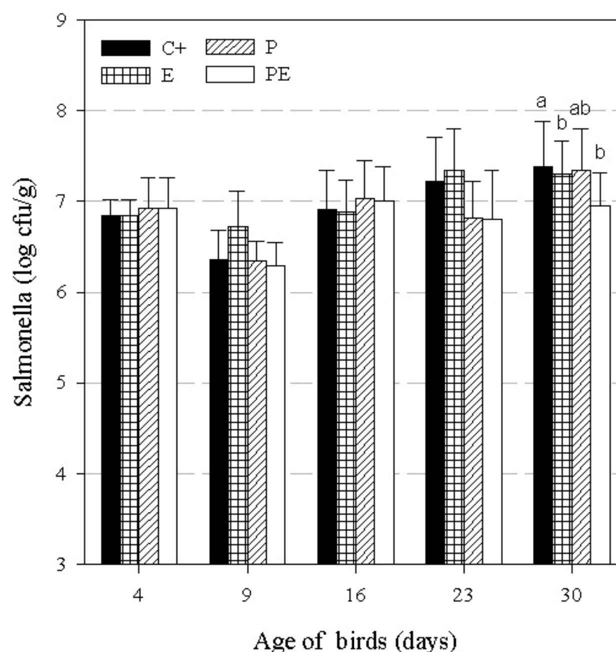

Figure 2. *S.* Typhimurium concentration in excreta at different time of male broiler chickens infected with 10^8 cfu *S.* Typhimurium CWBI-B1199 (experiment 2). C+: control wheat-based diet fed to infected chickens; E: control wheat-based diet supplemented with 0.1 g/kg of the xylanase Belfeed B1100MP (Beldem s.a., Andenne, Belgium); P: control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *L. plantarum* CWBI-B659; PE: control wheat-based diet supplemented with the combination of xylanase and probiotic. Bars represent mean log cfu per gram of excreta content, and error bars indicate the SD. For a given time, the values marked with a symbol above the bars are significantly different from the control group (** P< 0.05)

III.3. Experiment 3: Growth Performance, Mean Concentration of S. Typhimurium in Excreta and Nutrient Digestibilities

The effects of block and of the diet × block interaction on growth performance were not significant and only *P* values for diet effect were reported (Table 4). In comparison with uninfected birds fed the C- basal diet, infection of 3-d-old chicks with 10^5 cfu *S.* Typhimurium/chick induced, at 42 days, a significant decrease of the DWG by 29.8 % (43.9 *vs* 62.5 g/bird/d) and a significant increase of the FCR by 1.0 % (2.01 *vs* 1.99) of infected chickens fed the control basal diet.

Table 4. Growth performance from 7 to 42 d of age and nutrient digestibilities of a mixed population of broiler chickens infected with 10^5 cfu *S.* Typhimurium CWBI-B1199 according to the dietary treatment in experiment 3

Item	**Dietary treatment[1]**					**SEM**	***P* value**
	C-	**C+**	**E**	**P**	**PE**		
Body weight[2]							
Initial weight (g/bird) (7 days)	112.6^{a}	89.1^{d}	99.7^{b}	96.2^{c}	97.0^{c}	0.48	< 0.0001
Final weight (g/bird) (42 days)	2350^{a}	1572^{b}	1648^{b}	1672^{b}	1605^{b}	49.2	< 0.0001
DWG[3] (g/bird/d)							
28 days	48.9^{a}	33.5^{b}	33.6^{b}	33.9^{b}	34.4^{b}	0.51	< 0.0001
42 days	62.5^{a}	43.9^{b}	44.3^{b}	44.8^{b}	45.2^{b}	0.64	< 0.0001
Feed consumption[2]							
Feed intake (g DM/bird/d)							
28 days	68.1^{a}	46.4^{c}	45.6^{d}	47.9^{b}	46.2^{c}	0.13	< 0.0001
42 days	123.7^{a}	87.9^{b}	87.3^{b}	90.3^{b}	87.2^{b}	0.71	< 0.0001
FCR[3] (Feed intake/DWG)							
28 days	1.37^{b}	1.37^{b}	1.36^{b}	1.40^{a}	1.31^{c}	0.006	< 0.0001
42 days	1.99^{b}	2.01^{a}	1.97^{b}	2.02^{a}	1.93^{c}	0.006	< 0.0001
Digestibility (%)[4]							
Crude fibre	15.49^{c}	20.77^{abc}	26.22^{a}	17.03^{bc}	22.61^{ab}	1.996	0.012
Crude fat	89.01^{a}	86.92^{a}	79.57^{b}	78.34^{b}	78.95^{b}	0.863	<0.0001
Dry Matter (DM)	80.69	75.49	80.51	79.53	80.28	1.939	0.3893
Protein	85.12^{a}	59.93^{c}	74.17^{ab}	66.37^{bc}	78.56^{ab}	5.940	0.0638
AMEn (kcal/kg DM)	3,906	3,630	3,890	3,815	3,855	81.04	0.2837

[1] C-: control wheat-based diet fed to non-infected chickens; C+: control wheat-based diet fed to infected chickens; E: control wheat-based diet supplemented with 0.1g/kg of the xylanase Belfeed B1100MP ((Beldem s.a., Andenne, Belgium); P: control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *L. plantarum* CWBI-B659; PE: control wheat-based diet supplemented with the combination of xylanase and probiotic. Composition of the control wheat-based diet is given in Table 1. From 1 to 6 days of age, chickens were fed the starter control diet.

[2] Each mean represents 9 cages with 8 animals (4 males + 4 females) each.

[3] DWG: Daily Weight Gain; FCR: Feed Conversion Ratio

[4] Each mean represents 6 cages with 8 animals (4 males + 4 females) each.

[a-d] Means within a row lacking a common superscript are significantly different ($P < 0.05$).

The *L. plantarum* strain and the xylanase preparation, added separately to the C+ basal diet, not significantly improved the growth performance, in comparison with birds fed the C+ diet. In contrast, the *L. plantarum* CWBI-B659 strain combined with the xylanase Belfeed B1100MP significantly improved the FCR by an average of 4.0 % (1.93 *vs* 2.01) in comparison with birds fed the C+ diet. The FCR observed with the PE diet was also significantly decreased by 4.4 % (1.31 *vs* 1.37) and 3.0 % (1.93 *vs* 1.99), at 28 and 42 days of age respectively, in comparison with FCR values of non infected chickens given the C- basal diet.

Analysis of the digestibility data revealed that chickens infected with S. Typhimurium (treatment C+) had lower digestibilities of dry matter (75.5 *vs* 80.7 %), crude fat (86.9 *vs* 89 %, $P < 0.05$), and a reduced AMEn (3860 *vs* 4098 kcal/kg DM) of the diet compared to non infected chickens (treatment C-) (Table 4). Protein digestibility was also reduced but, in spite of a very large difference between digestibility values of infected and uninfected birds (59.9 *vs* 85.1 %), there was no significant difference between the treatments ($P = 0.0638$). Otherwise, infected chickens showed a higher digestibility of crude fibre by 34.1 % (20.77 *vs* 15.49 %), in comparison with healthy birds.

Chickens fed the xylanase-supplemented diet improved digestibilities of crude fibre, dry matter, protein, and the AMEn, by 26.2 % (26.22 *vs* 20.77 %), 6.7 % (80.51 *vs*75.49 %), 23.8 % (74.17 *vs* 59.93 %) and 6.5 % (3,890 *vs* 3,630 kcal/kg DM) respectively, as compared with the control birds fed the C+ diet. The probiotic strain *L. plantarum* CWBI-B659 added to the basal diet did not significantly affect the digestibilities of dry matter, protein, crude fibre, and AMEn. The PE diet did not significantly improve the digestibilities of crude fibre, dry matter and AMEn, but significantly increased protein digestibility by 31.1 % (78.56 *vs* 59.93 %), in comparison with the C+ treatment. All the dietary treatments led to a significantly decreased digestibility of crude fat by 8.5 to 9.9 % (78.34-79.57 *vs* 86.92 %), in comparison with the birds receiving the C+ diet.

The measurement of *S.* Typhimurium CWBI-B1199 concentration in excreta by microbiological methods showed variable results from 4 to 32 days of age. Moreover, the *Salmonella* concentration in excreta from 32 to 44 days of age increased by more than 1 log cfu/g, whatever the dietary treatment. At 44 days old, *Salmonella* concentration in excreta was 6.91 log cfu/g for the control and decreased to 5.06 log cfu/g, for the chickens fed the PE diet, which was similar for the E treatment, whereas the probiotic supplemented alone to the wheat-based diet showed a negligible reduction, in comparison with control chickens (Figure 3).

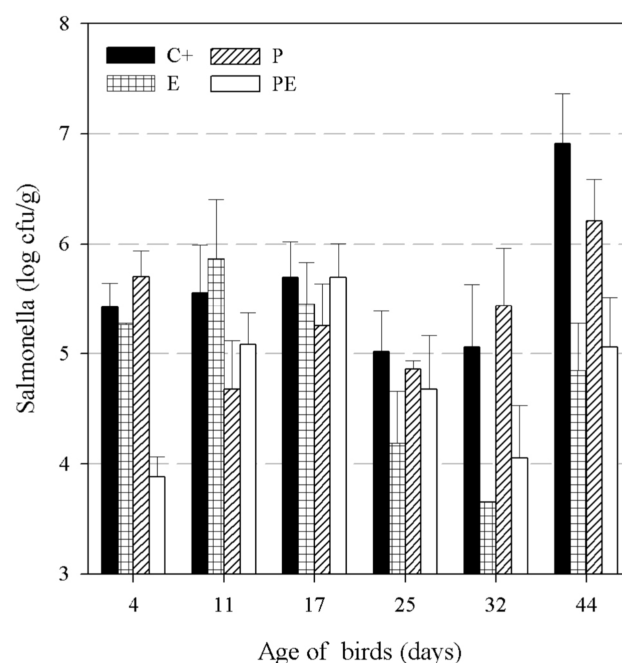

Figure 3. *S.* Typhimurium concentration in excreta at different time of a mixed population of broiler chickens infected with 10^5 cfu *S.* Typhimurium CWBI-B1199 (experiment 3). C+: control wheat-based diet fed to infected chickens; E: control wheat-based diet supplemented with 0.1 g/kg of the xylanase Belfeed B1100MP (Beldem s.a., Andenne, Belgium); P: control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *L. plantarum* CWBI-B659; PE: control wheat-based diet supplemented with the combination of xylanase and probiotic. Bars represent mean log cfu per gram of excreta content, and error bars indicate the SD

IV. Discussion

Colonization of the chicken gastro-intestinal tract by *Salmonella* strains produces a strong inflammatory response (Kaiser *et al.*, 2000), associated with harmful effects to the integrity of the intestinal epithelium (Desmidt *et al.*, 1998), that might potentially induce detrimental outcomes on nutrient digestibilities and growth performance. The effect of *Salmonella* colonisation on performance was reported by few authors (Nakamura *et al.*, 2002; Gupta *et al.*, 2005), and was confirmed in this study. Experimental infection of broiler 3-d-old chicks with *S.* Typhimurium induced a very pronounced reduction of performance by 20 to 30 % for DWG and 2 to 20 % for the FCR, which is seldom observed in industrial production. However, challenge models described in literature are generally carried out with high infection doses to study live birds interventions, and that may explain the marked effect observed in this study. Moreover, reported differences in infection effects on growth performance may be due to various infection doses and virulence of the pathogen strain used. Reduced growth performance is related to the decreased feed intake by infected chickens, as observed in this study. As bacterial pathogens like *Salmonella* have been postulated to cause mucosal damage by different mechanisms (Finlay and Cossart, 1997; Desmidt *et al.*, 1998), *Salmonella* colonization of the

intestinal gut at high infection dose might consequently impede nutrient absorption and digestion.

The negative effect of *Salmonella* infection on chicken performance may be related to a reduced diet absorption following pathogen invasion. Regarding the diet digestion, the results of this study pointed out a modification of nutrient digestibilities following *Salmonella* contamination. The competition by *Salmonella* with the host, combined to the inflammatory response and the activation of the immune system, have an energy cost which may lead to reduced energy for bird maintenance and growth at similar AME values of the diets. Moreover, until 19 days of age, *Salmonella* infection reduced the relative proportions of bifidobacteria and enterococci in comparison with normal microflora of uninfected chickens. Modification in the enteric population balance, like the increase of *Clostridium perfringens* numbers, was shown to be directly related to the reduction of growth performance in chickens (Fuller, 1984; Bomba *et al.*, 2006).

Exogenous xylanases are commonly added to wheat-based broiler diets to reduce the anti-nutritionnal effects of such ration due to the high content of AX in wheat. The anti-nutritionnal effect of wheat AX have been showed to be correlated mainly to the entrapment of nutrients in the polysaccharides structure, the so-called "cage effect" (Simon, 2000), and to the increased viscosity of the intestinal content in the lumen, in relation with increased bacterial populations of the gut (Mathlouthi *et al.*, 2002; Choct *et al.*, 2004).

By disrupting AX polymers, endo-xylanases as the Belfeed B1100MP may consequently make the nutrients more accessible for endogenous digestive enzymes, increasing the efficiency of diet digestion as observed by García *et al.* (2008) and Gutierrez del Alamo *et al.* (2008). However, these experiments were carried out with un-infected birds, whereas *Salmonella* infection may reduce the apparent digestibilities and AMEn so that the effect of exogenous enzyme in our study appeared to be more important than in these studies. Xylanase supplementation appeared particularly to exert a significant effect on the digestibility of crude fibre, which includes the AX fraction of the diet, in comparison with the uninfected birds. In contrast, the enzyme as well as the probiotic exerted a significant detrimental effect on fat digestibility, in comparison with the control diet fed to contaminated chickens. As Belfeed B1100MP was shown to improve fat digestibility in uninfected birds (Vandeplas *et al.*, unpublished data), decreased digestibility with the feed additives may be related to interaction with *Salmonella* contamination and increased hydrolysis activity of bile acids, as shown for the *L. plantarum* strain (Roblain *et al.*, 2002).

Moreover, xylanases may exert part of their response from changes in relative microbiota populations. By reducing the digesta viscosity, exogenous enzyme may lower total bacterial counts in intestinal contents, as already reported by several authors (Hübener *et al.*, 2002; Mathlouthi *et al.*, 2002), and especially gram-positive cocci and enterobacteria (Vahjen *et al.*, 1998; Hübener *et al.*, 2002) or populations of undesirable organisms such as *Clostridium perfringens* (Hübener *et al.*, 2002) which was observed in this study with the xylanase alone or in combination with the *Lactobacillus* strain. *Clostridium perfringens* infection of broiler chickens may cause subclinical or clinical disease associated with necrotic enteritis (Johansson, 2006), and thickening of the intestinal epithelium that may induce reduction of nutrients absorption (Mead, 2000). At the same time, degradation of AX by xylanase activity releases mono- and oligosaccharides in the intestinal lumen that may be fermented in the caeca by specific bacterial species. The development of these specific AX-fermenting populations, i.e. enterococci (Hübener *et al.* (2002), may lead to an increase of fatty acids production and a reduction of pH values (Engberg *et al.*, 2004) , which may inhibit the growth of gram-negative

pathogen bacteria such as *E. coli* (Mathlouthi *et al.*, 2002), or *Salmonella*. In this study, bifidobacteria were shown to be favored by xylanase alone or combined with the *L. plantarum* strain. Courtin *et al.* (2008) showed that arabinoxylooligosaccharides produced by AX hydrolysis increase the number of bifidobacteria in the caeca of broilers. The increased number of bifidobacteria in the caecum of chicken may be able to decrease the population of *Salmonella*. Indeed, bifidobacteria are known to stimulate butyric acid production in the caeca (Belenguer *et al.*, 2006) which have a detrimental effect on pathogen colonization in young chickens. As xylanase appeared to be effective on *Salmonella* counts only when the infection dose was low (Exp. 3 *vs* Exp. 2), it is hypothesized that such enzyme-favoured specific bacterial communities might only exert efficient competitive inhibition when the pathogen population is limited. The analysis of intestinal bacterial populations by the FISH technique demonstrated that the different dietary additives modified the microflora. However, further investigations have to be carried out with higher numbers of samples and targeted bacteria species in order to confirm the potential effect of the xylanase alone or with the probiotic. The denaturing gradient gel electrophoresis technique, for example, may help to evaluate the overall intestinal microbial profile (Hume *et al.*, 2003), rather than focusing on a few specific microorganisms.

Among the beneficial effect described for probiotic strains, the more important is the prevention or the reduction of intestinal colonization by bacterial pathogens. Briefly, Mead (2000) proposed 4 mechanisms by which probiotic cultures are able to exclude enteric pathogens: competition for receptor sites, competition for limiting nutrients, production of bacteriocins and production of volatile fatty acids that may inhibit certain enteric pathogens. Several studies have showed that the growth of *Enterobacteriaceae* like *Salmonella* spp. was inhibited *in vitro* (Tsai *et al.*, 2005) and *in vivo* (Higgins *et al.*, 2007; Wolfenden *et al.*, 2007) in the presence of *Lactobacillus* strains. Higgins *et al.* (2007) showed that oral inoculation of 7.5×10^5 cfu of a culture of a probiotic mixture, based on eleven lactic acid bacteria, induced a 2.4 $\log_{10}$ reduction of *Salmonella* in caeca of 1-d-old chicks inoculated with 4.5×10^3 cfu of *S. enteritidis*. However, oral gavage is not a practical way of administration in commercial hatcheries or poultry houses. Wolfenden *et al.* (2007) showed that treating day-of-hatch chicks challenged with 10^4 cfu/chick of *S.* Enteritidis with the same *Lactobacillus*-based probiotic by spray at 10^7 cfu/mL or in the drinking water at 10^6 cfu/mL during five days post-challenge induced a significant reduction of *S.* Enteritidis recovery for 15 to 65 % in comparison with the controls. In this study, the beneficial action of the *L. plantarum* probiotic on pathological effects induced by *Salmonella* infection was only significant when the strain was combined with the xylanase Belfeed B1100MP in the wheat-based diet. It was hypothesized that the survival and the growth of the *L. plantarum* strain was favoured by the xylanase activity, in the same way as other bacterial populations with probiotic potential like Bifidobacteria. The enhanced growth of probiotic strains may correspond to increased specific bacterial activities, leading to higher antagonistic effect against *Salmonella* (Jin *et al.*, 1997), to reduction of intestinal pathogen colonization and to decreased subsequent detrimental effects on growth performance. The upsurge of excreta *S.* Typhimurium concentrations at 44 days of age observed in experiment 3, whatever the dietary treatment applied, may be explained by adaptation of specific strains (Prouty *et al.*, 2004) and by increased pH values in the intestinal chime which may suppress partly the mechanisms of *Salmonella* inhibition by *Lactobacillus* strains (Fayol-Messaoudi *et al.*, 2005).

In conclusion, the results presented here revealed a potential complementary effect between a *L. plantarum* probiotic strain and an exogenous xylanase added to a wheat-based diet for broiler chickens experimentally infected at 3 days of age with *S.* Typhimurium. This complementary beneficial effect was partly expressed by FCR improvement, and by reduction of

S. Typhimurium concentrations in excreta which is more pronounced than with both additives supplemented alone. This complementary action was hypothesized to result mainly as well from reduced overall bacterial populations in the gut, which may decrease nutrients competition with the host, as from changes in composition and metabolic potential of bacterial populations towards specific genera, like enterococci, with notably probiotic properties. In order to increase the knowledge about the complementary mechanisms of xylanase and *L. plantarum* strain, further research could be conducted by analysing the ileal microflora, and by following AX degradation and environmental conditions in the gastrointestinal tract.

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Chapter VI. General discussion and future prospects

General discussion

Broiler flocks can be contaminated by enteric pathogens through several transmission routes. Vertical transmission from the hen to the chick was not assumed to be important in *Campylobacter* contamination (Sahin *et al.*, 2003). Moreover, vertical transmission of *Salmonella* is nowadays of less importance in industrial chicken production mainly due to the vaccination of breeder flocks (Collard *et al.*, 2008). Horizontal transmission is consequently the main determinative factor for colonization of broiler flocks by these bacteria. Drinking water was shown especially to be a real contamination vector for *Campylobacter* (Shanker *et al.*, 1990), while poultry feed can be a main transmission vector for *Salmonella* (Heyndrickx *et al.*, 2002; Davies and Wray, 1996).

Nevertheless, the most important contamination vector seems to be the outer environment. Animal bearing and fecal shedding of *Salmonella* and *Campylobacter* by farm animals and wild animals like birds and rodents have been actually pointed out in several studies as a potential origin of environmental contamination (Stanley and Jones, 2003; Hutchinson *et al.*, 2005; Oporto *et al.*, 2007). In the epidemiological study described in Chapter III (Article III), other animal productions like cattle on the farm were actually recorded as risk factors for *Campylobacter* infection of the broiler flocks. The pathogens are able to survive in the house surroundings soil (Zibilske and Weaver, 1978; Bull *et al.*, 2006). Friedman *et al.* (2000) showed that the presence of *Campylobacter* spp. in streams, rivers and ponds, can then be taken as a sign of faecal infection by livestock or wild animal and this can last up to four months (Rollins and Colwell, 1986; Hazeleger *et al.*, 1998). The open-air range to which broilers have access in free-range poultry production could therefore be a major environmental source for flock contamination. When campylobacters are isolated from the open-air range soil or from stagnant water, before the birds go out, the precedent flock may be responsible for the contamination. In our study, most samples found to be *Campylobacter*-positive were from the open-air range which actually appeared to be a major source of contamination. Such conclusion was also reported by Rivoal *et al.* (2005) who studied genomic diversity and sources of *Campylobacter* contamination in French free-range broiler farms. Among poultry farms sampled from 1996 to 1999, these authors found that flock contamination appeared from six weeks of age, at the time of outdoor rearing period. Nevertheless, the influence of the open-air range on the contamination is still debated. According to Huneau-Salaün *et al.* (2005), access to an open-air range could not be the main *Campylobacter* contamination route of free-range broiler production. The longer rearing period in these production systems can actually increase the risk of *Campylobacter* infection (Heuer *et al.*, 2001). As contamination of intensive reared broilers at slaughter age may be as frequent as in free-range broilers at the same age (Van Overbeke *et al.*, 2006), older broilers get more chance to become infected, without influence of the open-air range. The same hypothesis can be voiced for *Salmonella*. Bailey and Cosby (2005) actually shown that *Salmonella* prevalence at the slaughter age was higher in free-range chickens than in commercial chickens in the USA. Other potential sources of contamination recorder in the epidemiological study detailed in Chapter III were delivery tray, anteroom floor and water-lines. The contamination of the anteroom floor may actually suggest the possible infection of flock by the farmer, visitor, and equipments, through contact in the open-air range with wild birds and other animal faeces (Rodenburg *et al.*, 2004), but also through fans and dust, as stated by Berrang *et al.* (2003) and Berndtson *et al.* (1996). Nevertheless, there is an

assumption that *C. jejuni* cannot survive for long period within the dehydrating conditions of dust, which is confirmed by two studies that failed to isolate *Campylobacter*, contrary to *Salmonella*, from aerosols and dust from chicken houses (Saleha, 2004; Chinivasagam *et al.*, 2009). Unsuitable hygiene practices at the farm level, especially poor cleaning and disinfection of the poultry house and not dedicated protective clothing, could then be a major reason of *Campylobacter* contamination persistence in poultry flocks, as summarized by Allen and Newell (2005). Dedicated protective clothing is obligatory in Belgian broiler houses from standard and free-range production system. Footbaths are traditionnaly not required, and their effect against *Campylobacter* has not been demonstrated, although a temporary national legislation (Ministerial order, April 3, 2006) requires poultry farmers to use footbaths with disinfectants which are registrated by the AFSCA in order to face the avian influenza concern. Many authors have also reported that *Salmonella* can be carried into the house via boots, clothes and equipment (Bailey *et al.*, 2001; Heyndrickx *et al.*, 2002), associated with poor hygiene standards at poultry sites (Wray *et al.*, 1999). However, the carrying of *Campylobacter* from the environment and the open-air area into the house by human activity and environmental factors has yet to be proven by genotyping confirmation of strains from the environment and the open-air range which subsequently result in flock colonization. The analysis of risk factors in our study showed that cleaning and disinfection of water-line between flocks may help to reduce the risk of chicken colonization. Bailey *et al.* (2001) actually showed that the waterline and the water cup, as well as the drinking water, were contaminated with *Salmonella* in poultry houses with positive flocks. Sufficient chlorine is normally added to insure that the residual chlorine content of the treated water is in the range of 0.1 to less than 1 mg/l (0.1 to < 1 ppm) which is sufficient to provide *Salmonella* prevention. However, Phe *et al.* (2009) analysed the *in vitro* resistance of *S.* Typhimurium to different concentrations of chlorine with culture techniques and flow cytommetry, after staining the cells, and observed a subpopulation that was significantly reduced with 0.5 mg/l chlorine, but also a subpopulation that persisted physically at chlorine concentrations as high as 3 mg/l. Besides this resistance phenomenom, the development of biofilms in presence of other microorganisms like *Pseudomonas* was shown to increase the *Salmonella* resistance to chlorine (Leriche and Carpentier, 1995). According to Poppe *et al.* (1986), the level of free-available chlorine retained in the water of some type of drinkers can also be insuffIcent to exert a bactericidal effect against *Salmonella*. Newell and Fearnley (2003) pointed out that contamination of the water lines usually follows rather than precedes *Salmonella* infection of the flock, and it may be hypothesized that poor disinfection of water-line may be responsible for the pathogen-detection in the following flocks. However, Rose *et al.* (1999) considered that the drinking water source is a low risk factor for flock colonization by *Salmonella.*

Chickens usually are infected by oral uptake of bacteria. Bacteria are then able to survive gastric acidity and can therefore pass the stomach to reach the intestinal tract of the animal (Kwon and Ricke, 1998). In chicken, the caeca and the small intestine are the predominant sites of *Salmonella* and *Campylobacter* colonization (Desmidt *et al.*, 1997; Newell and Fearnley, 2003). The pathogenesis of the *Salmonella* species can be divided in two distinct phases, i.e. the intestinal and the systemic phase of the infection (Van Immerseel, 2004). The pathogen can first adhere to epithelial cells by specific adhesion-receptor interactions (Van Immerseel, 2004). Then, *Salmonella* induces actin cytoskeletal rearrangements at the site of bacterial-host cell contact that directs bacterial internalization. *Salmonella*-infected epithelial cells secrete chemokines and prostaglandins that act to recruit inflammatory to foci of infection. *Salmonella* interacts with inflammatory cells and stimulates the release of pro-inflammatory cytokines that enhance the inflammatory response. Infected enterocytes become extruded from the villus surface into the intestinal lumen, resulting in villus blunting and loss of absorptive surfaces.

Due to the pro-inflammatory cytokine production by epithelial cells, macrophages are attracted to the intestinal wall and can internalize *Salmonella* by phagocytosis. The pathogen can survive within and replicate in macrophages, which migrate to the draining lymphatics, carrying pathogenic bacteria to internal organs, such as liver and spleen (Barrow, 1999). Concerning *Campylobacter*, colonization mechanisms of poultry are not yet well defined. In the GIT, this pathogen was shown to freely invade the lumina of crypts without attachment to microvilli. *Campylobacter* is preferentially attracted to mucus-filled crypts because of the chemotactic behaviour of the microorganism toward mucin, a principal constituent of mucus (Beery *et al.*, 1988). Smith *et al.* (2008) demonstrated that *Campylobacter* can induce proinflammatory chemokine transcript in mucosal tissues, which was correlated with an influx of heterophils, but this phenomenon was not associated with any pathology. While *Campylobacter* remains in the mucus and generally does not invade epithelial cells, it can be recovered from liver or spleen and was detected in blood (Beery *et al.*, 1988), suggesting potential systemic infection. Some *in vitro* studies actually showed that *C. jejuni* strains were able to invade chicken epithelial cells (Byrne *et al.*, 2007; Van Deun *et al.*, 2008; Li *et al.*, 2008). Bacteria were not able to proliferate intracellularly, but quickly evaded from the cells.

Therefore, efficient measures to reduce *Salmonella* and *Campylobacter* contamination in broiler flocks may act at the environment or at the animal level. Reduction of pathogen population in the broiler house and/or on the open-air range, could prevent broiler infection, or at least could decrease the contamination level of carcasses at slaughter stage by reducing pathogen concentration in the chicken GIT. Both approaches were investigated in this PhD thesis but were based in the same way on a treatment combining Lactic Acid bacteria (LAB) with a polysaccharidase.

LAB are characterized by their potential to prevent the adherence, establishment, replication, and/or virulence of specific enteropathogens (Reuter, 2001). Their antagonistic effects against *Salmonella* and *Campylobacter* have been described *in vitro* (Jacobsen *et al.*, 1999; Chaveerach *et al.*, 2004; Van Coillie *et al.*, 2007; Nazef *et al.*, 2008; Musikasang *et al.*, 2009) and *in vivo* (Schoeni *et al.*, 1994; Stern *et al.*, 2005; Tsai *et al.*, 2005; Higgins *et al.*, 2007). Different antagonistic mechanisms have been proposed, including competition for receptor sites, competition for limiting nutrients, and production of antimicrobial metabolites (bacteriocins, VFA, hydrogen peroxide) that may inhibit certain enteric pathogens (Salminen *et al.*, 2004; Servin, 2004).

The *Lactobacillus plantarum* CWBI-B659, which was used as feed additive in the *in vivo* experiments presented in Chapter V (Article VI), was shown to adhere to gut epithelial tissue (Roblain *et al.* 2002). Such property can induce competition for receptor sites with pathogenic bacteria in the GIT, and is consequently an interesting feature for antibacterial effect. Some studies reviewed by Servin (2004) have demonstrated that *Lactobacillus* strains express adhesiveness properties that enable them to inhibit the adhesion of bacterial pathogens to host cells. Tsai *et al.* (2005) showed that *L. fermentum* LF33 from poultry caecum was able to adhere to Int-407 intestinal cells and inhibit the growth of *Salmonella* Typhimurium. Several strains were shown to impede pathogen adherence through their ability to increase the production of intestinal mucins (Servin, 2004), as observed by Mack *et al.* (1999) with *L. plantarum* 299v. However, these studied were performed with cultured human intestinal cells, but some research has been carried out with chicken intestinal cell lines and has also pointed out adhesion inhibition. Gusils *et al.* (2006) isolated chicken intestinal epithelium and demonstrated that *L. animalis* and *L. fermentum* were effective in reducing *in vitro* the attachment of *S.* Gallinarum, *S.* Pullorum and *S.* Enteritidis. Similarly Jin *et al.* (1996) showed that *L. acidophilus* I26 significantly reduced the attachment of *S.* Pullorum to ileal epithelial

cells of chicken by 19.2 % and 19.4 % under the conditions of exclusion and competition respectively, but not for displacement. Other studies have also demonstrated *in vitro* the antagonistic effect by *Lactobacillus* strains against the *Salmonella* adhesion to chicken intestinal mucus (Craven and Williams, 1997; Ma *et al.*, 2006).

In order to stimulate the antagonistic effect of LAB, the bacterial growth and metabolism can be enhanced by supplying specific easily metabolizable substrates. These can be produced by enzymatic activity from the environmental matrix where the antagonistic strain and the pathogen are brought together. The *in vivo* experiments presented in Article IV (Chapter IV) demonstrated that xylanase supplementation of a wheat-based diet improved nutrient digestibilities, and the AMEn of the diet. These results showed that hydrolysis of insoluble arabinoxylans at least partly breaks off the cage effect which causes the release of nutrients entrapped in the endosperm cell wall structure of wheat grain in the GIT. The nutrients contained in plant cells may consequently be more accessible for digestive enzymes, as well as for some specific bacterial species from the microflora. Xylanase addition to a wheat-based diet was shown to increase *Lactobacillus* spp. colony counts in intestinal mucus of chickens from 24h to 28 days of age, although luminal counts were unaffected in comparison with chickens fed the unsupplemented diet (Vahjen *et al.*, 1998). Hübener *et al.* (2002) showed that xylanase induced higher CFU for total anaerobic growth in the caeca in 7-days-old chicks, while gram-positive cocci increased in the caeca of chickens at 28 days of age in comparison with control birds. LAB can easily ferment carbohydrates as carbon source via the citric acid metabolism (Kandler, 1983). Fermentation consequently produces organic acids, which are known to show interesting antimicrobial activities against *Salmonella* and *Campylobacter* (Chaveerach *et al.*, 2002; Van Coillie *et al.*, 2007). Fermented carbohydrates are especially glucose and other monomers with 6 carbons, but also oligosaccharides of these sugars (Kontula *et al.*, 1998), and some LAB strains have also been shown to metabolize mono- and oligosaccharides based on 5 carbons-monomers, such as xylose (Kontula *et al.*, 1998; Moura *et al*., 2007). As *Salmonella* is also able to ferment glucose-based polysaccharides from the diet and xylo-oligosaccharides (Mortlock and Old, 1979), the antagonistic strain that was used as feed additive for *Salmonella* control was selected because of its very short generation time by metabolizing xylose and xylose-based oligosaccharides supplied by the supplemented xylanase. For developing the environmental treatment to control *Campylobacter* in free-range broiler production, competition for nutrient between the LAB strain and *Campylobacter* is easier to consider. *Campylobacter* is unable to utilize glucose as a carbon source (Velayudhan and Kelly, 2002). Adding an enzyme with cellulase activity in the environment should produce glucose and/or oligosaccharides, for example from the straw litter or from the open-air range grass. These hydrolysis products were then hypothesized to selectively favour growth and metabolism of LAB (Kailasapathy and Chin, 2000) to the detriment of *Campylobacter*. This enhancing effect of cellulase was demonstrated in the co-culture assays described in Chapter IV (Article V). Thus, LAB selection for *Campylobacter* control was not based on specific metabolic characteristics but only on its antagonistic potential.

Among LAB, *Lactobacillus* sp. have been widely studied for their antimicrobial properties. *Lactobacillus* sp. have been observed to produce various metabolites with antagonistic activities against enteropathogens like *Salmonella* and *Campylobacter* (Chaveerach *et al*., 2004; Tsai *et al*., 2005; Ammor *et al*., 2006). The most widely produced anti-microbial substances are organic acids (Ouwehand *et al*., 1999). Hydrogen peroxide and carbon dioxide may also be produced. Release of diacetyl, reuterin, pyroglutamic acid, and especially bacteriocins, has also been reported but is however not certain under *in vivo* conditions (Ouwehand *et al.*, 1999). *Lactobacillus* sp. are fermentative bacteria which produce organic acids when oxygen is not available as a terminal electron acceptor. The simplest fermentation is conversion of sugar to

lactate, and many lactobacilli have a scheme that is virtually homolactic when sugar is plentiful (Van Immerseel *et al.*, 2006). Lactic acid has been shown to exert antibacterial activity against *Salmonella* (Fayol-Messaoudi *et al.*, 2005; De Keersmaecker *et al.*, 2006; Van Coillie *et al.*, 2007) and *Campylobacter* (Cudjoe and Kapperud, 1991; Byrd *et al.*, 2001). Fayol-Messaoudi *et al.* (2005) conducted an experiment in order to distinguish between the killing activity attributable to the presence of lactic acid and that attributable to non-lactic acid molecule. They showed that viable *Salmonella* Typhimurium SL1344 decreased by 3.5 log CFU in MRS broth containing 60 mM D,L-lactic acid, in comparison with unsupplemented incubating medium. The antimicrobial effect is also positively correlated to the lactic acid concentration.

However, when sugars are scarce, all of these bacteria are capable of switching to a fermentation that produces acetate, formate and ethanol. In co-culture assays that we performed in order to select LAB strains with antagonistic effect against *Campylobacter* (Chapter IV, Article V), we showed that *Lactobacillus pentosus* CWBI B78 produced preferentially lactate during the first hours, until glucose is totally consumed. After 24h of *in vitro* culture, the lactate was progressively consumed and acetate was produced, probably because the antagonistic strain shifted hexose pathway to pentose pathway. This facultative heterofermentative scheme can be worthwhile because acetate presents a higher inhibitory potential against *Campylobacter* than lactic acid because of its pKa value (Heres *et al.*, 2004). Many enteropathogens exhibit different susceptibility according to the implicated organic acid. Van Immerseel *et al.* (2003) carried out *in vitro* assays to analyse the effect of formate, acetate, propionate and butyrate on invasion of *Salmonella* Enteritidis in avian intestinal epithelial cells. They observed that pre-incubation of the pathogen with propionate or butyrate resulted in decreased invasion, compared to bacteria preincubated in media supplemented with formate or acetate. However, the bacterial activity of organic acids is pH-dependent, as observed by Chaveerach *et al.* (2002), because pH affects the concentration of undissociated acid formed (Ricke, 2003). It has been assumed that undissociated forms can easily penetrate the lipid membrane of the bacterial cell and, once internalized, dissociate into anions and protons which can damage the cell. Another proposed mechanism is that organic acids could serve as uncouplers that dissipate pH and electrical gradient across cell membranes. The lactic acid produced by lactobacilli was actually shown to act as a permeabilizer of the Gram-negative bacterial outer membrane, allowing other antimicrobial substances produced by the host to penetrate and increasing the susceptibility of pathogens to these antimicrobial molecules (Alakomi *et al.*, 2000). In the chicken gut and in the outer environment, growth conditions of *Lactobacillus* strains, as well as of pathogens, could vary according to the physicochemical and pH conditions or to the season and climate, as suggested by Patrick *et al.* (2004). Production of antimicrobial metabolites providing antagonistic effects against *Salmonella* and *Campylobacter* could consequently fluctuate accordingly. It is noteworthy that enzyme activity is also pH- and temperature-dependent (Duarte *et al.*, 2000), so that extrapolating optimal conditions for the enzymatic and the bacteria metabolic activities is really difficult. As specific quantitative *in vitro* responses do not necessary translate to all *in vivo* possibilities for a particular organism, it is consequently necessary to validate each antagonistic treatment *in vivo*.

The first *in vivo* experiment, which is presented in Chapter V (Article VI), was carried out in order to study the effect of *L. plantarum* CWBI-B659 combined with the xylanase Belfeed B1100MP supplemented to a wheat-based diet on *Salmonella* colonization of broiler chickens. Results of this experiment showed cooperation between the enzyme and the antagonistic strain for reducing pathogen contamination. The combination also increased growth performance in a higher way than each component added alone, in comparison with the infected control birds fed the unsupplemented diet. Such cooperation can be related to the synbiotic concept (Schrezenmeir and de Vrese, 2001), in which probiotic strains and prebiotic substrate are

combined. In synbiotics, the non-digestible ingredient selectively favours the bacterial strain. This prebiotic effect induces increased antimicrobial activity of the probiotic, which results in advantages to the host (Collins and G. R. Gibson, 1999). Many studies have actually pointed out that combining probiotics and prebiotics has a synergistic effect on the fecal microflora of humans and rats (Gmeiner *et al.*, 2000.; Šušković, *et al.*, 2001; Liong and Shah, 2006), by increasing total anaerobes, aerobes, lactobacilli, and bifidobacteria counts, and by reducing negative bacteria, such as clostridia, *E. coli*, and *Enterobacteriaceae* comprising *Salmonella*. In Article VI, the FISH results showed that combining the xylanase with the *Lactobacillus* strain strongly increased the relative proportion of bifidobacteria in regard to total hybridized cells while the relative proportion of lactobacilli, which included the *L. plantarum* CWBI-B659 population, decreased. Assuming that the total hybridized cells increased with diet treatment, the population of lactobacilli in the microflora may be stable, or may increase with the *Lactobacillus*-supplemented and diets (Jin *et al.*, 1998; Lan *et al.*, 2003) but to a lesser extent than the Bifidobacteria population, which can explain the relative proportion reduction in lactobacilli. Concerning experiment performed with poultry, Nisbet *et al.* (1993) used a combination of dietary lactose and competitive exclusion flora to reduce *Salmonella* colonization in chickens, and reported that the combination was more effective in reducing *Salmonella* colonization than lactose or competitive flora alone. On the other hand, Fasina *et al.* (2008) failed to show any beneficial effect from the combination of pectin and a commercially available probiotic on cecal contamination of chickens infected at 4 days of age with 10^6 CFU of *Salmonella* Typhimurium.

Most data available for poultry also reported the effect of different synbiotics on growth performance and intestinal metabolism (Awad *et al.*, 2008; Buteikis *et al.*, 2008). In the study of Buteikis *et al.* (2008), the addition of dietary lactose with *Pediococcus acidilactici* MA 18/5M to a turkey basal diet negatively affected the growth rate of birds, while the probiotic added alone significantly improved body weight and feed utilization until 12 weeks of age. However, the symbiotic had positive effect on natural mortality, in comparison with the control turkeys. By supplementing a combination of *Enterococcus faecium* and a prebiotic derived from chicory rich in inulin to a corn-based diet, Awad and coworkers (2008) demonstrated that the synbiotic significantly improved the daily weight gain at 35 days of age and FCR by 6.1 % and 7.4 % respectively. The combination also improved intestinal morphology of broiler chickens, suggesting increased nutrient absorption. These results for performance and nutrient utilization are similar to the observations detailed in Article VI; but, contrary to these studies, the effect of the combination enzyme-probiotic was reported for infected birds. The daily weight gain was improved in comparison with control infected birds, and FCR was also improved compared to uninfected control chickens.

To our knowledge, no other studies are currently available investigating the effect of synbiotic on performance and nutrient digestibilities of chickens infected with *Salmonella*, in comparison with the probiotic and the prebiotic given alone to infected birds, and in comparison with uninfected birds. However, our challenge model (10^5 or 10^8 CFU/bird) induced a significant deterioration in growth performance in 3-days-old *Salmonella* challenged chickens. This observation is in contrast to the encoutered effect in commercial poultry flocks. Birds are generally asymptomatic carrier of this pathogen. The challenge dose experienced in our experiment is relatively high compared with that observed in commercial production, and this may partly explain the decreased performance. In the experimental design described in Article VI, a high infection dose was necessary because previous tests showed that infecting chicks with less 10^4 CFU/bird induced a rapid decrease of *Salmonella* colonization in the GIT. Alternative challenge models can be used., like the introduction of one infected bird used as seeder into the flock and leaving the chickens to contaminate themselves (Van Immerseel *et al.*,

2005) or the infection of older birds with preliminary antibiotic treatment (Marcq *et al.*, submitted). However, the first method is only applicable if the chickens are reared on litter, leading to cross-contamination through coprophagia. The broiler chickens that were treated with the second method also showed decreased performance, even if of minder extent.

In Article VI, the xylanase was substitute for the prebiotic. As non-digestible oligosaccharides, the enzyme can stimulate the growth and/or activity of bacteria species that are present in the GIT (Hübener *et al.*, 2002), by producing arabinoxylo-oligosaccharides specifically fermentable by some bacterial strains, as well as by releasing entrapped nutrients of the wheat grain, which are consequently more accessible as well for microflora fermentation as for host metabolism. The observed increase in broiler performance could then result from the higher nutrient availability and from beneficial microflora balance. The xylanase may actually favour the growth and the metabolism of *L. plantarum* CWBI-B659, which was shown to metabolize xylose, and many studies have shown the beneficial effect of *Lactobacillus* strains on poultry performance (Jin *et al.*, 1998; Lan *et al.*, 2003). Modification of bacterial populations in the caeca was actually observed in Article VI by Fluorescent In Situ Hybridization. Relative proportion, in regard to total hybridized cells of microbial communities, of bifidobacteria was especially increased with the xylanase alone or in combination. Courtin *et al.* (2008) actually showed that arabinoxylooligosaccharides increase the number of bifidobacteria in the caeca of broilers. As the probiotic strain, bifidobacteria may be able to decrease the population of *Salmonella*. Indeed, bifidobacteria are known to stimulate butyric acid production in the caeca (Belenguer *et al.*, 2006) which have a detrimental effect on pathogen colonization in young chickens. To a lesser extent, relative proportion of enterococci was also increased with enzyme supplementation, as observed by Hübener *et al.* (2002) who showed that they are able to ferment arabinoxylans. According to Engberg *et al.* (2004), development of enterococci may lead to an increase of fatty acids production and a reduction of pH values, which may inhibit the growth of gram-negative pathogen bacteria such as *Salmonella*.

In Article VI, supplementing the wheat-based diet with a *Lactobacillus* strain, in combination with a xylanase, decreased the *Salmonella* concentration in the caecal contents by 1.85 log CFU/g. The results presented in Article V also showed that a *Lactobacillus* strain, in combination with a cellulase, was able to reduce the *Campylobacter* concentration to 0 CFU *in vitro*. Of course, it is difficult to extrapolate what could be the *in vivo* efficiency of this bacterial strain from *in vitro* results. The European Regulation (EC) No 646/2007 specifies that the maximum percentage of broiler flocks remaining positive of *S.* Enteritidis and *S.* Typhimurium should be reduced to 1 % maximum or less by December 31, 2011 (Official Journal of the European Union, L 151). Low pathogen concentration in the GIT of live birds could not actually pose problem if the different steps of the slaughter process are implemented according to strict hygiene measures in order to reduce pathogen level from the live broiler to the final carcasses, as shown for *Salmonella* and *Campylobacter* by McCrea *et al.* (2006). Indeed, according to Regulation (EC) No 2160/2003, the absence of *Salmonella* must be only certified in 25 grams of fresh poultry meat from December 2010, to authorize the placing on the market for human consumption. In order to reduce the pathogen prevalence in broiler flocks to sufficiently low level, the only effective intervention strategy seems to be an integrated approach with multiple control measures along the poultry production chain. This purpose could be carried out by developing HACCP plans based on risk-assessment.

Future prospects

Studying in vivo the efficiency of the Lactobacillus pentosus CWBI B78 in combination with a cellulase against Campylobacter

The *in vitro* co-cultures experiments described in Article V provided information on the antagonistic potential of LAB strains in combination with a cellulase against *Campylobacter*. The next step is to evaluate the interest of this enzyme-bacterial strain formulation *in vivo*. Within the context of *Campylobacter* infection of free-range broiler flocks, the easier way to reduce bird colonization may be the incorporation of antimicrobial components in the broiler diet. By experimentally inoculating birds with the pathogen and by feeding them with a diet supplemented with the enzyme-bacteria formulation, we could directly compare the treatment effect on digestive, microbiological, and physiological parameters in the GIT. However, the use of feed additives is subjected to strict European legislations, with financial implications. Moreover, it was shown in Article III that the access to an open-air range could be associated with a higher *Campylobacter* prevalence. We have consequently decided to develop an environmental treatment of the open-air range and of the litter, based on the enzyme-bacterial strain formulation. Such treatment, which will be spread into the environment at regular frequency, could also be ingested by the broilers. In this case, the treatment impact in the GIT should be investigated through toxicologic studies. However, this hypothesis should be firstly proved or invalidated with the methodes explained in the following paragraph called "Determining LAB populations and enzyme activities in the environment". We have to take into account the fact that the antibacterial effect of the LAB strains that were selected in co-culture assays could also be influenced by the complex environment of the open-air range. The physico-chemical conditions and the microflora of the soil might especially influence the activity and the survival of the antagonistic strain and of the pathogen. Studies of such potential effect could be carried out according to the co-culture methods described in the following paragraph ("Identifying antimicrobial metabolites from LAB strains"), by using different soil types in the culture broth composition.

Identifying antimicrobial metabolites from LAB strains

The mechanism(s) of action of antagonistic strains on enteropathogens remain(s) to be clarified. This antagonism may result from the combined action of several factors, like the production of antimicrobial metabolites, or the competitive exclusion of the pathogen (Servin, 2004). The results of Article V suggested that *Campylobacter* reduction in the co-culture experiments could be related to the production of organic acids by *Lactobacillus pentosus* CWBI-B78 and *Enterococcus faecium* THT. Other molecules released by LAB may exert an antibacterial effect against pathogens, and their production is dependent on environmental conditions. Co-culture experiments, like those presented in Article V, could be used in order to quantify metabolite production in the broth medium, with or without polysaccharidase. Diacetyl, which is produced from the bacterial metabolism, can present some antagonistic activity against various pathogens (Jay, 1982; Lanciotti *et al.*, 2003). After being extracted from the co-culture medium on a *Solid Phase Extraction* column, diacetyl can be measured with GC-FID or GC-MS (Pejin *et al.*, 2006). Moreover, hydrogen peroxide H_2O_2 was also shown to have antimicrobial properties

(Batdorj *et al*., 2007; Pridmore *et al*., 2008) and its production can be quantify by colorimetric measurement, as explained by Marty-Teysset *et al.* (2000). Lactobacillus strains were also shown to produce bacteriocins (Sanni *et al*., 1999). These are ribosomally synthesized bioactive peptides or peptides complexes which have a bactericidal or bacteriostatic effect on other species. Stern *et al.* (2006) showed that bacteriocins from Lactobacillus strains can exert antagonistic activities against Campylobacter. Identification of potential bacteriocins in our Lactobacillus strains can be carried out by the Bio-industry Unit of the GxABT by means of molecular techniques.

Determining LAB populations and enzyme activities in the environment

In order to complement our data about enzyme-LAB strains formulation, as feed additive or as environmental treatment, it seems necessary to evaluate the evolution and the persistence of antagonistic strains, as well as the enzyme impact on these parameters, in the GIT and in the open-air range soil.

Classical microbiological techniques can not specifically differentiate one strain from other members of the same genus, so that a molecular approach could be investigated. As described by Brehm-Stecher *et al.* (2005), specific 16S rRNA-targeted molecular probes may be designed. 16S and 23S rDNA are frequently used for establishing bacterial phylogenetic classification and the 16S rDNA gene is the most often used gene for the study of bacterial populations (Huybens *et al.*, 2009). The molecular probe should label specifically the LAB strains selected in this thesis. Two probes have then to be designed: one targetting the 16S rDNA of the *Lactobacillus* genus and one targeting the specific gene of *L. plantarum* that express enzyme for xylose degradation. Specificity and sensitivity can be determined with *in silico* assays on DNA from different bacteria potentially present in the environment (Brehm-Stecher *et al.*, 2005). According to the hybridization technique applied, the probe may be labelled with biotin, which is revealed by enzymatic reaction (Leary *et al.*, 1983), or with a fluorochrome, which can be detected by immunocytochemical method (Serke and Pachmann, 1988), or by direct fluorescence (Schwarzacher and Heslop-Harrison, 1994). Bacterial identification can also be performed by Flurorescent *in situ* hybridization, which is a rapid, sensitive, and highly specific nucleic acid-based method. Fluorescently labelled nucleic acid probes complementary to genus- or species-specific rRNA sequences ara hybridized to whole bacterial cells, resulting in the selective staining of target cells (Moter and Gobel, 2000; Brehm-Stecher *et al.*, 2005). Tagged strain can also be constructed with the **GFP** (green fluorescent protein [GFP]) gene, as explained by Geoffroy *et al.* (2000). The advantage is that strains can be tagged before the injection into the environment (GIT or broiler house environment), representing a highly specific method. However, we must not forget that genetic engineering poses ethical problems which have to be taken into account.

The enzyme impact in the GIT or in the environment can be evaluated by measuring enzymatic activities. Some studies have estimated xylanase activity in the chicken gut (Dänicke *et al.*, 1997; Silva and Smithard, 2002; Billena *et al.*, 2008), and showed that the enzyme remains active in the small intestine. Estimation of enzyme activity in chicken digesta can be performed by several methods. In the study by Dänicke *et al.* (1997), digesta measurements were based on agar diffusion tests, with agar medium containing oat spent xylan. Silva and Smithard (2002) determined enzyme activity colorimetrically against buffer blank, using blue xylan (4-O-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue) as a substrate.The remaining

xylanase activity compared to the respective activity in diet can be calculated by employing the relations of xylanase:marker (titanium dioxide) in digesta and food, respectively (Dänicke et Smithard, 1997). Microbial xylanase activities produced in the digestible tract are detectable wether or not exogenous enzyme was supplied (Dänicke *et al.*, 1999) and need to be substracted from activity values measured in the gut. Similar colorimetric methods may be applied for the cellulase described in Article V with appropriate substrate, while taking into account that this enzymatic system contains three glycohydrolase activities (endoglucanase, cellobiohydrolase, and β-glucosidase).

Analysing the bacterial polysaccharide-degrading gene expression

The main aim of this work was to favour the growth and the metabolism of a specific bacterial strain to the detriment of pathogen strains. One apparent strategy for bacteria success in the ecosystem is to become proficient at using highly available energy-supplying susbstrate, which are mainly polysaccharides and derived polymers. In the intestinal environment, the bacterial strain should be able to use the dietary polysaccharides that the host consumes.

Results of Article V (Chapter IV) pointed out the antagonistic activity of *L. pentosus* CWBI B-78 against *Campylobacter in vitro*, even in the absence of celullase. In order to elucidate the mechanisms that are involved in this inhibition process, determining which susbstrate is used by this bacterial strain is an important parameter which could be evaluated by studying its bacterial transcriptome. The transcriptome is the set of all RNA molecules produced in one or a population of cells. Because it includes all mRNA transcripts in the cell, the transcriptome reflects the genes that are being actively expressed at any given time. Expression of gene coding for specific polysaccharide-degrading enzyme could give a global picture of the bacterial carbon metabolism. Such information can also be useful at selecting appropriate substrate in order to improve the bacterial vying for limited resources. Screening the transcriptome information of varied potential antagonistic strains should help to select interesting bacteria for similar applications as those described in this thesis. However, the different polysaccharidase activities that are expressed by bacteria are generally low and limited, so that supplying exogenous enzyme activity is probably necessary for complementary actions.

Research could also be directed towards other bacterial strains able to metabolize very specific polymers as a carbon source which can not be used by pathogens. The paper from Hehemann *et al.* (2010) described sulphated polysaccharides from seaweeds, called porphyrans, which can be metabolized by marine heterotrophic bacteria that produce specific carbohydrate-active enzymes. It could be interesting to supplement the broiler diet with seaweeds and the such heterotrophic bacteria in order to study the competitive advantage to the latter.

Feasibility studies and practical implementation

Feed additives like probiotics and enzymes, are subjected to strict European legislations. To be legally placed on the market and used, feed additives must especially be proved to have no harmful effect on animal health, human health or the environment. Therefore, additional toxicological studies are needed in order to validate the feed additive containing the *L. plantarum* strain and the Belfeed B1100MP, which was developed during this thesis. Furthermore, improving the production process and the packaging is necessary to give an economically sustainable feed additive to the producer and to reduce the production cost per

broiler. Production costs have been already optimized but the *L. plantarum* stability could still be improved with different processes (cryoprotectors, osmotic or temperature stress) for long term conservation at room temperature.

On the other hand, environmental treatment with bacterial strains and enzymes does not fall within the scope of the European rules for feed additives, and consequently could avoid the costs inherent to these autorisation procedures, if it can be proved that the product is not ingested by the birds. The product combining a *L. pentosus* and a cellulase, which was developed in this work in order to reduce *Campylobacter* prevalence in free-range broiler flocks, should be further studied for economical matters. Optimising the culture medium for scale-up production can be carried out using “corn steep”, which is a residual product of starch industry supplying carbohydrates, vitamins, nitrogen and minerals. Handling conditions of such environmental treatment have also to be clearly defined for the farmer. Previous *in vitro* tests showed that the *L. pentosus* strain is able to survive at least one week at sufficient levels in meadow and broiler litter. Thus, a treatment frequency of one week seems to be acceptable. The easier conditioning for treating the open-air range and the broiler litter would be solid form as powder. The atomisation process for strain protection and optimal survival has to be consequently further studied.

Conclusion

From the research presented in this PhD thesis, we can conclude that the antimicrobial effect of LAB strains can be enhanced by combination with a polysaccharidase-type enzyme, as shown *in vitro* against *Campylobacter* and *in vivo* against *Salmonella*. *Lactobacillus plantarum* CWBI-B659 was shown to reduce *Salmonella* colonization of broiler chickens when it was supplemented to wheat-based diet simultaneously with a xylanase. Previous *in vivo* experiments demonstrated that this xylanase was able to improve growth performance of birds. This advantage was probably related to the release of entrapped nutrients of the wheat grain by xylanase activity, which results in higher diet digestibility for the host but also in increased fermentation of undigestible nutrients by the microflora and by the *Lactobacillus* strain. Within the context of the reduction of *Campylobacter* colonization in broiler chickens, *in vitro* co-cultures assays were developed, by using a broth medium, based on dehydrated poultry excreta and straw. These studies demonstrated that *Lactobacillus pentosus* CWBI-B78, as well as *Enterococcus faecium* THT, exerted an antibacterial effect on *Campylobacter jejuni*, which was enhanced by adjunction of a cellulase. The inhibitory mechanism seemed to be based on the ability of LAB to produce organic acids. Regarding to these results, further research is required in order to study the efficiency *in vivo* of the antagonistic strain-cellulase formulation against *Campylobacter*. However, the *in vivo* efficiency of treatments combining antagonistic strain and polysaccharidases could probably never decrease pathogen colonization of broiler chickens to zero level by itself, but could reach this purpose when combining them with supplementary measures like sanitary barriers, organic acids and passive immunity for example. Of course, it is necessary to develop strategies combination that is financially viable for the farmers. Furthermore, in order to match the limits which are setted by the European legislation from 2010 for their absence in 25g of raw meat, the only effective intervention strategy seems to be an integrated approach with multiple control measures along the poultry production chain.

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