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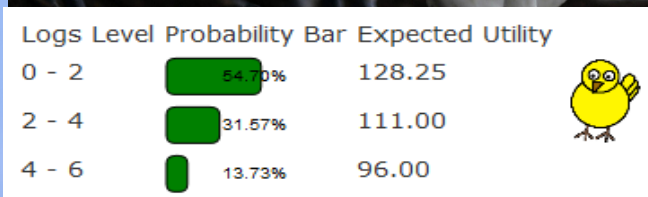
Campylobacter vaccination of poultry: Clinical trials, quantitative microbiological methods and decision support tools for the control of *Campylobacter* in poultry



Ana Belén García Clavero
PhD Thesis
2013

DTU Food
National Food Institute

Campylobacter vaccination of poultry: Clinical trials, quantitative microbiological methods and decision support tools for the control of *Campylobacter* in poultry

Ph.D. Thesis

Ana Belén García Clavero

2013

National Food Institute, Technical University of Denmark

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Preface

The work presented in this PhD thesis was carried out from August 2010 to August 2013 at the National Food Institute, DTU FOOD, Technical University of Denmark.

This PhD forms part of the research project CamVac (contract 09-067131) funded by the Danish Council of Strategic Research (Det Strategiske Forskningsråd)

The supervisors for this PhD project were Håkan Vigre (Division of Epidemiology and Microbial Genomics, National Food Institute, DTU FOOD, Denmark), Anders Madsen (HUGIN EXPERT A/S, Denmark & Department of Computer Science, Aalborg University, Denmark) and Laurids Siig Christensen (Division of Epidemiology and Microbial Genomics, National Food Institute, DTU FOOD, Denmark)

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I dedicate this thesis to all the beautiful souls (too many to mention them individually) who have accompanied me in this intriguing and exciting life journey, who have been with me through laughs and tears, through happiness and sorrows, sharing good moments and hardships, who offered me love, friendship and wisdom.

A very special loving mention to my grandmothers, Francisca and Maria, my dad Angel, my adoptive mum Rose Mary and my beautiful Persian cat Totti, all of them gifts from God, may their lovely souls rest in peace, without them, I am not sure I would have survived the adversities of life. A sweet thanks to all the beautiful pets who gave me unique unconditional love and companionship.

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"Feeling our own pain is human, feeling compassion for others is Divine; we cannot survive without love and compassion". "The power of knowledge and words is infinite; we should always use the power of knowledge and words to seek truth and love." **Ana Belen Garcia Clavero**

"Our prime purpose in this life is to help others. And if you can't help them, at least don't hurt them." "All suffering is caused by ignorance. People inflict pain on others in the selfish pursuit of their own happiness or satisfaction.""I find hope in the darkest of days, and focus in the brightest. I do not judge the universe." **Dalai Lama**

"Science is not only compatible with spirituality; it is a profound source of spirituality." **Carl Sagan**

Summary

Human campylobacteriosis represents an important public health problem and poultry has been identified as a significant source for human infections with *Campylobacter*. Nowadays, the implementation of effective controls to reduce the burden of disease in humans is considered a priority in many areas of the world. Consequently, the control of *Campylobacter* in poultry seems crucial for the reduction of human campylobacteriosis cases; this fact represents the fundamental inspiration for this PhD thesis. The term "poultry" is used in the title and throughout this PhD thesis as a synonym of chickens and broilers athough this term can refer to other birds bred for the production of meat or eggs. It was preferred to use the term poultry because some of the knowledge, information and/or reseach included in this thesis might refer to or could be applied to birds other than broilers.

The research presented in this thesis belongs to a larger project on vaccination of poultry against *Campylobacter*, the CamVac project, funded by the Danish Council of Strategic Research (Det Strategiske Forskningsråd). The overall aim of this research project is to support a cost-effective vaccination strategy able to reduce the numbers of *Campylobacter* in infected chickens which in turn will translate in a decrease of the numbers of *Campylobacter* in poultry products and a reduction in the associated public health risks to the consumers. The aim of my PhD research is to explore, investigate, research and/or develop different aspects related to the effect of vaccination strategies against *Campylobacter* in poultry.

In the beginning, a general and critical review of control strategies against *Campylobacter* in poultry production from farm to fork and public health implications is presented in this thesis. A variety of potential *Campylobacter* control measures are discussed with emphasis on vaccination strategies against *Campylobacter* and other zoonotic pathogens in poultry. In addition, information on *Campylobacter* risk assessments and the evaluation of the potential public health impact of controls against *Campylobacter* in poultry production are included.

Next, this PhD thesis presents results from an experimental inoculation and vaccination trial conducted for the investigation into *Campylobacter* colonization of poultry and for the assessment of the effectiveness of a *Campylobacter* vaccine candidate based on the protein ACE393 (the most promising candidate obtained in previous studies). Moreover, critical reflections related to the design of clinical vaccination trials and adequate data analyses are presented. The potential effect of the vaccine candidate ACE393 in poultry is assessed in this research based on the enumeration of

Campylobacter in chicken samples using different methods. Accurate and reliable quantitative microbiological data are crucial for quantitative risk assessment models and for the evaluation of the effectiveness of food safety control measures e.g. vaccination strategies. Therefore, conducted investigations related to molecular quantitative microbiological methods for the accurate, fast, direct and reliable enumeration of *Campylobacter* present in poultry fecal material are presented and discussed in this thesis.

The final part of this thesis describes and discusses the development and application of mathematical models and decision support systems that can integrate current knowledge to aid poultry producers in decision making regarding potential investments for the implementation of *Campylobacter* control strategies in poultry production.

Results from our inoculation and vaccination experiments based on a nested fixed block design and including 290 broilers indicate that the observed differences between vaccinated and placebo groups related to *Campylobacter* numbers could be attributed to "non-vaccine related" variation between birds within the same group and between groups. It is concluded that there is no statistically significant effect of the vaccine ACE 393 in broilers in this clinical trial under the experimental conditions applied. Despite years of extensive research, the availability of a cost-effective commercial vaccine against *Campylobacter* in poultry remains a major goal.

There seems to be no international consensus regarding the most appropriate sampling protocol to obtain accurate *Campylobacter* quantitative data from poultry flocks. The sampling protocol (including methods, sample size, sample origin, sample matrix, time of sampling and other aspects) will influence the quantitative microbiological data collected and data analyses results. Several of these aspects related to sampling protocols are explored further in this PhD work. A comparison of the main quantitative microbiological methods used in food safety (traditional culture and real-time PCR) is presented in this thesis. Although chicken faecal samples represent complex matrices for the quantification of *Campylobacter*, poultry faeces are often the sample of choice for the routine *Campylobacter* testing of poultry flocks. In this research, several DNA extraction methods are evaluated for *Campylobacter* DNA direct quantification (without enrichment) using real-time PCR and spiked chicken faecal samples. Subsequently, the DNA extraction methods Easy-DNA and MiniMAG are selected to quantify directly (without the use of enrichment) *Campylobacter* present in naturally infected chicken faecal samples. Results indicate that there are no statistically significant differences between culture and real-time PCR in these experiments. Results from

statistical analyses of *Campylobacter* quantitative data obtained from colonization studies and vaccination clinical trials show high variability between chickens in relation to the numbers of *Campylobacter* in individual chickens suggesting that individual factors may affect *Campylobacter* dynamics in poultry flocks. Remarkably, a significant correlation is observed between faecal and caecal *Campylobacter* concentrations at slaughter suggesting that *Campylobacter* counts from faecal samples at slaughter might be a good indicator of *Campylobacter* concentration in the caecum of slaughter chickens and supporting recommendations made related to the sampling of chickens closer to slaughter time.

Considering the relatively low profit margins in poultry production, *Campylobacter* control strategies that can be tested and/or applied at low cost are generally accepted but controls that require efforts and/or extra costs might not be welcome by poultry producers. For this reason, proposed controls should be backed up with strong evidence of effectiveness and a satisfactory viable cost-benefit balance. Mathematical models may provide poultry producers with valuable information related to the effectiveness of potential public health control strategies and the associated cost-benefit analyses. Even more, hypothetical controls such as the use of a commercial *Campylobacter* vaccine can be included in mathematical models. The flexibility of the mathematical models developed for this PhD thesis allows for the assessment of several *Campylobacter* control strategies in poultry and their potentially synergistic combinations. The models presented here integrate knowledge related to epidemiological, microbiological and financial factors for the control of *Campylobacter* in poultry. The selection of epidemiological and microbiological variables for model development can be complex. Challenges related to the selection of variables and quantitative data to be included in the models are discussed in this thesis. Results from the models include posterior probability distributions related to expected *Campylobacter* numbers in the flock (in logs) before and after the implementation of the decision/s and the expected cost-reward balance associated with each decision/s. Ideally, *Campylobacter* controls in poultry production should be cost-effective, reliable, easy to implement, easy to maintain and accepted by consumers. Consumers' preferences will influence the type of products available in the market. Socio-economic aspects are therefore crucial for the implementation of effective *Campylobacter* controls and are considered in this thesis.

The work presented in this PhD thesis provides an extensive review on *Campylobacter* controls along the food chain. Some of these *Campylobacter* control strategies could be integrated following

a “farm to fork” approach in mathematical models in future studies. The critical reflections related to the design of clinical vaccination trials and adequate data analyses presented here may prove useful for other researchers. Results obtained from the colonization study and vaccination clinical trial shed some light on complex issues related to microbiological sampling protocols in poultry and in relation to the assessment of vaccine effectiveness. Moreover, adequate *Campylobacter* testing of faecal samples from chickens just before slaughter will support producers in the implementation of relevant *Campylobacter* control strategies to reduce *Campylobacter* contamination of chicken products and accordingly decrease associated public health risks. The mathematical models developed and presented here may assist the poultry industry in the implementation of cost-effective *Campylobacter* control strategies under conditions of uncertainty. Even more, results from the mathematical models developed in this thesis could be integrated in risk assessment models in order to assess the public health impact of *Campylobacter* controls in poultry in terms of expected reduction of human campylobacteriosis cases. Hence, the food industry, scientists, researchers, government agencies and the society as a whole may benefit from the work presented in this PhD thesis related to the control of *Campylobacter* in poultry.

Sammendrag (Summary in Danish)

Infektioner med *Campylobacter*, campylobacteriose, hos mennesker udgør et betydeligt folkesundhedsproblem og fjerkræ er blevet identificeret som en væsentlig smittekilde. Implementering af bekæmpelsesstrategier for at mindske byrden af infektionssygdomme hos mennesker er i dag højt prioriteret i adskillige lande. For så vidt angår campylobacteriose hos mennesker anses bekæmpelse af *Campylobacter* i fjerkræ for at være afgørende for reduktion af antal tilfælde: denne antagelse udgør den grundlæggende inspiration for Ph.d. afhandlingen.

Forskningsarbejdet præsenteret i afhandlingen hører under et større projekt, der vedrører mulighederne for at udvikle en vaccine til fjerkræ mod *Campylobacter* (the CamVac projekt, 2010-2014). Projektet finansieres af det Strategiske Forskningsråd i Danmark. Hovedformålet med projektet er at støtte udviklingen af en omkostningseffektiv vaccinationsstrategi til fjerkræproduktionen for at reducere antallet af *Campylobacter* i inficerede kyllinger. En sådan reduktion antages at kunne afspejle sig i et fald i antallet af *Campylobacter* i fjerkræprodukter og dermed en reduktion af de tilknyttede sundhedsrisici for konsumenter. Formålet med min ph.d. forskning er at udforske, undersøge og udvikle vaccinationsstrategier over for *Campylobacter* i fjerkræ.

Ph.d. afhandlingen indledes med en generel og kritisk gennemgang af bekæmpelsesstrategier mod *Campylobacter* i fjerkræproduktionen fra jord til bord og konsekvenser for folkesundheden. Forskellige mulige bekæmpelsesstrategier mod *Campylobacter* diskuteres med vægt på vaccinationsstrategier mod *Campylobacter* og andre zoonotiske patogener i fjerkræ. Derudover diskuteres mulige folkesundhedsmæssige følger af bekæmpelsesstrategier mod *Campylobacter* i fjerkræproduktion.

Herefter præsenteres resultater fra et eksperimentelt podnings- og vaccinationsforsøg udført for at undersøge på hvilken måde *Campylobacter* kolonisering i fjerkræ påvirkes ved vaccinering med en *Campylobacter* vaccine kandidat baseret på proteinet ACE393 (selvsamme er i tidligere studier fundet at være den mest lovende kandidat). Desuden, på bagrund af forsøget diskuteres forskellige kritiske forhold omkring samspillet mellem forsøgsdesign, gennemførsel og analyse af vaccinationsforsøg generelt. Med udgangspunkt i forsøget foretages en kritisk gennemgang af udformningen af kliniske vaccinationsforsøg og fyldestgørende data analyser herom. Den

potentielle effekt af vaccinen måles ved brug af forskellige kvantificeringsmetoder for forekomst af *Campylobacter*. Nøjagtige og pålidelige kvantitative mikrobiologiske data er afgørende for kvantitative risikovurderingsmodeller og for evalueringen af effekten af bekæmpelsesstrategier i forhold til fødevaresikkerhed f.eks. vaccinationsstrategier. Følgelig præsenteres og diskuteres undersøgelser, relateret til molekylære kvantitative mikrobiologiske metoder for direkte, præcis, hurtig og pålidelig tælling af *Campylobacter* i fækalmateriale fra fjerkræ, i afhandlingen.

Den sidste del af afhandlingen beskriver og diskuterer udvikling og anvendelse af matematiske modeller som beslutningsværktøj, der kan integrere nuværende viden, for beslutningstager. Hensigten med beslutningsværktøjet er at hjælpe fjerkræproducenter i beslutningsprocessen i forhold til investering i bekæmpelsesstrategier mod *Campylobacter* i fjerkræproduktionen.

Resultater fra vores eksperimentelle podnings- og vaccinationsforsøg er baseret på et hierarkisk fastsat blok design med i alt 290 slagtekyllinger og indikerer, at de observerede forskelle i antal *Campylobacter* i tarmkanalen mellem vaccinerede og placebogruppen kan tilskrives "ikke – vaccine relaterede" variationer mellem individer og mellem isolater. Konklusionen er derfor, at der ikke er nogen statistisk signifikant virkning af vaccinen ACE 393 i slagtekyllinger i forhold til forsøgets forudsætninger. Trods flere års omfattende forgæves forskning, forbliver udviklingen af en omkostningseffektiv kommerciel vaccine mod *Campylobacter* i fjerkræ fortsat et vigtigt mål.

Der ser ikke ud til at være international konsensus om en bedst egnet prøvetagningsprotokol for nøjagtig kvantificering af *Campylobacter* i fjerkræflokke. Prøvetagningsprotokollen, herunder valg af metode, stikprøvestørrelsen, prøve oprindelse, prøvematrix samt tidspunkt for prøvetagning, vil have indflydelse på laboratorieresultater og på efterfølgende data analyser og konklusioner. Adskillige af disse aspekter er undersøgt nærmere i ph.d. arbejdet, deriblandt en sammenligning mellem de to væsentligste kvantitative mikrobiologiske metoder, der anvendes inden for fødevaresikkerhed (traditionel dyrkning og real-time PCR). Selvom gødningsprøver fra kyllinger udgør en kompleks matrix til kvantificering af *Campylobacter*, er det hyppigt fæces der vælges som prøvemateriale til rutinemæssig laboratorieundersøgelse. Ph.d. arbejdet omfatter tillige evaluering af forskellige DNA ekstraktionsmetoder, der kan bruges i forbindelse med direkte kvantificering (uden præopformering) ved hjælp af real -time PCR og spikede gødningsprøver fra kyllinger. Efterfølgende er DNA ekstraktionsmetoderne Easy- DNA og MiniMAG valgt til direkte kvantificering (uden brug af berigelse) af *Campylobacter* i gødningsprøver fra naturligt inficerede

kyllinger. Resultaterne viser at der ikke er statistisk signifikant forskel mellem dyrkning og real-time PCR i disse eksperimenter.

På basis af de kvantitative data for forekomst af *Campylobacter* i tarmkanalen hos inficerede kyllinger, fra det eksperimentelle podnings- og vaccinationsforsøget, påvises store variationer mellem kyllinger i samme isolator (flok) i forhold til antallet af *Campylobacter*, hvilket tyder på at individuelle faktorer muligvis påvirker dynamikken af spredningen af *Campylobacter* i en fjerkræflok. Data viser yderligere en signifikant sammenhæng mellem antallet af *Campylobacter* i gødningsprøver udtaget fra kloak samt blindtarm på slagtetidspunktet. Kvantificering af *Campylobacter* i gødningsprøver tæt på slagtning kan muligvis være en god indikator for antallet af *Campylobacter* i blindtarmen hos slagtekyllinger. Fundet understøtter nuværende anbefalinger, der tilråder prøveudtagning fra kyllinger tæt på slagtetidspunktet. I betragtning af det relativt lave dækningsbidrag per kylling i fjerkræproduktionen, vil fjerkræproducenter i højere grad være positivt indstillet overfor bekæmpelsesstrategier mod *Campylobacter*, der kræver begrænsede investeringer, i forhold til tilsvarende strategier der kræver større investeringer. Af samme grund bør bekæmpelsesstrategier understøttes af stærke beviser for effektivitet og fyldestgørende analyser for omkostningsgevinst. Matematiske modeller for spredning og kontrol af *Campylobacter* kan give fjerkræproducenter værdifulde oplysninger om effektiviteten af mulige sundhedsmæssige bekæmpelsesstrategier samt dertil hørende omkostningsgevinst analyse. Endvidere kan disse modeller efterligne effekten af hypotetiske bekæmpelsesstrategier, såsom anvendelse af en kommerciel *Campylobacter* vaccine. Fleksibiliteten i de matematiske modeller udviklet i forbindelse med ph.d. afhandlingen gør det muligt at vurdere flere bekæmpelsesstrategier mod *Campylobacter* i fjerkræ samt deres potentielt synergetiske kombinationer. Modellerne, der blev udviklet, integrerer viden om epidemiologiske, mikrobiologiske og økonomiske faktorer tilknyttet bekæmpelse af *Campylobacter* i fjerkræ. Selektionen af epidemiologiske og mikrobiologiske variabler samt kvantitative data i forbindelse med udvikling af modellen er kompleks, og udfordringer heri diskuteres i afhandlingen. Resultater fra modellerne omfatter posterior sandsynlighedsfordelinger for forventede antal *Campylobacter* (log CFU/gram) i en slagtekyllingeflok før og efter implementering af en bekæmpelsesstrategi (er), samt de forventede omkostninger og gevinster forbundet med hver strategi. Ideelt set bør bekæmpelsesstrategier mod *Campylobacter* i fjerkræproduktionen være omkostningseffektive, pålidelige, let at implementere, let at vedligeholde og accepteret af forbrugerne. Forbrugernes præferencer påvirker produkttyper tilgængelige på markedet. Socio -økonomiske aspekter er derfor afgørende for gennemførelse af

effektive bekæmpelsesstrategier mod *Campylobacter*, og overvejelser herom gennemgås i afhandlingen.

Afhandling giver en omfattende gennemgang af bekæmpelsesstrategier for *Campylobacter* i hele fødevarekæden, hvoraf flere kan integreres efter et "jord til bord"-princip i fremtidige studier af matematiske modeller. De kritiske refleksioner omkring design af vaccinationsforsøget under produktionslignende forhold, samt efterfølgende fyldestgørende analyse af data, der præsenteres i afhandlingen kan vise sig nyttige for andre forskere. Resultaterne fra det eksperimentelle podnings- og vaccinationsforsøget kastede lys over komplekse problemstillinger i forhold til mikrobiologiske prøveudtagningsprotokoller hos fjerkræ og i forhold til vurderingen af vaccine effektivitet.

En adækvat undersøgelse for *Campylobacter* i gødningsprøver fra kyllinger lige før slagtning vil støtte producenters beslutning for implementering af relevante bekæmpelsesstrategier mod *Campylobacter* og dermed mindske tilknyttede risici for folkesundheden. De matematiske modeller udviklet og præsenteret her, kan muligvis hjælpe fjerkræproducenter med at vurdere omkostningseffektivitet af forskellige bekæmpelsesstrategier, når vedkommende skal tage beslutning om implementering uden at have kendskab til flokkens *Campylobacter* status. Resultaterne fra de matematiske modeller, der blev udviklet i denne afhandling, kan højst sandsynlig integreres i risikovurderingsmodeller, der vurderer den offentlige sundhedsmæssige virkning af bekæmpelsesstrategier mod *Campylobacter* i fjerkræ, i form af forventet reduktion af menneskelige campylobacteriose tilfælde. Derfor kan fødevareindustrien; videnskabsfolk og forskere; offentlige institutioner og samfundet som helhed drage fordel af arbejdet, der præsenteres i ph.d. afhandlingen i forhold til kontrol af *Campylobacter* i fjerkræ.

List of abbreviations

AE: amplification efficiency

ASC: antibody secreting cells

C. jejuni: *Campylobacter jejuni*

C. lari: Campylobacter lari

C. upsaliensis: Campylobacter upsaliensis

C.coli: Campylobacter coli

C.I.: Confidence Interval

CamVac: Campylobacter Vaccination project

CFU: Colony Forming Units

CPS: polysaccharide capsule

Ct: threshold cycle

CWC: *Campylobacter* whole-cell

CWF: Compassion in World Farming

DAG: directed acyclic graph

Danish Kroners (DKK)

DANMAP: Danish Integrated Antimicrobial Resistance Monitoring and Research Program

EC: European Commission

EFSA: The European Food Safety Authority

EU: European Union

European Centre for Disease Prevention and Control [ECDC],

FAO: Food and Agriculture Organization

FSAI: Food Safety Authority of Ireland

FSC: Food Safety Criteria

GBS: Guillain-Barré Syndrome

GHP: Good Hygiene Practices

HACCP: Hazard Analysis and Critical Control Points

IAC: Internal Amplification Control

ICC: intra-cluster correlation coefficients

ICGFI: International Consultative Group on Food Irradiation

ICMSF: International Commission on Microbiological Specifications for Foods

ICTs: Information and Communication Technologies

IFR: Institute of Food Research

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

ISO: International standards

KGy: KiloGray

KM: Knowledge Management

LAB: lactic acid bacteria

mCCDA : modified charcoal cefoperazone deoxycholate agar

MLST: Multi Locus Sequence Typing

OR_s: Odds Ratios

PGMs: probabilistic graphical models

QMRA: quantitative microbiological risk assessment

R: regression parameter

ReA: reactive arthritis

ST: Sequence Type

UK: United Kingdom

USDA: United States Department of Agriculture

UV: Ultra Violet

VBNC: viable but non-culturable

WHO: World Health Organization

£: British Pound

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Manuscript I
Garcia AB, Bahrndorff S, Hald B, Hoorfar J, Madsen M, Vigre H. 2012. Design and data analysis of experimental trials to test vaccine candidates against zoonotic pathogens in animals: the case of a clinical trial against Campylobacter in broilers. Expert Rev. Vaccines 11(10): 1179-1188.

Manuscript II
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2. OUTLINE

This thesis' content is presented in four main chapters:

- Chapter 5 *Campylobacter*: public health aspects and control strategies

This Chapter includes background information on *Campylobacter*, human campylobacteriosis, *Campylobacter* sources and the epidemiology of *Campylobacter* in poultry. A review on *Campylobacter* control strategies that can be implemented in poultry production from farm to fork is presented. A variety of potential *Campylobacter* control strategies are considered with emphasis on vaccination against *Campylobacter* and other zoonotic pathogens in poultry. In addition, this Chapter includes background information on *Campylobacter* risk assessments and the evaluation of the potential public health impact of controls against *Campylobacter* in poultry production.

- Chapter 6 Campylobacter vaccination trials (manuscripts I and II)

Chapter 6 presents research conducted as part of the CamVac project such as vaccination clinical trials in chickens and investigations into sampling protocols to obtain accurate *Campylobacter* quantitative data. Accurate and reliable quantitative microbiological data are crucial for quantitative risk assessment models and for the evaluation of the effectiveness of food safety control measures. The sampling protocol (methods, sample size, sample origin, time of sampling and other aspects) will influence the quantitative microbiological data and data analyses results. Some of these sampling issues are explored further in this Chapter. Experimental infections and vaccination trials were conducted based on a nested fixed block design (no blinding) to test a Campylobacter vaccine candidate. The design of the experiments, data analyses, results and important considerations are included in this Chapter.

- Chapter 7 Microbiological technologies for quantitative assessment of Campylobacter (manuscripts III and IV)

The assessment of the effectiveness of vaccines and other control strategies that aim to reduce the numbers of *Campylobacter* in poultry depends on the quantitative microbiological techniques used. Chapter 7 presents research conducted to assess the complexity of obtaining accurate quantitative microbiological data related to the numbers of *Campylobacter* in chickens. Assessment of diverse quantitative microbiological techniques and quantitative data analyses to obtain reliable estimates of *Campylobacter* numbers from chicken fecal samples is presented in Chapter 7. Chicken fecal

samples represent complex matrices for the quantification of *Campylobacter*, still poultry feces are often the sample of choice for the routine testing of poultry flocks for *Campylobacter*. Quantitative risk assessment models, the evaluation of the effectiveness of control measures and mathematical models require accurate and reliable quantitative microbiological data.

- Chapter 8 Probabilistic Graphical Models designed for the control of *Campylobacter* in poultry (manuscripts V and VI)

Chapter 8 of this thesis focuses on the development of probabilistic graphical models (PGMs) to support decision making in order to control *Campylobacter* in poultry. The aim of PGMs is the efficient representation and integration of knowledge obtained from sources such as empirical observations, epidemiological data and expert opinions in order to support decision processes that have to be made under conditions of uncertainty. In many occasions, poultry producers need to make decisions regarding the implementation of control strategies before they even know if the flock will be infected or challenged with *Campylobacter*. PGMs may include many variables and represent complicated relationships among stochastic variables in an attractive, efficient and elegant way. The relationships of dependence or independence between the entities included in the models can be represented and the strength of the relationships can be defined using conditional probability distributions (Madsen *et al.*, 2012). This Chapter presents several PGMs that have been designed to integrate epidemiological knowledge and financial data in order to assist poultry managers in the selection of cost-effective strategies (such as vaccination of commercial broilers) for the control of *Campylobacter* in poultry.

3. INTRODUCTION

The term “poultry” is used in the title and throughout this PhD thesis as a synonym of chickens and broilers athough this term can refer to other birds bred for the production of meat or eggs. It was preferred to use the term poultry because some of the knowledge, information and/or reseach included in this thesis might refer to or could be applied to birds other than broilers.

Human campylobacteriosis is a general term used to describe bacterial disease in humans caused by several members of the genus *Campylobacter spp.* The bacteria *Campylobacter* has been recognized as the main etiological agent causing human bacterial gastrointestinal disease (Friedman *et al.*, 2000; Lindqvist *et al.*, 2001; Adak *et al.*, 2002; Lin, 2009; Hermans *et al.*, 2012). Children and adults can be severely affected by *Campylobacter* and the socioeconomic costs can be very high (Samuel *et al.*, 2004). Human infections with *Campylobacter* pathogenic strains are characterized by nausea, vomiting, stomachache, malaise, profuse watery diarrhea, blood in feces and high fever (Blaser *et al.*, 2008). The incubation period is usually 4 days but can vary from 2 to 10 days. Patients are advised to drink fluids and to follow antibiotic treatment when there is bacteremia or a serious underlying disease. In general, amoxicillin, tetracycline, erythromycin and fluoroquinolones are effective against campylobacteriosis if the pathogen is not resistant to these antibiotics (Moore *et al.*, 2006; Wassenar *et al.*, 2007). The disease is usually self-limited but complications may occur such as reactive arthritis, the Guillain-Barré syndrome (Carter and Hudson, 2009; Shahrizaila and Yuki, 2011; Baker *et al.*, 2012) and even death (Gradel *et al.*, 2008).

Campylobacter is usually associated with sporadic human cases of disease, however, outbreaks could be more common than previously thought (Gillespie *et al.*, 2003; Miller *et al.*, 2004; Fussing *et al.*, 2007). Outbreaks have been linked to contaminated chicken, water, milk and other food items (Allos, 2001; Frost *et al.*, 2002; Black *et al.*, 2006; Baker *et al.*, 2007). The epidemiology of *Campylobacter* remains poorly understood partly due to its widespread prevalence in the environment. It is known that livestock, domestic and wild animals (birds in particular) constitute important reservoirs, in fact, they may carry *Campylobacter* without the development of clinical signs which leads to the hypothesis that *Campylobacter* may be part of their natural intestinal microbiota (Whyte *et al.*, 2004; Young *et al.*, 2007; Ogden et al., 2009; Garcia *et al.*, 2010a; Jokinen *et al.*, 2011). There is increased evidence that, in many areas of the world, poultry, in particular broilers and chicken meat are the main contributors to human campylobacteriosis

(Wingstrand *et al.*, 2006; Wilson *et al.*, 2008; Mullner *et al.*, 2009; European Food Safety Authority [EFSA], 2010b; Friis *et al.*, 2010; EFSA, 2011a; Hermans *et al.*, 2012). The poultry reservoir has been identified as one of the main sources for human campylobacteriosis, actually it may account for 50% to 80% of human cases. In particular, according to the expert panel in EFSA, preparation and consumption of chicken meat could be the source for 20-30% of human campylobacteriosis cases (EFSA, 2010b). Contaminated poultry meat has been implicated in human campylobacteriosis outbreak investigations (Pebody *et al.*, 1997) and case-control studies (Studahl and Andersson, 2000; Kapperud *et al.*, 2003; Neimann *et al.*, 2003; Nielsen *et al.*, 2006; Stafford *et al.*, 2007; Doorduyn *et al.*, 2010).

Efforts have been directed towards the control of *Campylobacter* in chickens as a strategy to reduce the risk of human campylobacteriosis. There is a general belief that effective *Campylobacter* controls implemented throughout the food chain from poultry farms to the consumers will provide greater public health benefits than controls applied only later in the food chain because *Campylobacter* may infect humans via other pathways than chicken meat (EFSA, 2010a). However, despite a great number of research studies, it does not seem that an effective general strategy has been implemented in broiler farms to consistently produce *Campylobacter* free chickens (Hermans *et al.*, 2011). The production of *Campylobacter*-free broiler flocks is possible but often expensive and difficult to achieve due to the fact that considerable investments in control strategies that are difficult to maintain might be necessary (Loc Carrillo *et al.*, 2005; Wagenaar *et al.*, 2006; Wassenaar, 2011; Hermans *et al.*, 2012). Even when this aim is achieved, *Campylobacter*-free flocks might be contaminated at slaughter (Rivoal *et al.*, 2005).

The identification of important risk factors for the introduction of *Campylobacter* in broiler flocks may assist on the implementation of efficient controls. Strict bio-security may result in a significant reduction of the probability of *Campylobacter* infection of poultry flocks. Some studies have found a clear correlation between the level of biosecurity and flock infection with *Campylobacter* (Cardinale *et al.*, 2004; Johnsen *et al.*, 2006). Nonetheless, strict biosecurity might be difficult to achieve and maintain throughout poultry production operations. In order to reduce the public health risk, controls against *Campylobacter* should be implemented during the farming period but also during the transport of poultry, at slaughter and during the production of poultry products and by-products. Some control strategies will aim to prevent *Campylobacter* contamination of chickens and their products while other interventions such as vaccination will aim to reduce the numbers of *Campylobacter* in already contaminated animals, their products and by-products.

Chickens might carry *Campylobacter* in numbers as high as 10^{10} Colony Forming Units (CFU) per gram of faeces (Stas *et al.*, 1999; Sahin *et al.* 2002; Lütticken *et al*., 2007). Birds infected with *Campylobacter* will contaminate the food processing environment. The concentration of *Campylobacter* on chicken carcasses and *Campylobacter* numbers in caeca are positively correlated (Berrang *et al.*, 2004a; Reich *et al.*, 2008). In alignment with this knowledge, it can be assumed that a reduction of the amount of *Campylobacter* in the intestinal tract of chickens will result in a decrease of the numbers of *Campylobacter* present in chicken meat. In addition, risk assessment models indicate that a 2 log reduction of *Campylobacter* in chicken carcasses may translate into a decrease of human campylobacteriosis cases by 30 times (Rosenquist *et al*., 2003; Reich *et al*., 2008). The assumption that a reduction in the amount of *Campylobacter* in the intestinal tract of chickens will result in a decreased risk of human campylobacteriosis serves as basis for the research project (CamVac) that is the foundation for this Phd thesis. The CamVac project aims to develop a cost-effective vaccination strategy that can reduce the numbers of *Campylobacter* in infected chickens by at least 2 logs (CamVac, 2012). The work presented in this thesis has been conducted as part of the CamVac project and covers the following topics: clinical trials of vaccines, assessment of quantitative microbiological methods and development of decision support tools for the control of *Campylobacter* in poultry.

Nowadays, consumers demand safer food putting pressure on governments and food industries all over the world to improve food safety and reduce the risk of food-borne illnesses. Risk analysis (risk assessment, management and risk communication) is used by governments and public health agencies worldwide as a structured, science-based, integrated tool to reduce the risk of foodborne illness (Taylor and Hoffman, 2001). The food industry, government agencies and the society as a whole may benefit from the use of the assessment of quantitative microbiological techniques, vaccination trials and mathematical models developed in this thesis for the control of *Campylobacter* in poultry. Even though models are not perfect but limited representations of the reality, the models presented in this thesis may assist poultry producers in strategic decision making for the control of *Campylobacter*. In addition, efforts directed towards the control of an important public health issue such as *Campylobacter* will have a positive impact on consumers' perceptions related to food safety, the food industry and public health agencies.

4. OBJECTIVES

Human campylobacteriosis represents an important public health problem and reducing the burden of disease in humans is considered a priority in many areas of the world. Poultry has been identified as a significant source for human campylobacteriosis cases and consequently the control of *Campylobacter* in poultry is crucial for the reduction of human cases. The aim of this thesis was to assist in the control of *Campylobacter* in poultry. The main objectives were:

- To provide a general overview of control strategies against *Campylobacter* in poultry and public health implications
- To explore vaccination strategies against *Campylobacter* in poultry and to assess vaccine effectiveness in a particular clinical trial
- To investigate several quantitative microbiological methods for the accurate and fast enumeration of *Campylobacter* present in poultry fecal material
- To develop mathematical models and decision support systems that can integrate knowledge to aid on decision making regarding *Campylobacter* control strategies in poultry production.

5. *CAMPYLOBACTER*: PUBLIC HEALTH ASPECTS AND CONTROL STRATEGIES IN POULTRY PRODUCTION

5.1. Human campylobacteriosis

Human campylobacteriosis represents an important public health problem and the burden of disease is considerable even though the numbers of reported cases have decreased slightly in some areas of the world (Samuel *et al.*, 2004; Ailes *et al.*, 2008; European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2011). Human campylobacteriosis seems to be a particularly important problem in New Zealand where almost 400 cases per 100,000 habitants have been reported (Baker *et al.*, 2007; Baker and Wilson, 2007; French, 2008). The possibility that the high number of human campylobacteriosis cases in New Zealand may be due to an effective reporting system has been considered. Researchers have reported that the increase in human Campylobacteriosis cases in New Zealand is real and not only due to changes in the reporting system (McNicholas *et al*, 1995; Baker *et al.*, 2007). The overall number of reported campylobacteriosis cases in Europe was 45.6 cases per 100,000 persons in 2009 (EFSA and ECDC, 2011). However, it has been estimated that in Europe the true incidence of human campylobacteriosis can reach up to twenty million per year due to the effect of underreporting (EFSA, 2010a, 2011b). In addition to the personal consequences caused by the disease there are important socioeconomic costs associated with human campylobacteriosis caused by visits to the doctors, absence from work, hospitalizations and problems due non-diagnosed sequalae (Kemmeren *et al*., 2006).

The most frequently identified *Camplylobacter spp.* associated with human disease have been identified as *C. jejuni* and *C.coli* (Nachamkin and Blaser, 2000; Friedman *et al*., 2000; Allos, 2001; Gillespie *et al.*, 2002; Tam *et al.*, 2003; Lin, 2009; Hermans *et al*., 2012). In fact, it was observed that of the human campylobacteriosis cases characterized to species level in the EU in 2009, *C.jejuni* accounted for 90% of the cases, followed by *C. coli*, *C. lari* and *C. upsaliensis* corresponding to 2.5%, 0.2%, and 0.01% of the isolates respectively (EFSA and ECDC, 2011).
The disease is self-limited in most cases in adults and non-immune-compromised individuals. Still, complications may occur such as post-infection irritable bowel syndrome and reactive arthritis (ReA). The ReA syndrome can be characterized by conjunctivitis, urethritis and/or arthritis

(Altekruse *et al*., 1999; Allos, 2001; Carter and Hudson, 2009). Furthermore, the Guillain-Barré syndrome (GBS) may be developed, a neurological disorder that has been identified as the most frequent cause of acute neuromuscular paralysis in humans (Nachamkin *et al*., 1998, 2000; Jacobs *et al.*, 2008; Van Doorn *et al*., 2008; McGrogan *et al.*, 2009; Shahrizaila and Yuki, 2011; Baker *et al*., 2012). Reactive arthritis and the Guillain-Barré syndrome are considered to be caused by autoimmune responses to *Campylobacter* infections. Human campylobacteriosis may rarely result in long term disabilities or even death (Helms *et al*., 2003, 2006). Some persons are at higher risk of suffering severe symptoms (deriving in hospitalization and/or death) such as immunocompromised individuals, very young and very old persons (Helms *et al*., 2003; Gradel *et al*., 2008). Furthermore, *Campylobacter* strains that are resistant to the most commonly used antibiotics represent a challenge for the treatment of human campylobacteriosis (Moore *et al*., 2006). Recommendations regarding careful, safe and effective use of antibiotics in food-producing animals have been made to protect public health (WHO, 2000). In fact, a correlation between ciprofloxacin-resistant *Campylobacter* and poultry consumption has been observed. Additionally, associations between particular *Campylobacter* strains and antibiotic resistance have been detected (Kinana, 2006; Habib, 2009).

Human campylobacteriosis is hyperendemic in many developing areas of the world and the disease differs from campylobacteriosis in developed countries (Coker *et al*., 2002; Islam *et al.*, 2006). In developing areas, campylobacteriosis is predominantly a pediatric problem affecting children under the age of five while adults are generally less prone to the disease (Oberhelman and Taylor, 2000; Coker *et al*., 2002). Humans that are continuously challenged with *Campylobacter* might develop protection against clinical disease and become asymptomatic carriers (Blaser *et al*., 1985). A more favorable clinical outcome has been observed in human volunteer studies where humans have been re-challenged with the same *Campylobacter* strain (Black *et al*., 1988). Immune response against *Campylobacter* seems to differ between *Campylobacter* strains. Particular *C. jejuni* strains can induce immune response in the host to a higher degree than other strains (Pancorbo *et al.*, 2001). Additionally, differences in human hosts' immune reactions might explain disease outcomes such as the development of the Guillain-Barré Syndrome (Shahrizaila and Yuki, 2011).

5.2. *Campylobacter* reservoirs and sources

Campylobacter is frequently found in surface water and other environmental niches (Hanninen, 1998; Carrique-Mas *et al*., 2005; Sopwith *et al*., 2008; Jokinen *et al*., 2011). *Campylobacter* bacteria seem to be sensitive to heat (Waterman, 1982; Christopher *et al*., 1982; Sörquist, 1989), desiccation (Doyle and Roman, 1982), irradiation (Isohanni and Lyhs, 2009), freezing (Georgsson *et al*., 2006; Sandberg *et al*., 2005) and acids (Birk *et al*., 2010; Chaveerach *et al*., 2002, 2003). On the other hand, *Campylobacter* can survive in foods at chill temperatures of around 5°C (Lee *et al*., 1998; Solow *et al*., 2003; Bhaduri and Cottrell, 2004). *Campylobacters* are considered fragile bacteria but paradoxally they can survive in the environment outside hosts for long periods probably by developing survival mechanisms (Newell, 2002, Murphy *et al*., 2006). In fact, the presence of highly mutable areas on the genome of *C. jejuni* could explain survival and adaptation mechanisms (Jerome *et al*., 2011). Moreover, particular environments (such as fecal material and some foods) might represent protective vehicles for *Campylobacter*. Biofilm formation can play an important role in the epidemiology of *Campylobacter* infections (Gunther and Chen, 2009; Garcia and Percival, 2011). *Campylobacter* bacteria are ubiquitous and can be found widespread in the environment and animals. Multiple sources of *Campylobacter* and risk pathways can be associated with human exposure to this microorganism (EFSA, 2011b).

Birds are considered natural hosts for *Campylobacter* and may harbour *Campylobacter* in high numbers contributing to its survival and dissemination (Newell and Wagenaar, 2000; Waldenstrom *et al*., 2002). Molecular epidemiological studies have identified poultry as an important source for human campylobacteriosis but also ruminants and other sources (Sheppard *et al*., 2009a, 2009b; de Haan *et al*., 2010). *Campylobacter* is frequently present in the intestines of cattle and sheep and may contaminate the food processing environment and food products posing a public health risk for the consumers (Nachamkin and Blaser, 2000; Wesley *et al.* 2000; Dykes and Moorhead 2001; Garcia *et al*., 2010a/b). Numerous epidemiological studies have been conducted to identify potential sources for human campylobacteriosis, in fact, consumption and handling of poultry meat and direct contact with animals seem to be the most common and important sources (Kapperud *et al*., 1992; Eberhart-Phillips *et al*., 1997; Friedman *et al*., 2000; Studahl and Andersson, 2000; Rodrigues *et al*., 2001; Stafford *et al*., 2007, 2008; Tamm *et al*., 2009). Several risk factors have been identified as significant sources in a recent meta-analysis such as eating undercooked chicken, direct contact with farm animals, environmental sources and foreign travel (Dominguez *et al*., 2012).

Source attribution models developed based on Multi Locus Sequence Typing (MLST) data have identified poultry as one of the most important source of human campylobacteriosis (Sheppard *et al.*, 2009a/b; Mullner *et al.*, 2009; Mughini Gras *et al.*, 2012). In general, *C. jejuni* is the most commonly isolated species in birds of around six weeks of age. However, *C. coli* is more frequently identified in older animals and particularly from organic systems (El-Shibiny *et al.*, 2005). Poultry flocks and individual chickens might be infected with different *Campylobacter* strains at the same time (Jacobs-Reitsma *et al*., 1995; Stern *et al*., 1997; Rivoal *et al*., 1999). Furthermore, mixed infections can result in new strains through the exchange of genetic material (Jacobs-Reitsma *et al*., 1995; De Boer *et al.*, 2002; Hook *et al.*, 2005). In particular, *Campylobacter* strains resistant to antibiotics may interfere with the treatment of human campylobacteriosis (Moore *et al*., 2006). The use of vaccines in food producing animals could alleviate the problems related to antimicrobial resistance (Lütticken *et al.*, 2007).

5.3. The epidemiology of *Campylobacter* in chicken flocks

A harmonized baseline survey was conducted in EU in 2008 in order to estimate the prevalence of *Campylobacter* in broilers and on broiler meat (EFSA, 2010a). *Campylobacter* prevalence on broilers and their meat varied between countries (from 5 to 100%). A trend to obtain higher *Campylobacter* concentrations in broiler meat in geographical areas with a higher *Campylobacter* prevalence was observed (EFSA, 2010a). Broilers are considered *Campylobacter* free after hatching and in general, broiler flocks remain *Campylobacter* free for the first two weeks (Annan-Prah and Janc, 1988; Stern, 1992). Nonetheless, most chickens intended for human consumption are heavily and persistently colonised with *Campylobacter* representing an important public health risk. In modern poultry production systems, chickens grow to a slaughtering weight within four to six weeks, and during this relatively short period of time, *Campylobacter* may be introduced into the flocks and colonize chickens (Wagenaar *et al.*, 2006). Prevalence of *Campylobacter* in broiler flocks can vary between 3 and 91% (EFSA, 2010a). In chickens infected with *Campylobacter*, colonization and shedding patterns depend on a number of factors, such as the bacterial strain (Cawthraw *et al.*, 1996; Ringoir and Korolik, 2002; Conlan *et al.*, 2007; Janssen *et al.*, 2008). Several risk factors can result in the introduction of *Campylobacter* into the flocks making it difficult to keep chicken flocks free of *Campylobacter* throughout the rearing period. To increase the knowledge about why and how chicken flocks become infected with *Campylobacter* during the

rearing period, several observational studies have been carried out, focusing on different parts of the production processes and practices. Most epidemiological studies have focused on the outcome being the flock becoming infected, not considering the within flock prevalence nor the amount of *Campylobacter* in the infected chickens. Important risk factors for the introduction of *Campylobacter* into chicken flocks include season (Kapperud *et al.,* 1993; Jacobs-Reitsma *et al*., 1994; Refregier-Petton *et al*, 2001; Bouwknegt *et al.*, 2004; Barrios et al., 2006; Zweifel *et al.*, 2008; McDowell *et al*., 2008; Ellis-Iversen *et al*., 2009; Jore *et al*., 2010), the type of production system (Näther *et al*., 2009), age of the birds at the time of sampling (Evans and Sayers, 2000; Bouwknegt *et al*., 2004; Barrios *et al*., 2006; McDowell *et al*., 2008), partial depopulation practices (Hald *et al.*, 2000; Ellis-Iversen *et al.,* 2009), human traffic and farm equipment (Ramabu *et al*., 2004) size of the flock (Barrios *et al*., 2006; Nather *et al*., 2009), water from a private supply (Lyngstad *et al*., 2008), type of drinking water systems (Näther *et al*., 2009), the presence of other animals on farm or very close to the poultry farm (Bouwknegt *et al*. 2004; Lyngstad *et al*., 2008; Ellis-Iversen *et al*., 2009) and general farm hygiene (Hald *et al*., 2000; Evans and Sayers, 2000; McDowell *et al*., 2008). An association between *Campylobacter* status of broiler flocks and health and welfare of the birds has been suggested (Bull *et al*., 2008). The increased risk of *Campylobacter* introduction in poultry flocks attributable to partial depopulation practices could be partly explained by a confounding effect with age (Russa *et al.,* 2005). Nevertheless, partial depopulation was found to be a significant risk factor even after adjusting for confounding with age in a study conducted by Lawes *et al*., 2012. Human traffic on farm has also been reported as an important pathway for the introduction of *Campylobacter* into poultry flocks (Kapperud *et al*., 1993; Berndtson *et al*., 1996; Evans and Sayers, 2000; Hald *et al.,* 2000; Cardinale *et al.*, 2004; Hofshagen and Kruse, 2005). The number of poultry houses onsite has also been identified as a significant risk factor in some studies. The risk of *Campylobacter* infection increased with the presence of three or more houses on the same farm in France (Refregier-Petton *et al*., 2001) and five or more houses on the same poultry farm in the Netherlands (Bouwknegt *et al.* 2004). A strong association has been found between the presence of rodents in poultry farms and *Campylobacter* infection in poultry (Gregory *et al*., 1997; McDowell *et al.*, 2008).

The incidence and prevalence of *Campylobacter* in positive broiler flocks varies depending on geographical, farming and environmental conditions. Seasonality effects have been observed with a marked peak during summer much more noticeable in Northern Europe than in Southern Europe

(Nylen *et al.,* 2002). Seasonality effects could be explained by environmental factors such as humidity, temperature and sunlight that require further investigation (Wallace *et al.,* 1997; Arsenault *et al.,* 2007; Guerin *et al.,* 2008). The observed increased risk of *Campylobacter* introduction during the summer seemed to be more apparent in younger birds in a study conducted by Lawes *et al*. (2012). These authors suggested that broilers could be infected earlier during the summer due to increased pathogen survival in the environment. Seasonality effects have been detected regarding human campylobacteriosis cases (Nylen *et al.,* 2002; Bi *et al.,* 2008) and *Campylobacter* infections in chickens (Patrick *et al.,* 2004; Reich *et al.,* 2008; Jore *et al.,* 2010; Jorgersen *et al.,* 2011; Nichols *et al.,* 2012). Human infections with the clonal complexes ST-45 and ST-283 (both types have been frequently identified from chicken isolates) increase during early summer (Sopwith *et al.,* 2006; de Haan et al., 2010; McCarthy *et al.,* 2012). Pathogen infection pressure might increase during warmer periods in some areas of the world (Hald *et al.,* 2004, 2007) partly explaining an increase of *Campylobacter* prevalence in chickens and humans. Climate and environmental factors could partly explain seasonality effects (Louis *et al.,* 2005; Kovats *et al.,* 2005; Tam *et al.,* 2006). Remarkably, the increase in human cases can sometimes occur previous to infections in chickens suggesting that there might be a common risk factor responsible for the increase in *Campylobacter* cases. Flies can transmit *Campylobacter* to chickens and humans and they could partly explain the seasonality of human cases (Nichols, 2005; Ekdahl *et al.,* 2005; Hald *et al.,* 2004, 2007a, 2008; Nelson *et al.,* 2006; Guerin *et al.,* 2008; Nichols, 2010).

The numbers of *Campylobacter* in broilers may exceed 7.0 log cfu/gr of caecal content (Rosenquist *et al.,* 2006). In actual fact, the colonization level can be as high as 10^{10} CFU per g of faeces (Stas *et al.,* 1999; Sahin *et al.* 2002; Lütticken *et al.,* 2007). *Campylobacter* infective dose for chickens depends also on the colonizing strain. Transmission dynamics on the population are difficult to model mathematically (Conlan *et al.,* 2007). Though, it has been suggested that once a bird has been colonized by *Campylobacter*, the rest of the birds in the same house will be contaminated within one week (Jacobs-Reitsma, 1997). *Campylobacter* colonizes the chicken intestine, multiplies in the intestinal mucus layer being able to re-invade epithelial cells (Van Deun *et al.,* 2008). *Campylobacter* might be able to regulate gene expression of epithelial cells in chickens (Artis, 2008). Several *Campylobacter spp.* genomes have been sequenced suggesting a significant diversity between isolates (Parkhill *et al.,* 2000; Gundogdu *et al.,* 2007). Characterized *Campylobacter* colonization factors and pathogenic factors can be considered for use in vaccine development (Jagusztyn-Krynicka *et al.,* 2009). *Campylobacter* ability to control genetic expression for adaption

to different environmental conditions and the ability to form biofilms have been identified as important factors for chicken colonization (Kalmokoff *et al*., 2006; Van Deun *et al.*, 2008; Gunther and Chen, 2009; Garcia and Percival, 2011). Researchers have identified *Campylobacter*'s most critical metabolic pathways and the genes that regulate them which could serve as basis for the development of new antimicrobials and/or new vaccines (Institute of Food Research [IFR], 2012).

Campylobacter can be found in ceca, intestine and cloaca in very high numbers (Stas *et al*., 1999; Corry and Atabay, 2001; Sahin *et al.* 2002; Lütticken *et al*., 2007). The amount of faecal spillage during food processing will directly affect meat contamination with *Campylobacter* (Berrang *et al*., 2004a). Moreover, poultry processing can lead to cross/contamination of chicken carcases. There are specific areas or processes in food premises that can be considered higher risk for food contamination and might become critical control points. Food processing areas that constitute critical control points in poultry processing plants are usually scalding, defeathering and evisceration (Stern and Robach, 2003; Takahashi *et al*., 2006). Automated defeathering represents a high risk practice since cloacal contents can cause contamination of the carcases (Berrang *et al.*, 2001). The concentration of *Campylobacter* on chicken carcasses and *Campylobacter* numbers in caeca are positively correlated. In fact, small amount of cecal contents may increase the numbers of *Campylobacter* on carcasses (Berrang *et al.*, 2004a; Reich *et al.*, 2008).

A reduction of *Campylobacter* numbers in the intestinal tract of chickens will translate in a decrease of the numbers of *Campylobacter* present in chicken meat. This fact serves as basis for the development and implementation of vaccination strategies and other controls that aim to reduce the numbers of *Campylobacter* in the intestinal tract of chickens to achieve a reduction of *Campylobacter* in chicken meat which may translate on a decrease of human campylobacteriosis cases (Rosenquist *et al.*, 2003; Reich *et al*., 2008). A decline in reported human campylobacteriosis cases was observed in the Netherlands and Belgium when the consumption of chicken meat was temporarily limited. Furthermore, a decrease on human cases has been documented in Iceland and New Zealand following interventions against *Campylobacter* in poultry (Vellinga *et al.*, 2002; Stern *et al.*, 2003; EFSA, 2010a; Sears *et al.*, 2011). Therefore, it is possible that a reduction on chicken meat consumption and/or the effective implementation of *Campylobacter* controls in poultry will translate on a decrease of human campylobacteriosis cases.

5.4. *Campylobacter* control strategies in poultry production

Campylobacter control programmes have been implemented to reduce the prevalence of *Campylobacter* in broilers in order to decrease the burden of human campylobacteriosis. On the other hand, it seems difficult to compare the effectiveness of these controls between different countries due to a number of factors such as the use of different sampling and testing protocols. *Campylobacter* prevalence and concentration in chickens and their products can be high posing a public health risk (EFSA, 2010b). Effective controls should be implemented along the food chain in order to reduce *Campylobacter* concentration and prevalence in poultry (Figure 1).

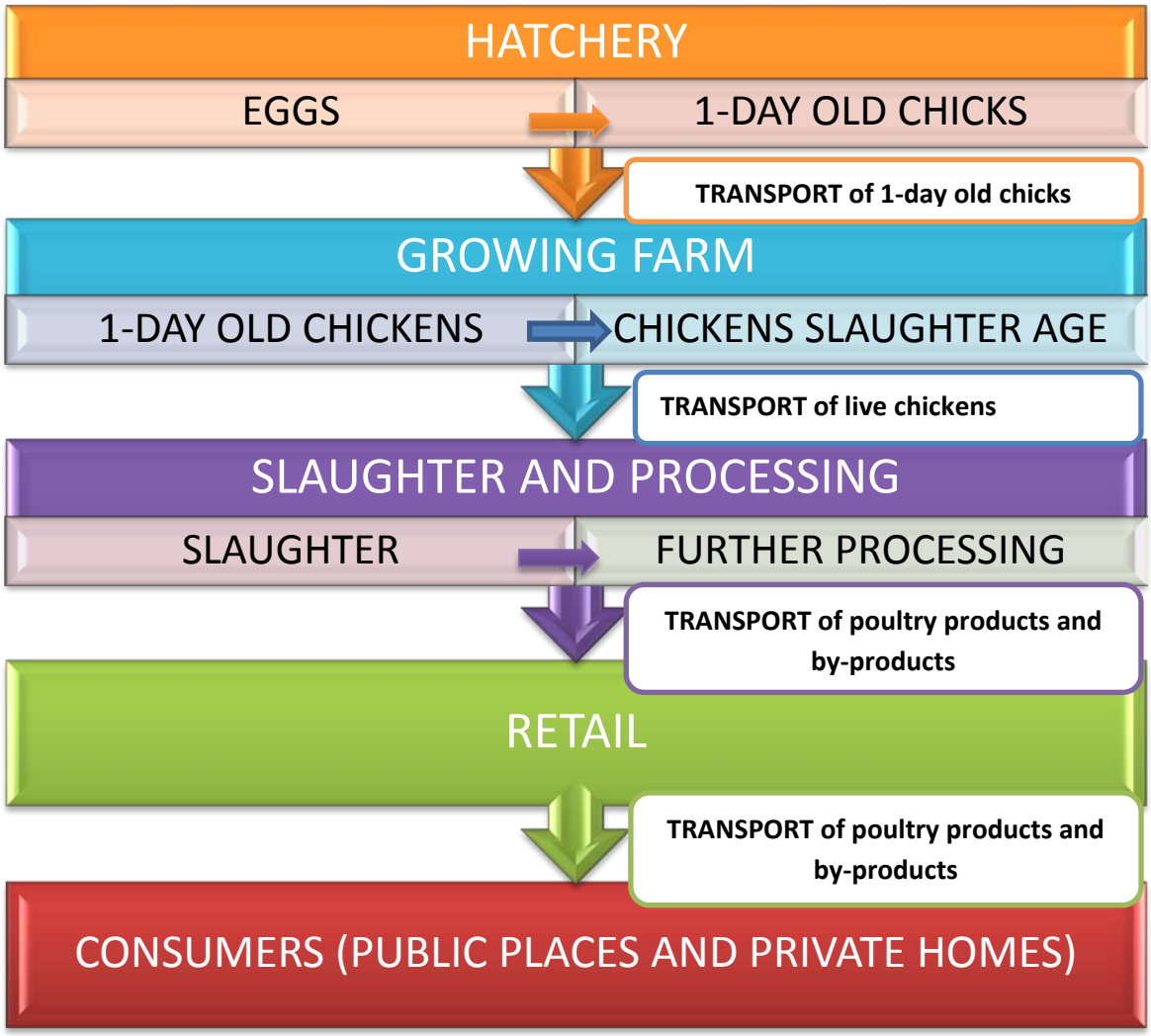

Figure 1 Graph illustrating poultry production stages from farm to fork.

Campylobacter control strategies should be implemented along the food chain (Reich *et al.*, 2008) and used synergistically in order to reduce the incidence of human campylobacteriosis. The synergistic effect of control measures implemented at all levels of the food chain should be properly assessed. *Campylobacter* control measures at farm level may include the identification of the most significant risk factors, increased bio-security, use of effective vaccines, use of phage therapy and husbandry measures (e.g. chlorinated water and/or food additives) to mention a few. *Campylobacter* control strategies in poultry farms based on increased biosecurity levels aim to reduce the probability of *Campylobacter* introduction in poultry flocks. Other controls such as the use of probiotics, effective vaccines or novel antibacterial treatments try to reduce *Campylobacter* burden in poultry (Newell *et al.*, 2011; Djenane and Roncalés, 2011).

Campylobacter controls should be implemented during transport of poultry, slaughter and processing. High levels of hygiene should be maintained during poultry processing to control *Campylobacter* contamination of chicken products. Cleaning and disinfection of poultry processing plants should be thorough and effective. In actual fact, *Campylobacter* may survive in surfaces after cleaning and disinfection of the processing plant posing an additional risk to meat contamination (Peyrat *et al.*, 2008). Decontamination treatments such as freezing can be implemented as a compliment to high levels of hygiene. Contamination of food products during transport and storage should be avoided. Effective controls such as high level of hygiene, decontamination techniques, freezing and the use of new technologies may assist in reducing *Campylobacter* numbers in chickens, their products and by-products. Some controls will aim to prevent contamination of *Campylobacter* free products while other measures will intent to reduce the numbers of *Campylobacter* in already contaminated animals and their products. Decontamination technologies should be a compliment to preventive control measures and good hygiene practices (GHP). The food industry needs to comply with legal requirements and the implementation of “Hazard Analysis and Critical Control Points” (HACCP), an internationally recognized food safety management tool. Nonetheless, food safety is everyone’s responsibility and consumers may get infected with *Campylobacter* at home via different routes: directly from contaminated hands or insufficiently cooked infected chicken, direct contamination of raw foods from chicken carrying *Campylobacter* and indirectly through contaminated kitchen surfaces and tools. A large number of food-borne disease cases may be due to unsafe handling of food in kitchens (Zhao *et al.*, 1998). Kitchen hygiene should be improved 30 times in order to reduce the incidence of human campylobacteriosis

(Rosenquist *et al.*, 2003). Eating raw chicken meat may translate in human exposure to *Campylobacter* up to 10^{10} times higher than the exposure level when the product is properly cooked (Uyttendaele *et al*., 2006). Consumer education is crucial to prevent human campylobacteriosis and it has been promoted by governments in many areas of the world.

5.4.1. Campylobacter controls in poultry primary production

Campylobacter control strategies implemented during poultry primary production, defined as the on-farm rearing of poultry, are crucial for the control of this significant public health issue. The identification of important risk factors for the introduction of *Campylobacter* in broiler flocks (see Thesis Introduction) will assist on the selection and implementation of efficient controls. It is interesting to notice that estimated *Campylobacter* prevalence in the environment around broiler houses from different farms seems to be quite similar independently of the biosecurity level (Hald *et al.*, 2000; Hansson *et al.*, 2007; Ridley *et al.*, 2011a). Therefore, *Campylobacter* must be carried from the environment into chicken houses somehow and human traffic has been identified as an important vehicle for this transmission (Kapperut *et al*. 1993; Berndtson *et al*., 1996; Evans and Sayers, 2000; Cardinale *et al*., 2004; Hofshagen and Kruse, 2005). *Campylobacter* strains isolated from hands, boots and clothes of farm staff, catchers and farm managers have been associated with *Campylobacter* strains present in broiler flocks (Herman *et al*., 2003; Ramabu *et al*., 2004; Johnsen *et al*., 2006; Ridley *et al.*, 2008b, 2011a/b). The number of staff members and the number of human visits to the poultry houses have been found to increase the risk of introducing *Campylobacter* into poultry flocks (Refregier-Petton *et al*., 2001; Huneau-Salaun *et al*., 2007; Chowdhury *et al*., 2012). *Campylobacter* can survive well in water (Blaser *et al.*, 1980) and a close association between rainy weather and *Campylobacter* prevalence in puddles or standing water around chicken houses has been reported (Hansson *et al.*, 2007). Additionally, *Campylobacter* strains isolated from soil and puddles around broiler houses in many cases can be identical to the strains isolated from the flocks supporting the hypothesis of *Campylobacter* transfer from the external environment into the broiler houses (Herman *et al.*, 2003; Bull *et al.*, 2006; Messens *et al*., 2009).

Campylobacter survives in poultry litter posing a risk for the infection of new flocks when poultry waste is stored on farm (Petersen *et al*., 2001; Rothrock *et al*., 2008). In fact, the risk of *Campylobacter* infection of flocks may increase significantly when the distance between the poultry house and used litter is less than 200 meters (Cardinale *et al*., 2004; Arsenault *et al*., 2007).

Consequently, adequate removal and treatment of used litter from the farm will potentially decrease the risk of *Campylobacter* infection in poultry. Removal of dead chickens from the house may also reduce the risk of a *Campylobacter* positive flock (Evans and Sayers, 2000).

The presence of other livestock on the same farm has been identified as a risk factor for the introduction of *Campylobacter* in poultry flocks in several studies (van de Giessen *et al.*, 1996; Cardinale *et al.*, 2004; Bouwknegt *et al*. 2004; Lyngstad *et al*., 2008; Ellis-Iversen *et al*., 2009). Recommendations have been made to minimize the presence of other livestock on poultry farms and/or to implement effective biosecurity barriers (Kapperud *et al.* 1993; Neubauer *et al.* 2005; Hald *et al*., 2007a/b). Biosecurity barriers should protect poultry by providing an effective physical separation between the "contaminated" environment outside the houses and the "protected" environment inside poultry houses. For example, an area at the entrance of a poultry house containing protective clothes, boots boot dips and hand washing facilities constitute a hygiene barrier. Nonetheless, the effectiveness of biosecurity barriers may vary between farms making the assessment of hygiene barriers as protective factors quite difficult (Neubauer *et al*., 2005). On the other hand, a significant reduction of the risk of *Campylobacter* infection of poultry flocks is possible to achieve by the effective use of biosecurity barriers specially when there are other animals on farm (van de Giessen *et al*., 1992; Berndtson *et al.*, 1996; van de Giessen *et al*., 1998; Evans and Sayers, 2000; Hald *et al.*, 2000).

The poultry house becomes contaminated with *Campylobacter* for a long time when a poultry flock becomes positive (Hiett *et al*., 2002; Herman *et al*., 2003; Johnsen *et al*., 2006). As a result, the presence of previous *Campylobacter* positive flocks in a house has been considered a risk factor for *Campylobacter* infection of new flocks (Refregier-Petton *et al*., 2001; Chowdhury *et al.*, 2012). Cleaning and disinfection of poultry houses should be effective inactivating *Campylobacter*.

Campylobacter does not seem to be present in clean litter (Jacobs-Reitsma *et al*., 1995) and feed (Mills *et al*., 2003) unless they become contaminated during transport and storage although these materials are very dry making *Campylobacter* survival difficult. Conversely, wet litter has been shown to be a significant risk factor increasing the risk of infection with *Campylobacter* (Berndtson *et al*., 1996). Poor quality of drinking water has been identified as a risk factor for *Campylobacter* in several epidemiological studies (Sparks, 2009). The use of unchlorinated water or failure of water treatments may introduce *Campylobacter* in poultry houses (Newell and Fearnley, 2003).

The application of hygienic measures and general biosecurity barriers such as the use of separate boots between houses and footbath disinfection when entering broiler houses between many others may reduce the risk of *Campylobacter* infections in birds considerably (van de Giessen *et al*., 1996; Evans and Sayers, 2000). Indeed, the prevalence of *Campylobacter* in broiler flocks decreased from 66% to 22% on a farm and from 100% to 42% in another broiler farm in the Netherlands due to the introduction of hygienic measures and biosecurity barriers such as the control of rodents and insects (van de Giessen *et al*., 1998). The use of house-specific clothes (Hald *et al*., 2000; Bouwknegt *et al*., 2004), boots (van de Giessen *et al*., 1996; Evans and Sayers, 2000; Bull *et al*., 2006) and boot dips (van de Giessen *et al*., 1996; Evans and Sayers, 2000; Gibbens *et al*., 2001; Bouwknegt *et al*., 2004; McDowell *et al*., 2008) and the application of overshoes (Puterflam *et al.*, 2005) can potentially reduce the risk of *Campylobacter* infection of poultry flocks.

Depopulation practices such as thinning have been identified as a significant risk factor for *Campylobacter* infection of poultry due to poor biosecurity maintained during these practices (Hald *et al*., 2001; Refregier-Petton *et al*., 2001; Bouwknegt *et al*., 2004; Puterflam *et al*., 2005; Barrios *et al*., 2006; EFSA, 2010a). The risk of introducing *Campylobacter* in poultry flocks during thinning was higher when the crews were larger (Puterflam *et al*., 2005). In a study conducted by Allen *et al.* (2008a) a *Campylobacter* strain was isolated from a farm following thinning and from the catcher's hand and it was very similar to the strain recovered from a crate used during thinning.
On the other hand, after adjusting depopulation practices for confounding with season and age, the importance of thinning in introducing *Campylobacter* in poultry flocks has been questioned (Russa *et al*., 2005). Nevertheless, the risk of introducing *Campylobacter* during depopulation practices could be reduced (Berndtson *et al*., 1996; Barrios *et al.*, 2006). A relationship between the time of depopulation and *Campylobacter* prevalence in poultry has been observed; *Campylobacter* prevalence might be higher when thinning occurs long time before slaughter (Allen *et al.*, 2008a). Increased biosecurity controls during depopulation practices could prevent *Campylobacter* infection in poultry. A clear correlation between the level of biosecurity and poultry flock infection with *Campylobacter* has been observed in Norway and Senegal (Cardinale *et al.*, 2004; Johnsen *et al.*, 2006). Increased biosecurity could be important at times of the year when the risk of introducing *Campylobacter* in broiler flocks is considered high due in part to seasonality effects (Kapperud *et al.* 1993; Jacobs-Reitsma *et al.*, 1994; Berndtson *et al*., 1996; Evans and Sawyers, 2000; Refregier-Petton *et al*., 2001; Bouwknegt *et al*., 2004; Hofshagen and Kruse, 2005; Puterflam *et al*., 2005;

Russa *et al*., 2005; Barrios *et al*., 2006; Johnsen *et al*., 2006; McCrea *et al*., 2006; Hansson *et al*., 2007; Huneau-Salaun *et al*., 2007; McDowell *et al*., 2008). Seasonality trends have been observed in many areas of the world with a seasonal peak usually happening during summer and/or early autumn although the extent and exact time of this peak varies between countries (EFSA, 2010a). The cause for this seasonality is not known but it could be related to the breeding of flies (Hald *et al*., 2007a/b). The role of insects in *Campylobacter* contamination of poultry houses is not clear. The presence of insects was not found a significant risk factor in some epidemiological studies (Berndtson *et al*., 1996; Refregier-Petton *et al*., 2001). Besides, in some studies *Campylobacter* was not recovered from insects or it was isolated only after the birds became infected with *Campylobacter* (Jones *et al*., 1991; Jacobs-Reitsma *et al*., 1995; Bates *et al*., 2004; Neubauer *et al*. 2005). Nevertheless, a number of researchers believe that flies can transmit *Campylobacter* to chickens and humans partly explaining the seasonality of human cases (Nichols, 2005; Ekdahl *et al*., 2005; Hald *et al*., 2004, 2007a,2007b, 2008; Nelson *et al*., 2006; Guerin *et al*., 2008; Nichols, 2010). In Denmark, flies have been identified as one of the most important risk factor for the introduction of *Campylobacter* in broiler flocks. In this country, *Campylobacter* prevalence in broiler flocks is highest during the summer. Studies conducted in Denmark showed that 70% of the flies captured around poultry houses carried *Campylobacter* and that the use of fly screens to prevent the access of flies to poultry houses reduced *Campylobacter* incidence in flocks from 51.4% to 15.4% during the summer (Hald *et al*., 2004, 2007a, 2007b).

Campylobacter might be present in poultry houses' air but it is believed that air contamination happens after flock colonization (Pearson *et al*., 1993). Though, some types of ventilation systems have been identified as risk factors for *Campylobacter* colonization of poultry such as horizontal (Barrios *et al*., 2006), static (Refregier-Petton *et al*., 2001) and nebulization systems. For example, a refrigeration system based on nebulization has been identified as the most important risk factor for the introduction of *Campylobacter* in broiler flocks in the South of Spain (personal communication).

Campylobacter spreads fast within poultry flocks (Jacobs-Reitsma, 1997) and consequently control measures that can impede *Campylobacter* spread, reduce the speed of transmission and/or decrease the numbers of *Campylobacter* in already contaminated chickens, flocks and poultry houses should be selected and implemented.

Campylobacter appears sensitive to acidic conditions, therefore, control strategies have been developed based on the acidification of feed (Line, 2002), water and the environment. The use of feed additives may assist on the control of *Campylobacter* in chickens. However, feed acidification seems to have a limited effect on the prevalence of *Campylobacter* in broiler flocks (Heres *et al.*, 2004; Line and Bailey, 2006; Solis de los Santos *et al.*, 2008). A reduction of *Campylobacter* colonization in broilers was obtained after the addition of a combination of 2% formic acid with 0.1% sorbate (Skånseng *et al.*, 2010) and the addition of fatty acids to the feed (van Gerwe *et al*., 2010). The use of enzymes as growth promoters alone or in combination with organic acids has been proposed (Anjum and Chaudhry, 2010).

Genetic selection of poultry with superior immunological responses to *Campylobacter* could be explored further (Kapperud *et al.*, 1993; Swaggerty *et al.*, 2009). Successful vaccines will probably be the most effective control against *Campylobacter* but the availability of a cost-effective commercial vaccine remains a major goal (Djenane and Roncalés, 2011: Garcia *et al.*, 2012). The use of antibodies against *Campylobacter* in poultry has been proposed. In fact, a strong protection against *C. jejuni* in chickens seemed to be induced by the oral administration of immunoglobins preparations from milk or eggs (Tsubokura *et al*., 1997).

Treatment of infected chickens with effective bacteriocins has been shown to reduce *C. jejuni* concentration levels substantially (Stern *et al*., 2008; Svetoch and Stern, 2010). The administration of purified encapsulated bacteriocins from *P. polymyxa* NRRL-B-30509 or *L. salivarius* NRRL B-30514 was successful in controlling *Campylobacter* colonization in young birds (seven to ten days of age). The use of bacteriocins BCN E 760 and BCN E 50-52 produced a considerable reduction on the numbers of *C. jejuni* in broilers close to slaughter age and naturally infected with *Campylobacter* (Svetoch and Stern, 2010).

Bacterial competitive exclusion can serve as basis for the control of *Campylobacter*, in effect, bacterial strains that colonise chicken caeca can produce anti-*C. jejuni* metabolites. The use of probiotics offers many potential benefits based on their ability to balance the intestinal microflora (Halfhide, 2003). Though, the effectiveness of prebiotics, probiotics and competitive exclusion products on protecting animals against *Campylobacter* depend on culture preparation techniques such as temperature and media used for preparation (Stern *et al*., 2001). *Campylobacter* colonisation of chickens was reduced in a study after using competitive exclusion cultures of *E. coli*, *Klebsiella pneumoniae* and *Citrobacter diversus* (Schoeni and Wong, 1994). A probiotic

including *Enterococcus faecium* and *Lactobacillus acidophilus* decreased colonization with *C. jejuni* and fecal shedding in broilers (Morishita *et al*., 1997). Many bacteria have been proven to be active against *Campylobacter* in vitro (Chang and Chen, 2000; Svetoch and Stern, 2010). In fact, a *Lactobacillus* strain isolated from a chicken proved to have bactericidal effect (through the production of organic acids) against *Campylobacter* in vitro (Chaveerach *et al*., 2004a). The inhibition of *C. jejuni* (below detection level) was obtained in vitro after 24h culture of *C. jejuni* with *Lactobacillus plantarum* or *Bifidobacterium bifidum* (Fooks and Gibson, 2002). The use of a characterized hyper-colonizing *C. jejuni* strain to displace other strains present in the chicken digestive tract has also been tested (Calderon-Gomez *et al*., 2009).

The use of antibiotics in animal feed for the only purpose of growth promotion of livestock has been officially banned in Europe since January 2006 (Compassion in World Farming [CWF], 2011; EFSA, 2012). Some antibiotics may be efficient in reducing *C. jejuni* concentrations in chickens (Farnell *et al.*, 2005; Hermans *et al*., 2010). However, antibiotics may only be used therapeutically when prescribed by a veterinarian. It has been suggested that the consumption of antibiotics in the veterinary sector has not decreased and there are huge concerns regarding antimicrobial resistance problems in humans and animals (CWF, 2011; European Medicines Agency, 2012). The development of antibiotic resistance may compromise treatment of human infections with *C.jejuni* (Dibner and Richards, 2005; Zhu *et al.*, 2006). As a result, the development of innovative methods for microbial inactivation such as the use of pulsed electric fields and high hydrostatic pressure has been investigated (Sagarzazu *et al*., 2010). The symbiotic effect of prebiotics and probiotics (the combination is known as synbiotics) may act as antimicrobial (Klewicki and Klewicka, 2004; Jones and Versalovic, 2009; O'Flaherty *et al*., 2010; Djenane and Roncalés, 2011). The design of novel antimicrobials could be based on the identification of *Campylobacter* critical metabolic pathways such as the shikimate pathway. The shikimate pathway is used by plants, fungi and bacteria to produce essential amino acids but is absent in mammals. In fact, this pathway has already been used to produce vaccine strains against other bacteria (IFR, 2012).

The treatment of drinking water may reduce *Campylobacter* numbers in infected chickens (Hermans *et al*., 2011). The addition of organic acids or chlorine to drinking water on poultry farms may be able to prevent *Campylobacter* infection and/or transmission (Chaveerach *et al*., 2002, 2004b; Ellis-Iversen *et al.*, 2009). The addition of lactic acid (Byrd *et al*., 2001), caprilic acid (Solis

de los Santos *et al.*, 2010) or monocaprin (Thormar *et al.*, 2006) to drinking water before slaughter may reduce *Campylobacter* counts in chickens (Hilmarsson *et al*., 2006). The addition of lactic acid to drinking water during the feed withdrawal before slaughter produced a significant reduction of *Campylobacter* present in crops of broilers at slaughter (from 85% to 62%). In addition, the application of lactic acid to the water did not affect animal health and welfare (Byrd *et al*., 2001). Several acids (propionic, acetic, hydrochloric and formic) were tested in vitro as additives to water and/or feed and their effect against *Campylobacter* seemed to be most effective when the pH was around 4.0 (Chaveerach *et al.*, 2002). Drinking water treatment with a combination of 0.1% sorbate and 1.5% formic acid produced a significant reduction of *C. jejuni* colonization in chickens (Skånseng *et al*., 2010).

Cecal colonization with *Campylobacter* could be reduced by feeding poultry with plant-protein-based feed instead of animal-protein-based feed (Udayamputhoor *et al.*, 2003). The use of large molecules in feed that can impede *Campylobacter* adhesion to the host cells has been proven to be successful *in vitro* (Wittschier *et al.*, 2007). On the other hand, additives may suffer metabolic breakdown in the chicken gastrointestinal tract and for that reason additives should be protected, encapsulated to avoid premature degradation and to succeed in preventing pathogen colonization (Van Immerseel *et al*., 2004). Hence, it seems difficult to conclude that the use of feed additives will always aid in the control of *Campylobacter in vivo*.

The use of bacteriophages to control *Campylobacter* colonization in poultry seems promising and immediate significant reductions in the numbers of *Campylobacter* in chickens after the use of bacteriophages have been reported (Wagenaar *et al*., 2005; El-Shibiny *et al*., 2009). Nevertheless, *Campylobacter* counts seemed to stabilize a few days after treatment with bacteriophages and for that reason the use of phage therapy just before slaughter has been recommended (Hermans *et al*., 2011). Carvalho *et al.* (2010) showed that the addition of phages in the feed was more efficient than oral administration to control *Campylobacter* in chickens. On the other hand, an increase in *Campylobacter* phage-resistant strains has been observed after phage therapy (El-Shibiny *et al*., 2009; Carvalho *et al.*, 2010). Vaccination strategies and the use of phage therapy are currently being tested (Goode *et al.*, 2003; Wagenaar *et al*., 2005; Loc Carrillo *et al*., 2005; Wagenaar *et al.*, 2006). New technologies such as nanotechnology and reverse vaccinology can be used to improve food safety (Malsch, 2005).

Strict biosecurity might be difficult to achieve and maintain and even when strict hygiene measures are successfully applied, *Campylobacter*-free flocks are almost always contaminated at slaughter

(Rivoal *et al*., 2005). Consequently, controls against *Campylobacter* should be implemented during transport of poultry, at slaughter and during the production of poultry products and by-products.

5.4.2. Controls during transport

The level of cross-contamination during transportation of poultry from farm to the abattoir can be very high because birds defecate over other birds and on the crates (Stern, 1992; Wesley *et al*., 2009). Transport crates are washed and used again for the transport of poultry from diverse farms. Still, crates and transport modules may remain contaminated after washing posing a risk for the transmission of *Campylobacter* and other pathogens to farms (McKenna *et al*., 2001; Berrang *et al*., 2004b; Hansson *et al*., 2005; Tinker *et al.*, 2005; Bull *et al.*, 2006; Allen *et al*., 2008a,b; Hastings *et al*., 2011). Hastings *et al.* (2011) demonstrated that *Campylobacter* could survive on crates for at least three hours after washing; the crates may have been used in many farms during that time indicating potential *Campylobacter* transmission. Moreover, *Campylobacter* strains isolated from transport crates may be the same as those recovered from poultry during holding at the abattoir (Hiett *et al.*, 2002; Slader *et al*., 2002; Rasschaert *et al*., 2007).

Different potential treatments of transport materials in order to reduce the risk of *Campylobacter* introduction into poultry flocks have been assessed. Spray washing transport materials can reduce *Campylobacter* numbers (Berrang and Northcutt, 2005). The storage of transport materials during 48 hours can reduce *Campylobacter* numbers but this control measure does not seem cost-effective (Berrang *et al.*, 2004b). The use of detergent during the washing of the crates might not always eliminate *Campylobacter* attached to crate surfaces (Slader *et al.*, 2002). Research into new methods for the effective cleaning and disinfection of transport materials and vehicles should be conducted in order to decrease or eliminate the risk of pathogen introduction into poultry farms this way.

5.4.3. Controls at slaughter, processing and consumption

Chicken meat and other poultry products can become contaminated with *Campylobacter* by cross-contamination during processing at the slaughter line (Allen *et al.*, 2008a). Logistic slaughter has been proposed to process *Campylobacter* free flocks before *Campylobacter* positive flocks in order to avoid contamination of meat and the processing environment. In order to achieve this aim, a

rigorous and accurate sampling protocol for *Campylobacter* should be in place to obtain reliable data from all flocks just before slaughter which might be difficult to perform. Studies have demonstrated that cross-contamination from positive flocks might contaminate with low *Campylobacter* concentration a number of chicken carcasses from negative flocks (Hermosilla, 2004; Johannessen *et al.*, 2007). On the other hand, risk assessment models have shown that logistic slaughter may have a limited effect on the reduction of the number of human cases (Havelaar *et al.*, 2007a/b). Thus the costs in terms of sampling and practical efforts for the implementation of logistic slaughter seem to surpass the public health benefits.

Scheduled slaughter consists on identifying *Campylobacter* positive flocks for subsequent decontamination treatment like freezing. Scheduled slaughter is applied in some countries such as Denmark, Norway and Iceland. Nonetheless, the efficiency of this system is highly dependent on the time of sampling and the use of rapid, simple tests for the detection of *Campylobacter* just before slaughter. For example, a study conducted in Norway showed that the percentage of *Campylobacter* positive flocks increased from 50% to 75% by moving the sampling time from one week to four days before slaughter (Hofshagen *et al.*, 2010). Moreover, scheduled slaughter and selective treatment of *Campylobacter* positive flocks requires complex practical methods and logistics that may not always be applicable. As a result, treatment of all positive birds might not be a realistic aim (EFSA, 2011a).

Poultry processing areas such as scalding, de-feathering, evisceration, washing and chilling are considered critical control points where controls could be most effective in preventing, eliminating or reducing food safety hazards (Mulder, 1999; Hafez, 1999; United States Department of Agriculture [USDA], 1999; Rosenquist *et al.*, 2006; Barros *et al.*, 2007). Water used in immersion scalding becomes heavily contaminated during processing increasing the risk for cross-contamination. The temperature generally used in scalding tanks is not high enough to ensure complete elimination of pathogens (Townsend, 2006). Cloacal contents may be released during automated de-feathering (Berrang *et al.*, 2001). In this way, poultry meat may become contaminated during the scalding process and also during the de-feathering step due to cross-contamination of the machinery and the production of aerosols (Izat *et al.*, 1988; Berrang and Dickens, 2000). Many studies have investigated the potential antimicrobial effect of scald additives such as chlorine, trisodium phosphate, sodium metabisulfite (Tambyln *et al.*, 1997), sodium hydroxide, propionic acid (Humphrey *et al.*, 1981), acetic acid (Okrend *et al.*, 1986; Lillard *et al.*, 1987; Tamblyn *et al.*, 1997) and a commercial additive known as RP Scald (Townsend, 2006). Evisceration is a critical

step because gastrointestinal tracts of chickens can harbor high numbers of *Campylobacter* that may be liberated during evisceration contaminating meat and the processing environment (International Commission on Microbiological Specifications for Foods [ICMSF], 1996; Perko-Mäkelä *et al*., 2009). Consequently, minimizing intestinal ruptures and preventing *Campylobacter* spread are very important control measures. A significant reduction in the numbers of *Campylobacter* present on chicken meat can be achieved by washing (Cudjoe *et al.*, 1991). But chicken skin protects *Campylobacter* (Atterbury *et al*., 2003) and *Campylobacter* may form or attach to biofilms in diverse surfaces, in chickens and the environment (Kalmokoff *et al*., 2006; Nguryen *et al*., 2011). Visceral rupture during meat processing has been significantly associated with increased *Campylobacter* contamination levels (Berrang *et al.*, 2004a; Boysen and Rosenquist, 2009). Hence, controls should be implemented to prevent visceral rupture and/or to remove fecal contamination during the slaughter process. In addition, changes to the poultry processing line such as altering the order of the processing steps have been proposed (e.g. performing evisceration prior to scalding) in order to reduce *Campylobacter* contamination of poultry products and by-products (Berrang *et al.*, 2011). Performing cloacal plugging before slaughter can be very effective in reducing *Campylobacter* numbers on poultry carcasses but the practical application of this control seems difficult and labor intensive (Musgrove *et al.*, 1997; Berrang *et al*., 2011).

Post-slaughter control measures such as chilling, freezing and the application of decontamination technologies such as the use of chlorinated water or irradiation of foods are currently used or tested (James *et al.*, 2007). On the other hand chicken skin might protect *Campylobacter* from some of these methods and environmental stresses (Atterbury *et al.*, 2003). *Campylobacter*-specific bacteriophages have been developed and applied to chicken skin successfully reducing the number of recovered *Campylobacter* by 1 log. Despite the success, a larger reduction of the numbers of viable *Campylobacter* present in chicken skin would be desirable. The combined use of phages and freezing rendered a significantly larger reduction in the concentration of *Campylobacter* in chicken skin (Atterbury *et al*., 2003). Freezing is widely used in some countries to control *Campylobacter* and other pathogens that may be present in chicken products (Sampers *et al*., 2010), in actual fact, *Campylobacter* numbers on chicken carcasses can be reduced by 1 to 3 logs by freezing (Rosenquist *et al*., 2006; Georgsson *et al*., 2006). Rapid freezing of chicken carcasses can be effective against *Campylobacter* (Sandberg *et al*., 2005). Freezing of chicken products has the advantage that meat quality and appearance is minimally affected although the use of freezing might translate on increase costs for the industry (EFSA, 2011a). Freezing of poultry meat from

Campylobacter positive flocks is a *Campylobacter* control strategy adopted by several EU countries (Hofshagen and Kruse, 2005; Tustin *et al.*, 2011). Freezing has been considered the most effective method in reducing *Campylobacter* contamination between several decontamination techniques (Sandberg *et al*., 2005; Georgsson *et al*., 2006). Other technologies such as crust freezing and forced air chilling can reduce *Campylobacter* numbers in poultry products but to a lesser extend (Boysen and Rosenquist, 2009).

The use of steam-ultrasound technologies to reduce *Campylobacter* numbers in poultry meat has been investigated. In a study conducted by Boysen and Rosenquist (2009) a considerable decrease of the numbers of *Campylobacter* was achieved in some samples (reduction of > 2.5 logs/sample) but as a result the meat appeared partially cooked. Thus, significant *Campylobacter* reductions can be difficult to obtain using steam-ultrasound alone without compromising the appearance and quality of the meat products (Whyte *et al*., 2003; James *et al*., 2007).

The application of organic acids into the cloaca of chicken carcasses during processing may decrease the numbers of *Campylobacter* in broiler meat (Berrang *et al*., 2006). The application of organic acids onto beef carcasses produced a decrease on the numbers of recovered bacteria (Bell *et al*., 1997; Dorsa *et al*., 1997; Castillo *et al*., 1998, 1999; Cutter, 1999). *Salmonella* numbers present on broiler carcasses can be reduced by spraying broiler carcasses with lactic acid (Li *et al*., 1997; Xiong *et al*., 1998; Yang *et al*., 1998). Organic acids can also reduce numbers of bacteria present in chicken meat when applied to scald water (Izat *et al*., 1990) as a spray during defeathering (Dickens and Whittemore, 1997) and before and after chilling (Izat *et al*., 1990; Dickens and Whittemore, 1994). Furthermore, lactic acid used in combination with modified atmosphere parking technology can preserve fresh chicken meat and increase the shelf life (Zeitoun and Debevere, 1992; Jimenez *et al*., 1999). The addition of acidifying bacteria particularly lactic acid bacteria (LAB) may preserve foods and several studies have demonstrated inhibitory effects against *Campylobacter* possibly due to pH reduction and/or the bactericidal effect of hydrogen peroxide (H2O2) generated by LAB when there is oxygen (Chaveerach *et al.*, 2004a; Svetoch *et al.*, 2005; Strus *et al*., 2006).

The effect of irradiation to eliminate pathogenic bacteria such as *Campylobacter* present in poultry meat has been investigated (Kampelmacher, 1984; Lacroix and Ouattara, 2000; Chun *et al*., 2010). The application of UV-C irradiation can be effective in reducing the numbers of *C. jejuni* in poultry meat and ready-to-eat meat (Chun *et al*., 2010). In reality, irradiation of foods is effective in the inactivation of pathogens and is permitted in some EU countries (Humphrey *et al*., 2007). Irradiation doses allowed in EU and a list of foodstuffs authorized for irradiation decontamination

can be found in the UE directive 1999/3/EC. Though, the use of gamma-irradiation is not accepted by EU consumers and therefore its use has been discouraged. The use of electron accelerators is more acceptable and currently applied for decontamination of chicken meat in Europe (Carry *et al*., 1995). Irradiation of poultry meat (at a maximum dose of 3 kGy) for the control pathogens such as *Campylobacter* is allowed in the United States (Keener *et al*., 2004).

Irradiation treatments might have detrimental effects on the organoleptic qualities of foods. Hanies *et al*., (1989) observed that off-flavors were detected in poultry meat irradiated above the 2.5 kGy threshold dose. On the other hand, the use of spices may control deterioration of irradiated foods due to their natural antioxidants, in fact, the joint effects of irradiation of poultry meat and the use of rosemary and thyme have been investigated (Ingram and Farkas, 1977; Monk *et al*., 1995).

Innovative technologies that produce safe foods conserving their nutritional and sensory properties are very attractive because consumers demand safe, natural, nutritious, high quality foods with extended shelf-life and natural flavor. Natural food preservatives may have antimicrobial properties against *Campylobacter* and other pathogens (Djenane *et al*., 2011a/b/c). In this way, consumer demands for natural foods, additives and preservatives have forced regulatory agencies and the food industry to investigate the use of natural antimicrobials in order to control the growth of spoilage microorganisms and pathogens in foods. Essential oils possess potential antimicrobial effects, however, their activity seems to be reduced by the presence of protein and fat that may protect bacteria (Tassou *et al*., 1995; Djenane *et al*., 2011a/b/c ; Burt, 2004). Essential oils could be used as antimicrobials in chicken meat but they seem to be less effective in chicken skin due to the skin's rough surface that may protect bacteria such as *Campylobacter* (Fisher and Philips, 2006). The antimicrobial effects of several essential oils against *C. jejuni* isolated from chicken meat have been demonstrated (Nannapaneni *et al*., 2009; Abdollah *et al*., 2010; Rattanachaikunsopon and Phumkhachorn, 2010; Djenane *et al*., 2011c) suggesting that essential oils could be used as safe and natural additives with antimicrobial activities against *Campylobacter*. In a study conducted by Aslim and Yucel (2008) the essential oil obtained from the plant *Origanum minutiflorum* displayed antimicrobial activity against ciprofloxacin-resistant *Campylobacter* strains. However, higher concentrations of essential oils might be necessary when applied to foods in comparison with the amounts used in vitro studies (Djenane *et al*., 2011a), especially when essential oils' vapours are used (Fisher and Philips, 2006).

Wine seems to be a hostile environment for *Campylobacter* (Gañan *et al.*, 2009) and the use of wine together with antimicrobial additives in meat marinades has been proposed for the control

Campylobacter in meat products (Isohanni *et al.,* 2010). Moreover, the potential use of the phenolic compounds found in wine as an alternative to the use of antimicrobials in chickens has been suggested (Djenane and Roncalés, 2011). The use of marinating ingredients can reduce *Campylobacter* numbers (Birk *et al*., 2010) but this technique can be applied only to a proportion of the produced chicken meat since consumers demand fresh chicken meat in most countries.
Novel methods for food preservation and packaging are under investigation. Active packaging such as oxygen scavengers, drip absorbent sheets and antioxidant packaging may extend the shelf life of foods especially of fresh "easily perishable" foods such as raw meat and fish (Camo *et al.,* 2011). Novel antimicrobial films have been developed using diverse concentrations of essential oils into chitosan and hydroxypropylmethylcellulose films (Sánchez-González *et al*., 2011). In addition, the application of natural antimicrobial additives together with novel technologies may improve pathogen control and therefore food safety and quality (Gálvez *et al*., 2010). Novel products and technologies are designed and developed in order to control foodborne pathogens in food premises and kitchens. Thormar and Hilmarsson (2010) demonstrated the efficiency of glycerol monocaprate (monocaprin) in reducing *Campylobacter* concentration on contaminated plastic and wooden cutting boards.

In addition to food safety controls, public health education programs have been developed in many areas of the world in order to educate consumers in food safety (Center for Science in the Public Interest, 2005). Public awareness campaigns contain clear messages (communicated through the media in form of advertisements, brochures, videos, web-sites and other education materials) designed to inform and educate consumers in food handling in order to decrease the number of food-borne illnesses (Partnership for Food Safety Education, 2010; Canadian Partnership for Consumer Food Safety Education, 2013). The World Health Organisation (WHO) has launched a global health message known as "The Five Keys to Safer Food" to communicate the basic food safety principles that every person in the world should follow to improve food safety and prevent food-borne illnesses (WHO, 2012).

5.4.4. Integration of controls against Campylobacter in poultry

Currently there is no one single decontamination technology alone capable to eliminate *Campylobacter* or reduce it to negligible levels in foods without altering food characteristics. For

that reason, an integrative approach must be followed in order to control *Campylobacter* in foods, implementing several effective control measures throughout the food chain. It has been demonstrated that the microbiological contamination of beef carcasses can be significantly reduced by the application of several sequential decontamination techniques (Bacon *et al*., 2000).

An integrated approach to the control of *Campylobacter* in poultry has been adopted in Denmark where increased biosecurity, allocation of meat from positive flocks to the production of frozen foods and consumer education campaigns have led to a significant decrease in *Campylobacter* prevalence in broiler flocks (from 43% in 2002 to 27% in 2007), a reduction of *Campylobacter*-positive samples of fresh broiler meat and a decrease in registered human cases by 12% from 2002 to 2007 (Rosenquist *et al.*, 2009).

The combination of decontamination technologies with appropriate storage conditions (low pH and low temperature) for the control of *C. jejuni* in foods has been investigated (Smigic *et al*., 2010). In fact, the synergistic effect against *Campylobacter* of the use of rosemary essential oil extract and freezing has been demonstrated (Piskernik *et al.*, 2011). Nevertheless, the effect of a *Campylobacter* control measure alone or in combination with other controls also depends on a number of factors related to the production conditions such as the type of equipment, type of processes, temperature, humidity and many others (Van *et al.*, 1995; Purnell *et al*., 2004). The step of the production where the control is applied will affect the effectiveness of this control measure, e.g., the effect of chemical products might be different when used in the scalding tank than when applied directly onto the meat at the end of the production process (Havelaar *et al*., 2007a).

5.4.5. Campylobacter risk assessments and evaluation of the public health impact of controls against Campylobacter in poultry production

Campylobacter risk assessments have been conducted nationally in some developed countries and internationally by WHO and FAO (Cahill, 2005). Risk assessment models may consider the whole of the food chain, from farm to the consumer following an integrated and multidisciplinary approach (Slorach *et al*., 2002). In particular, risk assessments conducted in Denmark (Rosenquist *et al.*, 2003), the Netherlands (Nauta *et al*., 2005), UK (Hartnett et al., 2001, 2002) and New Zealand (Lake *et al*., 2007) provide “farm-to-fork” estimations of the risk of human *Campylobacter* infection via the consumption of poultry meat. Risk assessments aim to assess the public health risks associated with the consumption of contaminated poultry meat but also to assess the

effectiveness of potential control measures (Rosenquist *et al*., 2003; Nauta *et al*., 2005). Despite the numerous high quality research outcomes regarding *Campylobacter* infection of broiler chickens, prevention and controls, important data gaps make risk assessment models incomplete (FAO, 2002).

The public health impact of the use of decontamination technologies against *Campylobacter* can be assessed using risk assessment models but accurate good quality data is necessary to obtain realistic and useful results. The synergistic application of effective control measures against *Campylobacter* such as freezing, irradiation and proper cooking could achieve a human risk reduction of 90-100% assuming that no re-contamination occurs (Havelaar *et al.*, 2007a/b; EFSA, 2011a). On the other hand, estimations of the effectiveness of controls against *Campylobacter* are uncertain, frequently based on limited data that might not be representative as concluded in a recent EFSA Expert Opinion: "Another general finding is that it is difficult to obtain good representative data that allow estimating the effect of specific control options in terms of reduction in *Campylobacter* concentration or prevalence. Quite often the effect estimates are based on one or a few published or unpublished laboratory experiments, or expert opinion, and they cannot always be correctly applied to conditions other than the specific ones under which they were designed. As a consequence their predicted effects on risk reduction are also highly uncertain" (EFSA, 2011a, page 53).

Data related to the effectiveness of decontamination technologies against *Campylobacter* obtained using naturally contaminated samples at an industrial scale are preferred to data produced in the laboratory using artificially inoculated samples (EFSA, 2011a). Many laboratory studies have investigated *Campylobacter* inactivation on artificially contaminated chicken meat but there is lack of data regarding the effects of using diverse strains and different inoculation levels. In real life situations, it is not uncommon that chickens are infected by several *Campylobacter* strains and that *Campylobacter* concentration varies between chickens. Accordingly, research data based on naturally infected birds and commercial production conditions are desirable. The effectiveness of decontamination technologies on reducing *Campylobacter* numbers might vary with the initial *Campylobacter* concentration present and also with different *Campylobacter* strains. For that reason, results obtained using one or few *Campylobacter* strains and/or concentrations might not produce representative data (Greer *et al.*, 1992; Birk *et al.*, 2010).

Quantitative microbiological risk assessments are used for risk management of food safety issues and to establish standards for the international trade of food. On the other hand, assessment of the

contribution of the diverse potential sources of disease is crucial for the implementation of effective control strategies (Havelaar *et al.*, 2007a). Source attribution models are valuable for the selection and prioritization of potential controls but the effects of interventions need to be assessed using different models such as risk assessment models. Source attribution models can fall into one of these two wide categories: epidemiologic approaches (based on public health data) or microbiologic approaches. These two approaches may complement each other; in fact, epidemiological data can be integrated in microbiologic models. Microbiologic approaches include risk assessment of specific pathogens, the Hald model (Hald *et al*., 2004; Mullner *et al*., 2009) and other models based on microbial source tracing methods which use molecular subtyping data from pathogens isolated from humans and potential sources. The use of epidemiological data alone will not be sufficient for a complete risk assessment (Haas *et al*., 1999). The source of disease in sporadic cases seems difficult to define, and particularly the identification of sources associated with endemic diseases becomes much more difficult (Riley, 2004). Moreover, the complexity can be overwhelming in cases when the epidemiology of the disease is complex involving diverse bacterial strains and many potential risk factors as it happens with human campylobacteriosis. Recent advances in molecular epidemiology have been incorporated in source attribution models providing a better understanding of the impact of particular sources and/or transmission routes on the human disease burden. Some source attribution models such as the Hald model (Hald *et al*., 2004), the Island model (Wilson *et al*., 2008) and STRUCTURE (Pritchard *et al.*, 2000) can attribute risks to sources directly. Other type of models such as the "Minimum Spanning Trees" based on clustering tools (Spratt *et al*., 2004) cannot provide risk estimations but they may increase our understanding of the epidemiology of the pathogen and of the relative importance of diverse sources. Knowledge obtained from diverse models and research areas can be integrated to increase our understanding of *Campylobacter* epidemiology, human infection sources and the effectiveness of potential controls. Nevertheless, disease models are based on assumptions in order to manage the complexity inherent to the disease and so models are limited and the results need to be interpreted carefully.

5.5. Vaccines against *Campylobacter* and other zoonotic pathogens

The development of vaccines to protect animals from zoonotic infections has important public health implications since approximately 75% of new emerging infections can be considered zoonoses (Lütticken *et al*., 2007). The benefits obtained from the development of successful vaccination strategies against zoonotic diseases can be considerably attractive. Nonetheless, the

implementation of successful vaccination strategies requires the collaboration of human doctors, veterinarians, epidemiologists, scientists and politicians (Lütticken *et al*., 2007). In general, when conducting vaccination trials, vaccine efficiency will be affected by many factors such as the type of vaccine, the microbial strain, the animal model used for testing and many others. Hoffelner *et al.* (2008) demonstrated differences on vaccine efficiency when using different strains of *Helicobacter pylori* and alternative animal models.

Enteric infections are considered one of the main causes for human disease and human casualties across the world. The development of effective vaccines to protect humans from the main pathogens associated with enteric infections is highly desirable. Rotaviruses have been considered the lead cause of enteric infections worldwide. Diagnosis, surveillance and control of enteric diseases can be difficult due to the variety of causative agents and to the lack of resources in developing countries. Efforts have been directed to share light into the causation of enteric diseases in developing countries and into the development of effective vaccines (Walker, 2005). Practical considerations regarding the use of vaccines against enteric pathogens need to be considered taking on account the target population. Vaccines intended to protect children in developing countries need to be safe for very young infants (Walker, 2005). The production of a combined vaccine to protect humans against the main pathogens responsible for intestinal disease has been proposed (Walker, 2005).

Vaccines are currently being used successfully to control avian diseases such as coccidiosis, Marek's disease and systemic salmonellosis (FAO, 1997). Vaccination is considered one of the main strategies to reduce the incidence of human Salmonellosis. Several vaccination strategies developed to control *Salmonella* in chickens and pigs seem to be effective such as the use of inactivated bacterins for the immunization of sows (Roesler *et al.,* 2006).The prevalence of *Salmonella* in laying hens can vary between 0% and 79% in EU countries (EFSA, 2007). In addition, vaccination of poultry for the control of *Salmonella* is compulsory in the EU for flocks of laying hens with a *S. Enteritidis* prevalence higher than 10% since 2008 (European Commission, 2006).

The development of a cost-efficient vaccine against *Campylobacter* has been identified as a priority especially for travelers, young children and the military (Girard *et al*., 2006). Vaccines against *Campylobacter* have been developed for humans (Baqar *et al.*, 1995a; Scott, 1997, Scott and Tribble 2000), chickens (Khuory and Meinersmann, 1995; Noor *et al.*, 1995; Widders *et al*., 1996;

Rice *et al*., 1997; Newell and Wagenaar, 2000) and other animals (Baqar *et al.*, 1995b). However, a general cost-efficient vaccine for the control of *Campylobacter* in chickens and humans has not been developed despite years of research (Jagusztyn-Krynicka *et al.*, 2009). A successful commercial vaccine should be safe, cost-effective and produced in large quantities. Conventional vaccines usually perform poorly when applied to chickens due to the interaction of *Campylobacter* with the intestinal niche in poultry (Ringoir and Korolik, 2003; Walker, 2005). Moreover, immunity against *Campylobacter* seems to be strain-specific. Hence, the development of a vaccine able to protect the host against all *Campylobacter* strains seems challenging.

A vaccine against *C. jejuni* infections in humans (ACE393) has been developed. A human trial confirmed that the injectable vaccine ACE393 was well tolerated and produced satisfactory immune responses in vaccinated individuals. In fact, when administered using a 250µg dose, ACE393 stimulated a four-fold increase in the production of IgG in all persons (Anon, 2007). Conversely, the vaccine ACE393 did not seem to produce a statistically significant reduction in *Campylobacter* concentration when administered intramuscularly in broilers under specific experimental conditions (Garcia *et al.*, 2012).

Animals have been used as models for *Campylobacter* pathogenesis studies and for testing new vaccine candidates (Islam *et al*., 2006). Chickens are obviously the preferred animal model to assess the efficacy of vaccines developed to reduce *Campylobacter* burden in birds (Davis and DiRita, 2008). Cell mediated immune response against *Campylobacter* has been observed in chickens three days after hatch (Noor *et al*., 1995; Smith *et al*., 2005). A significant level of anti-*Campylobacter* IgG antibodies from the maternal source can be detected in chicks younger than 3 weeks, the chickens become susceptible to infection later on in life (Sahin *et al*., 2002; Shoaf-Sweeney *et al*., 2008). Therefore, *Campylobacter* control strategies based on immunization of poultry might be feasible. On the other hand, the immune response against *Campylobacter* in chickens is generally moderate and the absence of a strong immune response has been identified as one of the main challenges for vaccine efficacy to control *Campylobacter* in chickens (de Zoete *et al*., 2007). What's more, the genetic diversity of *Campylobacter* might hamper controls based on immunization of chickens. *C. jejuni* strains differ on the ability to colonize chicken intestines and on the infective dose for chickens (Ziprin *et al*., 2002; Jagusztyn-Krynicka *et al.*, 2009). Genetic manipulation of specific *C. jejuni* genes (*dnaJ, cadF9*) can alter the ability of *C. jejuni* strains to colonize chicken caeca. Vaccination of chickens using a vaccine cocktail including viable non-colonising *C. jejuni*

strains was tested by Ziprin *et al*. (2002). However, their results found no statistically significant difference between vaccinated and control groups of chickens.

Vaccines are usually delivered orally but in-ovo vaccination has been developed (Negash *et al.*, 2004). *Campylobacter* vaccines have been tested using other experimental animals such as ferrets, mice and monkeys. Researchers have studied animals' immune responses to *Campylobacter* infections (Baqar *et al.*, 1995a/b; Rice *et al*., 1997; Islam *et al.*, 2006). Monkeys are generally used for developing human vaccine candidates due to similarities with human infections. In actual fact, monkeys seem to acquire immunity against *Campylobacter* infections (Islam *et al.*, 2006). In a study conducted by Islam *et al*. (2006) Rhesus macaque monkeys were challenged with *C. jejuni* strain 81-176 (doses of 10^7, 10^9 and 10^{11}). IgM-antibody secreting cells (ASC) responses in challenged monkeys were observed but no *C. jejuni*-specific IgA or IgG ASC responses. Though, some animals that recovered from infection seemed to be protected against re-infection. Rice *et al.* (1997) observed that increased titers of anti-*C.jejuni* IgA in bile corresponded to reductions in *C.jejuni* cecal colonization in poultry orally vaccinated with killed *C. jejuni* cells. In-ovo vaccination of chicks using heat-killed *C. jejuni* produced increased IgA antibodies (Noor *et al.*, 1995). The intraperitoneal administration of killed *C. jejuni* cells increased specific IgY in the intestine of chickens (Widders *et al*., 1996). Heat-killed and/ or formalin-killed bacteria have been used as oral vaccines inducing immune responses in ferrets, monkeys and mice (Rollwagen *et al*., 1993; Baqar *et al*., 1995a/b; Burr *et al*., 2005).

Diverse approaches can be followed for the development of *Campylobacter* vaccines:

- live attenuated vaccines,
- vaccines based on killed bacteria (heat-killed/formalin-killed) with or without adjuvants,
- subunit vaccines and
- live attenuated *Salmonella* strains expressing specific *Campylobacter* proteins

Live-attenuated vaccines against *Shigella* infections in humans have been developed (Venkatesan *et al*., 2006; Levine *et al.*, 2007; Phalipon *et al.*, 2008). But the use of a live-attenuated *Campylobacter* vaccine seems unlikely due to the risk of developing long-term sequelae such as GBS and genetic recombination of *Campylobacter spp.* (Jagusztyn-Krynicka *et al.*, 2009). Besides, the use of live vaccines for *Campylobacter* control can be hindered by the genomic instability of *Campylobacter* (de Zoete *et al.*, 2007; Ridley *et al.*, 2008a). Genetic immunization is a novel strategy that seems very promising; genes such as the gene flaA are considered very important for the pathogenicity of

C. jejuni. Vaccination of chickens with a protein including part of the *C. jejuni* FlaA proved successful in reducing *Campylobacter* colonization (Khoury and Meinersmann, 1995).

Campylobacter whole-cell (CWC) vaccines have been developed and tested (Baqar *et al.,* 1995a/b; Rice *et al.,* 1997). Nonetheless, the use of CWC seems difficult due to the genetic diversity of *Campylobacter* and to concerns regarding long-term sequaleae such as GBS syndrome despite the fact that volunteers infected with a CWC vaccine did not develop persistent antiganglioside antibodies (Prendergast *et al*., 2004). An inactivated whole cell *Campylobacter jejuni* vaccine was used to protect ferrets against *Campylobacter* (Burr *et al*., 2005). These authors observed that the use of adjuvant was not improving protection and that animals were protected against homologous and heterologous *Campylobacter* strains used in the study (*C. jejuni* strain 81–176, (Lior serotype 5) and strain CGL7 (Lior serotype 4)).

Campylobacter subunit vaccines using specific antigens are safer than CWC vaccines but less efficient. Nevertheless, the selection of the antigen(s) proves to be a crucial factor for vaccine success. The use of genomics and proteomics allows the identification of new antigens for vaccine development (Jagusztyn-Krynicka *et al.*, 2009). Metagenomic experiments and the production of a proteome-wide protein interaction map might provide relevant information regarding infection and colonization with *Campylobacter* (Parrish *et al*., 2007).

Campylobacter jejuni is able to produce a polysaccharide capsule (CPS) to protect its surface; in actual fact, a number of different CPS structures have been identified (Aspinall *et al*., 1995; Muldoon *et al*., 2002; Karlyshev *et al*., 2005; Chen *et al*., 2008). The role of CPS in the colonization and virulence properties of different *Campylobacter* strains has been suggested but this role is poorly understood (Champion *et al*., 2010; Guerry *et al*., 2012). Some researchers showed that *Campylobacter* strains with CPS performed better at colonizing chickens (Grant *et al*., 2005; Bachtiar *et al*., 2007). Vaccination strategies based on CPS have been developed to control diseases caused by encapsulated bacteria such as *H. influenzae* and *Streptococcus pneumoniae* (Lesinski and Westernick, 2001; Knuf *et al*., 2011). A capsule conjugate vaccine was able to protect non-human primates against enteric disease with *Campylobacter* (Monteiro *et al*., 2009). Genomics and proteomics are used to identify key *Campylobacter* proteins that may play an essential role in the stimulation of immune response in infected animals and could be included in vaccines (Prokhorova *et al*., 2006). The use of the flagella gene plasmid DNA has produced interesting results such as the reduction of number of *Campylobacter* in chickens by 2 logs (Wyszynska *et al*., 2004).

Immunization of chickens with flagella protein has proven to produce increased levels of IgG and IgM in serum and IgG in intestinal mucosal as well as reducing *C. jejuni* by 1-2 logs (Widders *et al.*, 1996). Vaccination of mice with flaA subunit vaccines stimulated immune responses (Lee *et al*., 1999). On the other hand, flagellin genes are highly variable and thus the efficacy of vaccines based on these particular genes might vary between *Campylobacter* strains. The selection of highly conservative areas of the *Campylobacter* genome for vaccine development may offer promising results. A *Campylobacter* gene (cjaA, common to diverse *Campylobacter* strains) which encodes a highly immunogenic lipoprotein was carried by an avirulent recombinant *Salmonella* strain and orally administered to chickens. As a result, production of mucosal IgA and serum IgY was observed and chickens seemed to be protected against cecal colonization with a different *C. jejuni* strain (Wyszynska *et al.*, 2004). The use of heterologous vaccines for the control of *Campylobacter* in poultry has been explored (Buckley *et al*., 2010; Layton *et al.*, 2011).

The identification of genes that are essential for *Campylobacter* colonization of chickens may be crucial for the development of an effective vaccine against *Campylobacter* in poultry. It has been suggested that inactivation of the gene cfrA may result in complete prevention of *Campylobacter* colonization in chickens and as a result this gene could be a very promising candidate for vaccine development (Zeng *et al*., 2009). The *C. jejuni* gene that encodes the *dps* protein plays an important role in cecal colonization of chickens and in biofilm formation. A *Salmonella*-based vaccine encoding the *Campylobacter* dps protein responsible for adherence to host cells seems promising and is under development at the University of Arizona, USA (Joens, 2012; Theoret *et al*., 2012). A vaccine candidate based on a live *Salmonella* vector expressing a linear peptide epitope from a *Campylobacter* protein (Cj0113 (Omp18/CjaD)) produced consistent immune responses and reduced *Campylobacter* numbers below detection level (Layton *et al.*, 2011). Intranasal delivery of Chitosan-pCAGCS-flaA nanoparticles as a vaccine for chickens successfully induced effective immune response with reductions of *Campylobacter* by 2-3 logs in large intestine and 2 logs in caecum of chickens. Chickens immunized with this vaccine showed significantly increased levels of intestinal mucosal antibody IgA and serum anti-*C. jejuni* IgG (Huang *et al.*, 2010). Chitosan, a natural bioadhesive product, is considered to be a good adjuvant for vaccines offering a few advantages as gene vectors. The efficiency of chitosan as gene vector was estimated to be more than 90% by using electron microscopy (Huang *et al.*, 2010).

The success of vaccination trials for the control of *Campylobacter* infections depends on a number of factors such as the vaccine candidate, the animal model, individual host factors, *Campylobacter*

strain, environmental factors and others. In addition, the choice of the study unit and clear objectives including the expectation for the successful vaccine candidate are crucial for the trials (Garcia *et al*., 2012). A particular vaccine might work on an specific trial (with an animal model for example) using a particular *Campylobacter* strain and controlled experimental conditions but the same vaccine might fail when using other strains, different animal models or diverse environments. Moreover, field studies to test vaccine efficiency carry additional complexity due to the lack of knowledge regarding some environmental factors. Additional factors to consider are microbiological methodologies used for isolation, characterization and quantification of *Campylobacter* and the choice of data analysis techniques. Uncertainty plays a key role in the studies and should be accounted for when developing mathematical or epidemiological models. It could be long and complex to examine and compare every study regarding vaccine development and vaccination strategies. Nevertheless, important epidemiological considerations can be discussed. The animal model is a crucial factor and chickens are the preferred animal model in *Campylobacter* vaccination trials (Cawthraw *et al.,* 1996; Davis and DiRita, 2008). Leghorn broilers have been used in *Campylobacter* colonization experiments and vaccine trials (Rice *et al.,* 1997; Stas *et al.,* 1999; Ziprin *et al*., 2002; Sahin *et al*., 2003; Shoaf-Sweeney, 2008**).** Most experimental protocols use three groups of chicken: vaccinated, experimentally colonized and control groups (Rice *et al.*, 1997). Time and age of animals can be an important factor when evaluating the efficacy of vaccines. The time of immunization, the age of the animals when being vaccinated, time at which samples are collected and analyzed will influence the results of the experiments. Rice *et al.* (1997) observed that in 31-day-old birds (21 days post-challenge) there was 81% reduction on *Campylobacter* numbers between vaccinated chickens and controls while the reduction was only 25% in 50-day-old chickens.

The challenge method is considered a crucial factor when conducting vaccination trials. The use of a challenge method that mimics natural transmission of *Campylobacter* in broiler flocks is highly desirable. More knowledge regarding the ecology of *Campylobacter* in commercial flocks will be advantageous. *Campylobacter* is rarely detected in commercial flocks with birds younger than two weeks of age (Annan-Prah and Janc, 1988; Stern, 1992). It has been suggested that this observation could be due to *Campylobacter* characteristics such as the presence of stressed *Campylobacter* cells that could still be infective (Rollins and Colwell, 1986; Jones *et al*., 1991; Oliver, 2005) or low numbers (below detection limit). Besides, young birds could be immunized against *Campylobacter* because a significant level of anti-*Campylobacter* IgG antibodies from the maternal source can be

detected in chicks younger than three weeks (Sahin *et al*., 2002; Shoaf-Sweeney *et al.*, 2008). It has been suggested that this *Campylobacter*-free "window" could be strategically used to introduce vaccination programs (Rice *et al*., 1997).

Sampling protocols and microbiological techniques for the detection and quantification of *Campylobacter* will influence the interpretation of the effect of vaccines in clinical trials. Quantitative microbiological data need to be properly analyzed. Sample size calculations and forecasted group effects need to be carefully considered during the experimental design phase. Additionally, data analysis methodologies should be carefully selected based on the experimental design (Garcia *et al*., 2012).

5.6. Discussion

During the last few years, risk assessment models have been developed to forecast the reduction of the number of human campylobacteriosis cases following successful implementation of *Campylobacter* controls. However, models are based on assumptions and uncertain data and consequently the results need to be interpreted carefully. In addition, the effectiveness of *Campylobacter* control strategies (one intervention alone or in combination with other controls) will depend on the presence and interaction with many factors as previously indicated in this thesis.

Nevertheless, it has been demonstrated that a reduction on the number of human campylobacteriosis cases is possible as a result of the successful implementation of effective interventions in poultry production. In New Zealand, reported human campylobacteriosis cases declined by 52% and hospitalizations due to GBS by 13% after successful interventions were implemented to reduce *Campylobacter* contamination of poultry meat indicating that additional public health benefits can be achieved by controlling foodborne campylobacteriosis (Baker *et al*., 2012).

Existing evidence indicates that efforts should be directed towards the successful implementation of the most promising interventions to control foodborne campylobacteriosis and particularly *Campylobacter* infections in poultry. Poultry has been identified as one of the main risk factors for human campylobacteriosis and extensive research has been conducted to identify the most effective *Campylobacter* control strategies that could be implemented during poultry production. Actually, the production of *Campylobacter*-free flocks has been achieved experimentally, but under commercial conditions it can be challenging although not impossible.

A considerable number of epidemiological studies have been conducted to identify significant risk factors for *Campylobacter* infections in poultry and to assess the effect of interventions for the control of *Campylobacter* in poultry from farm to fork. As an example, slaughter age has been identified as a significant risk factor for *Campylobacter* infection in many epidemiological studies and for that reason reducing the slaughter age of poultry may be an effective control strategy that could be used synergistically with other *Campylobacter* controls (Newell *et al.*, 2011). *Campylobacter* is widespread on the farm environment and thus the implementation of effective biosecurity measures seems crucial to prevent *Campylobacter* introduction into the poultry houses (Ridley *et al*., 2008b, 2011a, 2011b). On the other hand, it has been suggested that not all viable *Campylobacters* present in the environment can colonize chickens (Ridley *et al*., 2008b). Nevertheless, effective bio-security measures can prevent *Campylobacter* introduction into broiler flocks. On-farm controls based on identified risk factors for *Campylobacter* infection of poultry may be effective. Additionally, synergistic effects obtained from the implementation of several biosecurity control measures are expected. Although high levels of hygiene and biosecurity may not be sufficient to produce a *Campylobacter*-free flock, the risk of *Campylobacter* introduction into poultry flocks may decrease considerably (Gibbens *et al*., 2001). Human traffic, the number of staff members and the number of human visits to poultry houses have been identified as important vehicles for *Campylobacter* transmission into poultry flocks (Kapperut *et al*. 1993; Berndtson *et al*., 1996; Evans and Sayers, 2000; Refregier-Petton *et al*., 2001; Cardinale *et al.*, 2004; Hofshagen and Kruse, 2005; Huneau-Salaun *et al*., 2007; Chowdhury *et al.*, 2012). The poultry industry should implement and sustain best hygiene practices through adequate assessment, monitoring and staff education and motivation (Berndtson *et al*., 1996; van de Giessen *et al.*, 1998).

Seasonality trends suggest an increased *Campylobacter* infections risk caused by particular risk factors at specific times of the year. The seasonal peak of *Campylobacter* infections in poultry is much more evident in areas where *Campylobacter* prevalence is generally low (EFSA, 2010a). In places where *Campylobacter* prevalence in poultry flocks is high most of the time, non-seasonal risk factors predominate and constitute a priority for the control of *Campylobacter* in poultry.

There is no clear evidence of the introduction of *Campylobacter* in poultry houses via drinking water although the water source or disinfection regimes of water lines have been found significant risk factors in some studies (Evans and Sayers, 2000; Gibbens *et al.*, 2001; Herman *et al.*, 2003; Arsenault *et al.,* 2007). In particular, lack of cleaning of water lines could be confounded by poor general hygiene (Berndtson *et al.*, 1996). Although the presence of other livestock on poultry farms

has been identified as a risk factor, the evidence is limited and the impact on the overall risk for *Campylobacter* introduction may be low (Newell *et al.*, 2011), in actual fact, risk assessment models showed that removal of other livestock may only reduce *Campylobacter* prevalence from 44% to 41% (Katsma *et al*., 2007). Despite the suggested limited effect on the prevention of *Campylobacter* infection, effective distance between poultry houses and other livestock, effective bio-security barriers or poultry-only farms have been recommended (Kapperud *et al*., 1993; Neubauer *et al*., 2005; Hald *et al.*, 2007). Similarly, the role of wildlife in the introduction of *Campylobacter* in broiler flocks is not clear (Newell *et al*., 2011). In a study conducted by Hiett *et al.* (2002) the same *Campylobacter* strains recovered from wild bird feces on a farm were recovered from broilers. Conversely, other studies have found no significant links between *Campylobacter* strains from wildlife and broilers (Petersen *et al*., 2001; Colles *et al*., 2008). Rodents and flies have been found a significant risk factor in epidemiological studies (Hald *et al*., 2008; Hazeleger *et al*., 2008). Doubts regarding the importance of rodents as risk factors have been raised as *Campylobacter* has been rarely detected in captured rodents (Jones *et al*., 1991). Nevertheless, efficient rodent control on-farm is considered a protective factor (van de Giessen *et al*., 1998). On the other hand, rodent control was found a risk factor in a study conducted by Arsenault *et al.* (2007). This finding could be explained due to the low efficacy of vermin control (Huneau-Salaun *et al*., 2007) but also due to the risk of *Campylobacter* introduction being higher due to human traffic to control rodents than the risk due to the actual presence of rodents. The relevance of flies as vectors might vary between geographical areas and it seems difficult to assess (Newell *et al*., 2011). Further research is needed in this area especially on the relevant fly species, on the distance they can travel and *Campylobacter* carriage properties.

Epidemiological studies may be compromised by limitations in resources, non-adequate sampling, poor study design and data analysis. Uncertainty will always surround results from research studies and should be considered when interpreting results and implementing controls based on research findings. Some *Campylobacter* control strategies such as vaccination aim to reduce the numbers of *Campylobacter* in the intestinal tract of chickens to achieve a reduction of *Campylobacter* in chicken meat and in turn decrease the risk of human infections with *Campylobacter*. Promising vaccine candidates against *Campylobacter* have been developed but a commercial *Campylobacter* vaccine for poultry is not available at present. A lack of understanding of the immune system in chickens and the general absence of a strong immune response after vaccination against *Campylobacter* hampered vaccine development in some cases. Moreover, the induction of an

immune response in the host by a vaccine does not always correlate with a reduction of *Campylobacter* concentration in infected animals (Sizemore *et al*., 2006). Even when an immune response and a reduction in *Campylobacter* numbers have been achieved, other factors such as safety of the vaccine, efficiency under commercial conditions, costs and other practical considerations might make the commercialization of a potential vaccine candidate difficult.

The use of bacteriophages in broilers to control *Campylobacter* seems promising but there are concerns regarding long-term efficacy and consumer safety (Hagens and Loessner, 2010). The long-term efficacy of phage therapy seems questionable because *Campylobacter* phage-resistant strains may appear naturally and/or due to the use of bacteriophages. The oral administration of phages in humans seems to be harmless (Hermans *et al*., 2011) but the use of bioengineered modified foods can be controversial and not accepted by the consumers.

Field trials need to be conducted to examine the practical effects of the most promising *Campylobacter* control measures. It is important to consider that the effectiveness of some control strategies such as phage therapy, vaccination and competitive exclusion products may be influenced by the genomic instability of *Campylobacter* (Ridley *et al*., 2008a). The type of production system will also influence the results. *Campylobacter* prevalence in free-range poultry flocks is usually higher than in poultry flocks produced in intensive conditions (Lund *et al.,* 2003; Ring *et al*., 2005; McCrea *et al*., 2006). This observation may be explained by the extensive contact with the external environment and the fact that free-range birds are generally slaughtered at an older age than intensively produced poultry (Huneau-Salaun *et al*., 2007; Colles *et al*., 2008). Hence, the effectiveness of biosecurity barriers in preventing *Campylobacter* infection of free-range poultry is not clear indicating that the type of poultry production system is an important factor to consider for the control of *Campylobacter* and other pathogens.

The poultry industry needs to be highly integrated in order to maintain profit margins which are usually very low and to meet consumer demands. *Campylobacter* control measures that can be applied at low cost are generally accepted by the poultry industry although the consistency with which the controls are implemented may vary. On the other hand, controls that require efforts and/or extra costs are not usually welcome by the poultry industry. For this reason, proposed controls should be backed up with strong evidence of effectiveness and a satisfactory viable cost-benefit balance.

6. EXPERIMENTAL INFECTION WITH *CAMPYLOBACTER* AND A VACCINATION TRIAL AGAINST *CAMPYLOBACTER* IN BROILERS (Manuscripts I and II)

6.1. Introduction

Vaccines against *Campylobacter* have been developed for humans (Baqar *et al*., 1995; Scott, 1997; Scott and Tribble, 2000), chickens (Noor *et al*., 1995; Widders *et al*., 1996; Rice *et al*., 1997; Newell and Wagenaar, 2000) and other animals. Chickens have been considered the preferred animal model to assess vaccine efficiency to reduce *Campylobacter* numbers in birds (Davis and DiRita, 2008). Poultry might carry *Campylobacter* in numbers as high as 10 logs CFU per gram of faeces (Stas *et al*., 1999; Sahin *et al.* 2002; Lütticken *et al.*, 2007) posing a risk for public health. A large variation in the amount of *Campylobacter* spp. in cecae of broilers going for slaughter has been reported (Stern *et al.*, 2007; Hansson *et al.*, 2010). *Campylobacter* dynamics in poultry flocks are not fully understood but poultry genetics and the time of *Campylobacter* introduction in the flocks may affect *Campylobacter* prevalence and concentration in poultry flocks and individual birds (Stern *et al*., 1988, 1990; Boyd *et al*., 2005; Li *et al.*, 2008). Risk assessment models have predicted that the implementation of effective *Campylobacter* controls in poultry can translate in a decrease of human campylobacteriosis cases (Rosenquist *et al.*, 2003; Nauta *et al*., 2009; EFSA, 2011a). Based on these models, an expectation for a successful vaccine against *Campylobacter* in poultry could be based on a 2 logs reduction. A cost-effective vaccine against *Campylobacter* in poultry is not commercially available yet despite numerous attempts and years of research in this subject. Conventional vaccines usually perform poorly in chickens due to the interaction of *Campylobacter* with the intestinal niche in poultry and the absence of a strong immune response (Ringoir and Korolik, 2003; Walker, 2005; de Zoete *et al*., 2007). Moreover, immunity against *Campylobacter* seems to be strain-specific and consequently the development of a vaccine able to protect the host against all *Campylobacter* strains seems challenging. Nevertheless, an ideal successful commercial vaccine should protect poultry against all *Campylobacter* strains, should be not only cost-effective but also safe and produced in large quantities. Recently, a proteomic approach has been applied in order to identify new relevant antigens. The application of biochemical fractionation and mass spectrometry analysis has conducted to the identification of more than 110 surface polypeptides of *Campylobacter jejuni* (Prokhorova *et al.*, 2006). Following

this research, a vaccine against *Campylobacter* was developed based on the protein ACE 393. Colonization with *C. jejuni* was substantially reduced in mice vaccinated with ACE 393 protein (Prokhorova *et al.,* 2006; Schrotz-King *et al.,* 2007). A human trial confirmed that the injectable vaccine ACE393 stimulated a four-fold increase in the production of IgG in all persons when administered using a 250µg dose (Anon, 2007). These results prompted us to investigate if poultry vaccination with ACE393 protein (the most promising candidate obtained in the study by Prokhorova *et al.,* 2006) could also induce a protective response against gastrointestinal colonization of *C. jejuni* in broilers. The assessment of vaccine effectiveness based on quantitative reduction of pathogens is a complex task, which comprises: i) the statistical design of experimental trials, ii) the use of quantitative microbiological detection methods, iii) application of appropriate data analysis and finally the transformation of the vaccine effect in public health terms (e.g. reduced number of human cases). Likewise, the choice of study unit, sample size, sampling protocol and forecasted group effects as well as data analysis methodologies should be carefully selected as they might affect the results of vaccine trials (Manuscript I: Garcia *et al*., 2012).

6.2 Materials and methods

6.2.1. Animals and experiments

The experimental infections and vaccination trials were carried out at the National Veterinary Institute (Aarhus, Denmark) following Danish legislation for animal welfare and use of experimental animals. Experiments were conducted based on a nested fixed block design (no blinding) to test the vaccine candidate ACE 393 based on a *Campylobacter* surface polypeptide discovered by Prokhorova *et al*. (2006). The experiment used isolators (Montair Andersen B.V. HM 1500) with 13 broilers placed in each isolator (Figure 2). Commercial broiler chickens (Ross 308) of mixed sex obtained from a Danish hatchery (DanHatch A/S) were used for the experiments. Chicks were transferred directly from the hatchery to the experimental unit, tested free of *Campylobacter* at placement and placed in the isolators at random. Broilers sharing the same isolator were administered the same treatment (either vaccine or placebo). The required sample size was calculated based on the sample size for a simple random sample design (16), multiplied by the estimated design effect (7). Accordingly, it was necessary to include about 120 broilers in each

group. Eight isolators were available for the clinical trial and thereby four rotations were necessary to achieve enough number of chickens in the experiment.

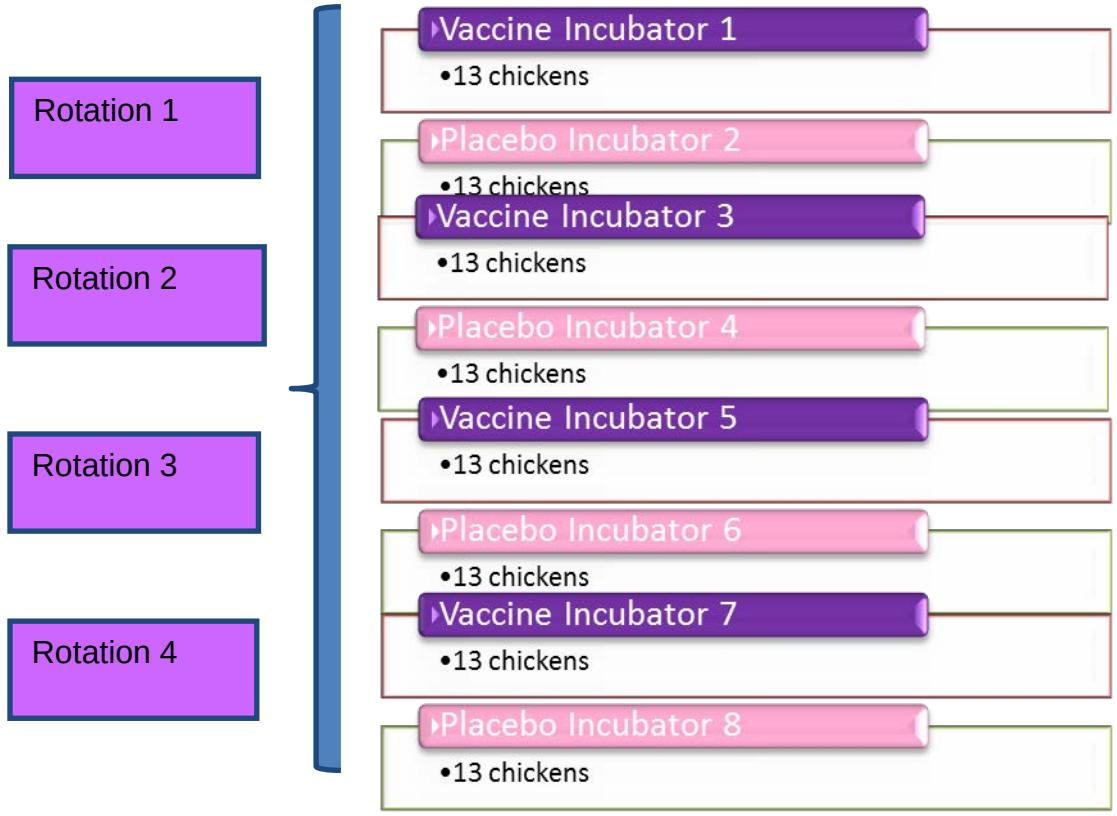

Figure 2 Graph representing the parallel group design used in the study.

Broilers received treatment (either vaccine or placebo) at day 14 (Figure 3). The vaccine ACE 393 (Prokhorova *et al.*, 2006) was administered via intramuscular using a dose of 50 µg of recombinant protein in 0.1 ml adjuvant (Alhydrogel 2%, Brenntag Biosector) per broiler (Garcia *et al.*, 2012). The same adjuvant (0.1 ml of Alhydrogel 2%, Brenntag Biosector) was given to the placebo chickens intramuscularly. Chickens were challenged with $(1.7 \pm 0.5) \times 10^4$ (mean ± SE) CFU/g of *Campylobacter jejuni* in 0.5 ml 0.9% saline solution at day 31 (Figure 3). The broiler chickens were inoculated individually by crop instillation, using a 1-ml syringe with an attached flexible tube (diameter 3 mm, length 10 cm). The *C. jejuni* strain used in the present study was a broiler strain (DVI-SC181) of the most common serotype (Penner serotype 2) and flaA type (1/1) in Denmark. Faecal samples were collected at day 35, 38 and 42 during rotations 2, 3 and 4 of the clinical trial (Figure 3). Birds were weighted at slaughter on day 42 and several samples were collected:

individual fecal and cecal samples for the microbiological studies and blood samples for immunological research (Manuscript I: Garcia *et al.*, 2012).

Figure 3 Experimental design time-line

Quantitative laboratory methods based on serial dilutions and selective culture were used to obtain *Campylobacter* quantitative data in order to assess vaccine effectiveness. Relevant calculations were performed and *Campylobacter jejuni* counts were obtained as CFU per gram of chicken caecum content or fecal mass.

Detailed Materials and Methods are described in Manuscript I (Garcia *et al.*, 2012) and Manuscript II.

6.2.2. Data analyses

Quantitative microbiological data often needs to be transformed for the statistical analyses; typically logarithmic transformation is used as in these experiments. Descriptive statistics and diverse statistical methods were performed utilizing data obtained from placebo and vaccinated chickens (Manuscript I: Garcia *et al.*, 2012 and Manuscript II). Data obtained from chickens administered placebo treatment were used to estimate the variability in *C. jejuni* numbers obtained from fecal samples over time and from cecal samples. Mixed linear models were used to estimate the contributions of rotation, isolator and broiler (residual) to the variation in *C. jejuni* counts obtained from faecal and caecal samples. Moreover, the numbers of *C. jejuni* obtained from pooled samples were compared with the mean of individual samples. A potential correlation between faecal concentrations of *C. jejuni* at day 35, 38, and 42 prior to slaughter with caecal load at slaughter was also investigated using Pearson correlation analysis (Manuscript II).

The potential effect of the vaccine was analyzed using diverse methods of increased complexity (see Manuscript I: Garcia *et al.*, 2012). Initially, t-tests were performed considering all data together and ignoring the experimental design. After that, t-tests were produced per rotation, analyzing the potential vaccine effect in every rotation. Finally, data obtained from all rotations were analyzed

together in a mixed effect model, which considers the hierarchical setup of the experiment (rotations, isolators within rotations, chickens within isolators within rotations). Mixed linear models were conducted in R using the lme4 package and the function lmer. Models were run separately for every set of samples (faecal samples at 35, 38 and 42 days, caecum samples at slaughter at 42 days). In the mixed models, the vaccine was the fixed effect while the random effect of rotations and isolators within rotations were both assumed to be normal distributed (N (0, σ^2)).

6.3 Results

Detailed results are presented in Manuscript I (Garcia *et al.*, 2012) and Manuscript II.

6.3.1. Results obtained from descriptive statistics

Results from the descriptive statistics are shown in Figure 4, in Appendix 1, Manuscript I (Garcia *et al.*, 2012) and in Manuscript II. The logs CFU of *C. jejuni* per gram of fecal or cecal mass varied substantially between broilers, isolators and rotations. Descriptive statistics indicated that the numbers of *C. jejuni* isolated from fecal material at day 35 ranged from 5.26 to 9.41 logs in the placebo (non-vaccinated group) and from 5.69 to 9.30 logs in the vaccinated group; the numbers of *C. jejuni* isolated from feces at day 38 varied from 4.90 to 8.84 logs in the placebo group and from 5.32 to 9.52 logs in the vaccinated group and at day 42 ranged from 4.04 to 9.38 logs in the placebo, non-vaccinated group and from 4.83 to 9.51 logs in the vaccinated group. The CFU counts of *C. jejuni* recovered from cecal contents varied from 4.81 to 9.30 logs in the non-vaccinated (placebo group) and 5.48 to 9.81 logs in the vaccinated group (Appendix 1). Therefore, counts in the vaccinated chickens seemed to be in general slightly higher than in the placebo group even though our initial hypothesis was based on a 2-log reduction of the numbers of *Campylobacter* in vaccinated chickens.

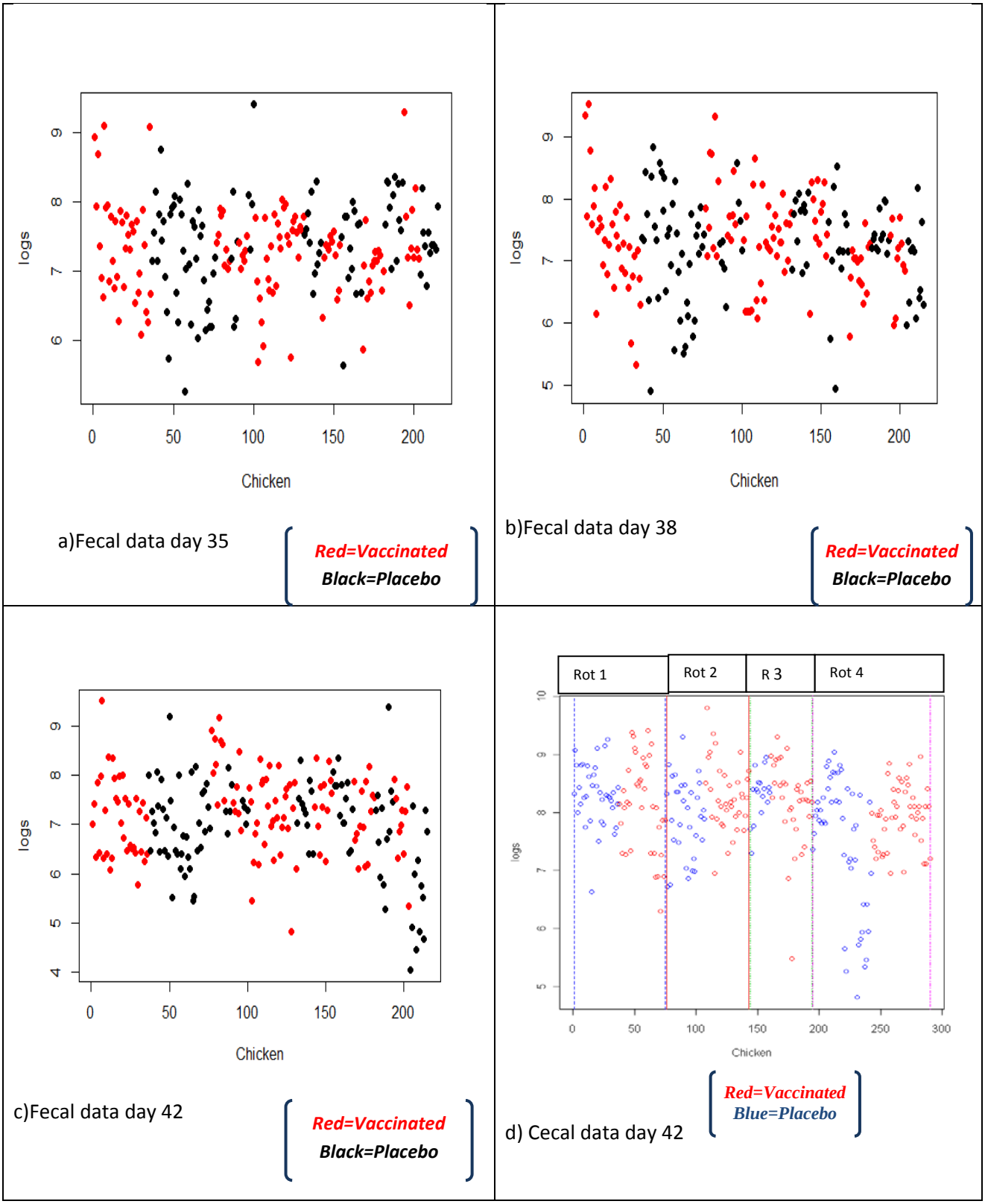

Figure 4 Scatterplots data regarding numbers of Campylobacter (logs) recovered from different samples from broilers.

6.3.2. Results from data analysis (placebo group only)

Analysis of quantitative data obtained from the placebo group only (Manuscript II) indicated a slight decrease in the numbers of *Campylobacter* obtained from faecal material over time. In fact, ANOVA analysis indicated that there was a significant effect ($p = 0.003$) of time related to the numbers of *Campylobacter* isolated from poultry feces. Additionally, pairwise t-test showed that faecal *Campylobacter* counts at day 42 were significantly lower than at day 35 ($p < 0.05$). Correlation analyses showed that there was a significant correlation between *Campylobacter* faecal counts at day 35 and 38 ($r = 0.3$; C.I. = 0.11 - 0.47) and at day 38 and 42 ($r = 0.2$; C.I. = 0.02 - 0.40). Likewise, a significant correlation was found between faecal and caecal CFU/g at day 42 ($r = 0.7$; C.I. = 0.5 – 0-8). When analyzing placebo data only (Manuscript II), a comparison of *Campylobacter* numbers obtained from pooled and individual samples revealed consistently lower counts in the pooled samples compared to the mean of the individual samples in all isolators except two of them. Results obtained from mixed linear models indicated that most variation was attributed mainly to individual chickens (residual), and a minor part to the effect of isolators.

6.3.3. Results from data analysis (vaccinated and placebo groups- Manuscript I)

Detailed results are shown in Manuscript I (Garcia *et al.*, 2012). Results from the t-tests performed based on the numbers of *C. jejuni* recovered from fecal and caecum samples obtained from birds at slaughter are presented in Table 1.

Table 1 Mean values and t-test p-values obtained from comparison of vaccinated and placebo groups

Campylobacter **Recovered At Slaughter (Logs)**	**Faecal Samples 42 days**			**Caecum Samples 42 days**		
	VACCINATED	PLACEBO	T-test p-value	VACCINATED	PLACEBO	T-test p-value
ALL ROTATIONS	7.26	6.95	0.013	8.12	7.93	0.04
ROTATION 1	N/A	N/A	N/A	8.14	8.31	0.28
ROTATION 2	7.12	6.90	0.31	8.28	7.92	0.02
ROTATION 3	7.55	7.31	0.19	8.19	8.30	0.55
ROTATION 4	7.16	6.85	0.11	7.93	7.52	0.03

The numbers of *C. jejuni* recovered from fecal samples at slaughter in all rotations when considered separately were higher for vaccinated broilers than for placebo but the differences were not statistically significant. The numbers of *C. jejuni* recovered from broilers belonging to the vaccinated groups were higher than numbers recovered from broilers in placebo groups when considering all rotations together. The difference was around 0.20-0.31 logs although statistically significant (p-values= 0.04; 0.013 when analyzing fecal samples and caecum samples at slaughter respectively) based on a significance level of p-value<0.05. On the other hand, vaccine effect was found not statistically significant when using mixed linear models (Table 2). Results obtained from the mixed models indicated high variability between birds. Variance distribution based on the results obtained from caecum and fecal contents are presented in Table 2.

Table 2 Variance distribution and p-values (vaccine effect) obtained from Mixed Linear Models using quantitative data from faecal and caecum samples

Samples	**Fecal**				**Cecal**	
	Day:	**35**	**38**	**42**	**Day:**	**42**
Rotation		0	0	0.01		0.02
Isolator		0.07	0.10	0.14		0.21
Residual		0.38	0.50	0.62		0.35
Total variance		0.45	0.60	0.77		0.58
p-value (vaccine effect)		0.70	0.42	0.14		0.40

The variance estimates obtained indicated a relatively high variability between birds. For both cecal content and fecal material, most variation was attributed to the broilers, but also a small part of the variation was due to the isolators. The results from the mixed linear models indicated that the differences between vaccinated and placebo broilers in terms of the numbers of *C. jejuni* recovered in every set of samples were not statistically significant, based on a significance level of p-value<0.05. Consequently, there was no statistically significant effect of the vaccine ACE 393 in this clinical trial in broilers under the experimental conditions applied. The "clustering effect" was estimated by calculating the intra-cluster correlation coefficients (ICC or ρ) and the results (Manuscript I: Garcia *et al.*, 2012) indicated that the effect of clustering could not be ignored. The lack of independence in the data (called the "design effect (DE)") associated to clustered data was obtained (DE= 4.34) and utilized to estimate the effective sample size (Snijders, 2005) which indicated the sample size taking on account the clustered design (Manuscript I: Garcia *et al.*, 2012). Interestingly, the effective sample size was reduced to 67 animals in the study due to the experimental design and clustering effect.

6.4. Discussion and conclusions

There seems to be no consensus regarding the most appropriate sampling protocol to obtain accurate *Campylobacter* quantitative data. The sampling protocol (including methods, sample size, sample origin, time of sampling and other aspects) will influence the quantitative microbiological data and data analyses results. For example, quantitative data related to the concentration of *Campylobacter* in chickens might differ between individual and pooled samples but samples obtained on farm and/or at the slaughterhouse are usually pooled for practical reasons. Moreover,

faecal samples collected on farm might not be a good predictor of the caecal load of *Campylobacter* in individual chickens going for slaughter (Hansson *et al.*, 2010). Hence further research seems necessary to explore and compare sampling protocols in order to obtain accurate *Campylobacter* quantitative data that can be used for risk assessment models and to assess the effectiveness of control strategies against *Campylobacter*. In our studies, a large variation between chickens related to the numbers of *C. jejuni* recovered from caecal samples and fecal samples at different time-points was observed. These results are in agreement with other findings showing that *Campylobacter* colonization levels differ between broiler chickens (Hansson *et al.*, 2010). Even more, in our studies, results from mixed linear models indicated that the variation can be attributed mainly to individual chickens and to a lesser extent to the isolators suggesting that in commercial situations, differences might be observed between flocks but even greater differences might be expected due to individual chickens. This observation suggests that individual factors such as chicken genetics may affect *Campylobacter* dynamics in poultry flocks (Stern *et al.*, 1990). What's more, in commercial farms chickens might be infected with *Campylobacter* at different times and diverse initial concentrations while in this study broilers were inoculated with the same dose of *C. jejuni* at the same time. In addition, the poultry digestive physiology might influence the intermittent excretion of *Campylobacter*. Previously mentioned aspects support concerns related to limited sampling of poultry flocks not being representative of the real *Campylobacter* situation in large flocks. Additionally, results from this study suggest that pooling of samples will probably lead to an underestimation of the numbers of *Campylobacter* in the flock. Data obtained from placebo chickens indicated that the mean concentration of *C. jejuni* recovered from caecum samples was 7.9 log CFU/g in agreement with other studies (Grant *et al.*, 1980; Stern and Robach, 2003; Hansson *et al.*, 2010). *Campylobacter jejuni* concentrations in fecal samples were slightly lower than in caecum samples, with mean concentrations decreasing from 7.4 log CFU/g on day 35 to 6.9 log CFU/g at day 42. Interestingly, this observation related to the decreased *C. jejuni* counts in fecal samples from day 35 to day 42 was found statistically significant ($p < 0.05$). Even more, a significant correlation was observed between faecal and caecal *C. jejuni* concentrations at slaughter ($r = 0.7$; C.I. = 0.5 – 0-8) suggesting that *Campylobacter* counts from fecal samples at slaughter might be a good indicator of *Campylobacter* concentration in the caecum of slaughter chickens. This significant correlation is in agreement with other studies (Fluckey *et al.*, 2003) supporting recommendations made related to the sampling of chickens closer to slaughter time (Hansson *et al.*, 2005). Moreover, if there is a significant positive correlation between the numbers of

Campylobacter in chickens at slaughter and the numbers of *Campylobacter* in carcasses as it has been suggested (Rosenquist *et al.*, 2003; Lindblad *et al.*, 2005; Reich *et al.*, 2008), *Campylobacter* testing of fecal samples from chickens just before slaughter will aid producers in the control of *Campylobacter* contamination of chicken products.

Results from our vaccination experiments indicated that the apparent observed differences between vaccinated and placebo groups related to *Campylobacter* counts could be attributed to the variation between birds in the same group and between groups. It is possible that poultry sharing the same environment re-infect each other with *Campylobacter* and that the micro-environmental conditions might also affect the numbers of *Campylobacter* in the groups. It seems important to consider the "clustering effect" when analyzing quantitative data and also when designing multilevel clinical trials. Although clustered designs can be more costly and require more individuals and more complex data analysis, they present some advantages. Ideally, an effective vaccine against *Campylobacter* for poultry will work under commercial farming conditions. Consequently, the vaccine should be effective when used with poultry belonging to different flocks and diverse farming systems. Nevertheless, the success of vaccine trials for the control of *Campylobacter* infections depends on many factors such as the vaccine candidate, the animal model, individual host factors, *Campylobacter* strain, environmental factors and others. A particular vaccine might work on a specific trial (with an animal model for example) using a particular *Campylobacter* strain and controlled experimental conditions but the same vaccine might produce results under expectations when using other strains, different animal models or environments. In fact, the vaccine ACE 393 substantially reduced colonization with *C. jejuni* in vaccinated mice (Prokhorova *et al.*, 2006; Schrotz-King *et al.*, 2007) while the same vaccine did not seem to work in this poultry trial. Field studies to test vaccine efficiency carry additional complexity due to the lack of knowledge regarding some environmental factors. The vaccine was considered a fixed effect in this study, however, in field studies it will be expected that the vaccine effect will vary. Moreover, the results from data analysis will depend on the methodologies employed for analysis but also on the raw quantitative microbiological data obtained during the experiments. Microbiological data will in turn be influenced by the choice of sampling site, sampling methodologies and laboratory protocols. The effect of the experimental design should not be ignored when analyzing experimental data (Garcia *et al*., 2012). In this study, the effective sample size was reduced to 67 animals due to the experimental design and clustering effect. This information can be useful when calculating the sample size required in an experiment with clustered sampling.

Manuscript I

Design and data analysis of experimental trials to test vaccine candidates against zoonotic pathogens in animals: the case of a clinical trial against *Campylobacter* in broilers

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EXPERT REVIEWS

Design and data analysis of experimental trials to test vaccine candidates against zoonotic pathogens in animals: the case of a clinical trial against campylobacter in broilers

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The development of effective vaccines against zoonotic pathogens represents a priority in public health protection programs. The design of clinical trials and appropriate data analysis of the experiments results are crucial for the assessment of vaccine effectiveness. This manuscript reviews important issues related to the assessment of the effectiveness of vaccines designed to obtain a quantitative reduction of the pathogen in animals or animal products. An effective vaccine will reduce the risk of human infections and therefore the number of human cases. Important considerations will be illustrated using a vaccination trial of a new campylobacter vaccine candidate developed to reduce the numbers of campylobacter in chickens and consequently the numbers of human campylobacteriosis cases. The design of the author's vaccination trial was based on the use of isolators, a parallel group design and several rotations. The effect of clustering or design effect was considered in the sample size calculations. Chickens were randomly assigned to different isolators (treatments) and challenged with *Campylobacter jejuni*. Samples were obtained at different intervals and processed in the laboratory. *C. jejuni* counts were determined as colony-forming unit-per-gram of chicken cecum or fecal mass in order to assess vaccine effectiveness. A desired vaccine effect of 2 logs reduction on the numbers of *C. jejuni* recovered from vaccinated chickens was selected. Sample-size calculations, desired vaccine effect, biological and epidemiological aspects, experimental design and appropriate statistical analysis of data considering group or clustering effects will be the focus of this manuscript.

Keywords: campylobacter • data analysis • experimental design • poultry • public health • vaccine effectiveness • vaccines • zoonoses

Important considerations that should be taken into account when designing randomized controlled veterinary vaccine trials have been reviewed [1]. A clear objective stating the expectation for the successful vaccine candidate and the choice of the study unit are crucial for the experiments. The sample size and forecasted group effects, as well as data analysis methodologies need to be selected carefully as they might affect the results of vaccine trials. Risk assessment models indicate that a reduction of the numbers of campylobacter in chickens intended for human consumption by 2 logs will translate on a reduction of the prevalence of human campylobacteriosis [2]. Therefore, the expectation for a successful vaccine against campylobacter could be based on a 2 logs reduction.

In contrast to vaccines with animal health benefits such as reducing clinical symptoms of infected animals, in recent years, an interest for veterinary vaccines against zoonotic pathogens of public health importance has emerged. The development of vaccines to protect animals from zoonotic infections will have important public

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health implications as approximately 75% of new emerging infections can be considered zoonoses [3,4]. In particular, enteric infections are considered one of the main causes for human disease and human casualties across the world. Therefore, the development of effective vaccines to protect humans from the main pathogens associated with zoonotic enteric infections is highly desirable.

Important issues related to the assessment of the effectiveness of vaccines aiming to result in a quantitative reduction of the pathogen in animals or animal products are examined in this manuscript. The assessment of vaccine effectiveness based on quantitative reduction of pathogens is a complex task, which comprises: the statistical design of experimental trials; the use of quantitative microbiological detection methods; application of appropriate data analysis; and finally the transformation of the vaccine effect in public health terms (e.g., reduced number of human cases).

This review discusses and illustrates the interaction between the experimental design and appropriate data analysis, and how different choice of approaches might influence the conclusions from vaccination studies. If the vaccine aims to reduce the number of pathogens, the vaccine effect will in turn reduce the risk of zoonotic infections and the number of human cases. As a case story, different aspects and considerations will be discussed based on a vaccination trial of a new campylobacter vaccine candidate aiming to reduce the numbers of campylobacter in chickens. The rapidly emerging knowledge of the biology of campylobacter in combination with advances in the fields of molecular vaccinology and immunology provide the setting for the development of efficient vaccines. Vaccines against campylobacter have been developed for humans [5–7], chickens [8–11] and other animals. However, no commercial campylobacter vaccine is currently available.

Poultry has been identified as the main risk factor associated with human campylobacteriosis [12,13]; chickens intended for human consumption can be heavily and persistently colonized with campylobacter representing an important public health risk. Prevalence of campylobacter in broiler flocks in Europe can vary between 3 and 91% [14]. In chicken infected with campylobacter, colonization and shedding patterns depend on a number of factors (such as the bacterial strain). The intestinal tract of chickens is a complex environment where different physical, physiological and biochemical factors can influence the colonization with campylobacter [15].

The concentration of campylobacter in the gastrointestinal tract of poultry can exceed 7.0 $\log^{10}$ colony-forming unit (CFU) per gram [16]. In fact, the colonization level can be as high as 10^{10} CFU per gram of feces [3,17,18]. Campylobacter can be found in high numbers in the large intestine, ceca and cloaca [19]. Small amounts of cecal contents can cause campylobacter contamination of broiler carcases [20]. Campylobacter can also originate from feces of infected chickens, contaminate the food processing environment and directly contaminate the meat; a positive correlation between numbers of campylobacter in ceca and numbers on chicken carcasses has been shown [2,21,22]. Therefore, a reduction of the numbers of campylobacter in poultry meat can be attained by reducing the number of campylobacters in poultry ceca [16,23], which in turn will reduce the risk of campylobacter infections in humans.

Experimental design & sample size considerations

The overall aim of vaccination against some zoonotic infections in animals is based on the reduction of quantitative exposure of pathogens to humans. Therefore, the outcome from the vaccination trial should be measured on a continuous scale, for instance the number of CFU per gram of feces or per cm^2 of the carcass. Traditionally, the sample size calculation in experiments with a continuous outcome is based on the comparison of two means. Data measured on a continuous scale, given a valid measurement, are mathematically more informative than a binomial outcome such as infected/noninfected and, in general, the size of the trial is smaller than a trial focusing on a yes or no outcome, for example, presence of disease. In our case study, the experiment was designed based on the use of isolators with 13 broilers placed in each isolator (Figure 1).

Campylobacter can spread quickly between chickens sharing the same environment by environmental contamination and coprophagy [24]. Broilers placed in the same isolator in our clinical trial shared community; calculations from data obtained in previous experiments using isolators indicated a 50% correlation on the numbers of campylobacter obtained from chickens within an isolator (results not shown). A design effect (DE) of seven was obtained in order to adjust the effect of correlation on the standard error (SE) [25]. The required sample size was calculated as the sample size for simple random sample design (16), multiplied by the DE seven, which equaled to approximately 120 chickens in each group. Eight isolators were available at the research facility, and thereby the authors had to run three to four rotations to achieve enough number of chickens in the experiment. The eight isolators were used during each rotation. Each incubator either contained only vaccinated or only nonvaccinated (placebo) chickens in order to design the experiment mimicking the infectious dynamics in intensive farming conditions. The infectious dynamics within each incubator were then similar to the infectious dynamics in production flocks, where campylobacter is spread between chickens by a sustained exposure to contaminated feces. The opportunity to mimic the realistic working mechanisms of a vaccine in the field conditions increased by using groups of animals where all the birds had been vaccinated. A vaccination effect was expected on the vaccinated animals by decreased susceptibility and also on the whole population as such by reduced population infectious pressure. In every rotation, four isolators with vaccinated chickens and four isolators with nonvaccinated chickens were managed in parallel (Figure 1).

Animals, experimental design timeline & treatments

The genetic makeup of animals included in the study might have an effect on the experimental results. Leghorn broilers have been used in campylobacter colonization experiments and vaccine trials [10,17,18,26–29]. Furthermore, inclusion and exclusion criteria should be defined during the experimental design process. In general, the animals used in an experimental study should be genetically as close as possible to the breed used in the commercial production systems. In this study, campylobacter-free 1-day-old broilers acquired from a commercial firm in Denmark were used. On arrival to the laboratory, the birds were tested to confirm

they were campylobacter free and placed in incubators at random [30]. Campylobacter is rarely detected in commercial flocks with birds younger than 2 weeks of age [30,31]. Therefore, this 2-week 'window' could be used strategically to introduce vaccination programs [10]. The aforementioned stated recommendation was followed in this study and broilers received treatment at day 14 (either vaccine or placebo). The vaccine ACE 393 based on a surface polypeptide discovered by Prokhorova *et al.* [32] was administered intramuscularly using a dose of 50 µg of recombinant protein in 0.1 ml adjuvant (Alhydrogel 2%, Brenntag Biosector, Frederikssund, Denmark) per broiler [30]. A dose of 0.1 ml of adjuvant (Alhydrogel 2%, Brenntag Biosector) was given to the placebo chickens. All birds were challenged with *Campylobacter jejuni* at day 31. The use of a challenge method that mimics natural transmission of campylobacter in broiler flocks is highly desirable in experiments. Fecal samples were collected during the trial (Figure 2).

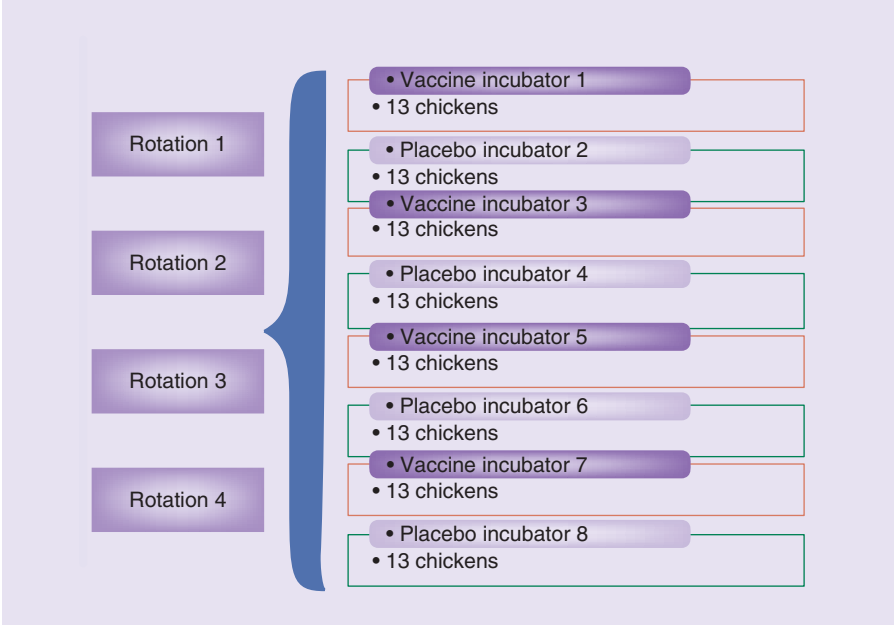

Figure 1. The parallel group design used in the study.

In this particular experimental vaccination trial, the outcome of interest was the number of Campylobacter in broilers during the experiment, but particularly at slaughtering time, which might pose a risk to the consumer. Birds were weighted at slaughter on day 42 and several samples were collected: blood samples for the immunological studies and individual fecal and cecal samples for the microbiological tests [30].

Measurement of outcome

Quantitative laboratory methods based on serial dilutions of samples followed by selective campylobacter cultivation were used in this trial to assess vaccine effectiveness. Relevant calculations were performed and *C. jejuni* counts were determined as CFU per gram of chicken cecum or fecal mass. However, several laboratory methods are currently available for the detection and quantification of campylobacter [33,34]. The choice of the laboratory methodologies should be included in the study design; furthermore, research results might be affected by the choice of laboratory methods and sampling matrices and protocols. Molecular methodologies such as quantitative PCR are based on DNA extraction, purification and quantification. The development of quantitative PCR seems promising for the near real-time detection of pathogens [35].

More efficient and reliable nonenrichment methods are under development in order to separate pathogen cells from complex sample matrices and concentrate the cells for quantification. However, complex sample matrices might present a challenge for the extraction and purification of campylobacter DNA. The concentration of bacterial pathogens from complex matrices (food, environmental samples and fecal material) can be difficult because most bacterial cells are fragile. Furthermore, good methodologies should be able to concentrate the pathogens while removing inhibitors present in the sample matrix. Inhibitory substances present in complex biological samples may reduce or even impede the amplification process [36]. Bile salts, DNase, complex polysaccharides, urea and proteinase present in fecal samples can act as inhibitors contributing to reduce the sensitivity of the PCR technique [37]. Sample quality has been identified as an important factor for PCR reliability [38]. An ideal method would also be applicable to multiple matrices and pathogens, rapid and inexpensive [33,39].

Quantitative microbiological data & data management

Reliable and accurate quantitative microbiological data should be obtained in order to assess vaccine effectiveness. Furthermore, reliable, rapid methods, high quality microbiological data and good food safety data management and traceability systems can play a key role in preventing diseases. In our case study, the quantification of campylobacter was crucial to conduct the assessment of vaccination strategies against campylobacter.

The choice of sampling site, sampling methodologies and laboratory protocols will influence quantitative data results. Challenges regarding these aspects need to be faced and decisions need to be made during the study design process. However, decision-making has to be usually performed under uncertainty and challenging conditions in terms of information access, time availability, practical limitations and other constraints. Due to the nature of sampling and microbiological processes, data might be missing in some instances (e.g., when there are too many colonies to be counted on a plate). Dealing with missing microbiological data can imply the use of complex mathematical methodologies. Furthermore, information regarding sensitivity and specificity of different microbiological methods might not always be available. New methods should offer increased sensitivity and

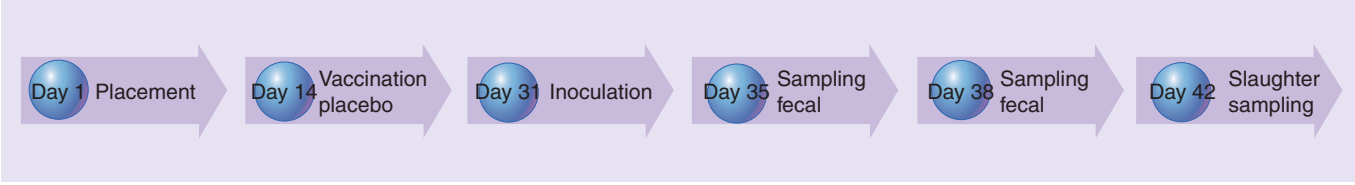

Figure 2. Experimental design time-line.

specificity in order to minimize false-positive and false-negative results that can have important financial and/or public health consequences. Therefore, it seems important to elucidate sensitivity and/or specificity of new methodologies. As an example, the diagnostic specificity of a recently developed real-time PCR for campylobacter in chicken feces was 0.96 and no statistical significant difference with selective enrichment techniques was observed [40].

The combination of advances in new technologies (e.g., quantitative real-time PCR), new or improved microbiological methodologies (e.g., optimal sample processing), new 'omic' technologies, bio-engineering (e.g., new biosensors), the use of mathematical models for epidemiological purposes and decision-making together with effective management and global validation systems seems crucial for the management of public health data. Decision makers face uncertainty and limitations (e.g., inaccurate and/or incomplete information, time constraints and lack of data) when making difficult decisions regarding disease control and global health. Therefore, accurate, reliable and rapid microbiological methods can help to decrease uncertainty around risk-based decisions.

Statistical analysis of quantitative data

Descriptive statistics are useful to explore the quantitative data in order to achieve preliminary conclusions regarding data distributions and decisions regarding appropriate data analysis methods. Furthermore, the choice of data analysis methodologies is very much dependent on the scale of measurement used to assess the effect of the vaccine, and how the results are distributed. For binomial outcomes, the data are analyzed using binomial models, whereas for continuous outcomes, typically linear methods are used. These methods are usually based on normal distributions of data; however, many biological continuous variables are not following normal distributions. Therefore, it might be necessary to carefully transform the data to use statistical methods correctly [101]. Microbiological data obtained from bacterial counts often need to be transformed to obtain useful distributions; typically logarithmic transformation is used, as was the case in our case study.

Different statistical software programs are available to manage, analyze quantitative data and design mathematical models. The authors used Microsoft Excel, R Statistical Software and Minitab® Statistical Software in their case study.

In a nested designed infectious animal experiment, a significant correlation was expected between the animals that share common features, for instance a common close environment such as an isolator, and this is usually expected to lead to dependence between the observations in a group. Clustering is not necessarily restricted to a single level, for instance, in our case study, the chickens are clustered within isolators, and the isolators are clustered within rotations. The clustering must be taken into account for obtaining valid estimates of the effect of a vaccine. This is because the assumption of independence inherent in most statistical models will be invalidated by the clustering [41]. The general effect of ignoring clustering is that the SE of the estimate of the effect of the vaccine will be too small. A reduction of the SE will also reduce the significance level, and therefore, there is a risk that the effect will be interpreted as significant, even if strictly speaking there is not enough power for that significance in the analysis [41].

To illustrate the importance of taking the structure of the trial into account in the data analysis, the effect of the vaccine was analyzed using methods with increased complexity. In all analyses, the effect of vaccine was estimated as a fixed effect. Initially, a t-test of all data, ignoring the physical setup of the trial, was performed. Subsequently, the t-test was stratified, analyzing each rotation separately. Finally, data from all rotations were analyzed together in a mixed effect model, taking into account the physical hierarchical setup of the trial (rotations, isolators within rotations, chickens within isolators within rotations). In the mixed model, the effect of rotation and isolator with rotation were both assumed to be normal distributed ($N\ [0, \sigma^2]$).

In this clinical trial, birds were clustered into groups in isolators and nested in rotations. The clustering effect can be described by the intracluster correlation coefficient (ICC or ρ), which compares the variance within clusters with the variance between clusters and therefore indicates the 'relatedness' of clustered data. A clustered design implicates loss of independence and data analysis needs to account for this lack of independence in the data (termed the DE). Furthermore, clustered sampling is not as statistically efficient as simple random sampling and therefore sample size calculations need to be adjusted to the clustered design. The effective sample size indicates the sample size taking on account the clustered design (in comparison with the number of individuals actually included in the study).

Results from descriptive statistics applied to the quantitative data obtained in our clinical trial

Results from quantitative data analysis revealed that recovered campylobacter numbers from fecal and cecal samples collected at slaughter varied between 4 and 10 logs (Figures 3 & 4). Quantitative data obtained from chickens included in placebo groups are represented in blue and from chickens included in vaccinated groups

are presented in red. Differences between rotations are also revealed in Figures 3 & 4.

Results obtained from t-tests (between vaccinated & placebo groups) conducted in our case study

The results of the initial crude and stratified t-tests performed based on the numbers of campylobacter (in logs) recovered from fecal and cecum samples taken from birds after slaughter are shown in Table 1. When considering all rotations together in the t-test, the numbers of *C. jejuni* recovered from broilers belonging to the vaccinated groups were higher than numbers recovered from broilers in placebo groups. The difference was approximately 0.20–0.31 logs although statistically significant (p-values = 0.04 and 0.013, respectively) based on a significance level of $p < 0.05$. However, the numbers of *C. jejuni* recovered from ceca from broilers in placebo groups in rotations one and three were higher when compared with vaccinated birds although the differences were not statistically significant. The numbers of *C. jejuni* recovered from fecal samples at slaughter in all rotations when considered separately were higher for vaccinated broilers than for placebo, but the differences were not statistically significant.

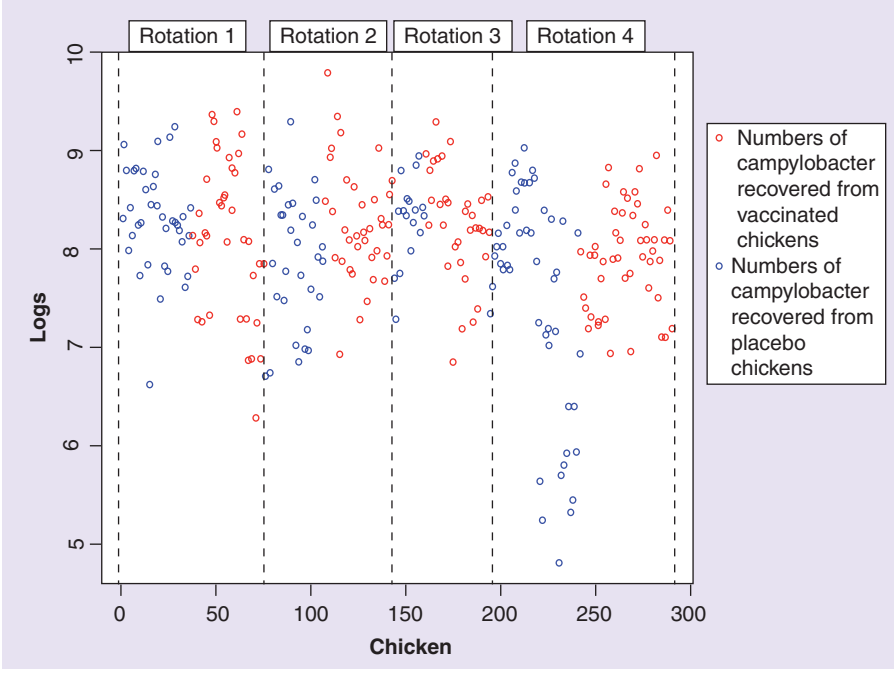

Figure 3. Recovered campylobacter numbers from cecum samples (at slaughter at 42 days).

Results from mixed linear models designed in case trial & the 'clustering effect'

The vaccine effect was found to be not statistically significant when using mixed linear models (Table 2). Results obtained from these models indicated high variability between birds. Variance distribution for the results obtained from cecum and fecal contents is presented in Table 2. For observations from both cecum and fecal contents, most variation was attributed to the individual chickens, but a relatively large part of the observed variation was also attributed to the isolators. The clustering effect was estimated by calculating the ICC presented in Table 3.

The results indicate that although some of the variation is within the groups, the effect of clustering should not be ignored. The lack of independence in the data (the DE) associated to clustered data was calculated. Furthermore, the effective sample size (ESS) can then be estimated [25] and indicates the sample size taking into account the clustered design (in comparison with the number of individuals actually included in the study). The design effect (DE) can be obtained using the following equation:

$$DE = 1 + r\,(m-1) = 1 + 0.40 \times (9.35-1) = 4.34$$

where ρ is the ICC for the statistic in question and m is the average size of the cluster (in this case between eight and 13 broilers were

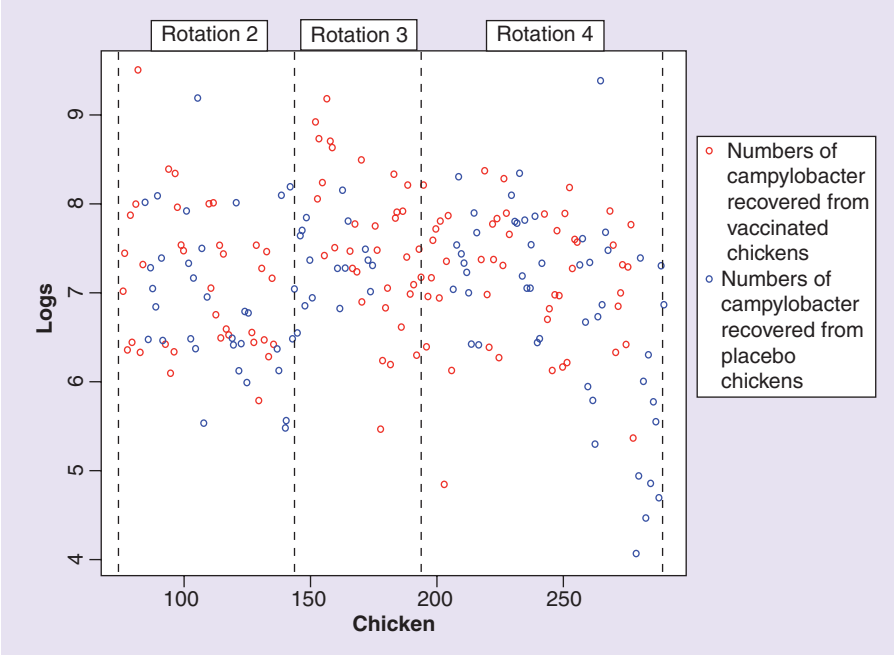

Figure 4. Recovered campylobacter numbers from fecal samples (at slaughter at 42 days).

Table 1. Mean values and t-test p-values obtained from comparison of vaccinated and placebo groups.

Rotations	Campylobacter recovered at slaughter (logs)					
	Fecal samples 42 days			*Cecum samples 42 days*		
	Vaccinated	*Placebo*	*t-test p-value*	*Vaccinated*	*Placebo*	*t-test p-value*
All rotations	7.26	6.95	0.013	8.12	7.93	0.04
Rotation 1	NA	NA	NA	8.14	8.31	0.28
Rotation 2	7.12	6.90	0.31	8.28	7.92	0.02
Rotation 3	7.55	7.31	0.19	8.19	8.30	0.55
Rotation 4	7.16	6.85	0.11	7.93	7.52	0.03

NA: Not applicable.

placed in the incubators, with an average size of 9.35). The ESS can be calculated considering the 290 birds included in the study.

$$ESS = 290/4.34 = 67$$

Therefore, the ESS was reduced to 67 animals in the study due to the experimental design and clustering effect. This information can be used when designing studies for sample size calculations considering clustered sampling.

The use of data analysis methods that ignore the clustering effect such as the t-test in our case study, translates on a small SE of the estimate of the effect of the vaccine [41]. This reduction of the SE will translate on the reduction of the significance level, and therefore there is a risk that the effect will be interpreted as significant, as illustrated in Table 4.

Expert commentary

It has been suggested that new vaccine candidates should be tested in randomized controlled trials in order to provide evidence of vaccine effectiveness [41,42]. An effort towards innovative designs of clinical experiments and the use of advances in systems biology could assist on the discovery of novel vaccine candidates and/or novel strategies for immunization [43]. Furthermore, a predetermined expectation of the vaccine effect necessary to reduce the public health impact of the disease should be considered [44,45]. In our clinical trial, the desired effect of the vaccine was defined as 2 logs reduction on the numbers of recovered *C. jejuni* from vaccinated chickens. Risk assessment models indicate that a reduction of the numbers of campylobacter in chickens intended for human consumption by 2 logs will translate on a reduction of the prevalence of human campylobacteriosis [2]. However, no previous studies had been found that indicated whether this aim was biologically possible. Experts within the research group made this decision regarding vaccine effectiveness. In general, it seems important to have a sound knowledge of the potential vaccine effect to avoid conducting an experiment based on impossible expectations. In conclusion, in this study, we obtained no evidence of 2 logs reduction on the numbers of *C. jejuni* isolated from infected vaccinated chickens. However, we observed diverging results of the effect of the vaccine depending on which approach we used in the statistical analysis of the quantitative data obtained from the clinical trials. In the crude analysis, when considering all rotations together, there were statistically significant results but the numbers of campylobacter were higher in vaccinated birds. A similar result was obtained in some of the rotations when we stratified the crude analysis per rotation.

Table 2. Variance distribution and p-values (vaccine effect) obtained from mixed linear models using quantitative data from fecal and cecum samples.

	Fecal (days)			Cecal (days)
	35	*38*	*42*	*42*
Rotation	0	0	0.01	0.02
Isolator	0.07	0.10	0.14	0.21
Residual	0.38	0.50	0.62	0.35
Total variance	0.45	0.60	0.77	0.58
p-value (vaccine effect)	0.70	0.42	0.14	0.40

There are many different studies conducted on vaccination research and diverse statistical analysis methods performed to assess vaccine effectiveness [46]. However, most assessments of vaccine trials have been generally based on data analysis using the student t-test [15,47]. The number of experimental studies where data are analyzed by adjusting for clustering seems to be limited. Mixed effect models can incorporate both fixed and random effects being suitable to analyze longitudinal data and clustered data [48–51]. Many standard statistical programs today have accessible options to account for clustering when analyzing quantitative data, but efforts should be made to adjust the analysis for clustering due to a nested setup of an animal experiment.

In our case study, we estimated the effect of vaccine as a fixed effect, assuming that the effect should be the same in all rotations and incubators. However, in the field you can expect that the effect of the vaccine will vary within different flocks and farming conditions. The selection of particular vaccination strategies will also vary depending on the factors considered [52]. In fact, the apparent observed differences between vaccinated and placebo groups in this study can be attributed to the variation between incubators, where chickens in the same incubator had more equal numbers of *C. jejuni* compared with chickens in other incubators. It is possible that chickens in the same incubator re-infect each other with campylobacter and that the microenvironmental conditions might also affect the numbers of campylobacter in the incubators. It is important to consider the clustering effect when analyzing

Table 3. Intracluster correlation coefficients.

	Data from fecal samples 35 days	Data from fecal samples 38 days	Data from fecal samples 42 days	Data from cecum samples 42 days
Correlation between chickens in the same isolator and same rotation	0.16	0.17	0.20	0.40
Correlation between chickens in the same rotation but different isolators	0	0	0.013	0.035
Correlation between means of two incubators of size 10 chickens per incubator	0	0	0.05	0.07

quantitative data and also when designing multilevel clinical trials [25]. The initial sample size calculations were based on a DE of 7 and a 50% ICC (ρ = 50%). The results obtained from the clinical trial indicated different values for ICC (ρ = 40%) and DE (4.34). The DE can be considerable, reducing the effective sample size (in this case, 63 animals instead of the 290 animals included). Although clustered designs can be more costly and require more individuals and more complex data analysis, they present some advantages. The design used in this trial was trying to emulate the clustering effect found in broiler flocks and farms. Ideally, an effective vaccine against campylobacter for broiler chickens will work under commercial farming conditions. Therefore, the vaccine should be effective when used with chickens belonging to different flocks and diverse farming systems.

However, the success of vaccine trials for the control of campylobacter infections depends on many factors such as the vaccine candidate, the animal model, individual host factors, campylobacter strain, environmental factors and others. A particular vaccine might work on an specific trial (with an animal model for example) using a particular campylobacter strain and controlled experimental conditions; however, the same vaccine might produce results under expectations when using other strains, different animal models or environments. Furthermore, field studies to test vaccine efficiency carry additional complexity due to the lack of knowledge regarding some environmental factors. Additional complexities to consider are microbiological methodologies used for isolation, characterization and quantification of campylobacter and the choice of data analysis techniques. The results from data analysis will depend on the methodologies used for analysis and also on the raw quantitative microbiological data obtained during the experiments. Microbiological data will in turn be influenced by the choice of sampling site, sampling methodologies and laboratory protocols. In our clinical trial, the numbers of campylobacter recovered from cecum samples were consistently higher than the numbers obtained from feces. This finding is in agreement with other studies [38]. However, the choice of laboratory methods (e.g., the choice of selective agar) might also influence the results obtained from fecal and cecal samples [53].

Uncertainty plays a key role in biological studies and should be accounted for the analysis and interpretation of laboratory data and when developing mathematical or epidemiological models [54–57]. Furthermore, the distinction between uncertainty and variability can influence research results and therefore should not be ignored [58,102].

Five-year view

Future advances in biotechnology and new technologies will assist public health experts, epidemiologists and workers in the fields of quantitative microbiology, vaccinology, immunology and mathematical modeling in providing efficient strategies for the control of zoonotic infections. Innovation in all areas is highly desirable for the control of important zoonoses. Furthermore, innovative integration of the different aspects will translate in more efficient controls for the protection of public health.

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Table 4. Comparison of the standard errors of estimated vaccine effects using different data analysis methods (t-tests and mixed linear models).

	Samples							
	Fecal 35 days		*Fecal 38 days*		*Fecal 42 days*		*Cecal 42 days*	
Estimates of the vaccine effect (logs, all rotations)	0.08 logs (higher in placebo)		0.10 logs (higher in vaccinated)		0.31 logs (higher in vaccinated)		0.19 logs (higher in vaccinated)	
Statistical methods	t-test	MLM	t-test	MLM	t-test	MLM	t-test	MLM
SE	0.10	0.14	0.11	0.17	0.12	0.19	0.09	0.09
p-value	0.39	0.70	0.37	0.42	0.013	0.14	0.04	0.40

MLM: Mixed linear model; SE: Standard error.

Key issues

- The safe use of effective vaccines against zoonotic pathogens represents a priority in public health protection programs.
- An effective vaccine will potentially reduce the risk of human infections and therefore the number of human cases.
- The assessment of vaccine effectiveness can be a complex task that requires careful consideration of experimental design and data analysis methodologies.
- The desired impact or effect of the vaccine needs to be selected during the trial design process.
- The design of the experiments needs to be carefully planned in order to maximize the research investment and to obtain accurate and useful results.
- Descriptive statistics can be used to explore the data in order to achieve preliminary conclusions regarding data distributions and to choose appropriate data analysis methods.
- Complex data analysis methods need to be selected based on the experimental design.
- The use of a clustered design implicates loss of independence and therefore data analysis needs to account for this effect (called the 'design effect'). The design effect can be considerable, reducing the effective sample size of the experiment.
- Vaccines against campylobacter have been developed; however, no commercial vaccine is yet available. A reduction in the numbers of campylobacter in chickens intended for human consumption will reduce the risk of campylobacter infections in humans.
- Field trials of vaccine effectiveness need to consider epidemiological factors. Mathematical models can be developed to assist in the assessment of vaccine effectiveness under different conditions.
- Uncertainty plays a key role in the studies and should be considered when developing mathematical or epidemiological models.

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Manuscript II

Intestinal colonization of *Campylobacter* spp. in broiler chickens in an experimental infection study

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Running head: Colonization of *Campylobacter* in chickens

Abstract

Consumption of poultry meat is considered as one of the main sources of human campylobacteriosis. Quantitative data on the *Campylobacter* spp. colonization dynamics in broiler chickens is thus crucial to implement effective control measures. We carried out four experimental infection trials (rotations) looking at the colonization of *Campylobacter jejuni* over time in individual broiler chickens. There were large differences between broiler chickens in the number of *C. jejuni* in caecal and faecal material. Faecal samples of *C. jejuni* ranged from 1.1×10^4 to 2.4×10^9 CFU/g and from 6.5×10^4 to 2.0×10^9 CFU/g in the caecae. There was a significant correlation between caecal and faecal CFU/g. Individual broiler chicken variation contributed significantly to the total variance of colonization, followed by isolators. Rotations did not contribute to the total variance. The results showed that pooled samples within isolators had lower CFU/g compared to the mean of the individual samples.

Keywords: Campylobacter**,** flocks, caecal, faecal, variation, poultry.

Introduction

Campylobacter spp. is the leading cause of bacterial gastroenteritis in the world, causing 2.4 million cases yearly in the United States [1]. In 2011 220,209 cases were reported in the European Union (EU) [2]. The total annual cost of campylobacteriosis in the EU is estimated to be 2.4 billion € [3]. Campylobacteriosis is largely perceived to be food-borne disease with poultry meat as the primary infection source. The incidence of campylobacteriosis in humans is correlated with the prevalence of *Campylobacter* spp. in chickens [4]. It is an international priority to eliminate *Campylobacter* spp. from broiler chickens to ensure better food safety [5,6].

There are large variations in the numbers of *Campylobacter* spp. in the cecae of broiler chicken flocks collected at slaughter plants [7,8]. Chicken lineage and time of infection in chickens seems to influence variability in colonisation of the chick intestine [9-12]. Despite the contribution of host genetics and time of introduction into the flock the dynamics of *Campylobacter* spp. in broiler chicken houses are not fully understood.

Efforts to reduce *Campylobacter* spp. flock prevalence and level of colonization include increased biosecurity [13-15], competitive exclusion, antibacterial agents, or phage-therapy [16,17], poultry vaccines [18-22] and improving the genetic resistance to *Campylobacter* spp. colonization in broiler chickens [10].

Risk assessment models have been developed to determine which strategies are the most efficient in reducing *Campylobacter* spp. flock prevalence and the number of cases of campylobacteriosis. [14,23].

66 At present there is no consensus regarding the most appropriate way of sampling a flock to provide
67 data that can be used in risk assessment models. Commonly a large (10-25) number of caecal
68 samples is taken at the slaughterhouse or faecal samples collected at the farm and pooled for
69 analysis. This can produce misleading results if CFU differ significantly between individual
70 numbers and pooled samples. Furthermore, it is unclear if faecal samples at the farm level is a good
71 predictor of the caecal load at the slaughter plant [7].
72
73 To increase our understanding of the dynamics of *Campylobacter* spp. in broiler chickens we
74 studied the numbers of *Campylobacter* in broiler chickens infected under controlled experimental
75 conditions and addressed the effect of pooling samples. The aims were to 1) estimate the variation
76 in number of *C. jejuni* in faecal and caecal samples over time in a conventional chicken broiler
77 breed (Ross 308) inoculated with the same dose of *C. jejuni*, 2) compare *C. jejuni* CFU/g in pooled
78 samples with the mean of individual samples, 3) evaluate any correlation between faecal loads of *C.*
79 *jejuni* at day 4, 7, and 12 post infection (PI), and with caecal loads at day 12 PI.
80
81 **Material and methods**
82 *Experimental birds*
83 The experimental infections were carried out at the National Veterinary Institute (Aarhus,
84 Denmark) following the Danish legislation for animal welfare and use of experimental animals and
85 approved by the Supervisory Authority on Animal Testing (2010/561-1803). Conventional broiler
86 chickens (Ross 308) of mixed sex were obtained from a Danish hatchery (DanHatch A/S). Chicks
87 were transferred directly from the hatchery to the experimental unit, where they were housed in

isolators (Montair Andersen B.V. HM 1500). All chicks were tested free of *Campylobacter* spp. at placement and before inoculation.

The chickens were killed by decapitation, and each chicken was sampled and examined individually at slaughter.

Experimental design

The placebo group described in the present paper was part of a larger vaccine study [24] and, due to the design of this study received 0.1 ml Alhydrogel (2% solution) adjuvant intramuscularly 17 days before *C. jejuni* challenge. In order for an Alhydrogel adjuvant to increase specific immunity against an antigen, in this case *C. jejuni* the antigen must be mixed with the adjuvant and injected as a mixed suspension and thus it is highly unlikely that the chickens of the placebo group have any specific immunity against *C. jejuni.*

The broiler chickens used were housed in isolators. All handling of chickens was done through the isolator gloves attached to the isolators. Four identical infection trials (rotations) were carried out in 2011, where only the flock of broiler chickens used differed between rotations. During each infection trial four identical isolators were used with an average of 9 birds per isolator. A total of 134 broiler chickens were infected.

Broiler chickens were challenged with a *C. jejuni* suspension at day 31. Faecal samples were collected on day 4, 7 and 12 PI, and caecal samples collected on day 12 PI. CFU/g of individual faecal samples was determined in samples from rotation 2, 3 and 4. Faecal and caecal samples were collected individually from each broiler chicken at each timepoint and kept separately in tubes and stored on ice until CFU determination was done. All birds were marked which ensured that faecal and caecal samples were only taken once from each bird. Faecal droppings were sampled by gentle anal stimulation and directly into a sterile falcon tube avoiding any cross contamination. CFU/g of pooled and individual samples was subsequently established.

Challenge with Campylobacter jejuni

On day 31 post hatch, all broiler chickens were challenged with $(1.7 \pm 0.5) \times 10^4$ (mean ± SE) CFU/g of *C. jejuni* in 0.5 ml 0.9% saline solution. The broiler chickens were inoculated individually by crop instillation, using a 1-ml syringe with an attached flexible tube (diameter 3 mm, length 10 cm).

Preparation of inoculum

The *C. jejuni* strain used in this study was a broiler strain (DVI-SC181) which belongs to the most common serotype (Penner serotype 2) and flaA type (1/1) [25]. This strain originated from a collection of *Campylobacter* spp. isolates obtained from faecal samples collected at the time of slaughter in Denmark [25]. Bacterial inoculum was prepared from cultures grown on blood agar base plates (Oxoid) supplemented with 5% (v/v) calf blood (BA) and incubated at 42 °C for 48 h under microaerobic conditions. Subsequently the bacteria were prepared by shaking of bacterial

material in 0.9% saline solutions at 4 °C. Before inoculation the bacterial suspension was adjusted to an optical density of approximately OD_{620} = 0.6 and diluted to the desired concentration (CFU/ml). The actual inoculation dose was determined by direct bacteria counting before and after inoculation.

Bacterial culture and counting

Quantification of *C. jejuni* followed the Nordic standard protocol for enumeration of thermotolerant *Campylobacter* [26]. One gram of caecal or faecal material was weighed and diluted 1:10 in 0.9% saline dilution series. The pooled samples were made out of 1 g from each individual sample within each isolator. Subsequently dilution series were streaked onto *Campylobacter* spp. selective Abeyta-Hunt-Bark agar plates (AHB) with 1% triphenyltetrazoliumchloride. The plates were incubated microaerobically at 42 °C for 48 h before being enumerated.

Statistics

A mixed linear model was used to estimate the contribution of rotation, isolator and broiler (residual) to the variation seen in the CFU/g found in individual faecal and caecal samples. Based on the estimated variances for rotation, isolator and broiler in the mixed linear model, the percentage of total variance that was due to rotation, isolator and broiler was calculated. The data obtained at the different timepoints were analysed separately. For each day of sampling (day 4, 7 and 12 PI, respectively), the data from all rotations were included in the model. At day 12 PI, CFU/g in faecal and caecal samples was analysed separately. CFU were log transformed log

(CFU/g) to normalize data. In the mixed model, the effect of rotation and isolator within rotation were both assumed to be normal distributed ($N(0, \sigma^2)$).

Distributions of number of *Campylobacter* spp. in individual faecal samples at different sampling timepoints (day 4, 7 and 12 PI, respectively) were diagrammed as box plots. A non-parametric ANOVA (Kruskal-Wallis test) based on ranks was used to test for the effect of time on CFU/g in the faecal samples and Dunn multiple comparisons was used to compare timepoints. The p-values were compared to the Bonferroni corrected significance level.

CFU of the pooled caecal samples was compared to the individual caecal samples from each isolator by taking the arithmetic mean of the individual caecal and comparing it with the pooled caecal samples. We expected that the CFU_{pool} would be equal to the arithmetic mean of the individual faecal samples, $(CFU_1 + CFU_2 + \ldots\ldots CFU_n) / n$.

Individual faecal and caecal CFU/g between timepoints is shown as a scatter plot with the regression line. Correlation analysis (Pearson) was used to evaluate the relationship between caecal and faecal counts at different timepoints.

Results

Concentrations of C. jejuni at different timepoints

There was a large difference between broiler chickens in gut content of *C. jejuni* at slaughter (Table 1). In the faecal samples number of *C. jejuni* ranged from 4.0 to 9.4 log CFU/g and for caecal samples from 4.8 to 9.3 log CFU/g. The mean number of *C. jejuni* detected in the caecal content of the broiler chickens was 7.9 log CFU/g and slightly lower for the faecal samples, with mean concentration decreasing from 7.4 on day 4 PI to 6.9 log CFU/g on day 12 PI.

When comparing the faecal CFU/g at day 4, 7 and 12 PI of each individual broiler chicken there was a slight decrease in CFU/g over time (Fig. 1). ANOVA analysis indicated that there was a significant effect of time on faecal CFU/g ($p = 0.003$) and pairwise test showed that faecal CFU/g at day 12 PI were significantly lower than at day 4 PI ($p < 0.001$), whereas neither CFU/g at day 4 and 7 ($p = 0.180$) or day 7 and 12 ($p = 0.041$) were significantly different when compared to the Bonferroni corrected significance level ($p = 0.0167$).

To further evaluate the change in CFU/g over time, variance contributions to the total variance were estimated at the different timepoints (Table 2). In the faecal samples, most variation was attributed to the broiler chicken (residual), and a minor part to the isolator whereas rotation did not affect the total variance. The total variance increased slightly with time, but the proportion of the different levels stayed the same at the different timepoints. For the caecal samples at day 12 PI the variance contributions looked similar to the faecal samples.

CFU/g of C. jejuni in pooled samples versus individual samples

When comparing the pooled and individual caecal samples from each isolator group there was a consistent lower CFU/g in the pooled samples compared to the arithmetic mean of the individual samples except in two isolators out of a total of 15 isolators during four rotations (Fig. 2). Pooled and individual faecal samples from each isolator group were only established in rotation 2, 3 and 4 and showed that CFU/g of pooled faecal samples taken at day 12 PI was lower or equal to in 8 out of a total of 11 isolators during three rotations (results not shown).

Correlation of CFU/g of faecal and caecal samples

The collection of faecal and caecal samples from each individual at multiple timepoints allowed us to compare CFU/g of faecal samples at different timepoints and also faecal with caecal samples (Fig. 3). There was a significant correlation between faecal CFU/g at day 4 and 7 PI (r = 0.3; C.I. = 0.11 - 0.47) and day 7 and 12 PI (r = 0.2; C.I. = 0.02 - 0.40). Likewise a significant correlation was found between faecal and caecal CFU/g at day 12 PI (r = 0.7; C.I. = 0.5 – 0-8).

Discussion

The results of the present study showed large variation in the load of *C. jejuni* in the caecal and faecal samples. The mean number of *C. jejuni* detected in the caecal content of the broiler chickens was 7.9 log CFU/g in the present study. This is slightly higher, but still within the range reported in other studies [7,27,28]. Faecal content was slightly lower than the caecal content, with mean concentration decreasing from 7.4 on day 4 to 6.9 log CFU/g at day 12 PI. In contrast to earlier studies broiler chickens in the present study were infected with the same dose of *C. jejuni* and at the same age. The results confirm that colonization differs between broiler chickens and support the

concern raised by Hansson *et al.* [7], suggesting that limited sampling for quantification of *Campylobacter* spp. in broiler chicken flocks will not be representative of large broiler chicken flocks.

The novel design of the present infection trials allowed the variance contributions to be established and show that most of the variation in colonization of *C. jejuni* could be attributed to factors such as broiler chickens and to lesser extent isolators and rotations. Furthermore, the total variation increased slightly with time in the faecal samples, but with the same factors attributing proportionally to the total variance. In a Swedish study results showed that slaughter groups that were tested positive at the farm level had mean number of *Campylobacter* spp. in carcass rinse samples 3 log units higher than the mean number in samples from slaughter groups in which *Campylobacter* spp. was first isolated at slaughter. In the present study the broiler chickens were inoculated with the same dose of *C. jejuni* and at the same time. Therefore the variation observed between individuals in our study is not due to the time of infection. This suggests that other factors in addition to the time of infection, such as the broiler chicken genetics [10] are involved in the *Campylobacter* spp. dynamics in broiler chicken flocks. The chicken intestinal physiology, most probably the caecal function may also cause an intermittent and fluctuating excretion of *Campylobacter* spp.

In the present study individual caecal samples were obtained allowing the CFU/g in both pooled and individual samples in each isolator to be established. To our knowledge no other studies have compared CFU/g of *Campylobacter* spp. in paired pooled and individual caecal samples. Based on the way the CFU of the pooled sampled was made up, we expected that the CFU_{pool} would be equal to the arithmetic mean of the individual faecal samples. However, in most of the cases, the

238 estimated arithmetic mean from the individual samples was higher than the obtained CFU/g in the
239 pooled samples although the difference was smaller than 1 log. One explanation for this could be
240 that the mean of a lognormal distribution is usually underestimated when it is based on sample data:
241 on average, the fewer samples taken, the lower the estimate. Of course, as we also see in this
242 experiment, the arithmetic mean can also be lower than the measured CFU/g in the pooled samples.
243 The results therefore suggest that pooling of samples will generally lead to an underestimate of
244 CFU/g compared to mean CFU/g of individual samples.
245
246 Human risk of campylobacteriosis from broiler chickens results predominantly from meat products
247 with high concentrations of *Campylobacter* spp. This is confirmed by data from Iceland [29] and
248 risk assessments [5,30-32]. It has therefore been suggested that the human incidence of
249 campylobacteriosis can be strongly reduced by aiming control strategies at products with relatively
250 high concentrations of *Campylobacter* spp. Several studies have therefore suggested that “testing
251 and scheduling” might be an efficient control strategy for *Campylobacter* spp. in broiler chicken
252 meat [33,34]. This strategy entails testing of broiler flocks at the farm shortly before transport to the
253 processing plant. Flocks with high concentrations of *Campylobacter* spp. at the farm can then be
254 diverted from the fresh meat production chain. For this approach to be successful there needs to be a
255 significant correlation between concentrations of *Campylobacter* spp. in the feces and on the meat
256 product. Earlier studies have shown a correlation between the proportion of positive cloacal and
257 caecal samples or the number in the caecal content and the number of *Campylobacter* spp. on
258 carcasses [5,35,36]. Our results showed that there was a significant correlation between CFU/g in
259 individual faecal and caecal samples before slaughter and that the caecal CFU/g was slightly higher
260 than the faecal CFU/g values. The significant correlation is in agreement with other studies [37] and
261 indeed suggest that “testing and scheduling” could be possible with faecal sampling before

slaughter. However, if faecal samples are taken earlier there is no or only a weak correlation. What could hamper the usefulness of "testing and scheduling" would be low variance of *Campylobacter* spp. concentrations between flocks and high variance of *Campylobacter* spp. concentrations between broiler chickens within flocks. Our results show that most of the variation in faecal or caecal load is indeed due to variation between broilers and not isolators or rotations. This indicates that most variation is between individuals and not flocks although in the present study the number of birds per isolator and rotation are far fewer then in natural broiler chicken systems.

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Declaration of interest: none

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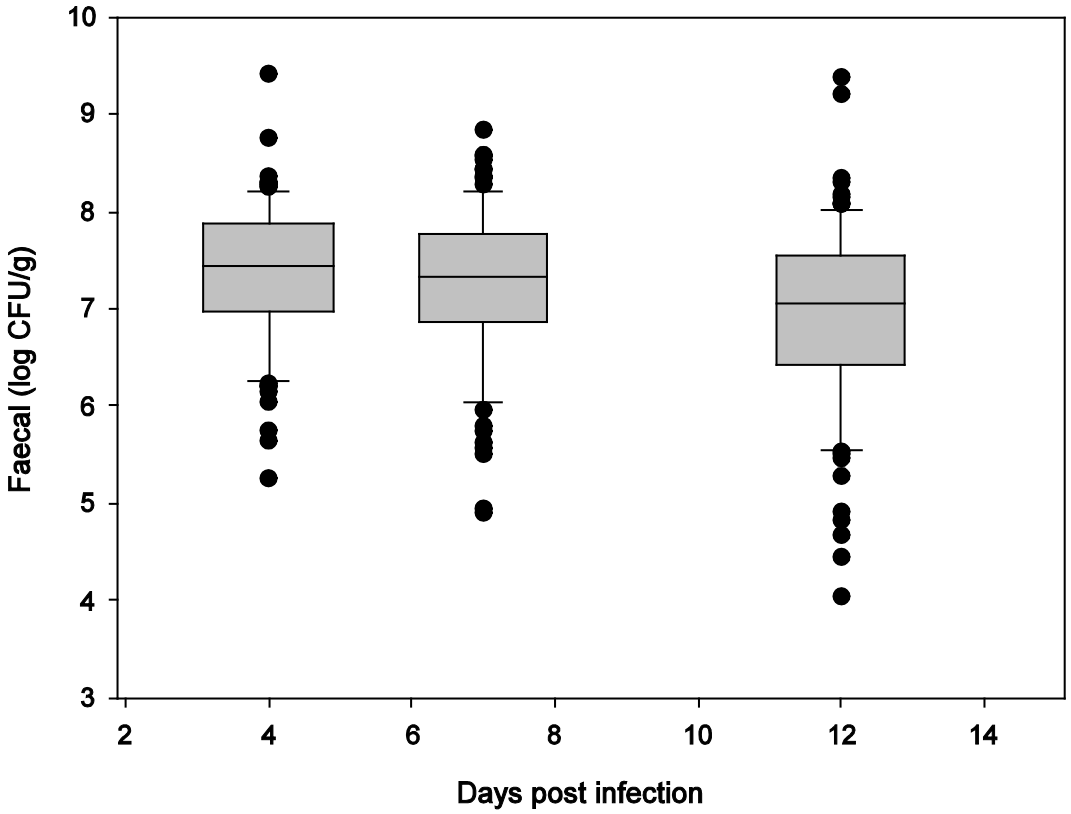
Figure legends

Figure 1: CFU/g of individual faecal samples from each broiler chicken at day 4, 7 and 42 post infection (n = 97). The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles and black dots the outliers.

Figure 2: CFU/g of pooled caecal samples (grey bars) from each isolator and the mean CFU/g of the individual caecal samples (open bars) obtained from each isolator. Samples were taken from each of four isolators during each of four rotations except in rotation four where samples were only obtained from three isolators.

Figure 3: Illustration of CFU/g of individual samples for a) faecal at day 4 and 7 post infection (PI), b) faecal at day 7 and 12 PI and c) faecal and caecal samples at day 12 PI (n = 97). The plotted line is the estimated regression line.



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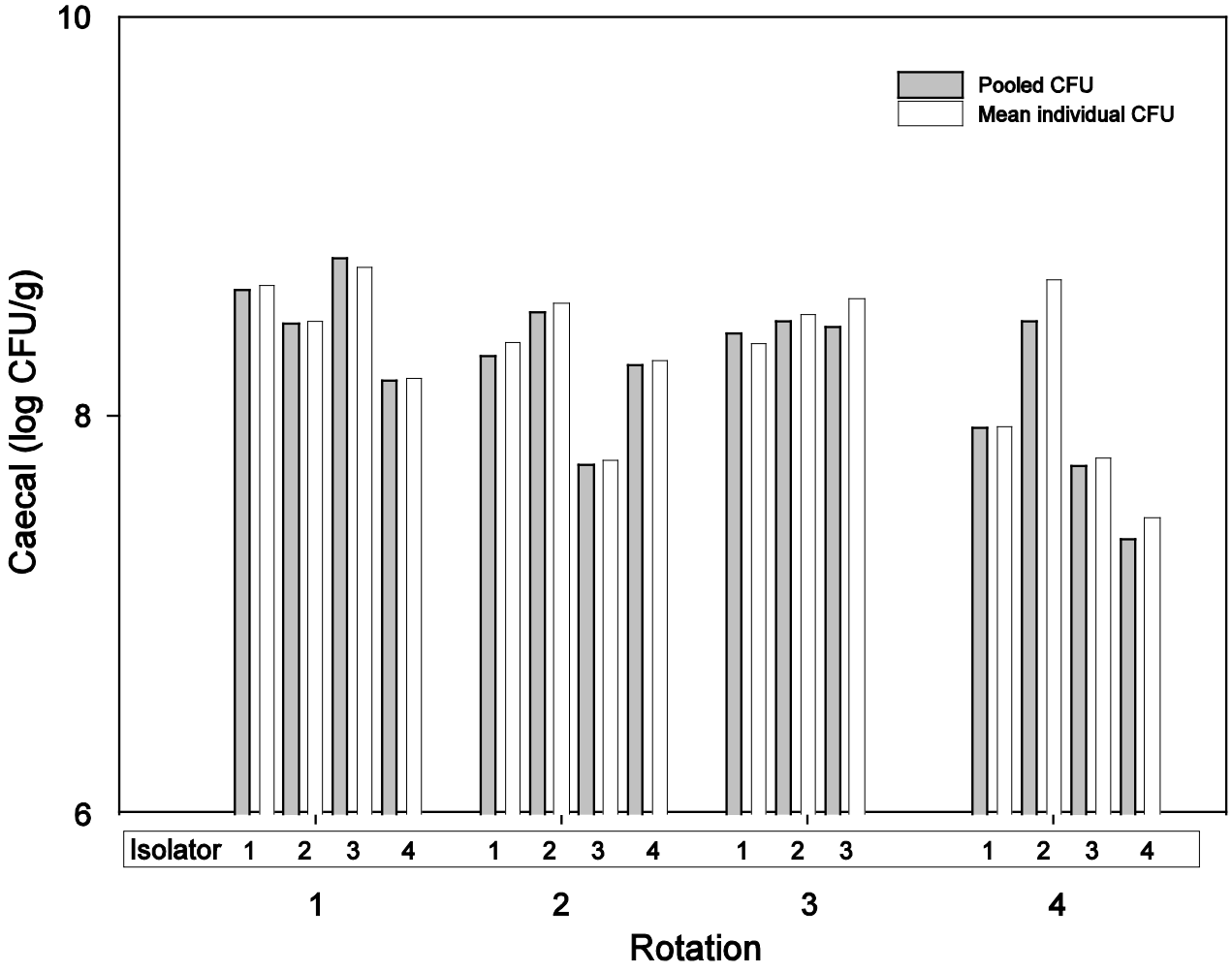
417 Fig. 1



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419 Fig. 2

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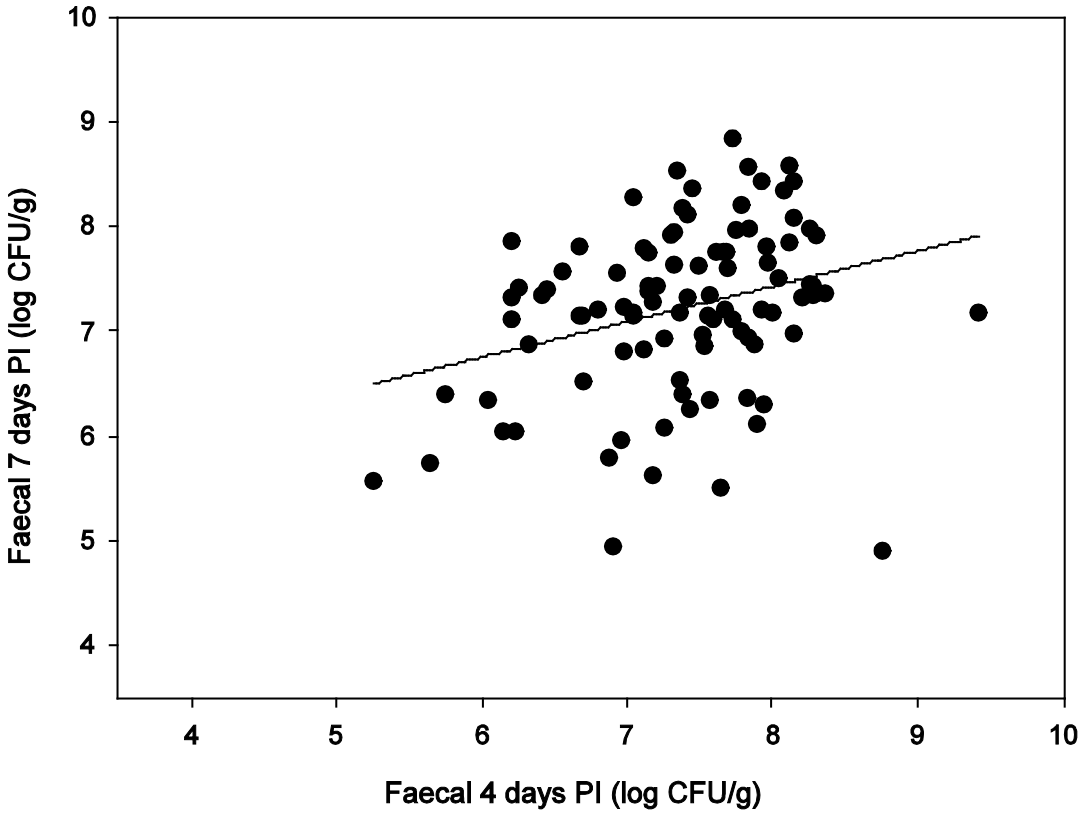

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426 Fig. 3 a

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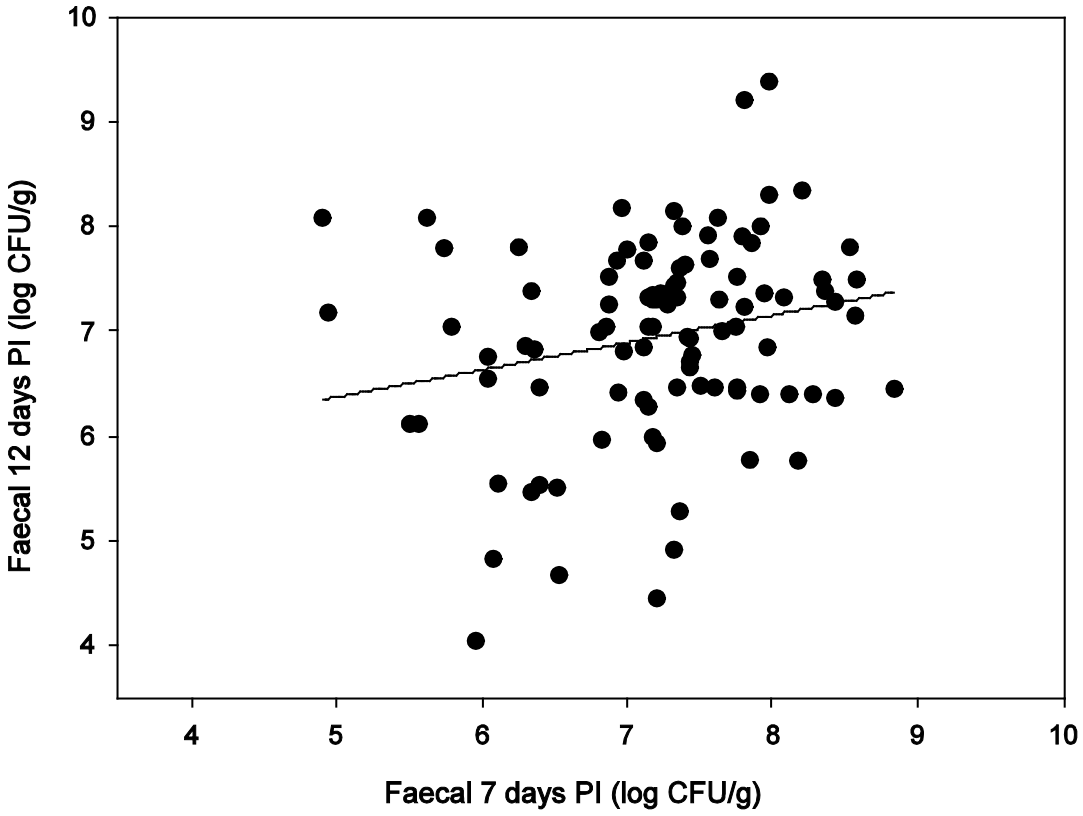
Faecal 12 days PI (log CFU/g)
Faecal 7 days PI (log CFU/g)
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9
8
7
6
5
4
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5
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7
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9
10

Fig. 3 b

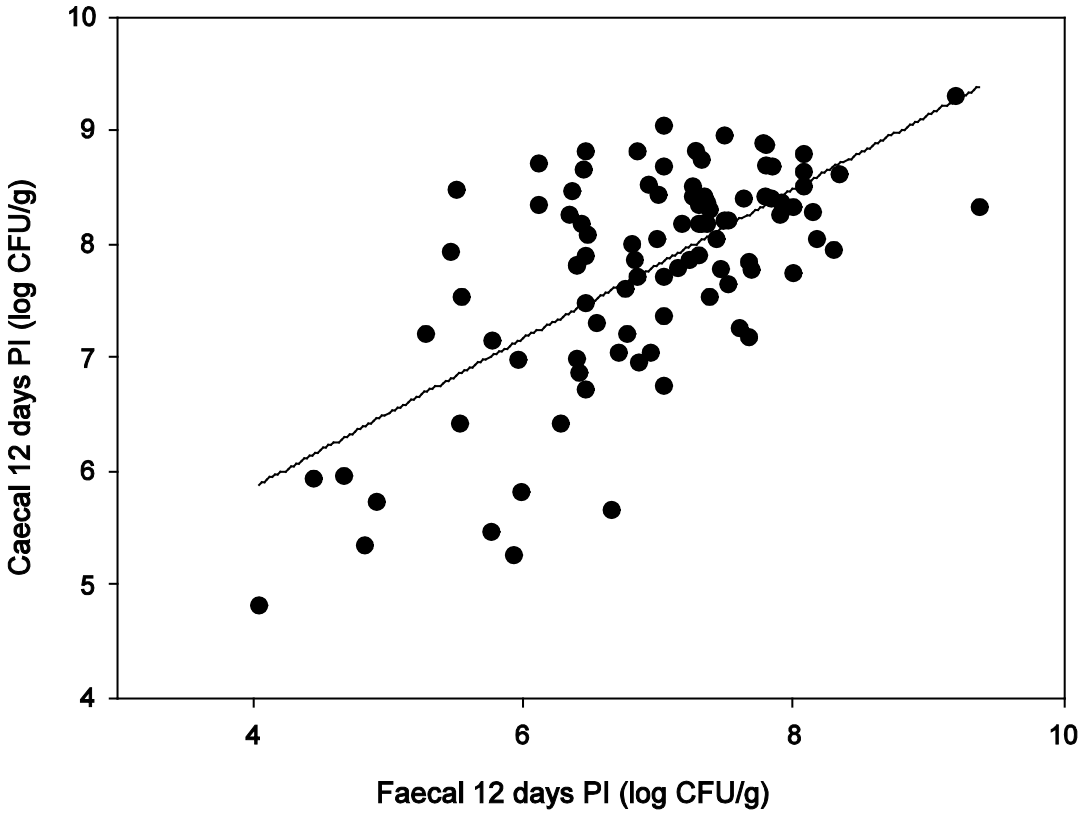
Caecal 12 days PI (log CFU/g)
Faecal 12 days PI (log CFU/g)
10
9
8
7
6
5
4
4
6
8
10

Fig. 3 c

Table 1: Colony forming units of *C. jejuni* in faecal and caecal material collected from broiler chickens at different timepoints post infection (PI) with *C. jejuni*. Table shows geometric mean ± SD CFU for individual samples and CFU of pooled samples (log CFU per gram faecal or caecal content). All samples used to establish individual and pooled CFU/g are paired. N is based on number of samples obtained from each isolator.

			Log CFU/g of faecal content							Log CFU/g of caecal content		
Rotation	Isolator	Days PI	4		7		12			12		
			Individual	Pooled	Individual	Pooled	Individual	Pooled	n	Individual	Pooled	n
1	1			8.0		7.8		8.0	9	8.5±0.4	8.6	9
	2			7.3		7.0		7.5	9	8.2±0.7	8.5	9
	3			7.5		7.5		7.9	10	8.4±0.6	8.8	10
	4			6.9		7.3		7.9	9	8.1±0.3	8.2	9
2	1		7.7±0.5	7.9	7.4±1.3	7.6	7.2±0.6	7.4	8	7.9±0.8	8.3	8
	2		7.1±0.9	6.7	7.6±0.8	8.0	7.2±1.1	8.3	9	8.2±0.7	8.5	9
	3		7.1±1.0	7.6	7.1±0.9	7.2	6.6±0.6	7.3	8	7.5±0.6	7.8	8
	4		7.4±0.6	7.7	6.5±0.8	7.2	6.6±1.1	7.2	8	8.1±0.4	8.3	7
3	1		6.6±0.4	7.5	7.1±0.7	7.5	7.2±0.5	7.3	8	8.2±0.5	8.4	8
	2		7.1±0.8	7.7	6.9±0.4	7.3	7.5±0.5	7.7	6	8.4±0.3	8.5	5
	3		8.2±0.9	7.9	7.8±0.6	7.8	7.3±0.2	7.3	4	8.5±0.3	8.5	4
	4		*									
4	1		7.5±0.5	7.9	7.6±0.5	7.9	7.3±0.6	7.4	11	7.9±0.3	7.9	11
	2		7.3±0.7	7.4	7.2±1.0	7.6	7.4±0.6	7.9	13	8.7±0.3	8.5	13
	3		7.9±0.5	8.0	7.5±0.3	7.7	7.0±1.1	7.6	10	7.2±1.0	7.8	12
	4		7.4±0.4	7.4	6.7±0.7	7.4	5.7±1.1	6.6	12	6.3±1.1	7.4	12

* Birds from this isolator were not included due to functional breakdown of the isolator. Grey areas indicate that individual samples were not taken during this rotation.

Table 2: Variance estimates (percentage) of the various levels in the infection trials using quantitative data from faecal and caecal samples.

	Faecal				Caecal	
	Days PI:	4	7	12	Days PI:	12
Rotation		0	0	0		0.03
Isolator		0.10	0.05	0.20		0.29
Residual		0.41	0.61	0.67		0.40
Total		0.51	0.66	0.87		0.72

7. MICROBIOLOGICAL TECHNOLOGIES FOR THE QUANTITATIVE ASSESSMENT OF *CAMPYLOBACTER* PRESENT IN POULTRY FECAL SAMPLES (Manuscripts III and IV)

7.1. Introduction

Direct, fast and accurate detection and quantification of pathogens such as *Campylobacter* that might be present in clinical samples and suspected sources of disease (e.g. food, water and environmental samples) is crucial for the investigation and control of disease cases and outbreaks and the protection of public health. Foodborne outbreaks caused by the presence of hazards such as chemical substances, toxins and pathogens are very common (WHO, 2007a). In 2005, 1.8 million persons died from enteric diseases mainly caused by contaminated food and water (WHO, 2007b). Global trading of food may increase the potential for outbreaks and consequently fast and sensitive detection of contaminated food is considered a priority in public health agendas all over the world. *Campylobacter* is the most frequent and important cause of foodborne diseases in some areas of the world, such as Ireland and New Zealand (Food Safety Authority of Ireland [FSAI], 2006; French, 2008; Sears *et al*., 2011). *Campylobacter* is also one of the most frequently identified pathogens in very young children suffering from diarrhoea in developing countries (Coker *et al*., 2002).

European legislation states that "foodstuffs should not contain microorganisms or their toxins or metabolites in quantities that present an unacceptable risk for human health" Regulation (EC) No 2073/2005" (FSAI, 2012). In order to comply with EU legislation, food safety controls based on risk assessment such as good manufacturing practices and hazard analysis and critical control point (HACCP) are implemented in the food industry (Mucchetti *et al*., 2008; Jin *et al*., 2008). On the other hand, prompt and accurate identification and quantification of pathogens that might be contaminating food is crucial to assist food safety controls. Conventional microbiological methodologies for the detection, identification and quantification of pathogens are commonly based on specific microbiological and biochemical characteristics of pathogens. These methods can be inexpensive and sensitive but they can also be time consuming and they usually rely on initial enrichment procedures that might introduce bias in the results. Some enrichment broths may select against specific *Campylobacter spp.* (Moore and Madden, 1998; Madden *et al.*, 2000; Nachamkin and Blaser, 2000). The use of enrichment broth might also increase the numbers of *Campylobacter*

in the sample depending on time and temperature of incubation (Sails *et al.*, 2003). As a result, it seems challenging to accurately quantify the initial numbers of pathogens present when using enrichment steps (Postollec *et al*., 2011).

Most common microbiological methods used for the detection and quantification of pathogens such as *Campylobacter* are based on selective culture. Nevertheless, the development of quantitative or real-time PCR seems promising for the near real-time detection and quantification of pathogens and beneficial populations such as probiotics (Stevens and Jaykus, 2004; Masco *et al.*, 2007; Malorny *et al*., 2008; Le Drèan *et al*., 2010). PCR offers some advantages in comparison with standard microbiological methods such as rapidity, specificity, sensitivity, the ability to detect small amounts of target DNA in samples and to quantify genes and gene expression (Toze, 1999; Nolan *et al*., 2006; Postollec *et al*., 2011). International standard (ISO) guidelines to detect pathogens by PCR are available (ISO 22174:2005, ISO 20838:2006). What's more, quantitative PCR with the use of adequate controls offers interesting applications in risk analysis and in gene expression (Bustin, 2009; Postollec *et al*., 2011). PCR detects viable and non-viable cells which could lead to an overestimation of the number of viable pathogens present in the initial sample. The use of an enrichment step can reduce PCR inhibition but can also lead to an overestimation of the numbers of pathogens initially present in the sample (Postollec *et al.*, 2011). On the other hand, PCR is a rapid and sensitive method for identification and quantification of pathogens that can be however limited by inhibitory substances. These inhibitory substances present in complex biological samples may reduce or even completely impede the amplification process (Lantz *et al.*, 1997). False negative PCR results may occur due to the presence of inhibitors but also due to DNA degradation or interference with the lysis needed for DNA extraction. For that reason, adequate treatment of samples prior to real-time PCR and the inclusion of appropriate controls in the PCR reactions are crucial to obtain reliable results (Rådström *et al*., 2003, 2004; Murphy *et al*., 2007). Sample treatment methodologies depend on the target organism and also on the sample matrix (Stevens and Jaykus, 2004). Poultry fecal samples represent complex matrices for the quantification of *Campylobacter*. Consequently, matrix preparation for the concentration and purification of *Campylobacter* is crucial for detection and quantification. Efficient and reliable non–enrichment methods should be developed in order to separate pathogen cells from the sample matrices and concentrate pathogen cells for quantification. An ideal method should be able to remove matrix-associated inhibitors without harming the bacterial cells and to concentrate pathogens. It should also

be universal (e.g., applicable to multiple food types and microorganisms), inexpensive, simple, fast and efficient.

Several PCR assays for the detection of *Campylobacter* in foods, milk, water and environmental samples have been developed (Ng *et al.*, 1997; Waage *et al*., 1999; O'Sullivan *et al*., 2000; Yang *et al*., 2003; Hong *et al*., 2007; Ridley *et al.*, 2008b; Rothrock *et al*., 2009; Josefsen *et al*., 2010; Schnider *et al.*, 2010; Leblanc-Maridor *et al.*, 2011a, Toplak *et al*., 2012). Researchers have applied real-time PCR for the quantification of *Campylobacter* spp. in food samples using no enrichment or a short enrichment procedure (Yang *et al.*, 2003; Botteldoorn *et al.* 2008). However, PCR detection and quantification of *Campylobacter* present in fecal samples might be hampered by the presence of inhibitors in fecal matrices. Several PCR methodologies have been described for the detection and quantification of *Campylobacter* in feces (Inglis and Kalischuk, 2004; Rudi *et al.*, 2004; Lagier *et al*., 2004; Jensen *et al.*, 2005; Leblanc-Maridor *et al.*, 2011a, 2011b). New or improved PCR methodologies for accurate, fast and direct detection and quantification of *Campylobacter spp.* should be tested using naturally infected samples and comparing the results with gold standard methods.

In our studies, several DNA extraction methods were tested for the quantification of *Campylobacter* (using real-time PCR) present in spiked poultry fecal samples. Subsequently, two methods were selected to extract *Campylobacter* DNA for real-time PCR quantification of *Campylobacter* present in naturally infected chicken fecal samples and the results were compared to data obtained using selective culture methods.

The main aim of these studies was to identify and improve (if possible) an efficient, accurate real-time PCR methodology for the quantification of *Campylobacter* present in poultry fecal samples directly without the use of enrichment steps. The following work was undertaken:

- Preparation of *Campylobacter* spiked chicken fecal samples. Comparison of real-time PCR results obtained from six commercially available DNA extraction methods using fecal samples spiked with *Campylobacter*. Standard curves were produced and methods were compared based on the results obtained from real-time PCR assays in terms of detection limit, limit of quantification, reproducibility (assessed by comparison of the obtained standard deviation between replicates), amplification efficiency (based on the slope of the standard curve), detection range (range of concentration levels detected) and precision (data fit to the standard curve).

- Preparation of *Campylobacter* naturally infected chicken fecal samples (using stomacher bags without filter and with number 6 filter) for the evaluation of two of the methods previously assessed using spiked fecal samples.
- Comparison of *Campylobacter* quantitative data obtained by selective culture and by real-time PCR (using two different DNA extraction methods) for the quantification of *Campylobacter* present in the naturally infected samples
- Quantitative microbiological data analysis

7.2. Materials and methods

7.2.1. Samples

Fecal samples from broilers confirmed to be *Campylobacter* negative were spiked with *Campylobacter jejuni* CCUG 11284 and used for the quantification of *Campylobacter* by real-time PCR. Six rapid DNA extraction methods were assessed in their performance and effectiveness for the direct quantification of *Campylobacter jejuni* in spiked chicken fecal samples using real-time PCR. Subsequently, naturally infected samples with *Campylobacter* were obtained and processed for the quantification of unknown concentrations of *Campylobacter* using two of the six DNA extraction methods previously assessed.

Detailed descriptions of the spiking protocols and the preparation of samples (spiked samples, naturally infected samples and negative controls) are provided in manuscripts III (Garcia *et al*., 2013a) and IV.

7.2.1.1. Spiked samples

Three different fecal samples obtained from broilers confirmed to be *Campylobacter* negative (by selective culture and PCR) were spiked with *C. jejuni* CCUG 11284 and used for the quantification of *Campylobacter* by real-time PCR. The spiked fecal samples (23.8 g, 21.3 g and 18.7 g) were mixed with saline (214.2 ml, 191.7 ml and 168.3 ml respectively) to produce the first ten-fold dilution. The produced biological replicates (Invitrogen, 2013) were placed into stomacher bags and homogenized for 30 s at average speed (400 Stomacher®, Seward Limited, London, UK). The culture used for spiking was prepared with a particular strain of *C.jejuni* CCUG 11284, inoculated on a modified charcoal cefoperazone deoxycholate agar (mCCDA) plate and incubated at 42ºC

overnight. Five colonies of the recovered *C. jejuni* were enriched in 10 mL Müeller Hinton broth and incubated at 42ºC in microaerobic conditions for approximately 18 hours. Serial dilutions (10^{-1} -10^{-8}) and culture on mCCDA plates allowed for the counts of the numbers of *C.jejuni* (CFU/mL) in order to determine the numbers of *C.jejuni* in the initial culture.

Homogenized fecal samples were spiked with *C. jejuni* CCUG 11284 (calculations were performed based on fecal sample volume and numbers of *C. jejuni* in the initial culture) to produce five spiking levels (10^{1}-10^{5}) and mixed thoroughly to promote equal distribution of *C. jejuni* in the samples. Validation of the correct dilutions of the samples spiked with *Campylobacter* was performed in the laboratory. Spiked fecal samples were alliquoted in 50 eppendorf tubes per dilution series. The remaining fecal sample that had not been spiked was homogenized and distributed in 20 eppendorf tubes to be used as negative controls during the experiments. Prepared samples (spiked samples and negative controls) were centrifuged at 5000 x *g* for 5 minutes, supernatants were discarded and the pellets kept for DNA extraction and quantification. Samples were stored at -20ºC until their use for *Campylobacter* DNA extraction and quantification with real-time PCR.

7.2.1.2. Naturally infected samples

Communication with one of the poultry processing companies in Denmark allowed us to obtain faecal samples from chickens known to be *Campylobacter* positive at a particular time of the year. Faecal samples from *Campylobacter* positive chickens were collected by abattoir personnel on the 18th of September 2012 and sent to the laboratory the same day. The received samples had been collected in four sterile pots; the pots were numbered 1 to 4 in the laboratory and the samples were processed within 30 hours. Two grams of fecal samples were taken from every pot: one gram was deposited in a stomacher bag without filter (samples A) and the other gram in a stomacher bag with number 6 filter corresponding to a pore size of 280 µm (samples B) as illustrated in Figure 5. Every gram of sample was diluted in 9 mL of sterile water and homogenized using a stomacher (Stomacher® 400 A.J. Seward & Co. Ltd., West Sussex, UK). The homogenized sample was transferred to a sterile tube (dilution 10^{-1}). One mL of the first dilution was transferred to another sterile tube containing 9 mL of sterile salted water (dilution 10^{-2}). The same protocol was used to produce dilutions 10^{-3} to 10^{-5} (Figure 5).

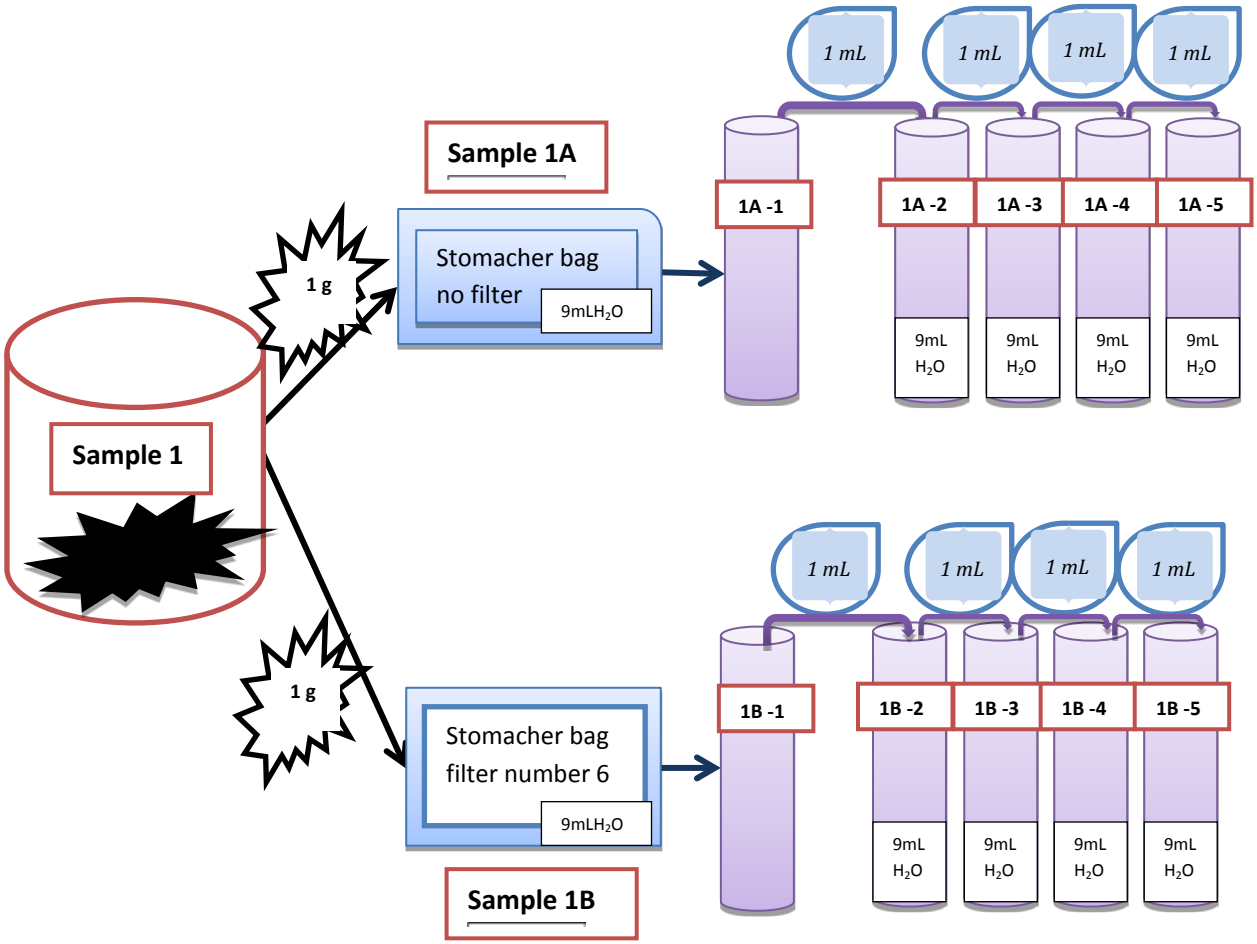

Figure 5 Sub-sampling and dilutions from naturally infected chicken fecal samples

The dilutions prepared this way (Figure 5) for every fecal sample (numbers 1-4) were subsequently processed in the following manner:

a. Conventional direct culture for *Campylobacter* quantification was performed within 30 hours from the collection of poultry fecal samples using *Campylobacter* selective agar, the modified charcoal cefoperazone deoxychocolate agar (product codes: CM0739, SR0155, Oxoid, Hampshire, UK). The previously prepared five dilutions from every sample (1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B) were mixed thoroughly trying to achieve equal distribution of the *Campylobacter* in the samples and 100 µL were then spread onto mCCDA agar. Inoculated plates were incubated microaerobically at 42°C for 48 hours (Forma Scientific Incubator from Thermo Fisher Scientific Inc. Waltham, MA, USA). After 48 hours, suspected *Campylobacter* colonies growing in every plate were counted and numbers recorded in Excel for further data analysis.

b. Tubes containing the dilutions prepared from every sample were stored at +5°C for future DNA extraction processing. Just before DNA extraction, every sample was mixed thoroughly and 1 mL transferred to a sterile Eppendorf tube. Samples were centrifuged at 117000 x *g* for 15 min, the supernatants were discarded and the pellets used for DNA extraction.

7.2.2. DNA extraction methodologies for Campylobacter quantification with real-time PCR

Samples spiked with *Campylobacter* (with *Campylobacter* concentrations from 10 to 10^5) had been previously prepared and stored at -20ºC. Required samples for every DNA extraction method were taken from the freezer and thawed before every DNA extraction method. Sample pellets were re-suspended and DNA extracted according to instructions found in every DNA extraction protocol (Manuscript III: Garcia *et al*., 2013a). The following six DNA extraction methods were evaluated:

1. Easy-DNA™ Kit For genomic DNA Isolation (Invitrogen, Leek, The Netherlands.).

2. MagneSil® KF, Genomic system (KingFisher®) (Promega, Madison, WI, USA).

3. SureFood® PREP *Campylobacter* (Congen Biotechnologie GmbH, Berlin, Germany).

4. QIAamp Qiagen DNA stool mini kit (Qiagen, Hilden, Germany).

5. NucliSENS® miniMAG® (bioMérieux sa, Lyon France).

6. NucleoSpin® Tissue (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

The final DNA elution volume was 100 µl for all methods except for QIAamp Qiagen DNA stool mini kit. In the Qiagen method, the purified, concentrated DNA was eluted from the spin column in 200 µl of low-salt elution buffer.

Two biological replicates were analyzed when using the NucliSENS® miniMAG method (due to protocol limitations) and three biological replicates were processed when using the rest of the methods. Furthermore, two real-time PCR replicates from one biological replicate (all dilutions) were included to evaluate variation attributable to the real-time PCR run. Therefore, a total of five replicates per sample (*Campylobacter* concentration level) were analyzed for every DNA extraction method except for NucliSENS® miniMAG (four replicates/per sample). The methods KingFisher

(Promega) and MiniMAG (BioMérieux) were partly automated, the rest of the extraction methods were manual. After DNA extraction, measurements (in duplicate or triplicate in case of large deviations) of DNA yield and purity were obtained using NanoDrop (Thermo Fisher Scientific Inc) spectrophotometer. Values related to DNA yield and purity obtained from DNA extracted using every protocol were recorded in Excell. Extracted DNA was stored at -20°C for real-time PCR assays.

7.2.3. Real-time PCR

Real-time PCR detects specific target DNA sequences as they are amplified. The amount of target DNA sequences theoretically doubles with every cycle (Figure 6). The copies of the target DNA sequence are measured through fluorescent signals in order to quantify the amplified products in "real time". It is possible to distinguish four phases in a real-time PCR cycle: the lag phase or baseline, the exponential phase, the linear phase and the plateau phase (Figure 7). The process can be followed on a computer screen during the real-time PCR run.

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000

Figure 6 Number of copies of the target DNA sequence obtained in every cycle of the real-time PCR (Hunt, 2010).

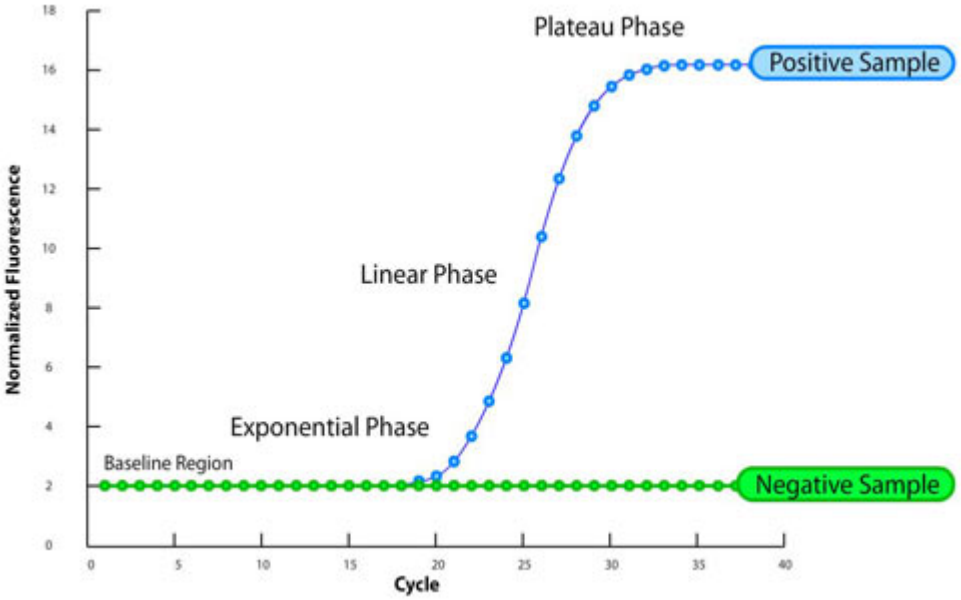

Figure 7 Real-time PCR cycle phases (Abbott Molecular, 2012)

Real-time PCR was performed using a real-time PCR thermo cycler Mx3005P™ (Strategene, La Jolla, USA). Samples and PCR mix were placed in the thermal cycler using MicroAmp Optical 96-well reaction plates (Applied Biosystems) covered with MicroAmp Optical caps (Applied Biosystems). The 25-µl real-time PCR mixture contained 1 U of *Tth* DNA polymerase (Roche A/S), 1 x PCR buffer for *Tth* DNA polymerase (Roche A/S, Hvidovre, Denmark), 0.6 mM deoxynucleoside triphosphate mixture (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), 2.5 mM MgCl2 (Applied Biosystems), 0.8 ml/l of glycerol (87%; Merck, Darmstadt, Germany), 0.5 µM forward primer OT-1559-5'-CTG CTT AAC ACA AGT TGA GTA GG-3', 0.5 µM reverse primer 18-1-5'- TTC CTT AGG TAC CGT CAG AA-3'(DNA Technology, Århus, Denmark; *C. jejuni* 16S rRNA; GenBank accession no. Y19244), 0.2 g/l bovine serum albumin (Roche A/S), 75 nM target locked nucleic acid (LNA) *Campylobacter* probe 5' [6FAM] CA[+T] CC[+T] CCA CGC GGC G[+T]T GC[BHQ1] 3' (Sigma-Aldrich), 60 nM internal amplification control (IAC) probe (5'-VIC-TTC ATG AGG ACA CCT GAG TTG A-TAMRA 3'; Applied Biosystems), 5 x 10^3 copies of IAC (124bp) and 10 µl of extracted template DNA.

The primers used (the forward primer OT-1559 and the reverse primer 18-1) amplify a 287 basepair fragment of the 16S rRNA gene of thermotolerant *C. jejuni, C. coli* and *C. lari* (Lübeck *et al.* 2003; Josefsen *et al.* 2004). The amplification products were detected by using the FAM (fluorescein

amidite) -labeled probe. Furthermore, an internal control (amplified with the target) was visualized using a HEX (hexachloro fluorescein) -labeled probe. The thermal profile included an initial denaturation step at 95°C for 3 min followed by 40 cycles consisting of denaturation at 95°C for 15 s, annealing at 60°C for 60 s and extension at 72°C for 30 s.

Adequate positive and negative controls need to be used in real-time PCR reactions. In our study, the following controls were included: (i) two positive controls, (DNA from C. jejuni, dilutions 1:100 and 1:1000) (ii) one negative control (DNA from E.coli, dilution 1:1000) and (iii) a non-template control (NTC) in duplicate. The inclusion of a non-template control (NTC) allowed for the identification of potential non-specific fluorescence signals (false positives).

7.2.4. Data analyses

Real-time PCR detects the increase in fluorescent signal throughout the PCR cycling process produced by all the samples (Figure 8). During the exponential phase and the linear phase of the real-time PCR assay the amount of fluorescence increases with the amount of the target DNA sequence amplified. Moreover, the rate at which the target DNA is amplified indicates the amount of target DNA in a particular sample (Edwards *et al.*, 2004).

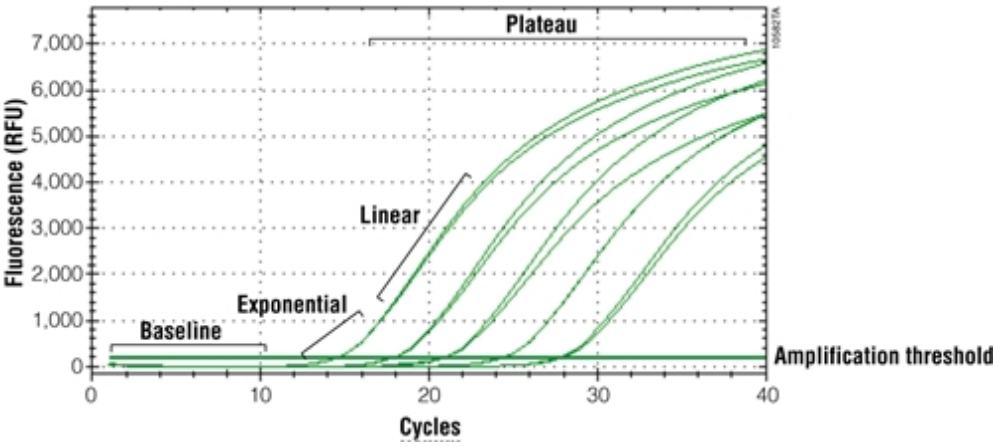

Figure 8 Amplification curves, showing terms commonly used in real-time PCR

The amplification threshold is usually set above the baseline. The cycle number corresponding to the point where the curve (for a positive sample) crosses the threshold line is called Ct value (threshold cycle). Ct values are used to evaluate the results of the experiments. The Δ*Ct* method is based on obtained Ct values for different samples in a real-time PCR run. Comparison of the

obtained Ct values with a standard curve designed from known amounts of the target gene will allow for quantification of the samples. Samples with known concentrations of the DNA target can be used to construct standard curves. Quantification of the amount of target DNA in unknown samples can be done indirectly by measuring corresponding Ct values and using the standard curve to determine initial numbers of DNA in the samples. The lower the Ct value for a given sample, the greater the amount of DNA initially present in the sample. Ideally, in experiments using dilution series, the dilution with the highest amount of target DNA should correspond to the lowest Ct value and Ct values should be 3.5 cycles apart for each of the 10-fold dilution series. The results obtained from real-time PCR using dilution series might indicate the degree of inhibitors present in the sample.

Most software programmes available with real-time PCR technologies can be used to calculate Ct values, prepare standard curves and for the determination of initial DNA concentration in samples. Amplification thresholds and Ct values can be obtained in three different ways when using real-time PCR Mx3005P (Stratagene, La Jolla, CA, USA): (i) default method set by the software (ii) background-based method (based on the background fluoresce of the experiment) and (iii) threshold manually set by the user (Stratagene, 2004). The selected threshold will determine the Ct values and consequently will influence the quantification results. In our experiments conducted to compare several DNA extraction methods (Manuscript III: Garcia *et al*., 2013a), real-time PCR data obtained from the six DNA extraction methods were analyzed all together in a common project using the MxPro-Mx3005P software (version 3.00, Stratagene, La Jolla, CA, USA). The amplification threshold was set using the software option “background-based threshold” which determines the standard deviation of all amplifications (from cycles 5 to 9) and multiplies them by a factor of 10 (default factor). A common baseline was set for all amplification plots obtained using an adaptive baseline and a non-adaptive baseline for comparison of the six DNA extraction methods (Stratagene, 2004). A common amplification threshold was generated and the threshold cycle values (Ct values) obtained were used to compare the different DNA extraction methodologies. In general, the lower the Ct values, the higher the DNA quantity obtained (Stratagene, 2004; Armbruster and Pry, 2008; Abdelwhab *et al*., 2010). Real-time PCR reproducibility was assessed by calculating the standard deviation (SD) between replicates, ideally the SD between replicates should be less than 0.5 (Eurogentec, 2012). The numbers of CFU (logs) obtained were plotted against the Ct values obtained in the real-time PCR runs and standard curves were produced by linear regression for all DNA extraction methods tested. Standard curves were used to determine the overall performance of

real-time PCR in terms of amplification efficiency, detection range (range of *C. jejuni* levels detected), limit of detection, limit of quantification and precision for every DNA extraction method. The slope of the standard curve indicates the amplification efficiency of the real-time PCR assay. The amplification efficiency (AE) was calculated based on this equation: $AE = 10^{(-1/slope)} - 1$ (Klein *et al.*, 1999). An amplification efficiency of 100% indicates perfect reactions where the amplicon doubles each cycle (Stratagene, 2004). An assessment of precision, linearity or data fit to the standard curve is produced using the parameter "R squared (R2)" which should be close to 1, in fact values ≥0.985 indicate good correlation (Stratagene, 2004).

Real-time PCR results (related to the direct quantification of *Campylobacter* in spiked chicken faecal samples) obtained from the six DNA extraction methods were carefully analyzed (Manuscript III: Garcia *et al*., 2013a) and two selected DNA extraction methods were tested with poultry fecal samples naturally infected with *Campylobacter* (manuscript IV). Results related to real-time PCR assays using the two selected DNA extraction methods and fecal samples naturally infected with *Campylobacter* were compared based on limit of detection, limit of quantification and real-time PCR amplification efficiency. Furthermore, estimates of the number of *Campylobacter* (present in naturally infected chicken fecal samples) obtained by real-time PCR when using the two different DNA extraction methods were compared with those obtained from selective culture. Correlation coefficients were obtained to assess agreement between the methods used in this study. Statistical significance of the differences observed between results from culture and from real-time PCR when using the two different DNA extraction methods were assessed using the multcomp package in the statistical program R (R Development Core Team, 2008).

7.3. Results

Detailed results can be found in manuscripts III (Garcia *et al*., 2013a) and IV.

7.3.1. Results obtained from the different methods used to extract Campylobacter DNA from spiked fecal samples

7.3.1.1. Results related to DNA yields and purity

Results related to DNA yield obtained for each of the six DNA extraction methods are presented in Manuscript III: Garcia *et al*., 2013a (Figure 1). The Easy-DNA™ Invitrogen method produced the

highest DNA yield (ranging from 188 ng/µl to 317 ng/µl) followed by KingFisher® (ranging from 54 ng/µl to 177 ng/µl). The method QIAamp (Qiagen) produced the lowest DNA yield (3 ng/µl-6 ng/µl).

Results related to DNA purity (absorbance ratio A260/280 values) were obtained for every method as follows: Easy-DNA kit (Invitrogen): 1.5-1.6, KingFisher (Promega): 1.1-1.8, miniMAG (bioMérieux): 1.0-1.4, SureFood (Congen): 0.9-4.9, NucleoSpin (Macherey-Nagel): 1.6-2.2 and QIAamp (Qiagen): 1.3-3.3. Purity ratios around 1.8 or higher are desirable (Thermo Fisher Scientific, 2011).

7.3.1.2. Results related to real-time PCR

Identical results (FAM Ct values) were obtained by selecting an adaptive baseline and a non-adaptive baseline (from cycles 3 to 12) with a common threshold (740) which was used for comparison of the six DNA extraction methods. Results related to the average FAM Ct values obtained from the real-time PCR experiments for the quantification of *Campylobacter jejuni* DNA extracted using the different extraction methods are presented in Manuscript III (Garcia *et al*., 2013a). All the controls included in real-time PCR assays produced the expected results. Briefly, FAM Ct values were obtained from the samples spiked with the lowest concentration (10 CFU/ml) only when using the NucleoSpin® Tissue DNA extraction method. No FAM Ct values were generated from the samples spiked with 10^2 CFU/ml when using the following methods: MagneSil® KingFisher, Easy-DNA Invitrogen and SureFood. Regarding the FAM Ct values generated from amplification signals produced by samples spiked with 10^2 CFU/ml, the NucleoSpin method produced the lowest FAM Ct value followed by miniMAG. Results related to samples spiked with 10^3 CFU/ml indicated that Easy-DNA Invitrogen produced the lowest FAM Ct values followed by miniMAG and MagneSil® KingFisher. Real-time PCR results obtained from samples spiked with 10^4 CFU/ml indicated that Easy-DNA Invitrogen generated the lowest FAM Ct values. SureFood produced the lowest FAM Ct value when using samples spiked with 10^5 CFU/ml followed by Easy-DNA Invitrogen. Overall, the Easy-DNA Invitrogen method generated the lowest FAM Ct values followed by the miniMAG method. SureFood generated a very low Ct value for the *Campylobacter* concentration level 10^5 CFU/ml; however, the variation between replicates was high as illustrated by a standard deviation of 2.5 cycles. The MagneSil® KingFisher method performed poorly in this study. DNA extraction methods Easy-DNA Invitrogen, miniMAG and NucleoSpin

offered general good real-time PCR reproducibility generating standard deviations from 0.3 to 0.8 between replicates.

7.3.1.3. Standard curves, amplification efficiency and linearity

Standard curves were generated and used to evaluate the amplification efficiency, detection range and precision of the DNA extraction methods tested. Generated amplification plots and standard curves are presented in Appendix 2. Results related to real-time PCR performance indicators used to evaluate the different DNA extraction methods can be found in manuscript III. The amplification efficiency of the real-time PCR assay was calculated based on the slope of the standard curve. Amplification efficiencies between 90% and 110% were considered acceptable (Stratagene, 2004). The methods Easy-DNA Invitrogen and QIAamp Qiagen generated the best amplification efficiencies (93.2% and 91.5% respectively). These two methods also produced R squared (R2) values close to 1 indicating good precision. The method NucleoSpin® Tissue was able to detect samples spiked with the lowest *Campylobacter* concentration level (10 CFU/ml), however, this extraction method generated higher Ct values for most concentration levels than the other methods and the amplification efficiency obtained was significantly above 100% (139.5%) possibly caused by inhibitors and/or experimental error (Stratagene, 2004).

Based on the results obtained (Manuscript III: Garcia *et al*., 2013a), the methods Easy-DNA and MiniMAG were selected to quantify directly (without enrichment) *Campylobacter* present in naturally infected chicken fecal samples (manuscript IV).

7.3.2. Results related to the quantification of Campylobacter present in naturally infected chicken fecal samples

7.3.2.1. Standard curves generated for absolute quantification

The standard curves designed from real-time PCR data from spiked chicken fecal samples for the extraction methods Easy-DNA and MiniMAG showed the methods to be linear in the range 10^3 - 10^5 CFU/mL (manuscript IV). Standard curves should be carefully designed (Whelan *et al.*, 2003; Leong *et al*., 2007; Malorny *et al*., 2008; Dhanasekaran *et al*., 2010). In this study, identical results were obtained by selecting an adaptive baseline and a non-adaptive baseline (from cycles 3 to 12) with a common threshold (740). The limit of detection when extracting DNA using MiniMAG was

10^2 CFU/ml; however, the standard curve did not seem to be linear below the 10^3 CFU/ml level and the limit of quantification was considered to be 10^3 CFU/ml. The amplification efficiencies obtained for the Easy-DNA and MiniMAG methods were 96.6% and 116.9% respectively. Data fit to the standard curves were assessed using R^2values which were 0.961 and 0.945 for the Easy-DNA and MiniMAG methods, respectively (manuscript IV).

7.3.2.2. Comparison of enumeration by culture and real-time-PCR

Results obtained from selective culture of the chicken fecal samples naturally infected with *Campylobacter* are presented in manuscript IV. Results indicated that samples 1 and 4 contained higher numbers of *Campylobacter* (in the order of 10^7 CFU/g) while samples 2 and 3 had lower numbers (in the order of 10^5 and 10^4 CFU/g respectively). The numbers of *Campylobacter* obtained by culture from samples B were consistently lower than those obtained from samples A (processed without filter during the sample homogenization step).

Generated standard curves were used for the real-time PCR direct quantification of *Campylobacter spp.* present in naturally infected chicken fecal samples. Quantification of samples with *Campylobacter* numbers higher than 10^5CFU/g was performed based on extrapolation of the standard curves (see manuscript IV). Obtained real-time PCR data were transformed for every dilution level and mean values were calculated as estimates of the numbers of *Campylobacter* present in every biological sample (manuscript IV). Real-time PCR data were obtained from all dilution levels when using the DNA extraction method MiniMAG, however, no real-time PCR results were obtained for the dilutions 10^{-4} and $10^{-5}$of samples 2, 3 and 4when extracting DNA with the Easy-DNA method. The mean estimates for the numbers of *Campylobacter* in every sample obtained by culture and by real-time PCR using the two different DNA extraction methods are presented in manuscript IV. Standard deviations were calculated and found to be lower when using the DNA extraction method MiniMAG. The numbers of *Campylobacter* obtained by real-time PCR when using MiniMAG for DNA extraction were in most cases higher than the numbers obtained by culture (for all samples except for Sample 3). Sample number 3 had the lowest *Campylobacter* concentration (below 5 logs) and the enumeration results obtained by culture were higher than those obtained using real-time PCR for this sample. In general, the numbers of *Campylobacter* obtained by real-time PCR when extracting DNA with the Easy-DNA method were lower or very similar to the numbers obtained by culture (manuscript IV). Agreement between methods was investigated

and the correlation coefficients obtained were 0.98 between culture and real-time PCR (both DNA extraction methods) and 0.99 between Easy-DNA and MiniMAG extraction methods.

The statistical significance of the differences between estimates of the numbers of *Campylobacter* obtained by culture and by real-time PCR using the two different DNA extraction methods was investigated. A result with a p-value ≤ 0.05 was considered a statistically significant result. The only difference found to be statistically significant was the one related to the estimates of the numbers of *Campylobacter* obtained for sample 1A by real-time PCR when using the two different DNA extraction methods (p-value = 0.02). The results from this study indicated that there were no statistically significant differences between culture and real-time PCR.

7.4. Discussion

Reliable quantification of pathogens is crucial to ensure food safety and consequently fast, sensitive and accurate methodologies and data analysis techniques need to be properly tested, improved or developed. Real-time PCR is widely used to detect and quantify pathogens or beneficial microbes (Masco *et al*., 2007; Malorny *et al*., 2008; Le Dréan *et al*., 2010). Real-time PCR also allows for the detection and quantification of viable but non-culturable microbial forms that might be of high relevance in some cases (Postollec *et al.*, 2011). In these studies, several DNA extraction methods were assessed in their effectiveness for the quantification of *Campylobacter jejuni* present in spiked chicken fecal samples using real-time PCR. Moreover, two of the methods were used to quantify *Campylobacter* (by real-time PCR) present in naturally infected chicken fecal samples and the results were compared to quantitative data obtained from traditional culture. Conventional microbiological methods for the detection, identification and quantification of *Campylobacter* can be time consuming, usually rely on initial enrichment procedures that might introduce bias (in relation to the strains identified and the numbers of pathogens) in the results (Velusamy *et al.*, 2010) and will not detect viable but non-culturable (VBNC) *Campylobacter* cells (Postollec *et al.*, 2011). On the other hand, real-time PCR might produce false negative results when no enrichment is used and the samples contain low numbers of bacteria. Real-time PCR quantifies DNA present in the samples; amplified DNA could be derived from live cells, viable but non-culturable microbial forms and dead cells (Botteldoorn *et al.*, 2008). Amplified DNA from dead cells may lead to an overestimation of the numbers of the target organism or even false-positive results (Wolffs *et al.*,

2005). As a result, it will be expected that quantification results from real-time PCR will be higher than those obtained by traditional culture. Despite the differences found between methods in these studies (manuscript IV), there was good agreement between real-time PCR methods and culture. The fact that chicken fecal samples were used for the preparation of spiked samples and construction of standard curves could partly explain this agreement because we already accounted for the effect of inhibitors that may be present in chicken fecal samples when building the standard curves. Several studies have compared quantification results obtained by culture and by real-time PCR with contradictory results. Some researchers found that the estimated numbers of target organisms were higher when using real-time PCR in comparison with traditional culture-based methods (Yang *et al.*, 2003; Lebuhn *et al.*, 2005; Pujol *et al.*, 2006; Lahtinen *et al.*, 2006; Hong *et al.*, 2007; Botteldoorn *et al.*, 2008; Reichert-Schwillinsky *et al.*, 2009 Löfström *et al.*, 2010; Converse *et al.*, 2012). However, other studies reported good agreement between the methods (Martín *et al.*, 2006; Josefsen *et al.*, 2010; Bui *et al.*, 2011) while others found an underestimation of the numbers of the target organism when using real-time PCR in comparison with culture (Pennacchia *et al.*, 2009; Noble *et al.*, 2010). Even when there is agreement between methods, the stability of this agreement might be dependent on other factors such as time, season and environmental factors (Shibata *et al.*, 2010; Converse *et al.*, 2012).

Direct quantification of *Campylobacter* present in fecal samples has proven to be difficult (Leblanc-Maridor *et al.*, 2011) and poultry faeces, in particular, represent complex samples for accurate quantification of *Campylobacter* (Rudi *et al.*, 2004). Inhibitory substances present in biological matrices may reduce the efficiency of real-time PCR assays significantly (Perch-Nielsen *et al.*, 2003; Guy *et al.*, 2003; Rådström *et al.*, 2004; Sunen *et al.*, 2004; Stratagene, 2004; Jiang *et al.*, 2005). In addition, the species of *Campylobacter* and the initial numbers of *Campylobacter* present in the naturally infected chicken fecal samples in this study were unknown. It seems difficult to be completely certain about the numbers of a given bacteria in a particular sample even when the methodologies used are very sensitive. The distribution of bacteria in samples might not be homogeneous (Griffith *et al.*, 2003) even though chicken fecal samples were homogenised using a stomacher in this study. What's more, microbiological methods might select against certain species of *Campylobacter* that may be present in the samples. In fact, CCDA agar is selective for *Campylobacter spp.* but some strains may fail to grow or grow poorly (Neogen, 2010). In general, CCDA agar plates (from Oxoid, Hampshire, UK) are selecting for *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari.* Chickens usually carry *C. jejuni, C. coli* and

*occasionally C. lari (*Newell and Fearnley, 2003; European Food Safety Authority and European Centre for Disease Control, 2011). Interestingly, *C. jejuni* and *C. coli* are the main species responsible for most human campylobacteriosis cases (Nachamkin and Blaser, 2000; Friedman *et al*., 2000; Allos, 2001; Gillespie *et al*., 2002; Tam *et al*., 2003). Furthermore, mixed infections with diverse *Campylobacter spp.* are not uncommon in poultry (Jacobs-Reitsma *et al*., 1995; De Boer *et al*., 2002).When using traditional microbiological methods for quantification it is important to bear on mind that culture selects for culturable bacterial cells only, other viable but non-culturable bacterial states could be missed in the quantification. Traditional microbiological methods for quantification rely on counting bacteria growing on plates; however, the results may differ between persons performing the enumeration of bacteria growing on a given plate in some occasions (US Food and Drug Administration, 2006). Furthermore, when using selective culture for *Campylobacter* it is generally assumed that colonies growing on plates are *Campylobacter* colonies. On the other hand, distinguishing *Campylobacter* colonies from other contaminants growing on plates may prove difficult (Line, 2001; Stern *et al.*, 2001).

The quantification results obtained in this study suggested that the use of filters during sample processing could translate on lower estimates of the numbers of *Campylobacter* in the samples independently of the quantification method applied (culture or real-time PCR). This finding is in agreement with other studies where the use of specific types of filters partly or completely inhibited PCR amplification due to target *Campylobacter* DNA binding to the filter membranes (Oyofo and Rollins,1993).

The presence of inhibitory substances in complex biological samples may reduce or even completely impede the amplification process (Nolan *et al*. 2006). The use of PCR facilitators has been recommended (Hedman and Rådström, 2013). The addition of bovine serum albumin (BSA) may help to overcome PCR inhibition in fecal samples and other types of samples such as blood and meat samples (Abu Al-Soud and Rådström 2000). The use of nonionic detergents such as detergents Tween 20 and Triton X-100 and polymers such as PEG and dextran has been shown to facilitate PCR amplification and reduce PCR inhibition in fecal samples (Abu Al-Soud and Rådström, 2000). The addition of phytase has been proposed to relieve inhibition caused by the presence of phytic acid in feces (Thornton and Passen, 2004). In this study, BSA was added to the PCR mix to facilitate PCR amplification. The selection of DNA polymerase might have an important effect on overcoming PCR inhibitors (Katcher and Schwartz, 1994; Abu Al-Soud and Rådström, 1998; Wolffs *et al.*, 2004; Bessetti, 2007). The *Tth* DNA polymerase was used in this

study. The *Tth* polymerase can significantly improve PCR amplification efficiency in comparison with the Taq DNA polymerase when processing feces or samples containing fecal material (Shames *et al.*, 1995; Abu Al-Soud and Rådström, 1998; Dahlenborg *et al.*, 2001). Additionally, the *Tth* buffer contains bovine serum albumin (BSA) and the detergent Tween 20 which facilitate PCR amplification (Hedman and Rådström, 2013).

Diverse combinations of DNA extraction methods may be used for the removal of PCR inhibitors and the concentration of target DNA in digesta and fecal samples (Yu and Morrison, 2004; Zoetendal *et al.*, 2001). DNA extraction methods can remove a significant amount of PCR inhibitors but they can be expensive and laborious (Rådström *et al.*, 2003). Advances in the development and improvement of DNA extraction methods can translate to fast, easier-to-use and cheaper methods. In these experiments, the methods Easy-DNA and MiniMAG were relatively fast, it was possible to process approximately 30 samples in less than 6 hours. In terms of cost per DNA extraction, the cheapest methods were Easy-DNA and KingFisher (17 DKK/DNA extraction) while the most expensive was MiniMAG (125 DKK/DNA extraction).

Real-time PCR data analyses can be complex and the comparison between real-time PCR results from different methodologies might be difficult and cumbersome (Karlen *et al*., 2007). Different real-time PCR data analysis methods have been described (Karlen *et al*., 2007) but no method seems to be fully characterized and completely reliable statistically (Karlen *et al.*, 2007). In fact, good statistical methods for thorough and rigorous Q-PCR data analyses have lagged behind the numerous applications of real-time PCR.

The amount of DNA will double at each real-time PCR cycle in a perfectly efficient reaction but this is difficult to achieve in experimental conditions. PCR efficiency depends primarily on the primers used and therefore careful design of primers seems necessary to obtain highly efficient PCR reactions (Tichopad *et al,* 2004; Tichopad *et al.*, 2010). The results from this study indicated that the method Easy-DNA Invitrogen produced the most optimal real-time PCR performance indicators when used with chicken fecal samples. On the other hand, the limit of detection obtained when using Easy-DNA Invitrogen was relatively high (10^3 CFU/ml). In a study conducted by Lund *et al.* (2004), a detection limit of 250 *Campylobacter* CFU/g of feces was obtained using the KingFisher method. In the study presented here, the KingFisher method did not seem to work very well with the spiked chicken fecal samples.

The limit of detection and the limit of quantification might differ because the limit of detection may be found at a concentration below the linear part of the standard curve (Armbruster and Pry, 2008; Leblanc-Maridor *et al*., 2011). It is generally accepted that real-time PCR may provide accurate quantification estimates when using samples with numbers of target organism exceeding 10^2-10^3 CFU/g or ml but not with lower concentrations due to the loss of target DNA during sample preparation and to the small volumes analyzed (Malorny *et al.*, 2008; Löfström *et al.*, 2010; Josefsen *et al*., 2010). Besides, the use of increased concentrations of target DNA might help to overcome the effect of PCR inhibitors (Rådström *et al.*, 2003; Lund *et al*., 2004; Roussel *et al*., 2005).

Quantification results depend on the sample matrix, sample preparation, DNA extraction method, real-time PCR reagents, real-time PCR experiments and real-time PCR data analysis. On the other hand, experimental variability can be very high even when the best methodologies are used and experiments are performed under very controlled conditions. Variability between different PCR plates or runs can be high; even when considering only one specific PCR plate, intra-plate variability can be significant (Karlen *et al.* 2007). In fact, biological variability between samples and replicates can also be high and partly explain different real-time PCR efficiencies.

Research results might also be different when using fresh samples for DNA extraction than when extracting DNA from frozen samples. Chicken feces present a semi-dry viscous consistency that might cause problems during sample processing and DNA extraction (Silkie and Nelson, 2009). It has been recommended that fecal samples are processed very soon after collection or alternatively, samples should be placed in the freezer (Nechvatal *et al.*, 2008). Sample storage conditions may affect detection and quantification of bacterial pathogens in fecal samples (Tang *et al.*, 2008; Barnard *et al.*, 2011). In this study, spiked samples and negative controls were immediately placed in the freezer (stored at -20ºC) after preparation as recommended (Qiagen, 2013) and used for DNA extractions within four months. However, it has been shown that freezing may affect DNA stability and produce false-negative results when using PCR for pathogen detection (Jensen *et al.*, 2004; Brinkman *et al.*, 2004). Further research should be conducted to assess the degree of *Campylobacter* DNA damage associated with freezing and/or other storage conditions. The naturally infected chicken fecal samples used in this study were processed within 30 hours from the time of collection and it is possible that *Campylobacter* cells with intact membranes survived well during that time which in turn could explain the statistical agreement between quantification results obtained by culture and by real-time PCR. *Campylobacter jejuni* can survive up to six days in

poultry feces (Ahmed *et al*., 2013). We could hypothesize that most *Campylobacter* cells present in our samples were in viable and culturable state because chicken fecal samples were fresh and processed within 30 hours of collection. It is therefore possible that not a great amount of stressed *Campylobacter* cells, VBNC *Campylobacter* states or free *Campylobacter* DNA were present in these samples. Novel and accurate methods able to discriminate between the different *Campylobacter* viable and non-viable states could share light in this matter.

In conclusion, there was good agreement between the *Campylobacter* direct quantification results obtained by selective culture and by real-time PCR when using two different DNA extraction methods in these studies which could indicate that the main aim to obtain reliable *Campylobacter* direct quantification results using chicken fecal samples was fulfilled.

Manuscript III

Direct Quantification of *Campylobacter jejuni* in Chicken Fecal Samples Using Real-Time PCR: Evaluation of Six Rapid DNA Extraction Methods

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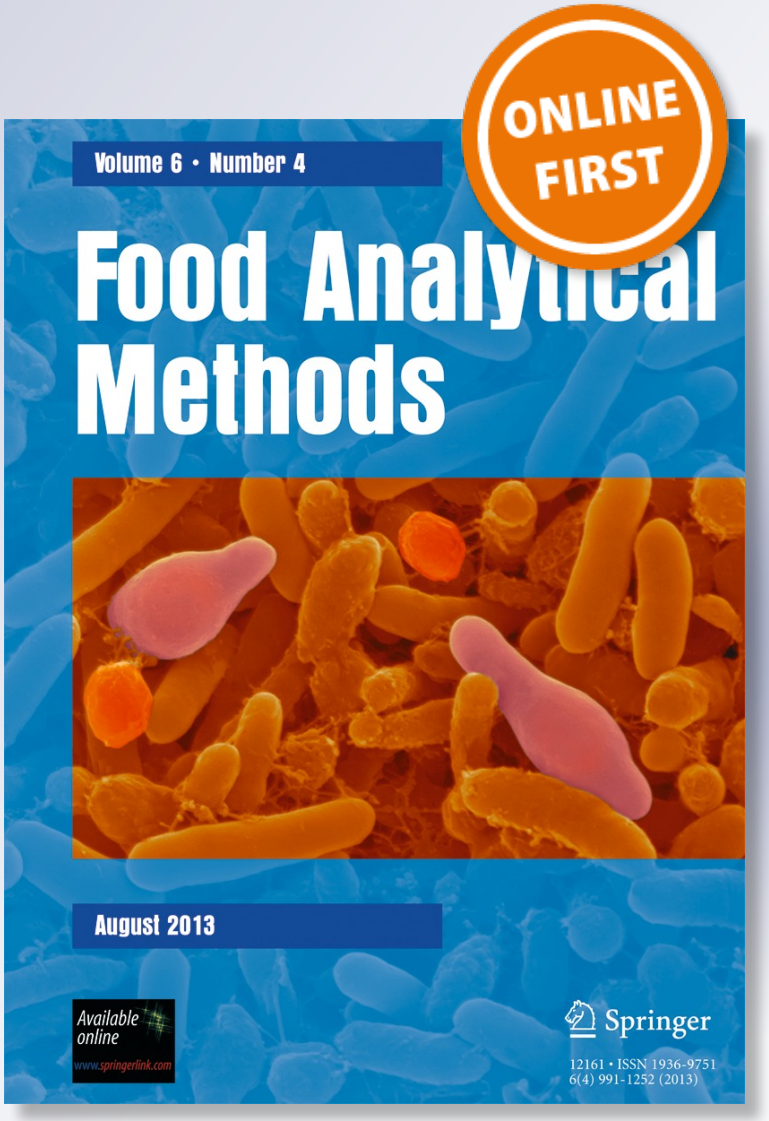

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Abstract Direct and accurate quantification of *Campylobacter* in poultry is crucial for the assessment of public health risks and the evaluation of the effectiveness of control measures against *Campylobacter* in poultry. The aim of this study was to assess several rapid DNA extraction methods for their effectiveness for the direct quantification (without enrichment) of *Campylobacter jejuni* in chicken fecal samples using real-time PCR. The presence of inhibitory substances in chicken fecal samples may reduce or even completely impede the PCR amplification process making quantification very difficult. Six rapid DNA extraction methods were compared based on their limit of detection, efficiency, reproducibility, and precision. Standard curves were designed for all the methods tested in order to assess their performance on the direct quantification of *C. jejuni* in chicken fecal samples. As a result of this study, the Easy-DNA (Invitrogen) method generated lower Ct values, the best amplification efficiency (AE=93.2 %) and good precision (*R* squared=0.996). The method NucleoSpin® Tissue was able to detect samples spiked with the lowest *Campylobacter* concentration level (10 CFU/ml) but the amplification efficiency was not optimal (AE=139.5 %). DNA extraction methods Easy-DNA Invitrogen, MiniMAG® and NucleoSpin® Tissue produced good real-time PCR reproducibility generating standard deviations from 0.3 to 0.8 between replicates.

Keywords *Campylobacter* · Quantification · Chickens · Real-time PCR · DNA extraction methods

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Introduction

Rapid and accurate detection and quantification of pathogens such as *Campylobacter* that might be present in clinical samples and suspected sources of disease (e.g., food, water, and environmental samples) are crucial for the investigation and control of disease cases and outbreaks and the protection of public health (World Health Organization 2007). *Campylobacter* is the most frequent cause of foodborne disease in many areas of the world (French 2008; Sears et al. 2011). Accurate methods for *Campylobacter* quantification may assist on the assessment of public health risks and the evaluation of control measures implemented during poultry production such as vaccination strategies (Garcia et al. 2012). Prompt and accurate identification and quantification of pathogens that might be contaminating food are crucial to assist food safety controls (Mucchetti et al. 2008; Jin *et al*. 2008). Most common microbiological methods for the detection and quantification of *Campylobacter* are based on selective culture. Conventional microbiological methodologies for the detection, identification, and quantification of pathogens are usually based on specific microbiological and biochemical characteristics of pathogens (Anonymous 2006; Velusamy et al. 2010). These methods can be inexpensive and sensitive but they can also be time consuming and they usually rely on initial enrichment procedures that might introduce bias (in relation to the strains identified and the numbers of pathogens) in the results. Some enrichment broths may select against specific *Campylobacter spp*. (Moore and Madden 1998; Madden et al. 2000; Nachamkin and Blaser 2000). The use of enrichment broth might also increase the numbers of *Campylobacter* in the sample depending on time and temperature of incubation (Sails et al. 2003). Therefore, it seems very difficult to accurately identify and/or quantify the initial numbers of pathogens present in the samples when using enrichment steps

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(Postollec et al. 2011). Furthermore, viable but non-culturable (VBNC) *Campylobacter* cells will not be detected using conventional microbiological techniques (Postollec et al. 2011).

The development of real-time PCR seems promising for the real-time detection and quantification of pathogens (Stevens and Jaykus 2004; Masco et al. 2007; Malorny et al. 2008). In general terms, real-time PCR offers some advantages in comparison with standard microbiological methods such as rapidity, the potential ability to detect small amounts of target DNA in samples and to quantify genes and gene expression (Toze 1999; Nolan et al. 2006; Postollec et al. 2011). However, direct quantification of *Campylobacter* (without the use of enrichment) by real-time PCR might prove difficult when low numbers of bacteria are present in samples. On the other hand, dead *Campylobacter* cells will be detected by real-time PCR which may produce an overestimation of the viable *Campylobacter* cells present in the samples. Real-time PCR can be extremely sensitive when pure target DNA is analyzed. However, the limit of detection, the limit of quantification and the amplification efficiency can be negatively affected by the presence of PCR inhibitors (Rådström et al. 2003; Hedman and Rådström 2013). Diverse compounds such as food degradation products, bilirubin (Kreader 1996), phytic acid (Thornton and Passen 2004), bile salts (Lantz et al. 1997; Abu Al-Soud et al. 2005), and complex polysaccharides (Demeke and Adams 1992; Lantz et al. 1997; Monteiro et al. 1997) present in feces have been identified as PCR inhibitors. Substances such as excess NaCl, KCl, and other salts, ionic detergents (Weyant *et al*. 1990), phenol (Katcher and Schwartz 1994), ethanol and isopropanol (Loffert et al. 1997), and other materials might also inhibit PCR. PCR inhibitors might originate from the samples and/or from materials and reagents used during sampling and sample preparation (Rossen et al. 1992; Wilson 1997; Bessetti 2007; Hedman and Rådström 2013). PCR inhibitors can interfere with target DNA and/or with DNA amplification reagents such as thermostable DNA polymerases and/or inhibit fluorescence (Bessetti, 2007; Hedman and Rådström 2013). DNA polymerases might be affected by compounds present in biological samples reducing PCR amplification efficiency (Rossen et al. 1992; Katcher and Schwartz 1994; Abu Al-Soud and Rådström 1998). In general, DNA extraction methods and DNA polymerases need to be carefully selected because their components might influence PCR reactions by inhibiting or facilitating DNA amplification. Furthermore, DNA extraction methods might work differently when using the same sample matrix resulting in different DNA extraction efficiencies. On the other hand, false negative PCR results may occur due to the presence of inhibitors in the samples but also due to DNA loss, DNA degradation or interference with the reagents needed for DNA extraction. Therefore, adequate treatment of samples prior to real-time PCR and appropriate controls should be included in the PCR reactions (Rådström et al. 2004; Cankar et al. 2006; Murphy et al. 2007). Sample treatment methodologies depend on the target organism and also on the sample matrix (Stevens and Jaykus 2004; Nolan et al. 2006). Poultry fecal samples represent complex matrices for the quantification of *Campylobacter*. Therefore, matrix preparation for the removal of inhibitory substances and for the concentration and purification of *Campylobacter* DNA is crucial for *Campylobacter* detection and quantification (Perch-Nielsen et al. 2003; Guy et al. 2003; Rådström et al. 2004; Sunen et al. 2004; Jiang et al. 2005).

The aim of this study was to assess six DNA extraction methods in their performance and effectiveness for the direct quantification of *Campylobacter jejuni* in spiked chicken fecal samples using real-time PCR. Standard curves were produced and methods were compared based on the results obtained from real-time PCR assays in terms of detection limit, limit of quantification, reproducibility (assessed by comparison of the obtained standard deviation between replicates), amplification efficiency (based on the slope of the standard curve), detection range (range of concentration levels detected), and precision (data fit to the standard curve).

Materials and Methods

Chicken Fecal Samples, Spiking Protocol, and Negative Controls

Three different fecal samples obtained from broilers were confirmed to be *Campylobacter* negative (by selective culture and PCR), spiked with *C. jejuni* CCUG 11284 and used for the quantification of *Campylobacter* by real-time PCR. The spiked fecal samples (23.8, 21.3, and 18.7 g) were mixed with saline (214.2, 191.7, and 168.3 ml, respectively) to produce the first tenfold dilution. The biological replicates produced this way (Invitrogen 2013) were placed into stomacher bags and homogenized for 30 s at average speed (400 Stomacher®, Seward Limited, London, UK).

The culture produced for spiking fecal samples was prepared from *C. jejuni* CCUG 11284, inoculated on a modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid, Greve, Denmark) plate and incubated at 42 °C in microaerobic conditions overnight. Five colonies of *C. jejuni* were enriched in 10 ml Müller Hinton broth (MH; Oxoid, Greve, Denmark) and incubated at 42 °C in microaerobic conditions for approximately 18 h. Culture of serial dilutions (10^{-1}–10^{-8}) on mCCDA plates allowed for enumeration of *C. jejuni* (CFU/ml) in the spiking culture.

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Homogenized fecal samples were spiked with *C. jejuni* CCUG 11284 (calculations were performed based on fecal sample volume and numbers of *C. jejuni* in the initial culture) to produce five spiking levels (10^1–10^5) and mixed thoroughly to promote equal distribution of *C. jejuni* in the samples. Validation of the spiking levels was performed in the laboratory. Aliquots of 1 ml were produced to obtain 50 replicates of each of the following spiking levels 10, 100, 1,000, 10,000, and 100,000. The remaining non-spiked fecal sample was homogenized and distributed in aliquots of 1 ml to produce negative controls for the real-time PCR experiments.

DNA Extraction Methods for the Quantification of Campylobacter Using Real-Time PCR

Prepared samples (spiked samples and negative controls) were centrifuged at 5,000×*g* for 5 min and the supernatants were discarded. Sample pellets were stored at −20 °C until their use for *Campylobacter* DNA extraction and quantification with real-time PCR.

Sample pellets were taken from the freezer and thawed at room temperature before DNA extraction. Sample pellets were re-suspended and DNA extracted according to instructions found in every DNA extraction protocol. The following six DNA extraction methods were evaluated:

1. Easy-DNA™ Kit for genomic DNA Isolation (Invitrogen, Leek, The Netherlands).

The published protocol #3 from the Easy- DNA™ Kit (Invitrogen) for the extraction of DNA from small amounts of cells, tissues, or plant leaves was followed. Samples (pellets) were re-suspended in 200 μl of 10 mM Phosphate Buffered Saline (PBS) buffer. The solutions and reagents included in the Kit were used together with chloroform and ethanol for the extraction of *Campylobacter* DNA. The approximate cost per DNA extraction (including laboratory materials) was 17 Danish Kroners (DKK).

2. MagneSil® KF, Genomic system (KingFisher®) (Promega, Madison, WI, USA).

The MagneSil® KF, Genomic system (KingFisher®) is based on the use of paramagnetic particles. Initially, sample pellets were re-suspended in 200 μl of lysis buffer. Samples were lysed allowing the DNA to bind to the paramagnetic particles. In the next steps, particles with DNA were washed (with salted water and alcohol) and air dried. In the final step, DNA was eluted and ready for PCR use. The approximate cost (including laboratory materials) was 17 DKK per DNA extraction.

3. SureFood® PREP *Campylobacter* (Congen Biotechnologie GmbH, Berlin, Germany).

Sample pellets were re-suspended in 400 μl of lysis buffer solution. The method SureFood® PREP *Campylobacter* used the spin column technique for the extraction of *Campylobacter* DNA. In the initial steps, cells were lysed by boiling allowing the DNA to bind to the column. Extracted DNA was washed and finally eluted. The approximate cost (including laboratory materials) was 49 DKK per DNA extraction.

4. QIAamp Qiagen DNA stool mini kit (Qiagen, Hilden, Germany).

Sample pellets were re-suspended in 1.4 ml of commercial buffer (buffer ASL).The protocol for the isolation of DNA from stool for pathogen detection from the QIAamp Qiagen DNA stool handbook was followed. This protocol indicated the use of buffer ASL and heat for cell lysis and the use of InhibitEX tablets for DNA purification. PCR inhibitors and substances that might damage DNA were absorbed using the InhibitEX matrix which was pelleted by centrifugation afterwards. The extracted DNA was further purified using QIAamp Mini spin columns which allowed for the digestion of proteins, DNA binding, washing, and finally, elution of pure DNA from the spin columns. The approximate cost per DNA extraction (including laboratory materials) for this method was 43 DKK.

5. NucliSENS® MiniMAG® (BioMérieux SA, Lyon France).

Sample pellets were re-suspended in 2 ml of 10 mM PBS buffer. The NucliSENS® miniMAG® method used magnetic silica particles for DNA extraction. Initially, cells were lysed using a lysis buffer and free DNA could bind the magnetic silica particles. After several washes with different buffers, DNA was eluted using an elution buffer and ready for further processing. The approximate cost (including laboratory materials) for this method was 125 DKK per DNA extraction.

6. NucleoSpin® Tissue (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

Sample pellets were re-suspended in 200 μl of lysis buffer T1 (part of the kit).The "support protocol for genomic DNA from stool" was used to extract *Campylobacter* DNA using the NucleoSpin® Tissue methodology. TE buffer, buffer T1, and proteinase K were used to prepare the samples. Samples were subsequently treated using buffer B3 and heat to lyse cells. The use of ethanol and NucleoSpin® Tissue Columns allowed for DNA binding. Extracted DNA was then washed with buffers from the kit and eluted using an elution buffer. The approximate cost per DNA extraction (including laboratory materials) for this method was 26 DKK.

The final DNA elution volume was 100 μl for all methods except for QIAamp Qiagen DNA stool mini kit. In the Qiagen method, the purified, concentrated DNA was eluted from the spin column in 200 μl of low-salt elution buffer.

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Two biological replicates were analyzed when using the NucliSENS® miniMAG method (due to protocol limitations) and three biological replicates were processed when using the rest of the methods. Furthermore, two real-time PCR replicates from one biological replicate (all dilutions) were included to evaluate variation attributable to the real-time PCR run. Therefore, a total of five replicates per sample (*Campylobacter* concentration level) were analyzed for every DNA extraction method except for NucliSENS® miniMAG (four replicates/per sample).

The methods MagneSil® KingFisher and NucliSENS® miniMAG were partly automated, the rest of the extraction methods were manual. After DNA extraction, measurements (in duplicate or triplicate in case of large deviations) of total DNA yield and quality were obtained using a NanoDrop (Thermo Fisher Scientific Inc., Delaware, USA) spectrophotometer. An absorbance ratio A260/280 of ~1.8 is generally accepted as "pure DNA" (Thermo Fisher Scientific 2011).

Extracted DNA was stored at −20 °C ready for real-time PCR assays.

Real-Time PCR

Real-time PCR was performed using a real-time PCR thermal cycler Mx3005P™ (Strategene, La Jolla, USA). Samples and PCR mix were placed in the thermal cycler using MicroAmp Optical 96-well reaction plates (Applied Biosystems) covered with MicroAmp Optical caps (Applied Biosystems).

The 25-μl real-time PCR mixture contained 1 U of *Tth* DNA polymerase (Roche A/S), 1×PCR buffer for *Tth* DNA polymerase (Roche A/S, Hvidovre, Denmark), 0.6 mM deoxynucleoside triphosphate mixture (Amersham Pharmacia Biotech, Buckinghamshire, UK), 2.5 mM MgCl2 (Applied Biosystems), 0.8 ml/l of glycerol (87 %; Merck, Darmstadt, Germany), 0.5 μM forward primer OT-1559-5′-CTG CTT AAC ACA AGT TGA GTA GG-3′, 0.5 μM reverse primer 18-1-5′-TTC CTT AGG TAC CGT CAG AA-3′ (DNA Technology, Århus, Denmark; *C. jejuni* 16S rRNA; GenBank accession no. Y19244), 0.2 g/l bovine serum albumin (Roche A/S), 75 nM target locked nucleic acid (LNA) *Campylobacter* probe 5′ [6FAM] CA[+T] CC[+T] CCA CGC GGC G[+T]T GC[BHQ1] 3′ (Sigma-Aldrich), 60 nM internal amplification control (IAC) probe (5′-VIC-TTC ATG AGG ACA CCT GAG TTG A-TAMRA 3′; Applied Biosystems), 5×10^3 copies of IAC (124 bp), and 10 μl of extracted template DNA.

The primers used (the forward primer OT-1559 and the reverse primer 18–1) amplify a 287-basepair fragment of the 16S rRNA gene of thermotolerant *C. jejuni*, *Campylobacter coli*, and *Campylobacter lari* (Lübeck et al. 2003; Josefsen et al. 2004). The amplification products were detected by using the FAM (fluorescein amidite)-labeled probe. Furthermore, an internal control (amplified with the target) was visualized using a HEX (hexachloro fluorescein)-labeled probe. The thermal profile included an initial denaturation step at 95 °C for 3 min followed by 40 cycles consisting of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s and extension at 72 °C for 30 s.

The following controls were included: (a) two positive controls (DNA from *C. jejuni* with concentrations: 100 and 1,000 CFU/ml), (b) a negative control (DNA from *Escherichia coli* with a concentration of 1,000 CFU/ml, and (c) a non-template control (NTC) in duplicate. The inclusion of a non-template control (NTC) allowed for assessment of master mix contamination.

Data Analysis Methodologies

Total DNA yields and quality values obtained from DNA extracted using every protocol were recorded in Excel. Real-time PCR data obtained from the six DNA extraction methods were analyzed all together in a common project using the MxPro-Mx3005P software (version 3.00, Stratagene, La Jolla, CA, USA). The amplification threshold was set using the software option "background-based threshold" which determines the standard deviation of all amplifications (from cycles 5 to 9) and multiplies them by a factor of 10 (default factor). A common baseline was set for all amplification plots obtained using an adaptive baseline and a non-adaptive baseline for comparison of the six DNA extraction methods (Stratagene 2004). A common amplification threshold was generated, and the threshold cycle values (Ct values) obtained were used to compare the different DNA extraction methodologies. In general, the lower the Ct values, the higher the DNA quantity obtained (Stratagene 2004; Armbruster and Pry 2008; Abdelwhab et al. 2010). Real-time PCR reproducibility can be assessed by calculating the standard deviation (SD) between replicates, ideally the SD between replicates should be less than 0.5 (Eurogentec 2012). The numbers of CFU (logs) obtained were plotted against the Ct values obtained in the real-time PCR runs, and standard curves were produced by linear regression for all DNA extraction methods tested. Standard curves were used to determine the overall performance of real-time PCR in terms of amplification efficiency, detection range (range of *C. jejuni* levels detected), limit of detection, limit of quantification and precision for every DNA extraction method. The slope of the standard curve indicates the amplification efficiency of the real-time PCR assay. The amplification efficiency (AE) was calculated based on this equation: $AE=10^{(-1/slope)}-1$ (Klein et al. 1999). The amplification efficiency is between 90 % and 110 % when the slope varies between −3.1 and −3.6. An amplification efficiency of 100 % indicates perfect reactions where the amplicon doubles each cycle (Stratagene 2004). An assessment of precision, linearity or data fit to the standard curve is produced using the

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parameter "R squared (R2)" which should be close to 1, in fact values ≥0.985 indicate good correlation (Stratagene 2004).

Results

Results Related to DNA Yield and Purity

Results related to DNA yield obtained for each of the six DNA extraction methods are presented in Fig. 1. The Easy-DNA™ Invitrogen method produced the highest DNA yield (ranging from 188 to 317 ng/μl) followed by KingFisher® (ranging from 54 to 177 ng/μl).

Results related to DNA purity (absorbance ratio A260/280 values) were obtained for every method as follows: Easy-DNA kit (Invitrogen), 1.5–1.6; KingFisher (Promega), 1.1–1.8; miniMAG (bioMérieux), 1.0–1.4; SureFood (Congen), 0.9–4.9; NucleoSpin (Macherey-Nagel), 1.6–2.2; and QIAamp (Qiagen), 1.3–3.3. Purity ratios around 1.8 or higher are desirable (Thermo Fisher Scientific 2011).

Results Related to Real-Time PCR

A common real-time PCR project was produced in order to analyze data obtained from all DNA extraction methods based on the same baseline and identical amplification threshold. Identical results (FAM Ct values) were obtained by selecting an adaptive baseline and a non-adaptive baseline (from cycles 3 to 12) with a common threshold (740) which was used for comparison of the six DNA extraction methods. Table 1 presents results related to the average FAM Ct values obtained from the real-time PCR experiments with *C. jejuni* DNA extracted using the different extraction methods. All the controls included in real-time PCR assays produced the expected results (data not shown).

FAM Ct values were obtained from the samples spiked with the lowest concentration (10 CFU/ml) only when using the NucleoSpin® Tissue DNA extraction method. No FAM Ct values were generated from the samples spiked with 10^2 CFU/ml when using the following methods: MagneSil® KingFisher, Easy-DNA Invitrogen and SureFood. Regarding the FAM Ct values generated from amplification signals produced by samples spiked with 10^2 CFU/ml, the NucleoSpin method produced the lowest FAM Ct value followed by miniMAG. Results related to samples spiked with 10^3 CFU/ml indicated that Easy-DNA Invitrogen produced the lowest FAM Ct values followed by miniMAG and MagneSil® KingFisher. Real-time PCR results obtained from samples spiked with 10^4 CFU/ml indicated that Easy-DNA Invitrogen generated the lowest FAM Ct values. SureFood produced the lowest FAM Ct value when using samples spiked with 10^5 CFU/ml followed by Easy-DNA Invitrogen.

The internal amplification control was visualized using a HEX signal which was detected from all samples except for the *Campylobacter* concentration levels 10^4 and 10^5 CFU/ml when using the MagneSil® KingFisher DNA extraction method.

Overall, the Easy-DNA Invitrogen method generated the lowest FAM Ct values followed by the miniMAG method. SureFood generated a very low Ct value for the *Campylobacter* concentration level 10^5 CFU/ml; however, the variation between replicates was high as illustrated by a standard deviation of 2.5 cycles. Standard deviations higher than 1 cycle were obtained when using the method QIAamp Qiagen with samples with a *Campylobacter* concentration level 10^2 CFU/ml. The MagneSil® KingFisher method performed poorly in this study. DNA extraction methods

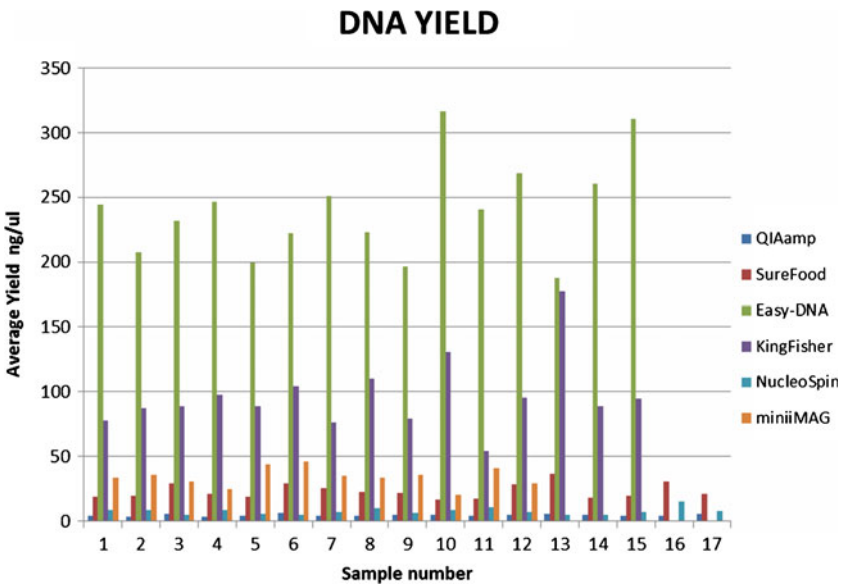

Fig. 1 DNA extracted from three different biological replicates using six different DNA extraction methods. Samples numbers *1–3* represent a spiking level of 10 CFU/ml, numbers *4–6* represent 10^2 CFU/ml, numbers *7–9* represent 10^3 CFU/ml, numbers *10–12* represent 10^4 CFU/ml, numbers *13–15* represent 10^5 CFU/ml, and numbers *16* and *17* are negative controls

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Table 1 FAM average Ct values (and standard deviations SD) obtained from real-time PCR results when using different DNA extraction methods

DNA extraction method	Campylobacter concentrations (CFU/ml)				
	10	10^2	10^3	10^4	10^5
Easy-DNA Invitrogen	NA	NA	28.0±0.4	24.3±0.7	21.1±0.6
MagneSil® King Fisher	NA	NA	30.6[a]	25.3[a]	26.3±1.0
NucliSENS® MiniMAG®	NA	35.7±0.3	29.2±0.8	26.0±0.5	23.4±0.6
SureFood® PREP	NA	NA	35.2±1.7	26.8±2.6	18.6±2.5
NucleoSpin® Tissue	37.8±0.8	34.5±0.4	33.1±0.6	29.2±0.8	27.4±0.5
QIAamp Qiagen	NA	36.3±1.2	33.1±0.6	29.7±0.6	25.5±0.5

NA no Ct values obtained

[a] Only one Ct value obtained

Table 2 Results related to real-time PCR performance indicators derived from standard curves generated for all six DNA extraction methods tested

DNA extraction method	Amplification efficiency (AE)	Slope	*R* squared	Detection range
Easy-DNA Invitrogen	93.2 %	−3.496	0.996	10^3–10^5
MagneSil® KingFisher	56.2 %	−5.160	0.774	10^3–10^5
NucliSENS® MiniMAG	79.8 %	−3.924	0.953	10^2–10^5
SureFood® PREP	31 %	−8.512	0.998	10^3–10^5
NucleoSpin® Tissue	139.5 %	−2.636	0.985	10–10^5
QIAamp Qiagen	91.5 %	−3.545	0.996	10^2–10^5

Easy-DNA Invitrogen, miniMAG and NucleoSpin offered general good real-time PCR reproducibility generating standard deviations from 0.3 to 0.8 between replicates.

Standard Curves, Amplification Efficiency, and Linearity

Standard curves were generated and used to evaluate the amplification efficiency, detection range, and precision of the DNA extraction methods tested. Results related to real-time PCR performance indicators obtained to evaluate the different DNA extraction methods are presented in Table 2. The amplification efficiency of the real-time PCR assay was calculated based on the slope of the standard curve. Amplification efficiencies between 90 % and 110 % were considered acceptable (Stratagene 2004).

The methods Easy-DNA Invitrogen and QIAamp Qiagen generated the best amplification efficiencies (93.2 % and 91.5 % respectively). These two methods also produced *R* squared (R^2) values close to 1 indicating good precision. The method NucleoSpin® Tissue was able to detect samples spiked with the lowest *Campylobacter* concentration level (10 CFU/ml), however, this extraction method generated higher Ct values for most concentration levels than the other methods and the amplification efficiency obtained was significantly above 100 % (139.5 %) possibly caused by inhibitors and/or experimental error (Stratagene 2004).

Conclusions and Discussion

The strain *C. jejuni* CCUG 11284 was used in this study to spike different chicken fecal samples in order to compare quantification results obtained when using six DNA extraction methods and real-time PCR. Overall, the Easy-DNA Invitrogen method seemed to offer the best amplification efficiency, low FAM Ct values and good precision and reproducibility when extracting DNA from chicken fecal samples spiked with *C. jejuni* CCUG 11284 for quantification using real-time PCR. Direct quantification of *Campylobacter* present in fecal samples has proven to be difficult (Leblanc-Maridor et al. 2011) and poultry faeces, in particular, represent complex samples for the accurate quantification of *Campylobacter* (Rudi et al. 2004).

Conventional microbiological methods for the detection, identification and quantification of *Campylobacter* can be time consuming, usually rely on initial enrichment procedures that might introduce bias (in relation to the strains identified and the numbers of pathogens) in the results (Velusamy et al. 2010) and will not detect viable but non-culturable (VBNC) *Campylobacter* cells (Postollec et al. 2011). On the other hand, real-time PCR might produce false negative results when no enrichment is used and the samples contain low numbers of bacteria. Real-time PCR quantifies DNA present in the samples; amplified DNA could be derived from live cells, viable but non-culturable microbial forms and dead cells (Botteldoorn et al. 2008). Amplified DNA from dead cells may lead to an overestimation of the numbers of the target organism or even false-positive results (Wolffs et al. 2005). Therefore, it will be expected that quantification results from real-time PCR will be higher than those obtained by traditional culture. In order to quantify bacterial DNA from viable cells only when using real-time PCR, the use of ethidium monoazide (EMA) and propidium monoazide (PMA) has been recommended (Rudi et al. 2005; Josefsen et al. 2010). However, these methods need to be evaluated further because the use of EMA resulted in an underestimation of viable cells of *C. jejuni* and *Stahylococcus spp*. in some studies (Flekna et al. 2007; Kobayashi et al.

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2009). Inhibitory substances present in biological matrices may reduce the efficiency of real-time PCR assays significantly (Perch-Nielsen et al. 2003; Guy et al. 2003; Rådström et al. 2004; Sunen et al. 2004; Jiang et al. 2005; Stratagene 2004).

The selection of DNA polymerase might have an important effect on overcoming PCR inhibitors (Katcher and Schwartz 1994; Abu Al-Soud and Rådström 1998; Wolffs et al. 2004; Bessetti 2007). The *Tth* DNA polymerase was used in this study. The *Tth* polymerase can significantly improve PCR amplification efficiency in comparison with the Taq DNA polymerase when processing feces or samples containing fecal material (Shames et al. 1995; Abu Al-Soud and Rådström 1998; Dahlenborg et al. 2001). Furthermore, the *Tth* polymerase has been shown to maintain DNA polymerase activity when 5 % of phenol is present in the sample (Katcher and Schwartz 1994).

The use of PCR facilitators has been recommended (Hedman and Rådström 2013). The addition of bovine serum albumin (BSA) may help to overcome PCR inhibition in fecal samples and other types of samples such as blood and meat samples (Abu Al-Soud and Rådström 2000). The use of nonionic detergents such as detergents Tween 20 and Triton X-100 and polymers such as PEG and dextran has been shown to facilitate PCR amplification and reduce PCR inhibition in fecal samples (Abu Al-Soud and Rådström 2000). The addition of phytase has been proposed to relieve inhibition caused by the presence of phytic acid in feces (Thornton and Passen 2004). In this study, BSA was added to the PCR mix to facilitate PCR amplification. Furthermore, the *Tth* buffer contains bovine serum albumin (BSA) and the detergent Tween 20 which facilitate PCR amplification (Hedman and Rådström 2013). The effect of PCR facilitators depends on their concentration. In fact, using high concentrations of facilitators (such as BSA, Tween 20, Triton X-100, formamide, and glycerol) might inhibit PCR amplification (Rossen et al. 1992; Ahokas and Erkkila 1993). Synergistic effects between facilitators are not clear and in fact, some combinations of facilitators may cause PCR inhibition (Ahokas and Erkkila 1993; Abu Al-Soud and Rådström 2000).

A thorough investigation into the different DNA extraction methods tested and a comparison of the results from diverse research studies when extracting *Campylobacter* DNA from different sample matrices are desirable but fall beyond the scope of this manuscript. Efficient, fast, and reliable non-enrichment methods should be assessed for their efficiency to separate pathogen cells from the sample matrices and concentrate the cells for quantification. An ideal method should be able to remove matrix-associated inhibitors without harming the bacterial cells in order to concentrate pathogens. Some commercial methods are specifically designed for their use with fecal samples, e.g., QIAamp® DNA stool purification kit (Holland et al. 2000; Gioffré et al. 2004). Diverse combinations of biochemical, physical, immunological, and commercially available DNA extraction methods may be used for the removal of PCR inhibitors and the concentration of target DNA in digesta and fecal samples (Yu and Morrison 2004; Zoetendal et al. 2001). DNA extraction methods can remove a significant amount of PCR inhibitors but they can be expensive and laborious (Rådström et al. 2003). Advances in the development and improvement of DNA extraction methods can translate to fast, easier-to-use and cheaper methods. In this study, the methods Easy-DNA and MiniMAG were relatively fast, it was possible to process approximately 30 samples in less than 6 h. In terms of cost per DNA extraction, the cheapest methods were Easy-DNA and KingFisher (17 DKK/DNA extraction), while the most expensive was MiniMAG (125 DKK/DNA extraction).

Ideal DNA extraction methods should ensure high DNA yield and quality and minimize interference with PCR reactions (Cankar et al. 2006). DNA extraction methodologies will extract DNA from diverse microorganisms present in the samples. The method Easy-DNA Invitrogen produced the highest DNA yield in this study; however, extracted DNA could be originating from other microorganisms (apart from *C. jejuni*) present in the chicken fecal samples. DNA purity ratios around 1.8 or higher are desirable, however, it has been suggested that "the best indicator of DNA quality is functionality in the application of interest" (Thermo Fisher Scientific 2011).

The results from this study indicated that the method Easy-DNA Invitrogen produced the most optimal real-time PCR performance indicators when used with chicken fecal samples. However, the limit of detection obtained when using Easy-DNA Invitrogen was relatively high (10^3 CFU/ml). In a study conducted by Lund et al. (2004), a detection limit of 250 *Campylobacter* CFU/g of feces was obtained using the KingFisher method. However, in the study presented here, the KingFisher method did not seem to work very well with the spiked chicken fecal samples. Leblanc-Maridor et al. (2011) extracted DNA from *C. jejuni* and *C. coli* present in swine feces using the DNA extraction method NucleoSpin® Tissue and obtained a quantification limit of 250 CFU/g of feces although the method was able to detect 10 genome copies. In this study, the method NucleoSpin® Tissue was also able to detect samples spiked with the lowest *Campylobacter* concentration level (10 CFU/ml) but the amplification efficiency was not optimal. A negative correlation between the detection limit and amplification efficiency has been previously reported (Rudi et al. 2004).

The limit of detection and the limit of quantification might differ because the limit of detection may be found at a concentration below the linear part of the standard curve (Armbruster and Pry 2008; Leblanc-Maridor et al. 2011). In fact, it is generally accepted that real-time PCR may provide accurate quantification estimates when using

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samples with numbers of target organism exceeding 10^2–10^3 CFU/g or ml but not with lower concentrations due to the loss of target DNA during sample preparation and to the small volumes analyzed (Malorny et al. 2008; Josefsen et al. 2010; Löfström et al. 2011). Furthermore, the use of increased concentrations of target DNA might help to overcome the effect of PCR inhibitors (Rådström et al. 2003; Lund et al. 2004; Roussel et al. 2005).

Quantification results depend on the sample matrix, sample preparation, DNA extraction method, real-time PCR reagents, real-time PCR experiments, and real-time PCR data analysis. Research results might also be different when using fresh samples for DNA extraction than when extracting DNA from frozen samples. Chicken feces present a semi-dry viscous consistency that might cause problems during sample processing and DNA extraction (Silkie and Nelson 2009). It has been recommended that fecal samples are processed very soon after collection or alternatively, samples should be placed in the freezer (Nechvatal et al. 2008). Sample storage conditions may affect detection and quantification of bacterial pathogens in fecal samples (Tang et al. 2008; Barnard et al. 2011). DNA is repaired efficiently in living cells, but DNA will degrade during the death of the organisms (Stivers and Kuchta 2006). Damaged DNA will hinder DNA amplification representing an important issue in many research areas. The degree of DNA damage depends on the environment to which DNA was exposed and on the DNA source (Lindahl 1993; Lehmann and Kreipe 2001; Wandeler et al. 2003; Paabo et al. 2004).

In this study, samples (spiked samples and negative controls) were immediately placed in the freezer (stored at −20 °C) after preparation as recommended (Qiagen 2013) and used for DNA extractions within 4 months. However, it has been shown that freezing may affect DNA stability and produce false-negative results when using PCR for pathogen detection (Jensen et al. 2004; Brinkman et al. 2004). Further research should be conducted to assess the degree of *Campylobacter* DNA damage associated with freezing and/or other storage conditions.

When analyzing real-time PCR data, the baseline should be set accurately in order to obtain reliable Ct values. In this study, a common baseline range was obtained by using an adaptive baseline and a non-adaptive baseline (from cycles 3 to 12) for comparison of the six DNA extraction methods. Amplification thresholds can be obtained in different ways when analyzing real-time PCR data. The threshold cycle values (Ct values) and therefore quantification results will depend partly on the amplification threshold selected in every case. In this study, a common amplification threshold (740) was obtained by selecting the software option "background-based threshold" when data analyses were performed using the MxPro-Mx3005P software (version 3.00). Standard curves were generated and used for comparison of all six DNA extraction methods tested. Standard curves may be used for quantification of unknown samples and therefore should be carefully designed (Whelan et al. 2003; Leong et al. 2007; Malorny et al. 2008; Dhanasekaran et al. 2010). Real-time PCR reactions producing very similar amplification efficiencies should be favoured for threshold-based quantification methodologies.

The routine use of complex mathematical methods for thorough and rigorous real-time PCR data analyses can prove challenging and lag behind the numerous practical applications of real-time PCR (Liu and Saint 2002). Many variables may affect real-time PCR efficiency. Inhibitors present in the samples, contaminants and differences in sample preparation protocols may also explain variability and different PCR efficiency estimations (Ståhlberg et al. 2003; Tichopad et al. 2004; Tichopad et al. 2010). Real-time PCR data can be used to determine the presence of inhibitors in samples (Lund et al. 2004; Kontanis and Reed 2006).

Real-time PCR efficiency also depends on the primers used and therefore careful design of primers is necessary to obtain highly efficient PCR reactions (Tichopad et al. 2004). A PCR efficiency of 100 % indicates a perfect reaction with doubling of amplicon during each cycle. An amplification efficiency of 100 % seems difficult to achieve when using complex biological matrices due to the presence of inhibitors (Rådström et al. 2004). Low amplification efficiencies indicate that the reaction is slowed down somehow due to the presence of inhibitors or suboptimal PCR reagents and/or conditions. Amplification efficiencies significantly higher than 100 % may indicate experimental error (Stratagene 2004).

Real-time PCR detects and quantifies DNA from viable and non-viable cells which could lead to an overestimation of the number of viable pathogens present in the initial sample. Real-time PCR also allows for the detection and quantification of viable but non-culturable microbial forms that might be of high relevance in some cases (Postollec et al. 2011). Real-time PCR is a rapid and sensitive method for the identification and quantification of pathogens that can be however limited by inhibitory substances. These inhibitory substances present in complex biological samples may reduce or even completely impede the amplification process (Lantz et al. 2000; Nolan et al. 2006). Therefore, overestimation of the numbers of viable pathogens in samples and underestimation of numbers of viable pathogens due to inhibition of the PCR reactions might produce results that diverge from the true numbers of pathogens, in this case, *C. jejuni* present in chicken fecal samples. Furthermore, the distribution of pathogens in samples might not be homogeneous. In fact, diverse non-uniform distributions of pathogens can be expected in biological samples and this should be considered when processing samples in the laboratory (Andrews and Hammack 2003; Van Schothorst et al. 2009). Samples should be thoroughly mixed when processed in the laboratory and biological replicates may be analyzed to assess biological variability in samples.

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A thorough investigation of the inhibitors present on particular sample matrices is desirable in order to design the best sample treatment and select the most appropriate DNA extraction methodology. Rigorous real-time PCR data analyses and accurate estimations of efficiencies of each real-time PCR reaction will be ideal. However, this approach might be demanding in terms of time and other resources. Furthermore, experimental variability can be very high even when the best methodologies are used and experiments are performed under very controlled conditions. Variability between different PCR plates or runs can be high; even when considering only one specific PCR plate, intra-plate variability can be significant (Karlen et al. 2007). In fact, biological variability between samples and replicates can also be high and partly explain different real-time PCR efficiencies. In future studies, novel and thorough analytical and/or statistical methods could be applied to accurately quantify viable pathogens such as *Campylobacter* present in biological samples.

Accurate and reliable enumeration of viable pathogens present in foods and/or environmental samples will assist exposure assessment and risk assessment models and the evaluation of the effectiveness of food safety measures and public health protection programs.

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Compliance with Ethics Requirements All institutional and national guidelines for the care and use of laboratory animals were followed.

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Manuscript IV

Towards the production of reliable quantitative microbiological data for risk assessment: direct quantification of *Campylobacter* in naturally infected chicken faecal samples using real-time PCR

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ABSTRACT

Poultry has been identified as a significant source for human campylobacteriosis which constitutes an important public health problem in many areas of the world. Rapid, direct and accurate quantification of *Campylobacter* in poultry is essential for the assessment of risks and control strategies associated with poultry production. The aim of this study was to compare estimates of the numbers of *Campylobacter spp*. in naturally infected chicken faecal samples obtained using direct quantification by selective culture and by real-time PCR. Absolute quantification of *Campylobacter* by real-time PCR was performed using standard curves designed for two different DNA extraction methods: Easy-DNA™ Kit from Invitrogen (Easy-DNA) and NucliSENS® MiniMAG® from bioMérieux (MiniMAG). Results indicated that the estimation of the numbers of *Campylobacter* present in eight chicken faecal samples was partly dependent on the methodologies used. In general, the numbers of *Campylobacter* obtained by real-time PCR when extracting DNA using the MiniMAG method were in most cases higher than the numbers of *Campylobacter* obtained by selective culture and by real-time PCR when using the Easy-DNA method. Although there were differences between the methods, the results indicated that there were no statistically significant differences between the estimates obtained by culture and by real-time PCR.

INTRODUCTION

Direct, fast and accurate detection and quantification of pathogens such as *Campylobacter* that might be present in suspected sources of disease (water, food and/or environmental sources) is essential for the investigation of the burden of disease, for disease control and for the protection of public health (Coleman and Marks, 1999; World Health Organization, 2007). The bacteria *Campylobacter* has been recognized as the main etiological agent causing human bacterial gastrointestinal disease (Lin, 2009; Hermans *et al.*, 2012). Poultry (including poultry products and by-products) has been identified as one of the most significant risk factors for human campylobacteriosis (Wingstrand *et al.*, 2006; Wilson *et al.*, 2008; Hermans *et al.*, 2012). Accurate methods for the enumeration of *Campylobacter* in poultry are essential for the assessment of public health risks and the evaluation of control strategies that might be implemented during poultry production such as vaccination (Garcia *et al.,* 2012). Risk assessment models indicate that the control of *Campylobacter* in poultry may reduce the human burden of disease. In actual fact, a reduction of 2 logs in chickens could translate in a significant decrease on the number of human cases (Rosenquist *et al*., 2003). Efficient and rapid direct methods for accurate identification and quantification of *Campylobacter* need to be improved or developed. The use of enrichment eliminates the possibility for accurate quantification and may select against specific *Campylobacter spp.* (Madden *et al*., 2000; Nachamkin and Blaser, 2000; Sails *et al*., 2003). Thus, it seems difficult to accurately identify and/or quantify pathogens when using enrichment steps (Postollec *et al.,* 2011).Traditional microbiological methods for the detection and quantification of *Campylobacter* based on selective culture can be time consuming and will not detect viable but non-culturable (VBNC) *Campylobacter* cells that might be infectious (Rollins and Colwell, 1986; Josefsen *et al*., 2010). The use of newer and faster technologies such as real-time PCR seems promising for the accurate detection and quantification of microorganisms (Stevens and Jaykus, 2004; Masco *et al*., 2007; Malorny *et al.,* 2008). Nevertheless, false negative results might be obtained when low numbers of bacteria are present in samples; in this case, direct quantification of *Campylobacter* (with no enrichment steps) by real-time PCR might prove difficult. On the

other hand, an overestimation of the numbers of *Campylobacter* present in samples might be produced by real-time PCR because dead *Campylobacter* cells will be detected. When pure target DNA is analysed real-time PCR can be very sensitive. On the other hand, the limit of detection, limit of quantification and efficiency of real-time PCR assays can be significantly reduced by the presence of inhibitors in biological samples (Perch-Nielsen *et al*., 2003; Rådström *et al.*, 2004; Sunen *et al*., 2004; Jiang *et al*., 2005). DNA extraction methods need to be carefully selected to remove inhibitors and to minimize interference with PCR in order to improve the efficiency of real-time PCR and to obtain accurate and reliable quantification. PCR controls and adequate treatment of samples prior to real-time PCR should be included in the assays (Rådström *et al*., 2004; Cankar *et al*., 2006; Murphy *et al*., 2007). Poultry faecal samples represent complex matrices and therefore, sample preparation for the removal of inhibitors and for *Campylobacter* DNA concentration and purification is crucial to obtain accurate quantification results (Perch-Nielsen *et al.*, 2003; Rådström *et al.*, 2004; Inglis *et al.*, 2010).

In this manuscript, estimates of the numbers of *Campylobacter* identified in naturally infected chicken faecal samples obtained by selective culture and by real-time PCR (using two different DNA extraction methods: Easy-DNA™ Kit from Invitrogen and NucliSENS® miniMAG® from bioMérieux) are presented and compared. Diverse aspects related to the direct quantification of *Campylobacter* in chicken faecal samples are discussed in this manuscript such as sample matrix characteristics, distribution of pathogens in samples, microbiological methods, real-time PCR performance and statistical agreement between methods.

RESULTS

Quantification by culture

The results obtained from selective culture of the faecal samples naturally infected with *Campylobacter* are presented in Table 1. Results indicated that samples 1 and 4 contained higher numbers of *Campylobacter* (in the order of 10^7 CFU/g) while samples 2 and 3 had lower numbers

(in the order of 10^5 and 10^4 CFU/g respectively). Variation in the appearance of *Campylobacter* colonies indicated the potential presence of diverse *Campylobacter* species. The numbers of *Campylobacter* obtained by culture from samples B were consistently lower than those obtained from samples A (processed without filter during the sample homogenization step).

Standard curves generated for absolute quantification

The standard curves designed from real-time PCR data from spiked chicken faecal samples for every DNA extraction method showed the methods to be linear in the range of 10^3 to 10^5 CFU/ml (Figure 2). Identical results were obtained by selecting an adaptive baseline and a non-adaptive baseline (from cycles 3 to 12) with a common threshold (740). The limit of detection when extracting DNA using MiniMAG was 10^2 CFU/ml; however, the standard curve did not seem to be linear below the 10^3 CFU/ml level and the limit of quantification was considered to be 10^3 CFU/ml. The amplification efficiencies obtained for the Easy-DNA and MiniMAG methods were 96.6% and 116.9% respectively. Data fit to the standard curves were assessed using R^2values which were 0.961 and 0.945 for the Easy-DNA and MiniMAG methods, respectively.

Comparison of enumeration by culture and real-time-PCR

Generated standard curves were used for the real-time PCR quantification of *Campylobacter spp.* present in naturally infected chicken faecal samples. Quantification of samples with *Campylobacter* numbers higher than 10^5CFU/g was performed based on extrapolation of the standard curves.

Obtained real-time PCR data were transformed for every dilution level and mean values were calculated as estimates of the numbers of *Campylobacter* present in every biological sample (Table 1). Real-time PCR data were obtained from all dilution levels when using the DNA extraction method MiniMAG, however, no real-time PCR results were obtained for the dilutions 10^{-4} and $10^{-5}$of samples 2, 3 and 4when extracting DNA with the Easy-DNA method. The mean estimates for the numbers of *Campylobacter* in every sample obtained by culture and by real-time

PCR using the two different DNA extraction methods are presented in Table 1 and in Figure 1. Standard deviations were calculated and found to be lower when using MiniMAG (results not shown).

Table 1*Comparison of results: mean values (CFU/g and in logs) obtained by culture and by real-time PCR using two DNA extraction methods (Easy-DNA and MiniMAG).*

SAMPLE ID	Culture CFU/g (logs)	Real-time PCR (Easy-DNA) * CFU/g (logs)	Real-time PCR (MiniMAG) * CFU/g (logs)
1A	2.7×10^7 (7.43)	8.5×10^6 (6.93)	7.9×10^7 (7.90)
1B	1.1×10^7 (7.04)	5.6×10^6 (6.75)	2.5×10^7 (7.40)
2A	1.8×10^5 (5.26)	2.1×10^5 (5.32)	5.4×10^5 (5.73)
2B	1.4×10^5 (5.15)	1.5×10^5 (5.18)	2.2×10^5 (5.34)
3A	5.4×10^4 (4.73)	1×10^4 (4.00)	1.2×10^4 (4.08)
3B	2.5×10^4 (4.40)	8.2×10^3 (3.91)	9.1×10^3 (3.96)
4A	1.6×10^7 (7.20)	2.1×10^7 (7.30)	4.5×10^7 (7.66)
4B	1.4×10^7 (7.15)	6.8×10^6 (6.80)	1.6×10^7 (7.21)

*A common real-time PCR threshold of 740 was applied

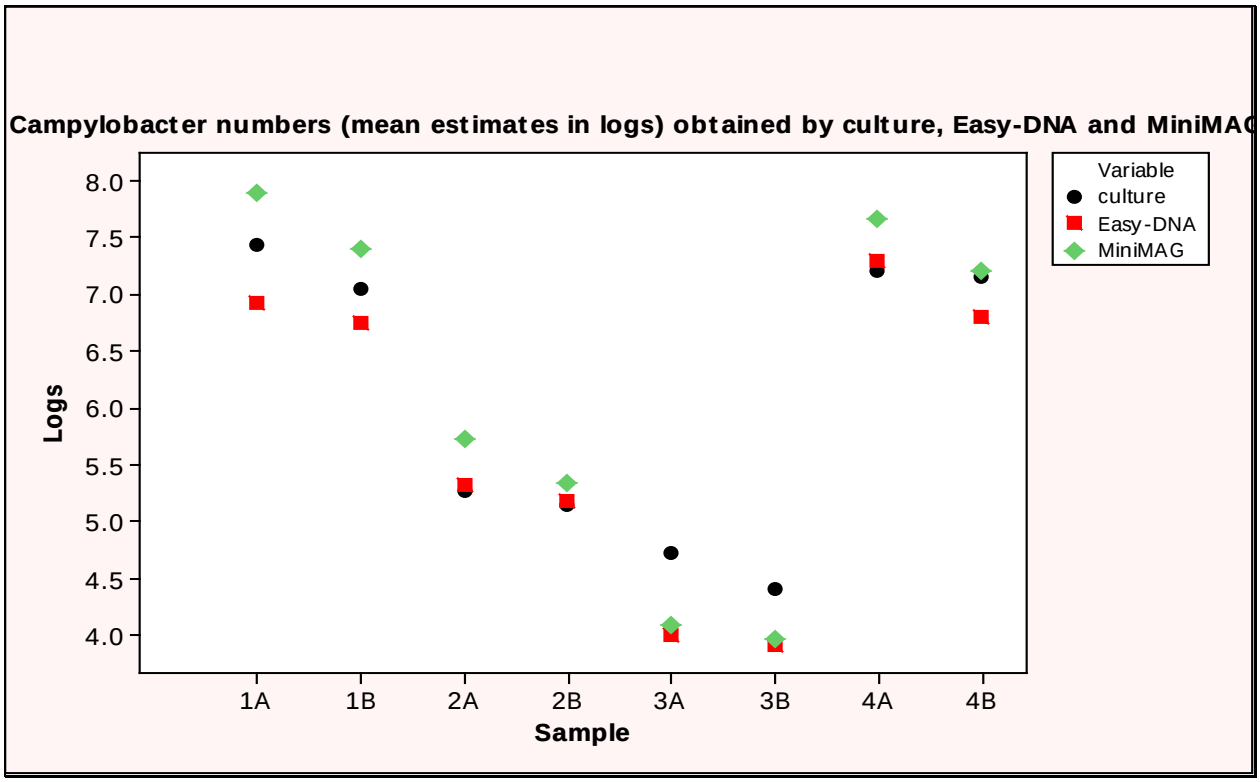

Figure 1 *Mean estimates (in logs) of the numbers of Campylobacter present in the naturally infected chicken faecal samples obtained by culture and by real-time PCR using two DNA extraction methods: Easy-DNA and MiniMAG*

The numbers of *Campylobacter* obtained by real-time PCR when using MiniMAG for DNA extraction were in most cases higher than the numbers obtained by culture (for all samples except for Sample 3). Sample number 3 had the lowest *Campylobacter* concentration (below 5 logs) and the enumeration results obtained by culture were higher than those obtained using real-time PCR for this sample. In general, the numbers of *Campylobacter* obtained by real-time PCR when extracting DNA with the Easy-DNA method were lower or very similar to the numbers obtained by culture (Table 1 and Figure 1). Agreement between methods was investigated and the correlation coefficients obtained were 0.98 between culture and real-time PCR (both DNA extraction methods) and 0.99 between Easy-DNA and MiniMAG extraction methods.

The statistical significance of the differences between estimates of the numbers of *Campylobacter* obtained by culture and by real-time PCR using the two different DNA extraction methods was

examined. A result with a p-value ≤ 0.05 was considered a statistically significant result. The only difference found to be statistically significant was the one related to the estimates of the numbers of *Campylobacter* obtained for sample 1A by real-time PCR when using the two different DNA extraction methods (p-value = 0.02).

Although there were differences between the methods the results indicate that there were no statistically significant differences between culture and real-time PCR. The differences between the estimates obtained when using MiniMAG and Easy-DNA methods were statistically significant only when quantifying *Campylobacter* present in sample 1A. In general, there was good agreement between the estimates obtained by culture and real-time PCR when extracting DNA with the two methods Easy-DNA and MiniMAG.

DISCUSSION

The species of *Campylobacter* and the initial numbers of *Campylobacter* present in the naturally infected chicken faecal samples in this study were unknown. It seems difficult to be completely certain about the numbers of a given bacteria in a particular sample even when the methodologies used are very sensitive. The distribution of bacteria in samples might not be homogeneous (Food Standards Agency, 2000; Griffith *et al*., 2003) even though chicken faecal samples were homogenised using a stomacher in this study. Furthermore, microbiological methods might select against certain species of *Campylobacter* that may be present in the samples. In fact, CCDA agar is selective for *Campylobacter spp.* but some strains may fail to grow or grow poorly (Neogen, 2010). In general, CCDA agar plates (from Oxoid, Hampshire, UK) are selecting for *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari.* Chickens usually carry *C. jejuni, C. coli* and occasionally *C. lari* (Newell and Fearnley, 2003). Interestingly, *C. jejuni* and *C. coli* are the main species responsible for most human campylobacteriosis cases (Allos, 2001; Gillespie *et al*., 2002; Tam *et al*., 2003). Furthermore, mixed infections with diverse *Campylobacter spp.* are not uncommon in poultry (Jacobs-Reitsma *et al*., 1995; De Boer *et al*., 2002). When using traditional microbiological methods for quantification it is important to bear

on mind that culture selects for culturable bacterial cells only, other viable but non-culturable bacterial states could be missed in the quantification. Furthermore, when using selective culture for *Campylobacter* it is generally assumed that colonies growing on plates are *Campylobacter* colonies. However, sometimes distinguishing *Campylobacter* colonies from other contaminants growing on plates may prove difficult (Line, 2001; Stern *et al*., 2001).

The quantification results obtained in this study suggested that the use of filters during sample processing could translate on lower estimates of the numbers of *Campylobacter* in the samples independently of the quantification method applied (culture or real-time PCR). This finding is in agreement with other studies where the use of specific types of filters partly or completely inhibited PCR amplification due to target *Campylobacter* DNA binding to the filter membranes (Oyofo and Rollins,1993).

In this study, the naturally infected chicken faecal samples were processed within 30 hours from the time of collection and it is possible that *Campylobacter* cells with intact membranes survived well during that time which in turn could explain the statistical agreement between quantification results obtained by culture and by real-time PCR. *Campylobacter jejuni* can survive up to six days in poultry faeces (Ahmed *et al*., 2013). *Campylobacter* DNA (in quantities equivalent to more than10^4 CFU/g of *C. jejuni*) has also been detected and proved to survive in bovine manure compost for more than nine months (Inglis *et al.,* 2010). These authors also concluded that most detected *Campylobacter* DNA originated from viable *Campylobacter* cells with intact membranes which were able to survive in compost (an environment considered inhospitable) for a long time.

The use of PCR for the real-time quantification of bacteria such as *Campylobacter* is considered promising (Josefsen *et al.,* 2010). Real-time PCR quantifies *Campylobacter* DNA present in the samples which could originate from viable but non-culturable states, live and dead cells (Yang *et al.,* 2003; Hong *et al.,* 2007; Botteldoorn *et al.,* 2008). Amplified DNA from dead cells may lead to false-positive results or to an overestimation of the numbers of the target organism (Wolffs *et al*., 2005). Thus, it will be expected that quantification results obtained using real-time PCR will

be higher than those generated by traditional culture. The use of ethidiummonoazide (EMA) and propidiummonoazide (PMA) has been recommended in order to quantify bacterial DNA derived only from viable cells when using real-time PCR (Rudi *et al.*, 2005; Delgado-Viscogliosi *et al*., 2009; Josefsen *et al.*, 2010). Nonetheless, these methods need to be evaluated further because the use of EMA resulted in an underestimation of viable cells of *C. jejuni* and *Stahylococcus spp*. in some studies (Flekna *et al.*, 2007; Kobayashi *et al.*, 2009). Furthermore, these reagents might not be effective when bacteria are embedded in biofilms (Pisz *et al*., 2007). *Campylobacters* can be present in biofilms, in actual fact, biofilms formed by *C. jejuni* can be found in the gastrointestinal tract of animals (Siringan *et al*., 2011) and hence *Campylobacter* may be present in biofilms in chicken faecal samples posing a risk for contamination of the food and the food processing environment (Hall-Stoodley *et al.*, 2004). *Campylobacter* cells embedded in biofilms can survive much longer under atmospheric conditions than planktonic *Campylobacter* cells (Garcia and Percival, 2011).

The presence of PCR inhibitors in complex samples has been identified as an important hindrance for quantification by real-time PCR (Rådström *et al.*, 2004; Jiang *et al.*, 2005). Thus, the selection of an adequate sample treatment and/or DNA extraction method for the quantification of *Campylobacter* in chicken faecal samples is crucial. In a previous study (Garcia *et al*., 2013), the Easy-DNA method produced the highest DNA yield when extracting DNA from chicken faecal samples spiked with *Campylobacter*. However, extracted DNA could also originate from other microorganisms present in the faecal samples. The total extracted DNA includes the target *Campylobacter* DNA and the non-target DNA (also called the “burden” DNA) which could interfere with the real-time PCR (Ariefdjohan *et al.*, 2010).

The method Easy-DNA uses chloroform which is a hazardous reagent and therefore health and safety precautions must be taken when using this method. In this study, the method Easy-DNA produced estimates of the numbers of *Campylobacter* present in naturally infected chicken faecal samples that were lower than the numbers recovered when using the DNA extraction method

MiniMAG and similar or lower than the numbers obtained by selective culture. Furthermore, no results were obtained from higher dilutions (10^{-4} and 10^{-5}) for most samples when extracting DNA with Easy-DNA. In contrast, the results from quantification by real-time PCR when using MiniMAG indicated that more quantitative microbiological data were obtained from all dilutions and that the standard deviations were lower suggesting that the method MiniMAG performed more robustly than Easy-DNA in this study. Both methods Easy-DNA and MiniMAG were relatively fast, it was possible to process approximately 30 samples in less than 6 hours. In terms of cost per DNA extraction, Easy-DNA was much cheaper (17 DKK/DNA extraction) than MiniMAG (125 DKK/DNA extraction).

The amplification efficiencies obtained when extracting DNA with the method Easy-DNA and when using MiniMAG were 96.6%and 116.9% respectively. Efficiencies between 90% and 110% are considered acceptable meaning that the amplicon doubles at each cycle (Stratagene, 2004). Several factors will influence PCR efficiency such as the master mix performance, the selected primers, type of DNA polymerase, sample quality, DNA extraction method, presence of inhibitors in the samples and the assay itself. Sample quality or consistency might hinder DNA extraction procedures (Bélanger *et al*., 2003; Forney *et al*., 2004). The sensitivity/specificity of primers is crucial to obtain good amplification efficiencies (Inglis *et al*., 2010). The combination of primers used in this study has been found to be selective for the detection of foodborne thermotolerant *Campylobacter* in a previous study (Lübeck *et al.*, 2003). Even when using these primers (the forward primer OT-1559 and the reverse primer 18-1), the detection level for *Campylobacter* obtained in this study was 10^2 CFU/mL which could be due to DNA loss during the DNA extraction process and/or the presence of inhibitors although the internal amplification control (IAC) indicated that no important inhibition was observed in this case. The choice of DNA polymerase might have an important effect on overcoming PCR inhibitors and might influence PCR amplification efficiencies (Wolffs *et al*., 2004; Hedman *et al*., 2009). The differences in amplification efficiencies when using different DNA polymerases could be partly due to the presence of PCR facilitators in their buffer systems (Wolffs *et al.*, 2004). The *Tth* DNA

polymerase was used in this study. The *Tth* polymerase can improve PCR amplification efficiency in comparison with the *Taq* DNA polymerase when processing samples containing faecal material (Dahlenborg *et al*., 2001).

The use of PCR facilitators has been recommended such as the addition of bovine serum albumin (BSA) that may help to overcome PCR inhibition in faecal samples, blood and meat samples (Abu Al-Soud and Rådström, 2000; Hedman and Rådström, 2013).In this study, BSA was included in the PCR mix to facilitate PCR amplification. Furthermore, the *Tth* buffer contained bovine serum albumin (BSA) and the detergent Tween 20 which facilitate PCR amplification (Hedman and Rådström, 2013).

Despite differences between methods, results obtained in this study indicated good agreement between real-time PCR methods and culture. Several studies have compared quantification results obtained by culture and by real-time PCR with contradictory results. Some researchers found that the estimated numbers of target organisms were higher when using real-time PCR in comparison with traditional culture-based methods (Lahtinen *et al*., 2006; Botteldoorn *et al*., 2008; Löfström *et al*., 2010; Converse *et al.*, 2012). On the contrary, other studies reported good agreement between the methods (Martín *et al.*, 2006; Josefsen *et al.*, 2010; Bui *et al.*, 2011) while others found an underestimation of the numbers of the target organism when using real-time PCR in comparison with culture (Pennacchia *et al.*, 2009; Noble *et al*., 2010). Even when agreement between methods is observed, the stability of this agreement might be dependent on other factors such as time, season and environmental factors (Shibata *et al*., 2010; Converse *et al*., 2012). Converse *et al.* (2012) demonstrated that the relationships between methods may vary temporally and spatially.

The chicken faecal samples naturally infected with *Campylobacter* used in this study seemed to contain high numbers of *Campylobacter* (in some cases higher than 10^5 CFU/g). The standard curves were constructed based on *Campylobacter* levels up to 10^5 CFU/g. Therefore, quantification of samples with levels higher than 10^5 CFU/g was obtained based on extrapolation

of the linear part of the standard curves. In this study, the enumeration results obtained by culture were higher than those obtained using real-time PCR when processing sample number 3 which contained a lower *Campylobacter* concentration. It is likely that real-time PCR methods work better with samples containing higher *Campylobacter* numbers because part of the target DNA might get lost during DNA extraction procedures. In this study, the limit of detection when extracting DNA using MiniMAG was 10^2 CFU/mL; however, the limit of quantification was considered to be 10^3 CFU/mL in agreement with other studies (Wolffs *et al*., 2005). It will be interesting to test the methods with samples with lower numbers of *Campylobacter* (lower than 10^3CFU/g which was the limit of quantification for the methods). However, it has been recognized that real-time PCR can be used to obtain accurate quantification estimates for samples with levels of target organism exceeding 10^2-10^3CFU/g but not for lower concentrations mainly due to the small volumes analysed and the loss of target DNA during sample preparation (Malorny *et al*., 2008; Löfström *et al.,* 2010; Josefsen *et al.*, 2010).

In conclusion, there was good agreement between the quantification results obtained by selective culture and by real-time PCR when using two different DNA extraction methods in this study. The fact that chicken faecal samples were used for the preparation of spiked samples and construction of standard curves could partly explain this agreement because we already accounted for the effect of inhibitors that may be present in chicken faecal samples when building the standard curves. We could also hypothesize that most *Campylobacter* cells present in our samples were in viable and culturable state because chicken faecal samples were fresh and processed within 30 hours of collection. It is therefore possible that not a great amount of stressed *Campylobacter* cells, VBNC *Campylobacter* states or free *Campylobacter* DNA were present in these samples. Novel and accurate methods able to discriminate between the different *Campylobacter* viable and non-viable states could share light in this matter.

EXPERIMENTAL PROCEDURES

Preparation of spiked chicken faecal samples and negative controls

Three different faecal samples from broilers confirmed to be *Campylobacter* negative (by selective culture and PCR) were spiked with known concentrations of *C. jejuni* CCUG 11284 for the generation of standard curves subsequently used for the quantification of *Campylobacter* present in naturally infected chicken faecal samples. Spiking of samples and production of negative controls were performed as described previously (Garcia *et al*., 2013). Fifty replicates of each of the following spiking levels 10, 100, 1000, 10000, 100000 were produced and the remaining non-spiked faecal samples were homogenized and distributed in aliquots of 1 ml to produce negative controls for the real-time PCR experiments. Samples were centrifuged at 5000 x *g* for 5 minutes, supernatants discarded and the pellets stored at -20 ºC for DNA extraction and quantification using real-time PCR.

Preparation of naturally infected chicken faecal samples

Chicken faecal samples confirmed to be *Campylobacter* positive were collected by abattoir personnel on the 18th of September 2012 and sent the same day at room temperature to the National Food Institute at the Technical University of Denmark, where they were processed within 30 hours from collection. The received samples had been collected in four sterile pots; the pots were numbered (1 to 4) in the laboratory. As illustrated in Figure 2, two grams of faecal samples were taken from every pot: one gram was deposited in a stomacher bag without filter (samples A) and the other gram in a stomacher bag with filter number 6 corresponding to a pore size of 280 µm (samples B). Every gram of sample was diluted in 9 mL of sterile water (dilution 10^{-1}) and homogenized using a stomacher (Stomacher® 400, Seward Limited, Worthing, UK). Dilution rows ranging to 10^{-5} were produced as shown in Figure 1.

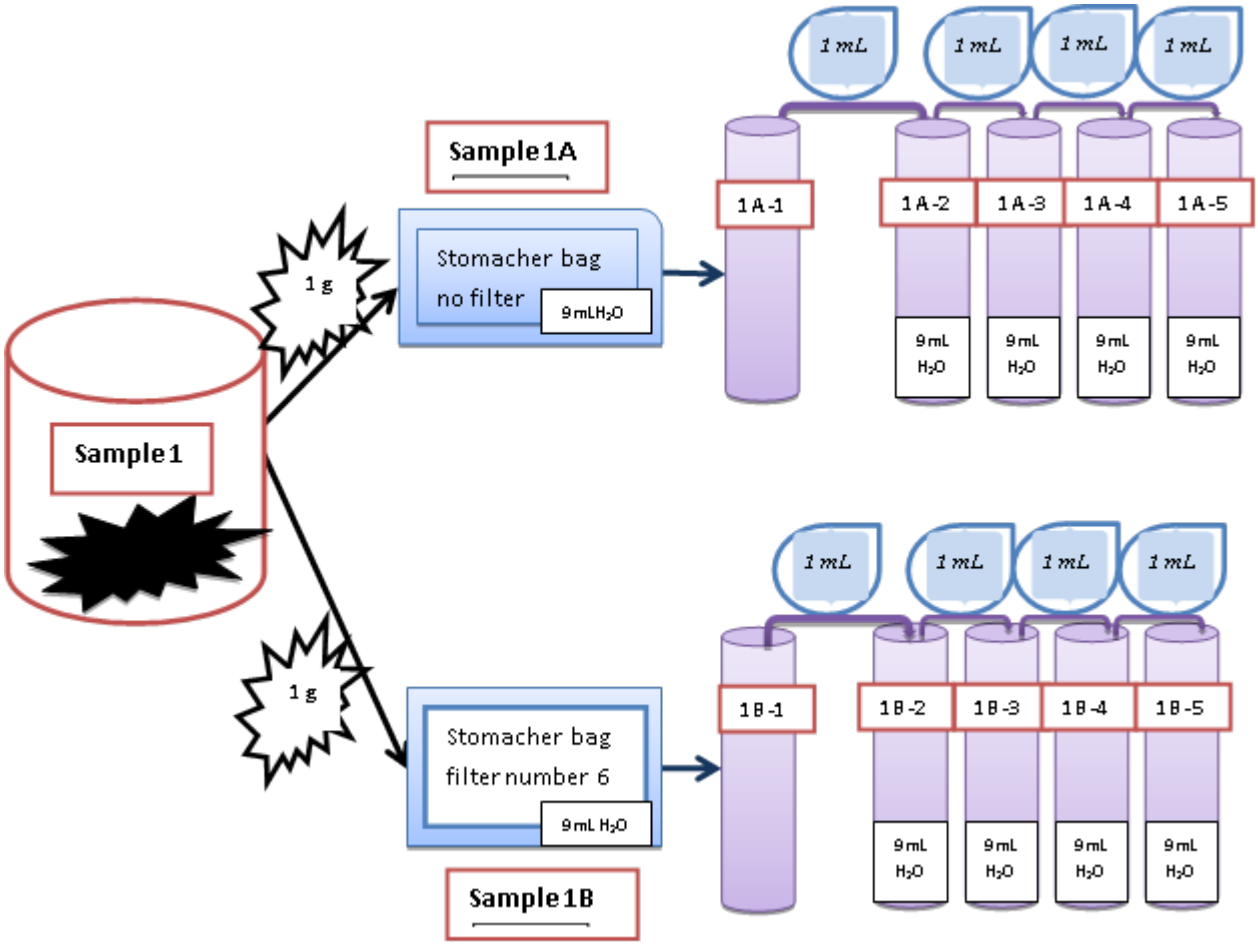

Figure 2 *Sub-sampling and dilutions from naturally infected chicken faecal samples*

The prepared dilutions for every faecal sample (Samples in pots 1-4) were processed in the following manner:

a. Direct culture for *Campylobacter* quantification was performed within 30 hours from the collection of poultry faecal samples. Direct culture was carried out using *Campylobacter* selective agar, the modified charcoal cefoperazonedeoxychocolate agar (mCCDA, product codes: CM0739, SR0155, Oxoid, Hampshire, UK). The previously prepared five dilutions from every sample (1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B) were mixed thoroughly to achieve equal distribution of *Campylobacter* in the samples and 100 µl were then spread onto mCCDA. Inoculated plates were incubated microaerobically at 42°C for 48 hours (Forma Scientific Incubator from Thermo Fisher Scientific Inc. Waltham, MA, USA). After 48 hours, *Campylobacter* colonies were counted and numbers recorded in Excel for further data analysis.

b. The sample dilutions were stored at 5°C. Prior to DNA extraction, every sample was thoroughly mixed, 1 ml of diluted sample was centrifuged at 117000 x *g* for 15 min, and the pellet used for DNA extraction.

DNA extraction

Pellets from spiked and naturally infected samples were thawed on the laboratory bench. The pellets were re-suspended and DNA extracted according to the kit manufacturers' instructions:

1. Easy-DNA™ Kit For genomic DNA Isolation (Invitrogen, Leek, The Netherlands).

The published protocol #3 from the Easy- DNA™ Kit (Invitrogen) for the extraction of DNA from small amounts of cells, tissues, or plant leaves was followed. Samples (pellets) were re-suspended in 200 µl of 10mM Phosphate Buffered Saline (PBS) buffer. This method is manual, the solutions and reagents included in the Kit were used together with chloroform and ethanol for the extraction of *Campylobacter* DNA. The final DNA elution volume was 100 µl and the approximate cost (including laboratory materials) was 17 Danish Kroners (DKK) per DNA extraction.

2. NucliSENS® MiniMAG® (bioMérieux SA, Lyon France).

Sample pellets were re-suspended in 2 ml of 10mM PBS buffer. The NucliSENS® MiniMAG® method required a machine and magnetic silica particles for DNA extraction. Initially, cells were lysed using a lysis buffer and the extracted DNA bound the magnetic silica particles. After several washes with different buffers, DNA was eluted using an elution buffer (final DNA elution volume was 100 µl) and ready for further processing. The approximate cost (including laboratory materials) for this method was 125 DKK per DNA extraction.

Biological and real-time PCR replicates from spiked samples were produced as described previously (Garcia *et al.*, 2013). When processing spiked samples, three biological replicates were analysed when using the Easy-DNA™ Kit and two biological replicates (two sub-samples from

each *Campylobacter* level) were processed when using the NucliSENS® miniMAG method (due to protocol limitations). In addition, two real-time PCR replicates from one biological replicate (all dilutions) were included to evaluate variation attributable to the real-time PCR experiment. Consequently, a total of five replicates per spiked sample (*Campylobacter* level) were analysed when DNA was extracted with the Easy-DNA™ method and four replicates per spiked sample when using the NucliSENS® miniMAG®.

Replicates from naturally infected chicken faecal samples were produced in the following manner: four different chicken faecal samples were sub-divided to obtain a total of eight biological replicates and three real-time PCR replicates from every biological replicate were included in real-time experiments for every dilution level.

Real-time PCR

A real-time PCR thermo cycler Mx3005P™ (Strategene, La Jolla,USA) was used, MicroAmp Optical 96-well reaction plates (Applied Biosystems) were available to place the samples with PCR master mix in the thermal cycler and covered with MicroAmp Optical caps (Applied Biosystems). The 25-µl real-time PCR mixture was prepared as previously described (Garcia *et al.*, 2013).

The primers included in this study (the forward primer OT-1559 and the reverse primer 18-1) amplify 287-basepair fragment of the 16S rRNA gene of thermotolerant *C. jejuni, C. coli* and *C. lari* (Lübeck *et al.*, 2003; Josefsen *et al*., 2004). The amplification products were detected using a FAM (fluorescein amidite) -labeled probe. An internal control (amplified with the target) was visualized using a HEX (hexachloro fluorescein) -labeled probe. The thermal profile consisted of an initial denaturation step at 95°C for 3 min followed by 40 cycles consisting of denaturation at 95°C for 15 sec, annealing at 60°C for 60 sec and extension at 72°C for 30 sec.

The following controls were included: (i) two positive controls (DNA from *C. jejuni* with concentrations: 100 and 1000CFU/mL), (ii) a negative control (DNA from *E. coli* with a

concentration of 1000 CFU/mL) and (iii) a non-template control (NTC) in duplicate for assessment of master mix contamination.

Data analyses

Data analyses were performed using the MxPro-Mx3005P software (version 3.00). Standard curves were generated for the two DNA extraction methods and used to quantify *Campylobacter* present in the naturally infected chicken faecal samples. The amplification threshold was set using the software option "background-based threshold" which determines the standard deviation of all amplifications (from cycles 5 to 9) and multiplies them by a factor of 10 (default factor). A common baseline was set for all amplification plots obtained using an adaptive baseline and a non-adaptive baseline (Stratagen, 2004) and a common amplification threshold was generated in this way. The slope of the standard curve indicated PCR amplification efficiency (AE) which can be calculated using the equation: $AE = 10^{(-1/slope)} - 1$.Amplification efficiency should be obtained for every PCR run and it is calculated from the slope of the linear regression between log10 of initial microbial concentration (CFU/ml) in known samples and the Ct values; an amplification efficiency of 100% indicates that the amplicon doubles each cycle in perfect reactions (Stratagene, 2004).Data fit to the standard curve is measured using the coefficient of determination R^2 which should be close to 1.00 (Stratagene, 2004).

Results related to real-time PCR assays using the two different DNA extraction methods were compared based on limit of detection, limit of quantification and real-time PCR amplification efficiency. Additionally, estimates of the number of *Campylobacter* (present in naturally infected chicken faecal samples) obtained by real-time PCR when using the two different DNA extraction methods were compared with those obtained from selective culture. Correlation coefficients were obtained to assess agreement between the methods used in this study. Statistical significance of the differences observed between results from culture and from real-time PCR when using the two different DNA extraction methods were assessed using the multcomp package in the statistical program R (R Development Core Team, 2008).

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8. THE USE OF PROBABILISTIC GRAPHICAL MODELS AND EXPERT SYSTEMS FOR THE CONTROL OF CAMPYLOBACTER IN POULTRY (Manuscripts V and VI).

8.1. Introduction

Probabilistic graphical models (PGMs) are increasingly and widely used to support knowledge management and decision making under conditions of uncertainty (Kjærulff & Madsen, 2008; Darwiche, 2009; Koller & Friedman, 2009; Madsen *et al.*, 2012). PGMs are extensively used in a variety of disciplines including medicine and epidemiology (Lucas *et al.*, 2000). The application of probability theory to complex problems involving many variables such as the development of Probabilistic Graphical Models (PGMs) is quite recent and increasingly popular. Probability theory sets the basis for modeling the diverse possible states of the parts of the world that we want to consider and to update the models with new evidence or knowledge. Decisions on vaccination and other public health controls have to be generally made under conditions of uncertainty. PGMs use Bayesian networks and other methods to include probability distributions in models that can involve hundreds or even thousands of variables. The aim of PGMs is the efficient representation and integration of knowledge obtained from sources such as epidemiological data, scientific knowledge, research data and expert opinions in order to support decision processes made under conditions of uncertainty. Knowledge is the product of complex and multifaceted processes. Furthermore, the creation and integration of knowledge seem crucial for Knowledge management (Wickramasinghe *et al.*, 2007). Stemke (2001:3) defines Knowledge Management (KM) as “the set of processes, technology and behaviors that deliver the right content to the right people at the right time and in the right context” so that they can make the best decisions and solve problems. In fact, the main objective of Knowledge Management is to produce better solutions (Firestone & McElroy, 2005; Garcia, 2012). The interaction between information, knowledge and technology seems crucial for innovation (Brelade & Harman, 2003). The use of Information and Communication Technologies (ICTs) and probabilistic graphical models has become an important and innovative tool for sustainable animal production and disease control strategies. Selective representation of important data and information is necessary for the efficient use of ICTs. The use of ICTs and selective objectification might be necessary in cases when the complexity of the reality we try to represent is very high. PGMs represent knowledge and relationships in structured models designed to represent

real situations where uncertainty plays an important role. The relationships of dependence or independence between the entities included in the models can be represented and the strength of the relationships can be defined using conditional probability distributions (Madsen *et al.*, 2012). The HUGIN tool is a commercial-off the-shelf software package designed for the construction, and deployment of probabilistic graphical models (PGMs). Engagement of different stakeholders in the PGMs development process is highly desirable. The use of sophisticated and complex computing interfaces and mathematical expressions and probabilities distributions needs to be reconciled with a simple and efficient tool that can be used by different stakeholders (Madsen *et al*., 2012). On the other hand, newer technologies seem to be more flexible in relation to supporting individuals' creativity and innovation.
Several PGMs designed using the HUGIN software in order to aid the poultry industry to make complex decisions regarding vaccination against *Campylobacter* are presented in this thesis.

Human campylobacteriosis is considered an important public health problem all over the world. Poultry has been identified as one of the main risk factors associated with human campylobacteriosis cases (Christenson *et al.*, 1983; Neimann *et al.*, 2003). In fact, seasonality effects have been detected regarding *Campylobacter* numbers in chickens and cases of human campylobacteriosis (Reich *et al.*, 2008). *Campylobacter* does not seem to induce health or welfare problems in chickens. Campylobacter colonizes the chicken intestine and quickly multiplies in the intestinal mucosa (Van Deun *et al.*, 2008). Furthermore, *Campylobacter* spreads fast within broiler flocks, once a bird has been colonized by *Campylobacter* it has been suggested that the rest of the birds in the same house will be infected within one week (Jacobs-Reitsma, 1997). Broilers might carry high numbers of *Campylobacter* in some cases exceeding 10^7 CFU/g of caecal content (Rosenquist *et al.*, 2006) and sometimes up to 10^{10} CFU/g of faeces (Stas *et al.* 1999, Sahin *et al.*, 2002; Lütticken *et al.*, 2007). *Campylobacter* present in feces of chickens going for slaughter might contaminate the food processing environment and the food products. Reducing the numbers of *Campylobacter* in chickens at farm level seems crucial to prevent *Campylobacter* contamination of chicken products. Furthermore, humans can be infected from poultry by other pathways than poultry products and therefore there are increased public health benefits associated with the implementation of effective controls of *Campylobacter* in primary production. The CamVac project aims to develop a cost effective vaccination strategy against *Campylobacter* in poultry in order to reduce the numbers of *Campylobacter* in poultry farms. Risk assessment studies have demonstrated

that a reduction of 2 logs on the numbers of *Campylobacter* in chickens can translate in a reduction of human *Campylobacter* cases by 30 times (Rosenquist *et al.*, 2003). *Campylobacter* control strategies should be implemented at all levels of the food chain. This part of the thesis focuses on the development of an expert system to support decision making on *Campylobacter* vaccination of poultry and particularly commercial broilers.

The type of PGM that we used for our models is an influence diagram (Howard & Matheson, 1981). An influence diagram formed by a set of variables has two components: a qualitative and a quantitative part. The qualitative part is represented by a directed acyclic graph (DAG) which includes diverse "nodes" such as variables, decision nodes and utility functions as well as arcs representing relationships between them. A decision node (drawn as a rectangle) defines decision alternative at a specific point in time, a chance node (drawn as an oval) represent a random variable and a utility node (drawn as a diamond) represents a reward or cost function, see Figure 1 for an example. Arcs directed into a decision node (e.g vaccination at two weeks in Figure 1) define the information that is known by the decision maker at the time that the decision needs to be made.

The quantitative part of the models encodes mathematical expressions and probability distributions associated with chance nodes and utility functions associated with the utility nodes as defined by the structure of the DAG. The solution of an influence diagram is a strategy consisting of a policy for each decision, i.e., a mapping from what the decision maker knows to the decision alternative. The strategy is determined using the principle of maximizing expected utility. The influence diagram is a powerful representation for supporting decision making under uncertainty. It represents the probabilistic structure of the complex problem such as vaccination decisions compactly and it facilitates communication between analysts and decision makers, i.e., farmers.

8.2. The design of probabilistic graphical models: epidemiological, microbiological and quantitative considerations

A very simple example of a PGM with just one input variable (that could be nonetheless the result of the interaction of many variables) is presented in Figure 9. The probabilistic dependence relationships between the variables are illustrated in Figure 9 using an influence diagram. An influence diagram formed by a set of variables has two components: a qualitative and a quantitative part. The qualitative part is represented by a directed acyclic graph (DAG) which includes diverse "nodes" such as variables, decision nodes and utility functions as well as arcs representing

relationships between them. A decision node (a rectangle in Figure 9) defines decision alternatives at a specific point in time, a chance node (an oval) represents a random variable and a utility node (a diamond in Figure 9) represents a reward or cost function. Arcs directed into a decision node define the information that is known by the decision maker at the time that the decision needs to be done. Each node includes a set of states or alternatives and the arcs represent the relationships between variables. Variables, decision nodes and utility functions need to be carefully selected in order to obtain reliable outcomes from the PGM.

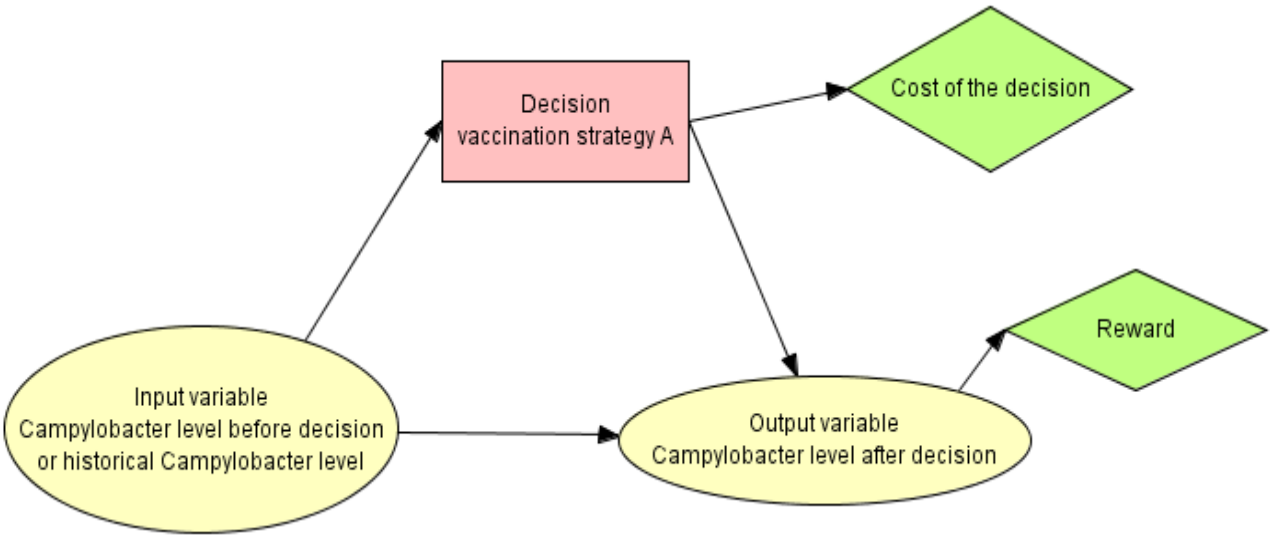

Figure 9 A simple PGM to assist on a decision related to vaccination of poultry against Campylobacter

The relationships of dependence or independence between the entities included in the models can be represented and the strength of the relationships can be defined using conditional probability distributions (Heckerman *et al.*, 1995). The quantitative part of the models encodes the mathematical expressions and probability distributions associated with the different states of the chance nodes and utility functions associated with the utility nodes as defined by the structure of the DAG. There are probability tables for each variable which include probabilities for every state of the variables. These tables will contain the prior probability distributions for variables without parents in the DAG and the conditional probabilities for each combination of states for variables with parents. The prior probability distributions should integrate knowledge obtained from sources such as empirical observations, epidemiological data and expert knowledge in order to obtain reliable outcomes from the decision support models. Bayesian inference and probability theory set

the basis for the quantitative outputs of the models. Decision support models offer flexibility and can be updated with new evidence, knowledge or information.

After careful design of the qualitative and the quantitative part of the models, the outcome of the models will include potential decisions related to *Campylobacter* control strategies that can be considered and selected for implementation. The solution of an influence diagram is a strategy consisting of a policy for each decision, for example, the use of vaccination strategy A (Figure 9). The strategy is determined using the principle of maximizing expected utility based on selecting a decision that will offer the decision maker the greatest expected reward. In this example, vaccination strategy A is able to reduce the expected numbers of *Campylobacter* in infected chickens. The results from the model will include posterior probability distributions (under the identified strategy) related to expected *Campylobacter* numbers in the flock (in logs) before and after the implementation of the decision/s and the expected cost-reward balance associated with each decision/s (Table 1).

Table 3 Hypothetical results from a Probabilistic Graphical Model (PGM) with one decision related to the use of Vaccination strategy A against Campylobacter in broilers.

No vaccination ***Posterior probabilities related to expected Campylobacter levels:***	***Vaccination strategy A*** ***Posterior probabilities related to expected Campylobacter levels:***
0-2 logs (7.00%) 2-4 logs (20%) 4-6 logs (23%) 6-8 logs (24%) 8-10 logs (26%)	0-2 logs (52%) 2-4 logs (18%) 4-6 logs (12%) 6-8 logs (10%) 8-10 logs (8%)
Expected cost-reward balance: +0.36 euros/chicken Expected cost-reward balance (gross profit) for an average flock with 20000 chickens: 7200 euros	***Expected cost-reward balance:*** + 0.44 euros/chicken Expected cost-reward balance (gross profit) for an average flock with 20000 chickens: 8800 euros

The model presented in Figure 9 is an influence diagram (Howard and Matheson, 1981) constructed around the decision on vaccination against *Campylobacter*; still, other control strategies could be considered in the models. The selection of factors, control strategies and quantitative data to be included in the models will obviously influence the final results. To be able to obtain financial results on the use of vaccines and/or other control strategies against *Campylobacter* in poultry the

models estimate the expected (average) utility on each decision. The flexibility of this methodology allows the user to consider different costs depending on the diverse strategies followed to control *Campylobacter*. Similarly, several reward strategies can be accounted for in the models. In the presented model, the reward system is based on the level of *Campylobacter* (logs) measured around slaughter time.

Diverse conceptual models (or qualitative parts of PGMs) have been designed to integrate existing knowledge in order to help poultry managers to decide whether to vaccinate poultry. The most general model (SimpleVac Model) is presented in Figure 10. Figure 11 shows the model referred to as ComBVac (vaccination of commercial broilers) which is an instantiation of the more general PGM SimpleVac Model. A more complex model defined as CampyCVac Model is presented in Figure 12. These influence diagrams have been constructed around the decision on vaccination but other control strategies could be included in the models. The outputs of the complete PGMs (after the quantitative parts have been developed) will be obtained as distributions of the expected numbers of Campylobacter in the flock and expected financial balances (that will be influenced by the cost-reward function and the rest of factors in the model). Consequently, the selection of factors and quantitative data included in the models will obviously influence the final results of the PGMs.

The models presented in Figures 10-12 share the following similarities:

1- Cost-reward functions

A cost-reward function is included in all the models in order to obtain financial results on the use of vaccines and/or other control strategies against *Campylobacter* in poultry. The flexibility of this methodology allows the users to consider different costs depending on the diverse strategies followed to control *Campylobacter*. Similarly, several reward strategies can be accounted for in the models.

2- The decision node is based on performing vaccination against *Campylobacter* in broilers at 2 weeks of age.

Campylobacter is not usually detected in birds younger than two weeks of age (Annan-Prah and Janc, 1988; Stern, 1992). It has been suggested that this “two weeks window” could be strategically used to introduce vaccination programs (Rice *et al.*, 1997). Therefore, vaccination is usually performed in chickens around the 2 weeks of age (except in-ovo vaccination). On the other hand, the immune response against *Campylobacter* in poultry is generally low or moderate. The absence

of a strong immune response has been identified as one of the main challenges for the development of an efficient vaccine to control *Campylobacter* in poultry (de Zoete *et al.*, 2007).
The decision about vaccination in poultry needs to be made usually before *Campylobacter* is introduced in the flock. Even more, there is uncertainty regarding the introduction of *Campylobacter* into the flock that needs to be taken into account in the decision making process. Historical farm data regarding *Campylobacter* status could be accounted for in the models and in fact, it has been included in the PGMs presented in Figures 10-12.

3- Measured and/or observed *Campylobacter* at 2 weeks of age and at slaughter time. Vaccination impact (based on logs reduction of *Campylobacter*)

Microbiological methods for the detection and quantification of *Campylobacter* can be used to assess the *Campylobacter* status of birds. However, it seems important to distinguish between the true numbers of Campylobacter in birds and the detected or measured numbers. There are several microbiological techniques available for the detection and enumeration of *Campylobacter spp.* from different sample matrices. Some techniques are still under development and the detection limit of most methodologies seems to be 100 CFU/g (depending on sample preparation). Hence, a negative result might actually indicate very low numbers of *Campylobacter* (1 to 100 CFU). In addition, it may not be possible to assess *Campylobacter* status at 2 weeks of age or even before slaughter time due to husbandry or farm management practices. Nevertheless, it will be useful to quantify *Campylobacter* before slaughter and at slaughter time in order to assess *Campylobacter* status and any potential vaccine (and/or other control strategies) effect.

PGMs can be extended and/or modified to adapt to different real circumstances. For example, the time of slaughter might vary depending on the final product. Nevertheless, *Campylobacter* quantification at slaughter time should be performed in order to assess the effectiveness of vaccines and/or other control strategies. In the models presented in Figures 10-12, the vaccination impact (in terms of reduction of *Campylobacter* numbers) has been included as a node between the quantification of *Campylobacter* at 2 weeks of age and at slaughter time.

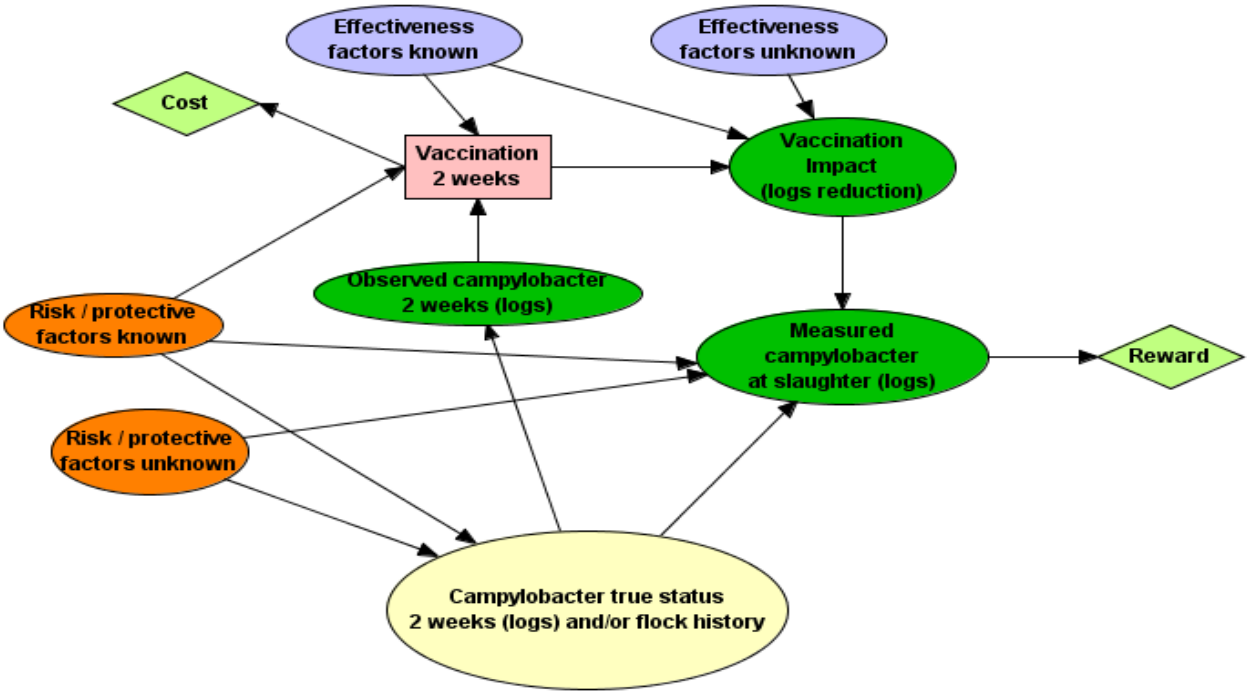

Figure 10 The Simple Vaccination (SimpleVac) Model

The SimpleVac Model includes very general biological, epidemiological and husbandry factors (known and unknown) that might affect the numbers of *Campylobacter* (in logs) in poultry primary production at 2 weeks of age and at slaughter time. This general model also includes groups of factors that might influence vaccine effectiveness (known and unknown factors). The general SimpleVac Model offers a reliable representation of the *Campylobacter* control in poultry. On the other hand, due to the intangible nature of the unknown factors, the quantitative part of the model seems difficult to perform. Consequently, a more specific model was developed, the Commercial Broilers Vaccination (ComBVac) model (Figure 11) which includes epidemiological factors selected from published epidemiological data (EFSA, 2011a) that significantly influence the numbers of *Campylobacter* in broilers produced for human consumption.

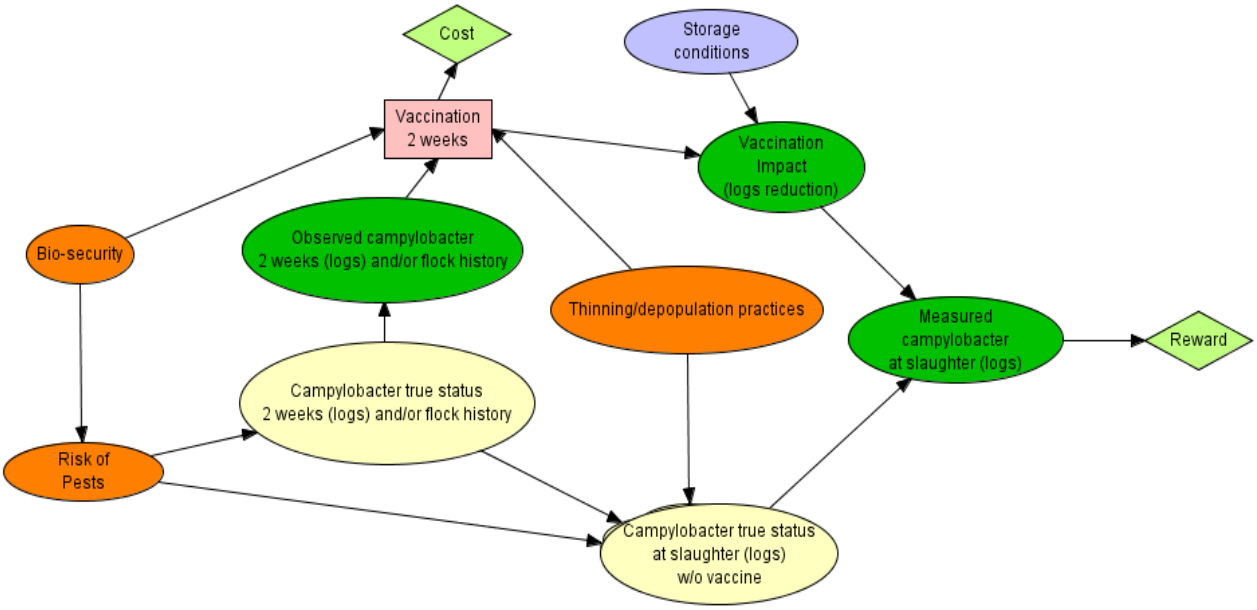

Figure 11 Commercial Broilers Vaccination (ComBVac) model

The epidemiological factors bio-security, risk of pests and thinning/depopulation practices have been included in the ComBVac model. Several definitions of bio-security have been proposed but in practice it should include effective measures taken by food producers to protect food producing animals from disease and zoonotic pathogens (Permin and Detmer, 2007). Bio-security has been identified as a crucial factor for the introduction or control of Campylobacter in poultry farms (Berndtson *et al*., 1996; Gibbens *et al*., 2001; Rivoal *et al.*, 2005; Johnsen *et al.*, 2006). An effective and comprehensive biosecurity plan is recommended to protect food producing animals from disease and infection with zoonotic pathogens (Segal, 2011). The importance of a good biosecurity program has been highlighted in a Swedish study where the contamination level in the environment surrounding farms was found similar for *Campylobacter* negative and positive flocks (Hansson *et al.*, 2007). Strict on-farm biosecurity can prevent *Campylobacter* colonization in poultry, in particular, restricting the access of pests (e.g., rodents and flies) into chicken houses will protect against *Campylobacter* colonization (Hald *et al*., 2008; McDowell *et al.*, 2008). Ventilation has also been identified as an important factor (Newell and Fearnley, 2003; Guerin *et al.*, 2007; Rushton *et al.*, 2009). Thinning (a depopulation practice consisting on removing a number of birds from the flock) has been identified in many studies as a significant risk factor for the introduction of *Campylobacter* into chicken houses (Wedderkopp *et al.*, 2000; Hald *et al.*, 2001; Refregier-Petton

et al., 2001; Bouwknegt *et al.*, 2004; Adkin *et al*., 2006 Allen *et al.*, 2008a; Hansson *et al.,* 2010). A study conducted by Puterflam *et al.* (2005) indicated that this risk was higher when the thinning was performed by large crews.

There are many factors that might affect vaccine effectiveness, but their assessment can be complex in many cases (it will depend on vaccine design, delivery method, dose, particular *Campylobacter* strains, animal genetics and other factors). In the models presented in Figures 11 and 12, only the storage conditions have been considered to affect vaccine effectiveness. Nonetheless, diverse vaccine candidates could be tested under different conditions and the information related to its general effectiveness should be included in the models as "vaccination impact". What's more, the flexibility of these methodologies allows the users to expand and/or modify the information contain in the node named "vaccination impact" and/or include several options (with different probabilities or probability distributions).

PGMs can be modified and adapted to diverse farming conditions and/or new knowledge. Many different risk or protective factors could be potentially included in the models. A humble, preliminary review of the literature on epidemiological data related to risk/protective factors that might significantly influence *Campylobacter* in chickens was performed (Table 4).

Table 4 Risk Factors (From Selected Publications) Significantly Associated With The Presence Of Campylobacter In Poultry

Geographical area/reference	**Production system**	**Selected Risk factors**
Reunion Island, Indian Ocean (Henry *et al.*, 2011)	50 broiler flocks	Several houses on-farm Cleaning with no detergent
Shiraz, Iran (Ansari-Lari *et al.*, 2011)	100 broiler flocks	Age at slaughter >45 days Use of antibiotics Level of owner's education
Sweden (Hansson *et al.*, 2010)	37 producers, 90 broiler houses	Presence of other livestock on farm, presence of other livestock within 1 km., poor hygiene, thinning, farm workers changing footwear once only
Germany (Näther *et al.*, 2009)	146 broiler flocks	Free-range and organic farms Flocks with less than 15000 birds and more than 25000 Use of nipple drinkers with trays
Great Britain (Ellis-Iversen *et al.*, 2009)	603 broiler flocks from 137 farms	Time of the year (July, August, Sept) Cattle present or near Non-chlorinated water Flocks closed to each other
Norway (Lyngstad *et al.*, 2008)	131 broiler farms	Private water supply Pigs closer than 2 km Transport personnel Less than 9 days between depopulation and restocking Multiple houses on farm
Denmark (Hald *et al.*, 2008)	5 broiler farms	Large numbers of flies
Iceland (Guerin *et al.*, 2008)	792 broiler flocks	Temperature-related risk factors. Higher risk when the cumulative-degree days (CDD) WAS >139 and temperature >8.9 degrees 2-4 weeks before slaughter
Northern Ireland (McDowell *et al.*, 2008)	88 broiler farms,388 flocks	Rodents on farm Age of birds at sampling Season (summer) 3 or more broiler houses Frequency of footbath disinfectant changes General hygiene

Table 4 (cont.) Risk Factors (From Selected Publications) Significantly Associated With The Presence Of Campylobacter In Poultry

Geographical area/reference	**Production system**	**Selected Risk factors**
Iceland (Guerin *et al..*, 2007)	1425 broiler flocks (analyses included 792 flocks)	Vertical ventilation in-house Vertical and horizontal ventilation Cleaning and disinfection of boots Cleaning with geothermal water Increasing flock size Using manure on-farm Increased number of broiler houses on-farm
Canada (Arsenault *et al.*, 2007)	81 broiler flocks and 59 turkey flocks	Farms with rodent control Manure 200 m from chickens Number of birds on farm Age at slaughter
Great Britain (Adkin *et al.*, 2006)	Data extracted from 159 research papers	Depopulation events Another house on-farm On-farm staff Other animals on-farm
Senegal (Cardinale *et al.*, 2004)	70 broiler farms	Other animals on-farm Staff not wearing protective clothing Uncemented house floors Use of cartons to transport chicks as feed plates Protective factors: cleaning and disinfection, manure outside the farm
Netherlands (Bouwknegt *et al.*, 2004)	495 broiler flocks	Age =5 broiler houses on farm Other animals on farm Animals on farms within 1 km Summer Fall Children entering broiler house
Netherlands (Van de Giessen *et al.*,1996)	20 broiler farms, 112 flocks	Other farm animals Protective factors: hygiene measures
Red- numbers of animals/houses on farm, age related factors Orange- type of farm Blue- biosecurity related factors (including thinning and depopulation events, rodents and people) Green- Other animals on farm Purple- time of the year, temperature-related factors		

Many variables have to be considered when designing epidemiological studies and mathematical models. Furthermore, material, human, knowledge and time limitations will influence the

epidemiological studies and in turn the results from the models. *Campylobacter* prevalence, quantification and strains of *Campylobacter* might vary with the geographical area and animal, husbandry and microbiological factors. Similarly, the significance of the considered risk/protective factors in every study will be influenced by many variables. As human beings, we are restricted by a limited capacity to understand the complex subjects. Classifications and representations can help us to understand the complex world we face. Therefore, the factors identified in Table 4 have been classified in five different groups to be included in the models. A *Campylobacter* Complex Vaccination (CampyCVac) Model has been produced including these five groups of risk/protective factors (Figure12).

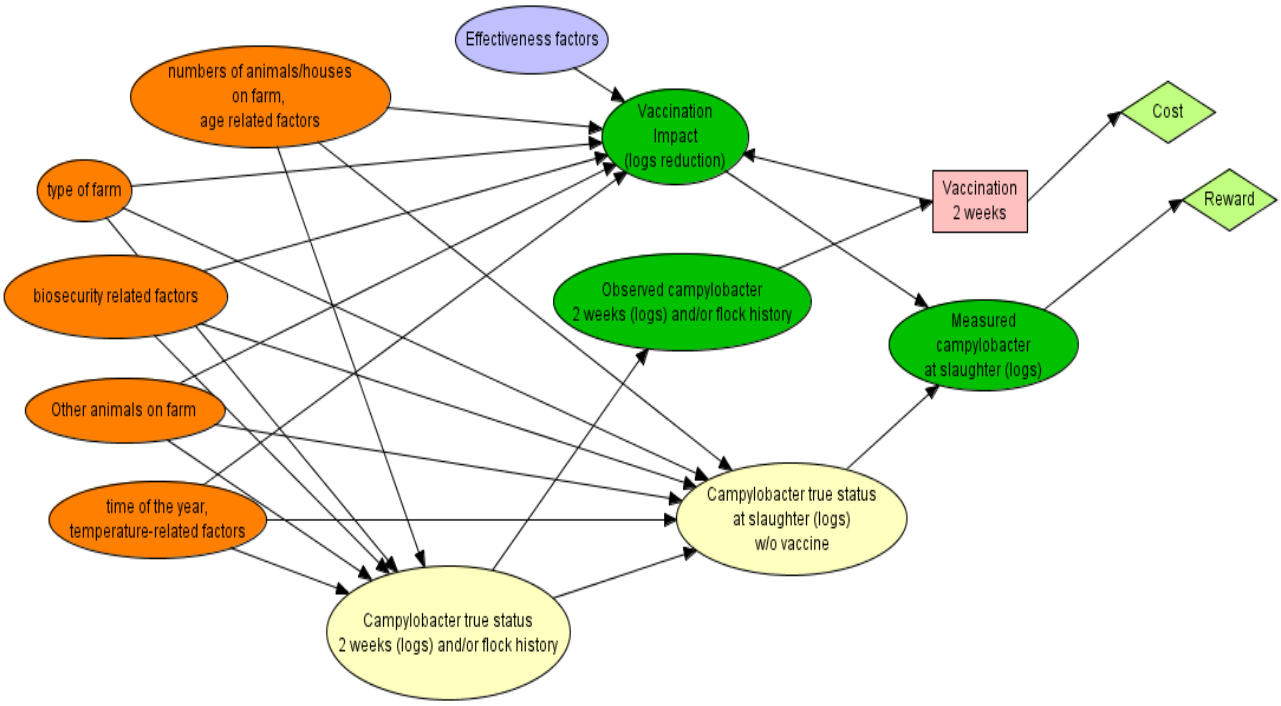

Figure 12 Campylobacter Complex Vaccination (CampyCVac) model

The CampyCVac Model is based on the general SimpleVac model but it considers only known and quantifiable factors (risk, protective and effectiveness factors). These groups of factors include different quantifiable factors so the user can select the ones that are more relevant or applicable to a particular real life situation for the control of *Campylobacter* in poultry.

PGMs take on account uncertainty and use probability functions to fulfill its purpose. Quantitative models offer flexibility by allowing the user to change probability functions to adapt the models to the different conditions that might be present in real life situations. The general SimpleVac Model

presented in Figure 10 offers a good representation of the Campylobacter control in poultry but due to the intangible nature of the unknown factors, the quantitative part of the model does not seem feasible. A number of risk factors were selected to design the ComBVac Model (Figure 11). Important considerations related to the selection of risk factors to be included in models have been previously mentioned. On the other hand, it has been recognized that while the construction of conceptual models is feasible, the quantitative part represents a hard task (Renooij, 2001). Crucial challenges regarding the quantitative part of our models after selection of factors are related to:

- A "selected risk factor" such as biosecurity could be influenced by many other factors or variables and as a result it may be very difficult to select "one number or defined distribution" to represent the group of factors. Additionally, some of these factors may well be protective instead of risk factors based on particular epidemiological studies. In fact, results related to the same factor can be contradictory in different studies (e.g. pest control has been found to be a risk factor instead of a protective factor in some papers). The presence of potential cofounders could explain some findings making the analysis and the models more complex.
- Epidemiological studies are conducted in different areas of the world, diverse conditions, farming systems, sample sizes, sampling protocols to name a few. Accordingly, it seems challenging to design a general PGM that could be applied in all circumstances to support decision making. In fact, the quantitative part of the model should be based on one "standardized measure of risk"; however, epidemiological studies use different measurements or parameters to represent the concept of "increased or decreased risk" due to the factor considered. Even when the parameter used is the same (e.g. Odds Ratio) the quantitative values associated with a particular risk factor can be very different between studies. Although many epidemiological studies use the Odds Ratio as a measurement of risk attributable to the factor considered, this mathematical value cannot be used in the models as such. It is necessary to transform the Odds Ratio value to a fixed probability value or a specific distribution of potential values to be included in the quantitative part of the PGMs.

In summary, different PGMs can be constructed to assist poultry farmers in decision making regarding *Campylobacter* vaccination of poultry. However, it seems challenging to design a general model (qualitative and quantitative) that could be applied to all situations, poultry farming

conditions and geographical areas. For that reason, our suggestion is that several specific PGMs may be constructed or adapted to address particular decision making processes under specific circumstances. Moreover, the conditions, selection of factors, different parts of the models, quantitative data should be clearly specified to add value and perspective to the decision support system designed in every case.

We have designed several PGMs to assist poultry farmers in making decisions related to *Campylobacter* controls based on published data from United Kingdom (Manuscript V: Garcia *et al.*, 2013b), Denmark (manuscript VI) and Spain (not shown).

8.3. The development of a decision support model for the control of *Campylobacter* in poultry farms in the United Kingdom (Manuscript V: Garcia et al., 2013b)

8.3.1. Introduction

The estimated prevalence values of *Campylobacter* in UK broilers (caecal samples) and UK broiler carcasses (samples taken from skin) were 75% and 86% respectively in the EU baseline survey carried out in 2008 (European Food Safety Authority, 2010a). These values exceeded the mean EU prevalence percentages of 71% and 77% respectively. Quantitative data indicated that *Campylobacter* numbers on broiler carcasses varied widely; the numbers of *Campylobacter* in UK broiler carcasses were reported as less than 100 *Campylobacter* per gram (cfu/g) in 42% of the samples and more than 1,000 *Campylobacter* per gram (cfu/g) in 27% of the samples. Even when contaminating with low numbers of Campylobacter, infected chickens going for human consumption pose a public health risk. Consequently, efforts should be directed to control *Campylobacter* in chickens and to improve food safety. A *Campylobacter* Risk Management Program has been developed in the UK in order to reduce foodborne illnesses due to campylobacteriosis (Food Safety Authority, 2010a/b). An important reduction in human campylobacteriosis cases in the UK by 2015 is desirable and therefore reducing the level of *Campylobacter* in UK chickens is considered a priority. Working in partnership with the poultry and food industries and engaging stakeholders (including the consumers) is considered crucial to achieve these aims. Furthermore, a coordinated program based on *Campylobacter* research has been developed because a greater understanding of the microbiology and epidemiology of *Campylobacter* infections is necessary to control campylobacteriosis. Effective controls and

interventions should be implemented and realistic targets defined. The UK target aims to reduce the percentage of UK produced chickens with highest levels of Campylobacter (more than 1000 cfu per gram) from 27% in 2008 to 10% by 2015 (after chilling). A reduction of the *Campylobacter* numbers and/or prevalence in the least contaminated chickens is also expected as a result of the implementation of effective control programs.

8.3.2. Materials and Methods

Two PGMs have been designed to aid on decision making regarding *Campylobacter* vaccination of UK broiler flocks (Figures 13 and 14). The risk factors and epidemiological quantitative data included in the models were selected based on published data from the UK (Lawes *et al.*, 2012). These authors conducted epidemiological studies based on twenty-nine risk factors that could be potentially associated with *Campylobacter* status in broilers. The following risk factors were found significantly associated with *Campylobacter* positive flocks in the study: previous depopulation practices, higher recent flock mortality, increasing age at slaughter and slaughter in the summer months. We have included these risk factors for the presence of *Campylobacter* in UK broilers at slaughter in the PGMs (Figures 13 and 14). The quantitative part (probabilities of events or states of the variables) of the PGM (Manuscript V: Garcia *et al.*, 2013b) was obtained by a mathematical transformation of odds ratio values presented in the study from the UK (Lawes *et al.*, 2012). Furthermore, probabilities related to Campylobacter introduction in the flock due to the presence of risk factors are conditional to a "baseline level" of Campylobacter (lowest level of Campylobacter in broilers close to slaughter time found in the literature). In these models, the "baseline Campylobacter flock prevalence" in the UK considered was 28.8% based on data from a study conducted by the Food Standards Agency (2009).

The formula applied to calculate probabilities of the diverse states of risk factors (P(s)) based on the baseline Campylobacter flock prevalence (b_p) and odds ratios (OR_s) was:

$$P(s) = \frac{\exp(\ln(b_p/(1-b_p)) + \ln(OR_s))}{1+\exp(\ln(b_p/(1-b_p)) + \ln(OR_s))}$$

A cost-reward function was included in the models in order to assess the financial consequences of every decision that the farmer might consider to control *Campylobacter* in chickens. Financial data related to the UK poultry industry was obtained from a farm business survey from 2009/2010

(Crane *et al.*, 2011). There is no commercial *Campylobacter* vaccine at present and thus a commercial Campylobacter vaccine price is not available. The cost of a hypothetical vaccine against *Campylobacter* in broilers could be considered to be between 2 and 6 Euro cents based on prices of other vaccines used in poultry production (DIANOVA, 2013). The vaccine effectiveness or vaccine impact was also hypothetical in these models. We decided to consider a hypothetical vaccine B against *Campylobacter* in broilers able to decrease *Campylobacter* numbers from 2 to 6 logs in 20% of the broilers and less than 2 logs in 80% of the chickens with a cost of 0.025 £/chicken (UK). The reward system has been designed based on the reported average gross profit of 0.36 £/per chicken for UK farmers in 2010 (Crane *et al.*, 2011). Based on this hypothetical reward system (Manuscript V: Garcia *et al.*, 2013b), farmers producing chickens with numbers of *Campylobacter* lower than 4 logs will get higher gross profits (+20% extra with respect to other *Campylobacter* levels) while farmers delivering chickens carrying high numbers of *Campylobacter* (higher than 6 logs) will get lower gross profits (-20% between Campylobacter levels). It was assumed that an average broiler chicken from a positive flock in the UK will carry *Campylobacter* in a concentration of 4-6 log CFU/g or ml of sample (from the digestive tract).

The two models designed to aid on decision making regarding *Campylobacter* vaccination of UK broiler flocks share similarities in the conceptual design but the quantitative components of the models differ mainly due to the lack of data regarding how the risk factors may affect the numbers of *Campylobacter* in broilers over time during the rearing period. Epidemiological studies usually provide insight regarding the risk of *Campylobacter* introduction attributable to particular risk factors in specified conditions. Conversely, there seems to be lack of data regarding the numbers of *Campylobacter* carried by broilers throughout the farming period in relation to particular risk factors. Besides, in the models, the vaccination impact and the cost-reward functions are based on a log-scale because the objective is to develop a vaccination strategy able to reduce the numbers of *Campylobacter* in commercial broilers by 2 logs (CamVac, 2012). As a result, a mathematical transformation needs to be performed, data related to the effect of risk factors on *Campylobacter* status of the broilers are based on positive/negative results (significant or non-significant effects) and they need to be translated to a log scale. The main challenge resides on where this mathematical transformation should take place in the models. There are at least two possible alternatives as illustrated by the two models presented in Figures 13 and 14.

The model named Commercial Broilers Vaccination UK in Logs (ComBVacUK_ Logs) utilizes a log-scale (from 0 to 10 logs) from the risk factors part of the model and it is referred to as the *logs*-model (Figures 13 and 15). However, the Commercial Broilers Vaccination UK Positive Negative (ComBVacUK_PN) uses a positive/negative-scale at the risk factors part of the model assuming detection above 2 logs for a positive result and it is referred to at the *pn*-model (Figure 14). A posterior mathematical transformation is performed to transform the positive/negative part of the model to the log scale part of the model (Figure 16).

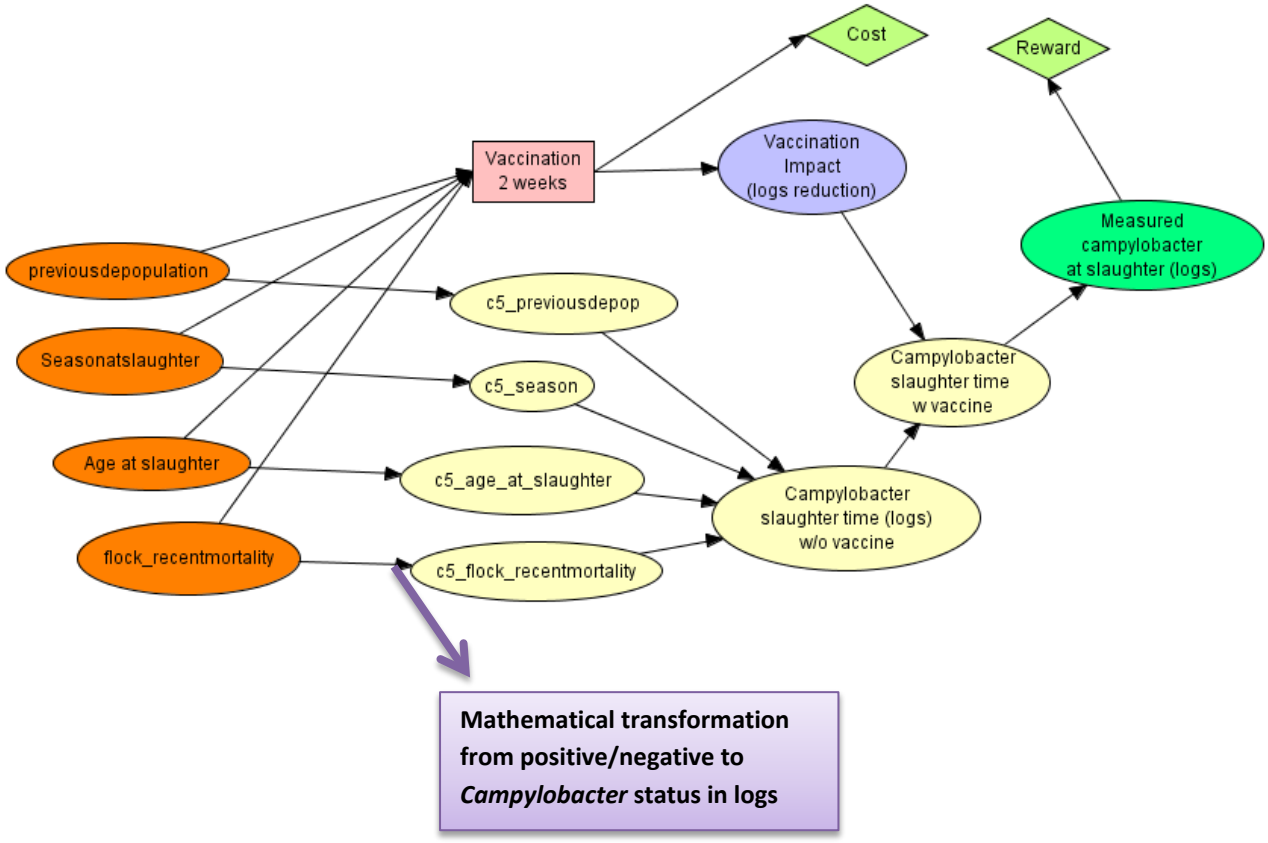

Figure 13 Commercial Broilers Vaccination UK in Logs (ComBVacUK _Logs) Model

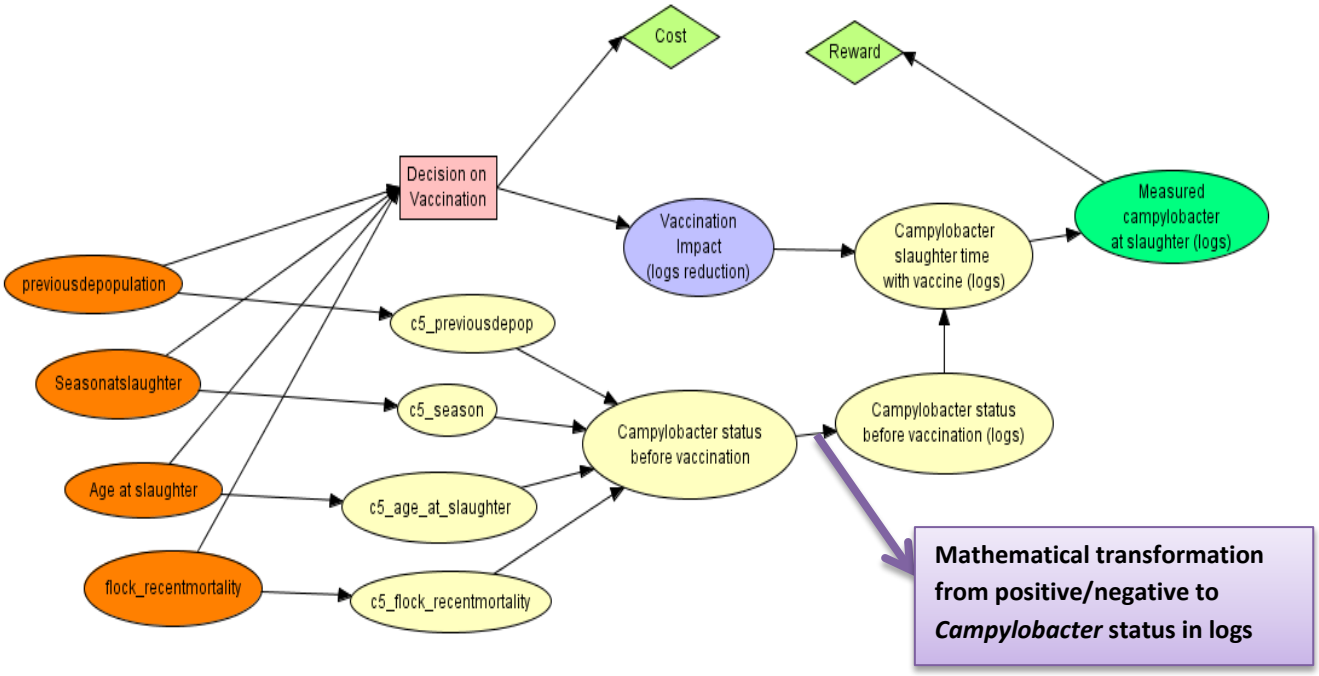

Figure 14 Commercial Broilers Vaccination UK Positive Negative (ComBVacUK _PN) Model

The assumptions consider when building the models are as follows:

- The assumed detection limit for *Campylobacter* is 2 logs and the maximum level that can be found in chickens is considered 10 logs. Consequently, a negative result means 0-2 logs and a positive result indicates 2 to 10 logs.
- The contributions from different risk factors to the level of *Campylobacter* are independent.
- Figure 15 illustrates the mathematical transformation from the positive/negative part of the ComBVacUK _Logs model to the log scale part of the model at the risk factors level (risk factor "age at slaughter" as an example). The distribution over the positive results for each risk factor is uniform as illustrated in Figure 15.

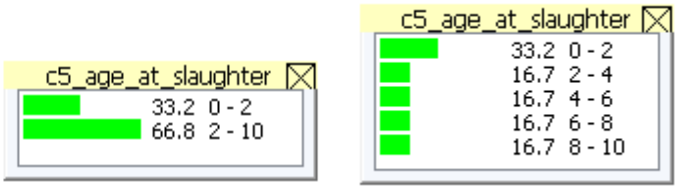

Figure 15 A positive/negative scale for the risk factor "age at slaughter" is presented on the left side and a log-scale based on a flat or uniform distribution is shown on the right side

- The effectiveness levels of the vaccines are the same for the two models. The impacts of the vaccinations are specified in the conditional probability distribution of node *Vaccination Impact (logs reduction).*
- The accuracy of the measurement of *Campylobacter* is specified in the conditional probability distribution of node "Measured *Campylobacter* at slaughter in logs". The measurement is provided on a log-scale and it is used to define the reward system, i.e., the reward system is based on the level of *Campylobacter* (in logs).
- A mathematical transformation from positive/negative to the logs-scale is performed in the model ComBVacUK _PN by introducing the node "Campylobacter status before vaccination (logs)". The posterior distribution of a positive result is transformed to a uniform distribution over the different *Campylobacter* levels (in logs) as illustrated in Figure 16.

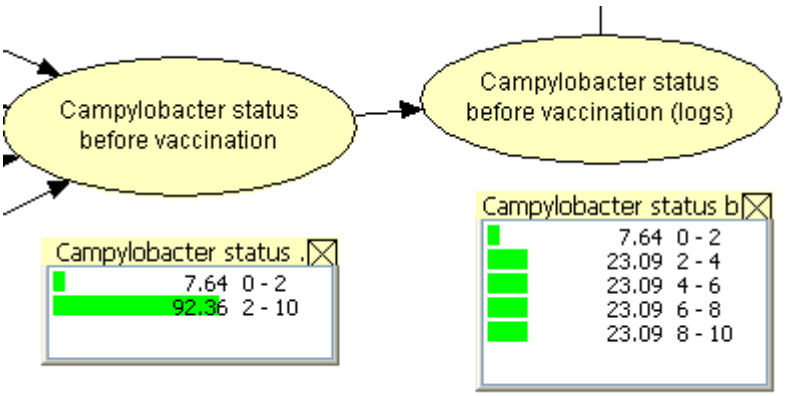

Figure 16 Illustration of the part of the model showing the Campylobacter status before vaccination in positive/negative format (0-2 logs is considered negative and 2-10 logs translates on a positive result) and in logs format (distribution of the different levels of Campylobacter in logs).

Sensitivity analyses were conducted using the ComBVacUK _PN model (Manuscript V: Garcia *et al.*, 2013b) to determine the sensitivity of the vaccination decision under different evidence scenarios with respect to single parameters of the models. In this particular case, two very different reward systems and a hypothetical vaccine C were included in the models. Reward system 2 was based on an extra payment for chickens testing *Campylobacter* negative of 2.5 times the normal price while reward system 3 was based on the existing reward systems in Denmark which is based on an extra payment of about 2% for flocks testing negative for *Campylobacter* and in Norway and Sweden where the payment is reduced by about 4% for flocks that are tested positive for

Campylobacter (personal communication). A hypothetical vaccine C able to reduce 2-6 logs the level of *Campylobacter* in 90% of the chickens was considered with a cost of 0.03 £/chicken (Manuscript V: Garcia *et al.*, 2013b).

8.3.3. Results and discussion

The results from the models can be visualized by selecting diverse combinations of "nodes states" and obtaining the output in terms of the expected distribution of probabilities related to *Campylobacter* levels and expected cost-reward balance in every case. A high number of potential combinations or scenarios may be considered and so, it is up to the user to select the relevant combination of factors. We used three combinations in order to illustrate the potential outputs of the model (Manuscript V): most likely combination (based on UK epidemiological data from Lawes *et al.*, (2012)), the worst scenario (based on states of risk factors found to increase significantly the risk of *Campylobacter* infection in chickens) and the best scenario (opposite risk factors' states from the worst scenario).

Detailed results obtained from the ComBVacUK _PN model are presented in Manuscript V: (Garcia *et al.*, 2013b). Results obtained from both models ComBVacUK _PN model and ComBVacUK _Logs model were very similar (data not shown).

Results generated from the model ComBVacUK _PN indicated that in the best-case scenario the farmer will not gain financially when using vaccine B although the posterior probabilities related to the introduction of *Campylobacter* in the flock will be slightly reduced (approximately from 75% to 65%). However, in the worst-case scenario the best option will be to use vaccine B because it produces the maximum cost-reward balance (0.34 £/chicken) and a reduction on the probabilities related to expected high numbers of *Campylobacter* in the flock. Similarly, results obtained when considering the "most-likely" scenario based on study data (Lawes *et al.*, 2012) indicated that the best option will be to use vaccine B. However, the results showed that the financial differences between diverse strategies were very small mainly due to the narrow differences between the levels of the reward system. The results from the model ComBVacUK _PN indicated that the posterior probability of introduction of *Campylobacter* into the UK poultry flock in the most likely scenario before vaccination was approximately 93% based on the assumptions and data previously specified. The posterior probability of *Campylobacter* introduction into the flock decreased significantly by

the use of a hypothetical vaccine B (to approximately 81%) and even more when using a much more effective hypothetical vaccine C (to approximately 46%).

Sensitivity analyses performed indicated that the factors that influenced the results to a greater extent were the financial variables (cost/reward functions) and the effectiveness of the control strategy, e.g. vaccination impact. The results indicated that when applying the reward system 2 (a system with higher differences between gross benefits obtained by farmers delivering chickens *Campylobacter* negative or with low *Campylobacter* numbers) the best solution in terms of maximum expected benefit will be using the vaccine C (very effective and not expensive) in all case-scenarios. However, when implementing reward system 3 (closer to real reward systems currently employed in several countries) the best solution in financial terms will be "not vaccinating" even though the use of vaccine C could potentially reduce the expected posterior probabilities related to high numbers of *Campylobacter* in the flock significantly (Manuscript V: Garcia *et al.*, 2013b).

The results indicated that the public health impact of the control strategies will depend on the effectiveness of the controls. The assessment of the effectiveness of diverse control strategies might prove challenging e.g. the assessment of vaccine effectiveness (Garcia *et al.*, 2012). *Campylobacter* control strategies that can significantly reduce the probability of *Campylobacter* introduction into a flock and/or the numbers of *Campylobacter* in already infected chickens should be implemented from a public health perspective. However, the producers will usually base their strategic decisions on financial gains and consequently a reward system that can translate on an attractive cost-reward balance will be a good incentive for poultry producers to implement *Campylobacter* control strategies. The cost-reward functions are crucial drivers for the selection of the optimal decision which is determined based on the principle of maximum benefit (cost-reward balance). The cost function included in the models relates only to the cost of the control measure and does not include any other additional costs such as those related to microbiological testing. The reward system might not be in place in most parts of the world, as a result it should be hypothesized and tailor-made based on the gross profit/per chicken for farmers in specific areas and/or production systems (e.g. organic farmers might obtain a higher gross profit/chicken than farmers producing commercial broilers). Financial gain will also depend on the effectiveness of the vaccine (and/or other control strategies) and the costs associated with the controls. A cost-efficient vaccine against *Campylobacter* in chickens is not commercially available at present. We considered that the market

price of a cost-effective vaccine against *Campylobacter* in chickens should be less than 10% of the gross profit per chicken to be competitive. However, the market price could be higher depending on the effectiveness of the vaccine and the reward system. The flexibility of PGMs allows for the inclusion of more than one vaccine and other control measures and more than one reward system.

8.4. The development of a decision support model for the control of *Campylobacter* in poultry farms in Denmark (Manuscript VI)

8.4.1. Introduction

Human campylobacteriosis has been notifiable in Denmark since 1980. Fresh chicken meat has been identified as one of the most important risk factors for human campylobacteriosis in Denmark (Wingstrand *et al*., 2006). The first initiatives to control *Campylobacter* in Danish poultry were adopted in the 1990s based on research conducted in Sweden (Berndtson, 1996). A risk profile for pathogenic *Campylobacter* was conducted in 1998 (Anon, 1998). Broiler flocks have been tested for *Campylobacter* at slaughter in Denmark since 1998. The publication of a quantitative microbiological risk assessment of *Campylobacter* in broilers and chicken meat in 2001 lead to the first Danish Action plan against *Campylobacter* in broilers in 2003 (Christensen *et al*., 2001). In Denmark, an integrated approach for the control of *Campylobacter* in poultry has been adopted where increased biosecurity, allocation of meat from positive flocks to the production of frozen foods and consumer education campaigns have led to a significant decrease in *Campylobacter* prevalence in broiler flocks (from 43% in 2002 to 27% in 2007), a reduction of *Campylobacter*-positive samples of fresh broiler meat (from 18% in 2004 to 8% in 2007) and a decrease in registered human cases by 12% from 2002 to 2007 (Rosenquist *et al.*, 2009). These authors suggested that the coincidental decrease in the number of reported human campylobacteriosis cases was partly due to the implemented *Campylobacter* control strategies in broiler flocks. One of the main intervention strategies implemented was to use *Campylobacter*-positive broilers for frozen products because freezing is known to reduce *Campylobacter* numbers by around 2 logs (Sandberg *et al.*, 2005; Georgsson *et al*., 2006; Havelaar *et al*., 2007a). A new four-year action plan was adopted in Denmark in 2008 in order to further reduce the prevalence and concentration of *Campylobacter* in chickens and their products. The new plan intensified already implemented control strategies and introduced new interventions such as the use of fly screens in broiler houses. A *Campylobacter* prevalence level of 10% in broilers could potentially result in a reduction of human cases by nearly 50% (Nauta *et al.*, 2009; EFSA, 2011b). A recent EFSA report reported a

prevalence of 19.2% for *Campylobacter* in Danish broiler flocks (EFSA, 2011a). Chowdhury *et al.* (2012) observed that 14% of Danish broiler flocks included in the study were positive to *Campylobacter* during the study period December 2009 to November 2010. Even more, *Campylobacter* prevalence in Danish fresh chicken meat is affected by the prevalence of *Campylobacter* in Danish broiler flocks and seasonality effects (Boysen *et al*., 2011). In conclusion, *Campylobacter* prevalence in broiler flocks and chicken meat in Denmark seems to be decreasing, but the prevalence can be higher than 14% especially during summer due to seasonality effects. For this reason, the most promising control strategy seems to be the use of fly screens in broiler houses to reduce the prevalence of *Campylobacter* in chickens especially during the summer (Hald *et al.*, 2007b; Boysen *et al*., 2011).

8.4.2. Materials and methods

A PGM (Figure 17) has been developed to assist poultry producers in decision making related to the implementation of two different *Campylobacter* controls: hypothetical *Campylobacter* vaccines and/or the use of fly screens for the control of *Campylobacter* in broiler flocks based on epidemiological and financial data from Denmark. The solution of the developed PGM provides posterior probabilities related to expected *Campylobacter* numbers in chickens and expected cost-benefit analyses for each decision considered in the model. Poultry producers can then select the most optimal decision/s in every case.

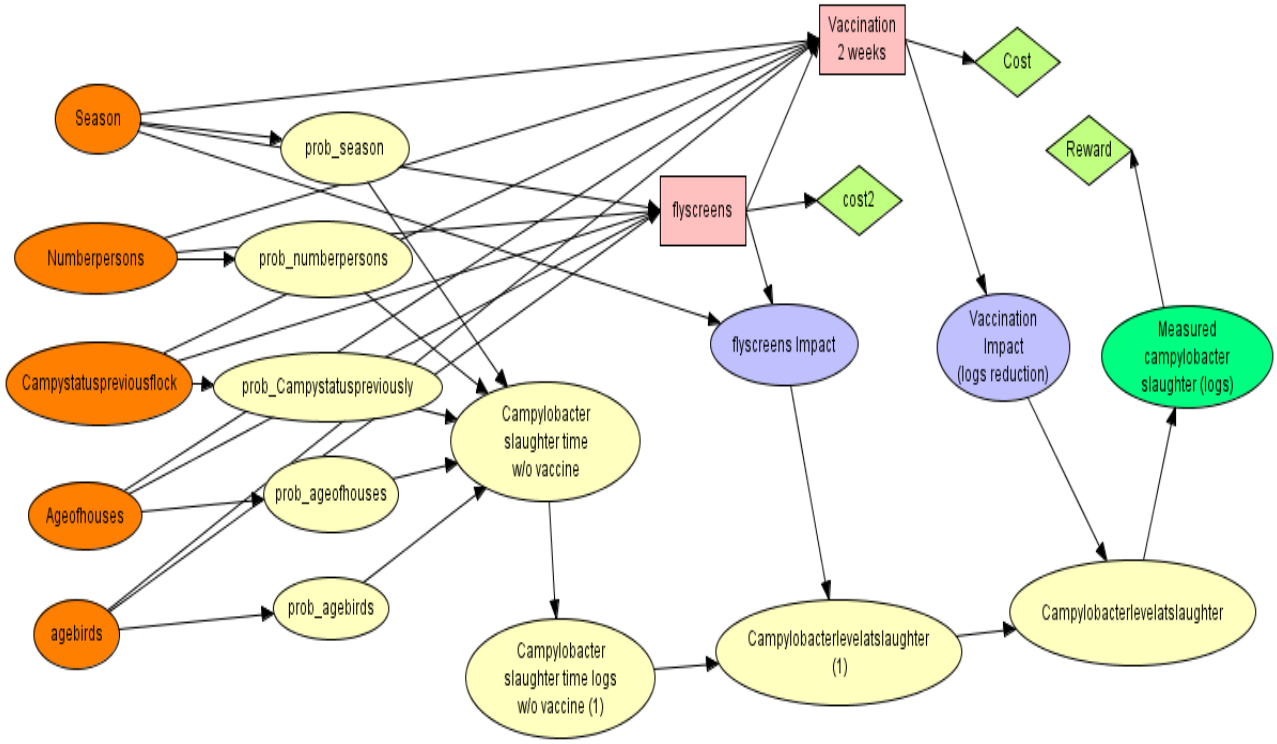

Figure 17 The commercial broilers DK (ComBDK) model designed to control Campylobacter in Danish broiler flocks using hypothetical Campylobacter vaccines and/or the use of fly screens

The risk factors and epidemiological quantitative data included for the ComBDK model were selected based on results from a study conducted by Chowdhury *et al*. (2012). These authors analysed data from the Quality Assurance System in Danish Broiler Production (Kvalitetsikring i kyllingeproduktionen: abbreviated as KIK system) in order to identify farm related risk factors for *Campylobacter* infection in broiler flocks in Denmark. In their study, data related to the time period December 2009 to November 2010 from 187 farms and 2835 flocks were considered. These authors observed that out of the 36 variables initially considered, primary factors like season and increasing age of the birds and other risk factors such as the age of the poultry houses, previous *Campylobacter* positive flocks in the same houses and the number of persons entering the poultry houses were significantly associated with Campylobacter infection of broilers. These risk factors and related epidemiological quantitative data were considered for the development of the ComBDK model (manuscript VI).

The quantitative data (probabilities of events or states of the variables) of the PGM (manuscript VI) were obtained by a mathematical transformation of odds ratio values presented in the study conducted by Chowdhury *et al*. (2012). In addition, probabilities related to *Campylobacter*

introduction in the flock due to the presence of selected risk factors were conditional to a "baseline level" of *Campylobacter* (lowest level of *Campylobacter* in broilers close to slaughter time found in the literature). In the ComBDK model, the "baseline *Campylobacter* broiler flock prevalence" in Denmark considered was 14% based on data from the same study by Chowdhury *et al*. (2012). In this study, *Campylobacter* status of the flocks was assessed by PCR tests using sock samples collected on farm 7 to 10 days prior to slaughter.

The same formula previously shown in this Chapter for the models based on UK data was applied to calculate probabilities of the diverse states of risk factors (P(s)) based on the baseline Campylobacter flock prevalence (b_p) and odds ratios (OR_s).

A cost-reward function was included in the ComBDK model in order to obtain cost-benefit analyses related to every decision that the farmer might consider to control *Campylobacter* in Danish broiler flocks. The decisions included in the ComBDK model were related to the use of hypothetical vaccines against *Campylobacter* and the use of fly screens. No commercial vaccine against *Campylobacter* in poultry is currently available and for that reason a real price related to a commercial *Campylobacter* vaccine does not exist at present. A hypothetical vaccine price can be considered to be around 0.15-0.50 Danish Kroners (DKK) based on prices of other vaccines used to control diseases in poultry (DIANOVA, 2013). Two hypothetical *Campylobacter* vaccines were included in the model with different vaccine effectiveness and costs (manuscript VI). Flies are considered important vectors for the introduction of *Campylobacter* in poultry flocks (Rosef *et al*., 1983; Shane *et al.*, 1985; Berndtson *et al*., 1996). As a consequence, the use of fly screens in poultry houses in order to control *Campylobacter* has been recommended (Hald *et al.*, 2004, 2007b; Barhndorff *et al*., 2013b). Fly screens should be placed in all openings of a poultry house such as doors, windows and chimneys to prevent the introduction of *Campylobacter* into the flocks by flies. The average cost (including capital investments and variable expenses) of implementing fly screens on broiler farms has been considered to be 0.13 DKK/chicken (Lawson *et al*., 2009) and included in the model (manuscript VI).

The reward system was designed around an "average" gross profit (for farmers producing chickens carrying an "average" number of *Campylobacter*) reported to be 2.92 (DKK/chicken) based on financial data from 2013 (Farmtal Online, 2013). The reward system was designed in relation to a real system implemented in Denmark where poultry producers get an extra payment when the flock is identified as *Campylobacter* negative before slaughter (personal communication). In the model,

an extra payment (around 2%) is given to farmers producing chickens carrying *Campylobacter* in numbers less than 4 logs and a reduced payment (around 4% less) is given to farmers producing broilers that carry *Campylobacter* in numbers higher than 6 logs before slaughter (manuscript VI).

In the ComBDK model, the control measures and the cost-reward functions are based on a log-scale but the risk factors affect the probabilities related to *Campylobacter* status (positive/negative) of the flock in the first part of the model. As a result, a mathematical transformation needs to be performed, data related to the effect of risk factors on *Campylobacter* status of the broilers are based on positive/negative results (significant or non-significant effects) and they need to be translated to a log scale (manuscript VI).

The Commercial Broilers in Denmark (ComBDK) model (Figure 17) was designed based on the following assumptions:

- A microbiological detection limit of *Campylobacter* was considered to be 2 logs CFU/g or ml of sample while the maximum colonization level was considered to be 10 logs CFU/g or ml of sample. Thus, in this model, a *Campylobacter* level of 0-2 logs will give a negative result while a positive result suggests *Campylobacter* numbers in the samples from 2 to 10 logs. Intervals for bacterial concentration with two log widths (e.g.0-2 logs, 2-4 logs, 4-6 logs, 6-8 logs and 8-10 logs) were included in the model.
- The contributions from different risk factors to the level of *Campylobacter* are independent.
- The "measured *Campylobacter* at slaughter (logs)" will depend on the microbiological quantitative methods used and the "true numbers" of *Campylobacter* in chickens. In the model, a hypothetical nearly-perfect quantitative method was considered.
- The Campylobacter controls included in the model are: the use of fly screens and hypothetical Campylobacter vaccines A and B. Fly screens are only used during the summer and therefore their effect will be null during the rest of the year although the costs associated with the use of fly screens are always considered.

Sensitivity analyses were performed in order to determine the sensitivity of the decisions considered in the model under diverse evidence scenarios with respect to single parameters of the models. In this model, the following were included: a different reward system 2 (based on an extra payment of 2.5 times the normal price for chickens testing Campylobacter negative) and a cost-effective hypothetical vaccine C (with an effectiveness level of 90% and a cost of 0.26 DKK/ chicken).

8.4.3. Results and discussion

Results from the ComBDK model including the distribution of expected probabilities related to *Campylobacter* levels and expected cost-reward balance in every case were obtained by selecting diverse combinations of "nodes states" or scenarios. Detailed results from the ComBDK model are presented in Manuscript VI.

Results obtained from the model based on the previously described assumptions suggested that the best solution in financial terms will be to use the fly screens alone in the worst case scenario and not to implement the controls under the assumed conditions in the "most likely" and best case scenarios. Nevertheless, the best solution from a public health point of view will be the use of fly screens and vaccine B synergistically. Based on the results from the model, this strategy can decrease significantly the posterior probability related to expected Campylobacter positive results in all scenarios (from 72% to 29% in the most likely scenario; from 55% to 30% in the best scenario and from 87% to 9% in the worst case-scenario) although the implementation of this strategy will translate on a decrease of expected gross profit (around 0.50 DKK/chicken). During the summer, the use of fly screens alone and/or synergistically with vaccines A or B may be able to reduce the posterior probability related to expected Campylobacter positive results below the considered "baseline level of 14%". In fact, using fly screens alone during the summer will reduce the posterior probability related to expected Campylobacter positive results from 87% to 13% resulting also in a small increase of the expected gross profit (based on the cost-reward balance).

Sensitivity analyses performed showed that the financial variables (cost/reward functions) and to a lesser extend the effectiveness of the control measures (e.g. vaccination impact) drive the model's results (manuscript VI). As a result of implementing reward system 2 (based on an extra payment of 2.5 times the normal price for chickens testing Campylobacter negative) the farmers will potentially obtain higher payments and the differences between implementing diverse controls will be much more pronounced. The effectiveness of vaccines B and C are very similar but vaccine C is more cost-effective and desirable for this reason. From a public health perspective, the best Campylobacter control strategy in all case-scenarios will be the use fly screens together with vaccine B or C. However, from an economic point of view, the best solution will depend on the scenario considered, for example, in the most likely scenario, the best solution is using fly screens and vaccine C while during the summer the use of fly screens alone is most rewarding financially.

The use of vaccine C alone in the best case scenario seems to be the most cost-efficient strategy based on the results from the model.

The effectiveness of the use of fly screens against *Campylobacter* in poultry has been reported in terms of decreased *Campylobacter* prevalence in broiler flocks in Denmark. In a study conducted by Hald *et al*. (2007b), the use of fly screens during the 2006 summer (June–October) produced a statistically significant decrease from 51.4% to 15.4% of *Campylobacter* positive flocks in comparison with control houses. A recently published paper (Bahrndorff *et al*., 2013) reports data related to the long-term effect of the use of fly screens on the prevalence of *Campylobacter* in broiler flocks in Denmark collected over the years 2006-2009. These authors reported a statistically significant decrease in *Campylobacter* prevalence from 41.4% in 2003–2005 (before the use of fly screens) to 10.3% in 2006–2009 in agreement with the results obtained by Hald *et al*., (2007b). The use of fly screens was tested on poultry farms in Iceland during the summer of 2008. Reductions on *Campylobacter* prevalence were observed: from 48.3% to 25.6% among flocks in 19 houses from one company and from 31.3% to 17.2% in 16 houses from another poultry firm (Lowman *et al*., 2008). Comparison of the results related to the use of fly screens on broiler farms previously reported (Hald *et al*., 2007; Lowman *et al.*, 2008; Barndorff *et al*., 2013) with the results from the ComBDK model presented here are not straight forward. Results from the experimental studies previously mentioned relate to average *Campylobacter* prevalence values obtained from diverse farms and flocks and therefore different farming conditions. Results from the ComBDK model are based on one flock level and the expected posterior probability related to a *Campylobacter* positive result for that flock under specific conditions. Even more, a "nearly perfect" quantitative microbiological test has been considered in the model and consequently results related to the expected probability of a *Campylobacter* positive result might be higher than the actual result if a quantitative *Campylobacter* test was performed in that particular flock (the detection limit of most microbiological test are around 2 logs). Nonetheless, the flexibility of the model allows the user to test diverse controls, different farming conditions, microbiological protocols and reward systems. Sensitivity analyses performed indicated that the factors that influenced the results to a greater extent were the financial variables (cost/reward functions) and the effectiveness of the *Campylobacter* control strategies (manuscript VI). Obviously, cost-effective control measures will be preferred by poultry producers and a reward system that can translate on an attractive cost-reward balance will be a good incentive for poultry producers to implement *Campylobacter* control strategies.

Manuscript V

Integration of Epidemiological Evidence in a Decision Support Model for the Control of Campylobacter in Poultry Production

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Article

Integration of Epidemiological Evidence in a Decision Support Model for the Control of *Campylobacter* in Poultry Production

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Abstract: The control of human Campylobacteriosis is a priority in public health agendas all over the world. Poultry is considered a significant risk factor for human infections with *Campylobacter* and risk assessment models indicate that the successful implementation of *Campylobacter* control strategies in poultry will translate on a reduction of human Campylobacteriosis cases. Efficient control strategies implemented during primary production will reduce the risk of *Campylobacter* introduction in chicken houses and/or decrease *Campylobacter* concentration in infected chickens and their products. Consequently, poultry producers need to make difficult decisions under conditions of uncertainty regarding the implementation of *Campylobacter* control strategies. This manuscript presents the development of probabilistic graphical models to support decision making in order to control *Campylobacter* in poultry. The decision support systems are constructed as probabilistic graphical models (PGMs) which integrate knowledge and use Bayesian methods to deal with uncertainty. This paper presents a specific model designed to integrate epidemiological knowledge from the United Kingdom (UK model) in order to assist poultry managers in specific decisions related to vaccination of commercial broilers for the control of *Campylobacter*. Epidemiological considerations and other crucial aspects including challenges associated with the quantitative part of the models are discussed in this manuscript. The outcome of the PGMs will depend on the qualitative and quantitative

data included in the models. Results from the UK model and sensitivity analyses indicated that the financial variables (cost/reward functions) and the effectiveness of the control strategies considered in the UK model were driving the results. In fact, there were no or only small financial gains when using a hypothetical vaccine B (able to decrease *Campylobacter* numbers from two to six logs in 20% of the chickens with a cost of 0.025 £/chicken) and reward system 1 (based on similar gross profits in relation to *Campylobacter* levels) under the specific assumptions considered in the UK model. In contrast, significant reductions in expected *Campylobacter* numbers and substantial associated expected financial gains were obtained from this model when considering the reward system 2 (based on quite different gross profits in relation to *Campylobacter* levels) and the use of a hypothetical cost-effective vaccine C (able to reduce the level of *Campylobacter* from two to six logs in 90% of the chickens with a cost of 0.03 £/chicken). The flexibility of probabilistic graphical models allows for the inclusion of more than one *Campylobacter* vaccination strategy and more than one reward system and consequently, diverse potential solutions for the control of *Campylobacter* may be considered. Cost-effective *Campylobacter* control strategies that can significantly reduce the probability of *Campylobacter* introduction into a flock and/or the numbers of *Campylobacter* in already infected chickens, and translate to an attractive cost-reward balance will be preferred by poultry producers.

Keywords: *Campylobacter* control; epidemiology; poultry; public health; probabilistic graphical models; decision support systems

1. Introduction

Human infections with *Campylobacter* are considered an important public health problem all over the world and poultry has been identified as one of the most significant sources for human Campylobacteriosis [1–10]. *Campylobacter* can break through biosecurity barriers and enter poultry houses, colonizing the chicken intestine and quickly multiplying in the intestinal mucosa. However, *Campylobacter* does not induce health or welfare problems in chickens [11]. After introduction, *Campylobacter* spreads fast within broiler flocks and almost all birds in the same house will be infected within one week [12]. Broilers might carry high numbers of *Campylobacter* in some cases exceeding 10^7 colony forming units per gram (CFU/g) of caecal content [13] and sometimes up to 10^{10} CFU/g of faeces [14–16]. *Campylobacter* present in the intestinal tract of chickens going for slaughter might contaminate the slaughtering and food processing environment and the food products representing a public health risk for the consumers. *Campylobacter* seems to be highly infectious and humans may develop clinical disease with the ingestion of a *Campylobacter* dose as low as 500 CFU [17,18]. Furthermore, humans can be infected from poultry by pathways other than poultry products and therefore increased public health benefits can be associated with the implementation of effective controls against *Campylobacter* in primary poultry production. Vaccination of chickens against *Campylobacter* has been proposed as a promising *Campylobacter* control measure [19].

A previous risk assessment study has shown that a reduction of two logs on the numbers of *Campylobacter* in chickens can translate in a reduction of human cases by 30 times [20]. Consequently, decreasing the numbers of *Campylobacter* in chickens at the farm level seems crucial to prevent *Campylobacter* contamination of chicken products, which in turn will reduce the risk of human infections with *Campylobacter*. In the last few years, research studies have focused on the reduction of the probability of *Campylobacter* introduction in broiler flocks [3,21–24] but recently some studies have focused on the development of vaccination and other control strategies with the aim to reduce the concentration of *Campylobacter* in the intestines of already infected chickens [25–29].

Poultry producers need to make important decisions and sometimes expensive investments to control *Campylobacter*. Incentives to differentiate the payment to poultry producers are implemented in some countries in order to improve the safety of poultry products regarding *Campylobacter*. For instance, in Denmark, when the microbiological test identifies a flock as *Campylobacter* negative a few days before slaughter, the producer gets an extra payment (around 2%) while in Norway and Sweden the payment is reduced by about 4% for flocks that test positive for *Campylobacter* [30]. In this way, poultry producers need to make decisions under conditions of uncertainty mainly related to the possibility of the flock being infected with *Campylobacter*. Furthermore, there is always uncertainty around existing knowledge and the generalization of results from specific studies further increase the uncertainty surrounding the knowledge decisions are based on. Mathematical models can be used to simulate the effectiveness and economic impact of diverse control measures. The decision support systems presented in this manuscript are constructed as probabilistic graphical models (PGMs) which integrate knowledge in one representation and use a Bayesian approach to handle uncertainty. Due to the inclusion of uncertain variables in the models (with diverse "states" or alternatives) and the use of probability distributions, using a Bayesian inference seems logic when making decisions under conditions of uncertainty and in situations that require statistical inference [31]. The integration of prior evidence (prior probabilities) can be used to infer the probabilities of other variables (or states) that are not known (posterior probabilities) using a Bayesian approach.

This manuscript describes the development of decision support models for poultry producers, focusing on the integration of qualitative and quantitative epidemiological data related to the effect of different management factors, in order to select optimal decisions regarding the cost-efficient controls that could be implemented to reduce *Campylobacter* concentration in chickens at farm level. The development is exemplified by a model designed using data from the United Kingdom (UK) to assist decision-making related to the control of *Campylobacter* in chicken farms using vaccination strategies. Human Campylobacteriosis represents an important problem in the UK causing significant morbidity and socio-economic costs [32,33]. The number of reported human Campylobacteriosis cases in 2009 was 57,772 in England and Wales, however, it has been estimated that the burden of human infection in 2009 could be closer to 400,000 [34]. An overall *Campylobacter* spp. prevalence of 79.2% in UK broilers going for slaughter was obtained in a stratified randomized survey conducted during 2007–2009, including data from the EU baseline survey of 2008 [35].

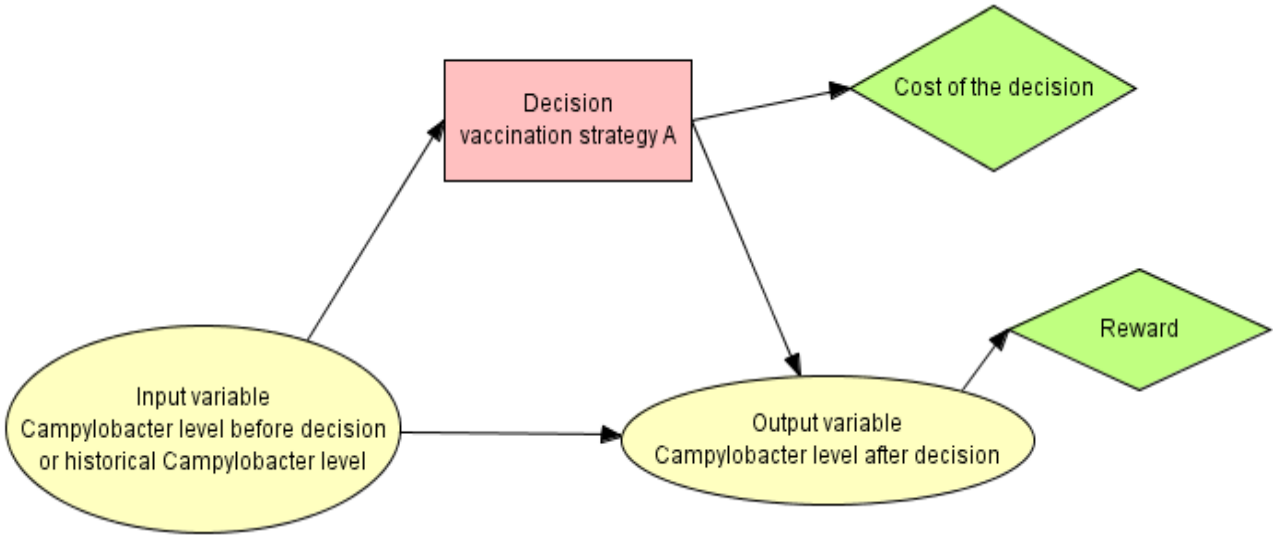
2. Materials and Methods

2.1. Probabilistic Graphical Models (PGMs)

Poultry producers need to make important decisions related to the implementation of interventions against *Campylobacter* in poultry flocks before they know for sure if the flock will be challenged or infected with *Campylobacter*. Probabilistic graphical models (PGMs) may assist poultry producers in these crucial decisions made under conditions of uncertainty. The probabilistic graphical models presented in this manuscript have been designed using the HUGIN tool which is a commercial off-the-shelf software package created for the construction and deployment of probabilistic graphical models. A very simple example of a PGM with just one input variable (that could be however the result of the interaction of many variables) is presented in Figure 1.

In Figure 1, the probabilistic dependence relationships between a set of variables are illustrated using a probabilistic graphical model (formed by a set of variables) which has two components; a qualitative and a quantitative part. The qualitative part is represented by a directed acyclic graph (DAG) which includes diverse "nodes" such as variables, decision nodes and utility functions as well as arcs representing relationships between them. A decision node (a rectangle in Figure 1) defines decision alternatives at a specific point in time, a chance node (an oval) represents a random variable and a utility node (a diamond in Figure 1) represents a reward or cost function. Arcs directed into a decision node define the information that is known by the decision maker at the time that the decision needs to be done. Each node includes a set of states or alternatives and the arcs represent the relationships between variables. The strength of the relationships between the entities included in the models can be defined using conditional probability distributions [36]. Variables, decision nodes and utility functions need to be carefully selected in order to obtain reliable outcomes.

Figure 1. A simple probabilistic graphical model (PGM) to assist in a decision related to vaccination of poultry against *Campylobacter*.



Crucial challenges that might be encountered when developing the quantitative part of the models may be related to the following:

(1) A selected random variable such as "biosecurity" could be influenced by many other factors or variables and for that reason it may be difficult to select one defined probability distribution to represent the group of factors. Furthermore, some of these factors may well be protective instead of risk factors based on particular epidemiological studies. In fact, results related to the same factor can be contradictory in different studies (e.g., pest control has been found to be a risk factor instead of a protective factor [5]). The presence of potential confounders could explain some epidemiological findings making the analysis and the models more complex.

(2) Epidemiological studies are conducted in different areas of the world, diverse conditions, farming systems, sample sizes, sampling protocols, *etc.* Consequently, it seems challenging to design a general PGM that could be applied in all circumstances to support decision-making for *Campylobacter* vaccination of poultry. In fact, the quantitative part of the model should be based on one "standardized measure of risk"; however, epidemiological studies use different measurements or parameters to represent the concept of "increased or decreased risk" due to the factor/s considered in every case. Even when the parameter used is the same (e.g., Odds Ratio) the quantitative values can be very different between epidemiological studies. The statistical combination of results from two or more studies can be referred to as meta-analysis and needs to be produced with care [37].

(3) Although many epidemiological studies use the Odds Ratio as a measurement of risk attributable to the factor considered, this mathematical expression cannot be used in the PGMs as such. It is necessary to transform the Odds Ratio value to a fixed probability value or a specific distribution of potential values to be included in the quantitative part of the Bayesian models. The selection and in some cases the combination of different odds ratios or probabilities for their use in PGMs need to be carefully performed. Moreover, the use of sensitivity analysis has been recommended [38].

After careful design of the qualitative and the quantitative part of the models, the outcome of the models will include potential decisions related to *Campylobacter* control strategies that can be considered and selected for implementation. The solution of an influence diagram is a strategy consisting of a policy for each decision, for example, the use of vaccination strategy A (Figure 1). The strategy is determined using the principle of maximizing expected utility based on selecting a decision that will offer the decision maker the greatest expected reward. In this example, vaccination strategy A is able to reduce the expected numbers of *Campylobacter* in infected chickens. The results from the model will include posterior probability distributions (under the identified strategy) related to expected *Campylobacter* numbers in the flock (in logs) before and after the implementation of the decision/s and the expected cost-reward balance associated with each decision/s (Table 1).

Table 1. Hypothetical results from a PGM with one decision related to the use of Vaccination strategy A against *Campylobacter* in broilers.

No vaccination	**Vaccination strategy A**
Posterior probabilities related to expected *Campylobacter* levels:	**Posterior probabilities related to expected *Campylobacter* levels:**
0–2 logs (7%)	0–2 logs (52%)
2–4 logs (20%)	2–4 logs (18%)
4–6 logs (23%)	4–6 logs (12%)
6–8 logs (24%)	6–8 logs (10%)
8–10 logs (26%)	8–10 logs (8%)
Expected cost-reward balance:	**Expected cost-reward balance:**
+0.36 euros/chicken	+0.44 euros/chicken
Expected cost-reward balance (gross profit) for an average flock with 20,000 chickens: 7200 euros	Expected cost-reward balance (gross profit) for an average flock with 20,000 chickens: 8800 euros

The model presented in Figure 1 is an influence diagram [39] constructed around the decision on vaccination against *Campylobacter* but other control strategies could be considered in the models. The flexibility of this methodology allows the user to consider different costs depending on the diverse strategies used to control *Campylobacter*. Similarly, several reward strategies can be accounted for in the models. In the presented model, the reward is based on the level of *Campylobacter* (logs) around slaughter time.

In the model presented in Figure 1, the decision node is based on performing vaccination against *Campylobacter* in broilers at two weeks of age. *Campylobacter* is not usually detected in birds younger than two weeks [40,41]. It has been suggested that this "two weeks window" could be strategically used to introduce vaccination programs [42]. Therefore, the decision about vaccination in poultry needs to be made usually before *Campylobacter* is introduced into the flock, and there is uncertainty regarding the introduction of *Campylobacter* into the flock that needs to be taken into account in the decision-making process. For this reason, historical farm data related to previous *Campylobacter* status could be accounted for in the models.

2.2. Case Study Model

2.2.1. Current Knowledge Related to Poultry Management Factors

Here, we present a decision model we have developed based on the results from an observational study on risk factors that could be associated with *Campylobacter* in broilers in the UK [35]. These authors conducted epidemiological studies based on 29 risk factors that could be potentially associated with *Campylobacter* status in broilers. The following risk factors were found significantly associated with *Campylobacter* positive flocks in the study: previous depopulation practices, higher recent flock mortality, increasing age at slaughter and slaughter in the summer months. We have included these risk factors for the presence of *Campylobacter* in UK broilers at slaughter in a probabilistic graphical model. The quantitative part (probabilities of events or states of the variables) of the PGM (Table 2)

was obtained by a mathematical transformation of odds ratio values presented in the study from the UK [35]. Additionally, probabilities related to *Campylobacter* introduction in the flock due to the presence of risk factors are conditional to a "baseline level" of *Campylobacter* (lowest level of *Campylobacter* in broilers close to slaughter time found in the literature). In these models, the "baseline *Campylobacter* flock prevalence" in the UK considered was 28.8% based on data from a study conducted by the Food Standards Agency [43].

The formula applied to calculate probabilities of the diverse states of risk factors (P(s)) based on the baseline *Campylobacter* flock prevalence (b_p) and odds ratios (OR_s) was:

$$P(s) = \frac{\exp(\ln(b_p/(1-b_p)) + \ln(OR_s))}{1+\exp(\ln(b_p/(1-b_p)) + \ln(OR_s))} \quad (1)$$

Table 2. Significant risk factors, frequency of occurrence [35] and associated probability of *Campylobacter* introduction in UK broiler flocks.

Risk factor and frequency of occurrence	**Odds Ratio (95% CI)**	**Probability of a *Campylobacter* positive flock * due to the presence of specific risk factors**
Season		
Summer (26.32%)	14.27 (7.83–26.02)	0.85
Autumn (25.38%)	1.70 (1.21–2.37)	0.41
Spring or winter [a] (48.3%)	1	
Age of broilers		
≥46 days (19.59%)	13.43 (7.40–24.35)	0.85
42–45 days (15.67%)	3.56 (2.39–5.29)	0.59
40–41 days (18.57%)	3.18 (1.42–7.12)	0.57
36–39 days (21.98%)	1.25 (0.86–1.81)	0.34
<36 days [a] (24.19%)	1	
Flock recent mortality		
>1.49% (32.22%)	2.74 (1.18–6.40)	0.53
1.00%–1.49% (29.35%)	1.57 (1.12–2.21)	0.39
<1.00% [a] (38.43%)	1	
Previous partial depopulation		
Yes (64.94%)	5.21 (2.89–9.38)	0.68
No [a] (35.06%)	1	

[a] Reference category (mathematical models); * Based on a baseline level of *Campylobacter* of 28.8% [44].

2.2.2. Cost-Reward Function

Accurate cost-benefit analyses of potential control measures against a particular disease play a crucial role in the implementation of successful disease control programs. A cost-reward function was included in this model in order to assess the financial consequences of every decision that the farmer might consider to control *Campylobacter* in chickens. Financial data related to the UK poultry industry was obtained from a farm business survey from 2009/2010 [44]. There is no commercial *Campylobacter* vaccine at present and thus a commercial *Campylobacter* vaccine price is not

available. The cost of a hypothetical vaccine against *Campylobacter* in broilers could be considered to be between 2 and 6 Euro cents based on prices of other vaccines used in poultry production [45]. The vaccine effectiveness or vaccine impact was also hypothetical in these models. We decided to consider a hypothetical vaccine B against *Campylobacter* in broilers able to decrease *Campylobacter* numbers from two to six logs in 20% of the broilers and less than two logs in 80% of the chickens with a cost of 0.025 £/chicken (UK). The reward system has been designed based on the reported average gross profit of 0.36 £/per chicken for UK farmers in 2010 [44]. Based on this hypothetical reward system (Table 3), farmers producing chickens with numbers of *Campylobacter* lower than four logs will get higher gross profits (+20% extra with respect to other *Campylobacter* levels) while farmers delivering chickens carrying high numbers of *Campylobacter* (more than six logs) will get lower gross profits (−20% between *Campylobacter* levels). It was assumed that an average broiler chicken from a positive flock in the UK will carry *Campylobacter* in a concentration of 4–6 log CFU/g or mL of sample (from the digestive tract).

Table 3. Reward system 1 considered in the model.

***Campylobacter* numbers (logs)**	**0–2**	**2–4**	**4–6**	**6–8**	**8–10**
Gross profit (£/chicken)	0.52	0.43	0.36	0.29	0.23

2.2.3. Designing the PGM

The model we present in this case study (Figure 2) was designed based on the following assumptions:

(1) The contributions from different risk factors to the level of *Campylobacter* are independent.

(2) It is considered that the detection level of *Campylobacter* is 2 logs CFU/g or mL of sample and the maximum colonization level is 10 logs CFU/g or mL of sample. This means that a *Campylobacter* level of 0–2 logs will give a negative result while a positive result includes *Campylobacter* numbers from 2 to 10 logs. In this model, we use intervals for bacterial concentration with two log widths (e.g., 0–2 logs, 2–4 logs, 4–6 logs, 6–8 logs and 8–10 logs).

(3) Vaccination impact is based on log-reduction of the numbers of *Campylobacter* in chickens and therefore the numbers of *Campylobacter* in broilers going for slaughter will be lower after vaccination.

(4) The "measured *Campylobacter* numbers at slaughter" will depend on the "true numbers" and the microbiological quantitative methods used. In these models, we assume a nearly-perfect quantitative method so the obtained *Campylobacter* numbers in the lab are closer to the numbers in reality.

Epidemiological studies provide insight regarding the risk of *Campylobacter* introduction attributable to particular risk factors in specified conditions. However, there seems to be lack of data regarding the numbers of *Campylobacter* carried by broilers throughout the farming period in relation to particular risk factors. In the models presented here, the vaccination impact and the cost-reward functions are based on a log-scale because the objective is to develop a control strategy (e.g. vaccination strategy) able to reduce the numbers of *Campylobacter* in commercial broilers. In order to obtain reliable results from the model, data must be on the same scale. Data related to the effect of risk factors on the *Campylobacter* status of the flock are based on positive (2–10 logs)/negative (0–2 logs) results and they need to be translated to the expected distribution of

probabilities related to *Campylobacter* levels in the flock (Figure 3). The nodes "*Campylobacter* status before vaccination" and "*Campylobacter* status before vaccination (logs)" in this model specify the transformation from positive/negative to the diverse *Campylobacter* levels (in logs) scale (Figure 3). A flat distribution is used in this case to transform a general *Campylobacter* probability (e.g., 92.36%) into a distribution of equal probabilities for different levels of *Campylobacter* as illustrated in Figure 3.

Figure 2. The model Commercial Broilers Vaccination (ComBVacUK) based on epidemiological and financial data from the UK.

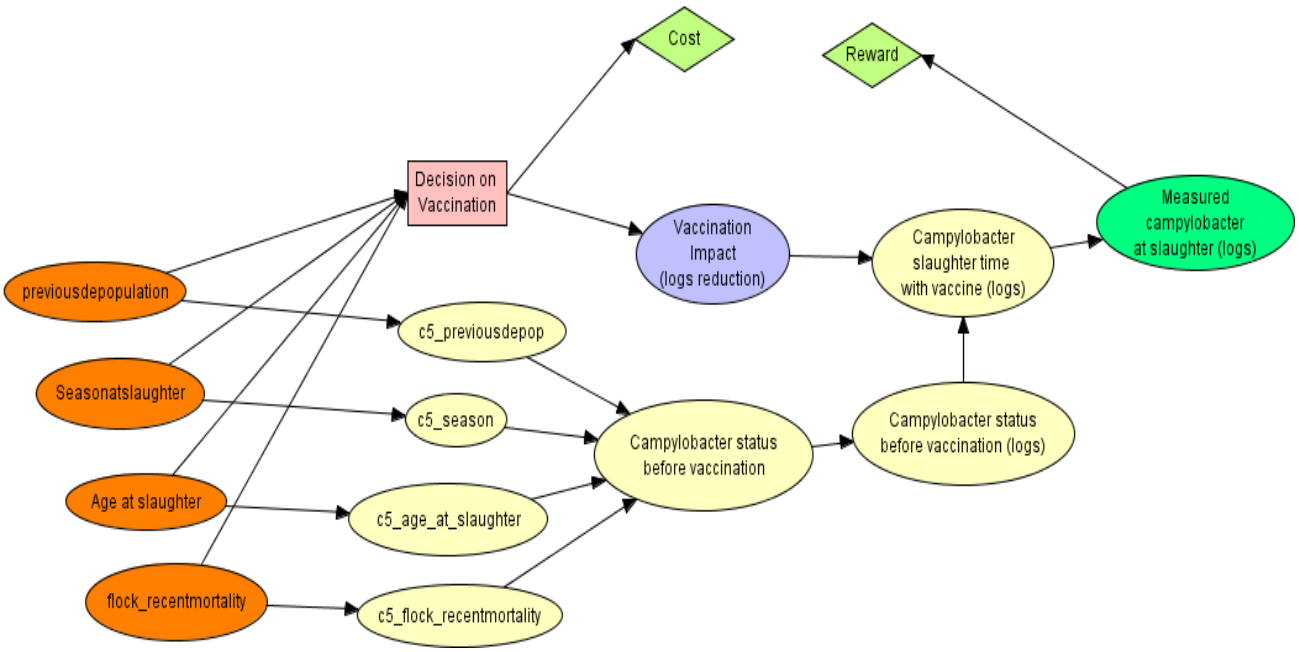
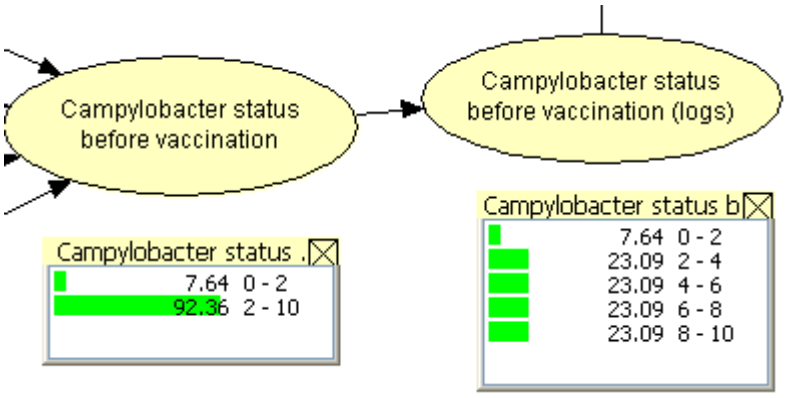


Figure 3. Illustration of the part of the model showing the *Campylobacter* status before vaccination in positive/negative format (0–2 logs is considered negative and 2–10 logs translates on a positive result) and in logs format (distribution of the different levels of *Campylobacter* in logs).



The quantitative part of the models encodes the mathematical expressions and probability distributions associated with the different states of the chance variables and utility functions associated with the utility nodes as defined by the structure of the influence diagram. For example, the following mathematical expression: "max (prob_season, prob_previousdepop, prob_flock_recentmortality,

prob_age_at_slaughter)" is introduced in the variable "*Campylobacter* status before vaccination" to calculate the posterior conditional probabilities based on probability distributions from the parent variables (Figure 4). There are probability tables for each variable which include probabilities for every state of the variables. These tables will contain the prior probability distributions for variables without parents in the model and the conditional probability distributions for variables with parents. Figure 4 illustrates probability tables for the variables: prob_season, prob_previousdepop, prob_flock_recentmortality and prob_age_at_slaughter in the Commercial Broilers Vaccination (ComBVacUK) model.

Figure 4. Probability tables for the variables: prob_season, prob_previousdepop, prob_flock_recentmortality and prob_age_at_slaughter.

prob_age_at_slaughter(prob_age_at_slaughter)

age at slaughte	>=46 days	42-45 days	40-41 days	36-39 days	<36 days
0 - 2	0.1555	0.4098	0.4374	0.6642	0.712
2 - 10	0.8445	0.5902	0.5626	0.3358	0.228

prob_flock_recentmortality(prob_flock_recentmortality)

flock recentmo	<1%	1-1.49%	>1.49%
0 - 2	0.712	0.6116	0.4743
2 - 4	0.228	0.3884	0.5257

prob_season(prob_season)

seasonatslaught	summer	autumn	winter or sp
0 - 2	0.1477	0.5925	0.712
2 - 10	0.8523	0.4075	0.288

prob_previousdepop(prob_previousdepop)

previousdepopı	no	yes
0 - 2	0.712	0.3218
2 - 10	0.288	0.6782

The prior probability distributions should integrate knowledge obtained from sources such as empirical observations, epidemiological data and experts in order to obtain reliable outcomes from the decision support models. Bayesian inference and probability theory set the basis for the quantitative outputs of the models. Decision support models can be updated with new evidence, knowledge or information.

2.2.4. Sensitivity Analyses

The aim of performing sensitivity analyses is to determine the sensitivity of the vaccination decision under different evidence scenarios with respect to single parameters of the models. In this particular case, two very different reward systems and a hypothetical vaccine C were included in the models. Reward system 2 was based on an extra payment for chickens testing *Campylobacter* negative of 2.5 times the normal price while reward system 3 was based on the existing reward systems in Denmark which is based on an extra payment of about 2% for flocks testing negative for *Campylobacter* and in Norway and Sweden where the payment is reduced by about 4% for flocks that are tested positive for *Campylobacter* (personal communication). A cost-efficient hypothetical vaccine

C able to reduce 2–6 logs the level of *Campylobacter* in 90% of the chickens was considered with a cost of 0.03 £/chicken.

3. Results

3.1. Results from the Model Commercial Broilers Vaccination UK Model (ComBVacUK)

The results from the model can be visualized by selecting diverse combinations of "nodes states" and obtaining the output in terms of the expected distribution of probabilities related to *Campylobacter* levels and expected cost-reward balance in every case. A high number of potential combinations or scenarios can be considered and therefore it is up to the user to select the relevant combination of present factors. In Table 4, we have described three combinations in order to illustrate the potential outputs of the model.

Table 4. Scenarios considered in the model; risk factors and their frequency of occurrence in every scenario.

Best-case scenario	**Worst-case scenario**	**"Most likely" scenario (based on study data [35])**
Spring or winter (100%)	Summer (100%)	Season Summer (26.32%) Autumn (25.38%) Spring or winter [a] (48.3%)
Age of broilers ≤36 days (100%)	Age of broilers ≥46 days (100%)	Age of broilers ≥46 days (19.59%) 42–45 days (15.67%) 40–41 days (18.57%) 36–39 days (21.98%) <36 days [a] (24.19%)
Flock recent mortality <1.00% (100%)	Flock recent mortality >1.49% (100%)	Flock recent mortality >1.49% (32.22%) 1.00%–1.49% (29.35%) <1.00% [a] (38.43%)
Previous partial depopulation No (100%)	Previous partial depopulation Yes (100%)	Previous partial depopulation Yes (64.94%) No [a] (35.06%)

[a] Reference category (mathematical models).

Results from the models (based on prior probabilities shown in Table 4) are included in Table 5 where expected posterior probabilities and expected cost-reward financial balances are presented.

Results from the model indicate that the financial results are relatively insensitive to choices in this case. There are no or only small financial gains when using vaccine B and reward system 1 under the specific assumptions considered in the model. Actually, in the best-case scenario the farmer will not gain financially when using vaccine B although the posterior probabilities related to expected high numbers of *Campylobacter* in the flock will be reduced. On the contrary, in the worst-case scenario the best option will be to use vaccine B because it produces the maximum cost-reward balance

(0.34 £/chicken) and a reduction on the probabilities related to expected high numbers of *Campylobacter* in the flock. Similarly, results obtained when considering the "most-likely" scenario based on study data [35] indicate that the best option will be to use vaccine B.

Table 5. Results based on the model Commercial Broilers Vaccination (ComBVacUK) using reward system1 (Table 3) and a hypothetical *Campylobacter* vaccine B.

	Scenarios		
	Best-case scenario	*Worst-case scenario*	*"Most likely" scenario (based on study data from the UK* [35]*)*
Posterior probabilities related to expected Campylobacter levels when implementing no additional protective measure	0–2 logs (25.70%) 2–4 logs (18.58%) 4–6 logs (18.58%) 6–8 logs (18.58%) 8–10 logs (18.58%) Cost-reward balance: 0.38 £/chicken Flock with 50,000 chickens = 19,000 £	0–2 logs (0.35%) 2–4 logs (24.91%) 4–6 logs (24.91%) 6–8 logs (24.91%) 8–10 logs (24.91%) Cost-reward balance: 0.33 £/chicken Flock with 50,000 chickens = 16,500 £	0–2 logs (7.27%) 2–4 logs (23.18%) 4–6 logs (23.18%) 6–8 logs (23.18%) 8–10 logs (23.18%) Cost-reward balance: 0.34 £/chicken Flock with 50,000 chickens = 17,000 £
Posterior probabilities related to expected Campylobacter levels after the implementation of a decision (Vaccine B)	*Vaccine B* 0–2 logs (35%) 2–4 logs (18.59%) 4–6 logs (17.67%) 6–8 logs (15.81%) 8–10 logs (12.93%) Expected cost-reward balance: 0.38 £/chicken Flock with 50,000 chickens = 19,000 £	*Vaccine B* 0–2 logs (12.82%) 2–4 logs (24.94%) 4–6 logs (23.70%) 6–8 logs (21.21%) 8–10 logs (17.34%) Expected cost-reward balance: 0.34 £/chicken Flock with 50,000 chickens = 17,000 £	*Vaccine B* 0–2 logs (18.87%) 2–4 logs (23.21%) 4–6 logs (22.05%) 6–8 logs (19.74%) 8–10 logs (16.14%) Expected cost-reward balance: 0.35 £/chicken Flock with 50,000 chickens = 17,500 £

3.2. Results from the Sensitivity Analyses

Sensitivity analyses performed indicated that the factors that influenced the results to a greater extent were the financial variables (cost/reward functions) and the effectiveness of the control strategy, e.g. vaccination impact. On the other hand, the results showed that the financial differences between diverse strategies were very small mainly due to the narrow differences between the levels of the reward system. The results indicated that when applying the reward system 2 (a system with higher differences between gross benefits obtained by farmers delivering chickens *Campylobacter* negative or with low *Campylobacter* numbers), the best solution in terms of maximum expected benefit would be using the vaccine C in all case-scenarios. Significant reductions in expected *Campylobacter* levels and substantial associated expected financial gains were obtained from this model when considering the reward system 2 and the use of vaccine C; for example, in the most-likely scenario, the expected benefit increased from 0.34 £/chicken to 0.69 £/chicken (translated to a flock with 50,000 chickens, from 17,000 £ to 34,500 £). However, when implementing reward system 3 (closer to real reward

systems currently employed in several countries) the best solution in financial terms will be "not vaccinating" even though the use of vaccine C could potentially reduce the expected posterior probabilities related to high numbers of *Campylobacter* in the flock significantly. In fact, the use of a hypothetical vaccine C in the most-likely scenario could reduce the probability of *Campylobacter* introduction into the flock from around 93% to approximately 46%.

4. Discussion

Poultry producers need to make important, complex decisions and related investments for the sustainability of their businesses. Increased consumer concerns related to food safety put pressure on food producers to implement food safety assurance systems. In particular, poultry producers should implement effective controls against *Campylobacter* in poultry to increase food safety and to reduce the burden of human Campylobacteriosis.

Different PGMs can be developed to assist in decision-making regarding *Campylobacter* vaccination of poultry and/or other *Campylobacter* control strategies. The graphical nature and decomposition into variables and relationships of PGMs make it possible to create a common generic model to assess diverse strategies for the control of *Campylobacter* in poultry. Nevertheless, it seems challenging to design a general model (qualitative and quantitative) that could be applied to all situations, poultry farming conditions and geographical areas. Furthermore, the conditions, selection of factors or variables, different parts of the models, and quantitative data need to be clearly specified to add value and perspective to the decision support system designed in every case. Tailor-made properly developed PGMs will help poultry managers make important decisions in order to solve complex problems such as the control of *Campylobacter*. PGMs can be extended and/or modified to adapt to different real circumstances. For example, the time of slaughter might vary depending on the final product. In addition, the assumption about independence between factors gives flexibility to include supplementary factors or new knowledge in the model.

Microbiological methods for the detection and quantification of *Campylobacter* can be used to assess the *Campylobacter* status of birds. However, it seems important to distinguish between the true numbers of *Campylobacter* in birds and the detected or measured numbers. There are several microbiological techniques available for the detection and enumeration of *Campylobacter* spp. from different sample matrices. However, some techniques are still under development and the detection limit of most methodologies seems to be 100 CFU/g or mL (depending on sample type and sample preparation). Therefore, a negative result might actually indicate very low numbers of *Campylobacter* (1–100 CFU/g or mL). Moreover, microbiological sampling and processing methods will not be perfect and in reality, the sampling procedures and microbiological techniques will affect the estimates of the true numbers of *Campylobacter* in chickens and in poultry flocks. In this model, we assumed a nearly-perfect quantitative method but other tests and/or other uncertainties related to microbiological sampling could be considered in the models. Similarly, diverse sources of contamination of broiler flocks could be included. Sources of *Campylobacter* contamination might be implicit in some risk factors (e.g., biosecurity). In this model, the presence of flies (a potential source of *Campylobacter* contamination) could be a confounder with the risk factor "season: summer". Nevertheless, other potential *Campylobacter* sources could be considered, increasing the complexity of the models.

In the UK, human Campylobacteriosis represents an important public health problem [32,33]. Estimates of *Campylobacter* prevalence in UK poultry flocks can be found in the literature, e.g., 75% in the EU baseline survey carried out in 2008 [46] and 79.2% in the considered study from the UK [35] which are average prevalence values obtained from sampling a number of poultry flocks for human consumption. The introduction of *Campylobacter* in the food processing environment poses a risk for the contamination of food products; in fact, in the EU baseline survey carried out in 2008, 86% of the UK poultry carcasses tested were found positive for *Campylobacter*. It seems crucial to reduce the number of *Campylobacter* positive flocks and the numbers of *Campylobacter* in chickens and their products. The control of *Campylobacter* in poultry could translate to a decrease in the incidence of human Campylobacteriosis cases in the UK.

The results from the model presented here indicated that the posterior probability of introduction of *Campylobacter* into the UK poultry flock in the most likely scenario before vaccination was 92.36% based on the assumptions and data specified in this manuscript. The posterior probability of *Campylobacter* introduction into the flock decreased significantly by the use of a hypothetical vaccine B (to approximately 81%) and even more when using a much more effective hypothetical vaccine C (to approximately 46%). The results indicated that the public health impact of the control strategies will depend on the effectiveness of the controls. However, the assessment of the effectiveness of diverse control strategies might prove challenging in some cases, e.g., the assessment of vaccine effectiveness [29]. In any case, decreasing the probability of *Campylobacter* introduction into poultry flocks is highly desirable. The EU baseline survey carried out in 2008 identified a trend in countries with higher prevalence of *Campylobacter* positive poultry flocks to produce poultry carcasses with high numbers of *Campylobacter* due to *Campylobacter* in the intestines of infected chickens contaminating the food processing environment and the poultry products [46]. In fact, high numbers of *Campylobacter* in the cecum of chickens for slaughter can correlate with high numbers of *Campylobacter* on chicken carcasses [47]. *Campylobacter* control strategies that can significantly reduce the probability of *Campylobacter* introduction into a flock and/or the numbers of *Campylobacter* in already infected chickens should be implemented from a public health perspective. On the other hand, poultry producers will usually make strategic decisions based on financial gains and therefore a reward system that can translate to an attractive cost-reward balance will be a good incentive for poultry producers to implement *Campylobacter* control strategies. In actual fact, the financial results obtained from the model when using the reward system 1 and a hypothetical *Campylobacter* vaccine B indicated that the expected financial gains might be too small to justify the use of vaccine B in this case. Nevertheless, this type of information might prove very valuable and it is likely that producers will find this decision-making tool more beneficial at times when the consequences from implementing alternative decisions for the control of *Campylobacter* are not very clear. In contrast, when considering the reward system 2 and the use of vaccine C, significant reductions in expected *Campylobacter* levels and substantial expected financial gains were obtained. Sensitivity analyses can be used to test diverse hypothetical vaccines and reward systems in order to compare them and their combinations.

The aim of the sensitivity analyses was to determine the sensitivity of the vaccination decision under different evidence scenarios with respect to single parameters of the models but we did not perform sensitivity analyses on the probabilistic quantification of the model. The cost-reward functions are crucial drivers for the selection of the optimal decision which is determined based on the principle

of maximum benefit (cost-reward balance). It is important to bear in mind that in the model the cost function relates only to the cost of the control measure and does not include any other additional costs such as those related to microbiological testing.

Financial data considered in the models should be as accurate as possible (e.g., cost of a specific *Campylobacter* control). The reward system might not be in place in most parts of the world, therefore it should be hypothesized and tailor-made based on the gross profit/per chicken for farmers in specific areas and/or production systems (e.g., organic farmers might obtain a higher gross profit/chicken than farmers producing commercial broilers). The reward system currently used in Denmark is based on an extra payment of about 2% for flocks testing negative for *Campylobacter* while in Norway and Sweden the payment is reduced by about 4% for flocks that test positive for *Campylobacter* (personal communication). The results from the model presented here indicate that it might be useful for the reward system to be based on an increased extra payment for flocks testing negative for *Campylobacter* in order to justify financially the use of a commercial vaccine against *Campylobacter*. However, financial gain will depend on the effectiveness of the vaccine (and/or other control strategies) and the costs associated with the controls. A cost-efficient vaccine against *Campylobacter* in chickens is not commercially available at present. We considered that the market price of a cost-effective vaccine against *Campylobacter* in chickens should be less than 10% of the gross profit per chicken to be competitive. Nonetheless, the market price could be higher depending on the effectiveness of the vaccine and the reward system.

There are many potential strategies for the control of *Campylobacter* in poultry that could be included in the models but the complexity of the models will increase significantly. *Campylobacter* vaccination strategies have been considered in the models presented in this paper but the authors are working on different models where three *Campylobacter* control strategies (and their combinations) are included. Consequently, the selection of *Campylobacter* control strategies in poultry will become more and more complex due to the increased number of possibilities, and poultry producers may benefit from the use of decision support models. The flexibility of PGMs allows for the inclusion of more than one hypothetical *Campylobacter* vaccine and other control measures and more than one reward system. The users might then obtain a range of potential solutions for the control of *Campylobacter* in poultry. The most profitable solutions will be more attractive for poultry farmers, although they might not be feasible in the real world. On the other hand, some producers may be inclined to implement food safety controls (even when there is little financial reward involved) if the controls improve the image of their brands and/or the producers feel pressure from consumers and/or governments.

From a public health perspective, results from the model in terms of expected reductions in the numbers of *Campylobacter* in chickens after the implementation of controls could be translated into the expected decrease in human Campylobacteriosis cases and expected reductions in associated health care costs using mathematical models. However, at present, a risk assessment model to estimate the number of human cases based on the occurrence of *Campylobacter* in chickens sent for slaughter does not seem to be available. Information related to public health benefits that could be obtained from the implementation of cost-effective *Campylobacter* controls in poultry will prove very useful, for example, when considering future reward systems.

PGMs represent knowledge and probabilistic conditional relationships in structured models designed to represent real situations where uncertainty plays an important role. The integration of information, knowledge and technology is crucial to discover new/better solutions to complex problems [48,49] and may aid poultry farmers to make optimal decisions on the implementation of controls against *Campylobacter*. In addition, engagement of different stakeholders in the PGMs' development process is highly desirable. The use of sophisticated and complex computing interfaces, mathematical expressions and probability distributions needs to be reconciled with a simple and efficient tool that can be used by different stakeholders [50,51]. Considerations regarding the epidemiological and microbiological factors to be included in the models together with important challenges for the development of the quantitative part of the models have been presented in this manuscript.

5. Conclusions

Poultry producers should implement cost-effective *Campylobacter* control strategies in order to protect public health and to reduce the burden of human Campylobacteriosis. Decision support tools such as probabilistic graphical models (PGMs) will aid poultry producers to select cost-effective *Campylobacter* control strategies. The cost-reward functions and the effectiveness of the control strategies integrated in the models are crucial drivers for the selection of optimal decision/s. The public health impact of the control strategies depends on the effectiveness of the controls. The model's optimal decision in every case is determined based on the principle of maximum benefit (cost-reward balance). Poultry producers will be able to choose from a range of potential solutions for the control of *Campylobacter* in poultry. Some decisions might be ideal from a public health perspective but may be costly for producers. The flexibility of PGMs allows for the consideration of diverse real-life circumstances, the integration of new knowledge, the inclusion of more than one *Campylobacter* control measures and more than one reward system. Nonetheless, the selection of epidemiological evidence, qualitative and quantitative data needs to be clearly specified to add value and perspective to the decision support system designed in every case.

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Conflicts of Interest

The author declares that there are no conflicts of interest.

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Manuscript VI

Prevention of human campylobacteriosis and a decision support system for the control of *Campylobacter* in chickens, Denmark

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Abstract

The control of *Campylobacter* in poultry is considered a public health priority and some intervention strategies have been implemented in Denmark. Nonetheless, *Campylobacter* infection in poultry can still be considerable particularly during the summer when the most promising *Campylobacter* control strategy seems to be the use of fly screens. The use of cost-effective vaccines against *Campylobacter* is also desirable. Poultry producers need to make crucial decisions related to the control of *Campylobacter* under conditions of uncertainty. This manuscript describes a decision support model (for *Campylobacter* control in poultry flocks) which integrates knowledge and use a Bayesian approach to handle uncertainty. The model integrates epidemiological data, microbiological considerations, financial information and potential control strategies (the use of fly screens and hypothetical vaccines). The results from the model and sensitivity analyses indicate that the financial variables (cost/reward functions) and the effectiveness of the control measures drive the model's results.

Introduction

Human campylobacteriosis represents an important public health issue and it has been notifiable in Denmark since 1980. Fresh chicken meat is considered one of the most important risk factors for human infections with *Campylobacter* in Denmark (1). A risk profile for pathogenic *Campylobacter* was conducted in 1998 (2) and ever since broiler flocks have been tested for *Campylobacter* at slaughter. The first Danish Action plan against *Campylobacter* in broilers was established in 2003 (3). A more recent action plan to control *Campylobacter* in broilers and broiler meat was implemented in Denmark in 2008. Between 2001 and 2009, *Campylobacter* prevalence in Danish broiler flocks before slaughter decreased from around 40% to about 30% (4). A recent report from the European Food Safety Authority stated an average prevalence of 19.2% for *Campylobacter* in Danish broiler flocks (5). Nevertheless, *Campylobacter* prevalence in broiler flocks during the summer months in Denmark can be as high as 51.4% (6). Researchers have reported several risk factors significantly associated with *Campylobacter* infection of broilers in Denmark such as late introduction of whole wheat in the chickens' diet, improper storage of whole wheat, age of birds at slaughter, old broiler houses, number of chimneys on the broiler house, improper rodent control, number of broiler houses on farm and the location of the poultry farm in relation to cattle farms (7-9). A recent Danish study (10) reports that season, increasing age of the birds and several factors related to biosecurity (age of the poultry houses, previous *Campylobacter* positive flocks in the same houses and the number of persons entering the houses) were significantly associated with *Campylobacter* infection of broilers. Field trials conducted in Denmark showed that the use of fly screens in broiler houses during the summer can reduce the prevalence of *Campylobacter* in Danish broilers (6, 11). A recommendation of using fly-nets in all the houses on a farm has been made because transmission of *Campylobacter* from the non-netted houses to the netted houses has been considered probable (5, 12). In fact, the use of fly screens on farm may decrease *Campylobacter*

prevalence in broilers by 10-30% (6) which in turn may translate in a significant reduction of human campylobacteriosis cases.

Alternative *Campylobacter* control measures that can reduce the numbers in already infected chickens such as vaccination strategies might also be considered. Effective vaccination is highly desirable; however, a cost-efficient vaccination strategy against *Campylobacter* in poultry is not commercially available at present. In the last couple of years, we have conducted studies aiming to reduce the numbers of *Campylobacter* in chickens and their products by developing a vaccination strategy against *Campylobacter* in poultry (13). In addition, a decision support tool has been developed to assist poultry producers to make complex decisions (sometimes expensive investments) under conditions of uncertainty for the control of *Campylobacter* in poultry flocks (14, 15).

The decision support system presented in this manuscript is constructed as a probabilistic graphical model (PGM) which integrates prior knowledge related to the farm as well as the expected cost-effect of two different *Campylobacter* controls on-farm: hypothetical *Campylobacter* vaccines and/or the use of fly screens. The integration of prior knowledge (prior probabilities) can be used to infer the probabilities of other variables that are not known (posterior probabilities) using a Bayesian approach. The prior probability distributions should integrate knowledge obtained from diverse reliable sources such as epidemiological data, empirical observations and expert knowledge in order to obtain reliable outcomes from the models. Epidemiological data, microbiological considerations, financial information and diverse control strategies have been integrated in the model. The solution of the developed PGM offers posterior probabilities related to expected *Campylobacter* levels in chickens before and after implementation of controls and a cost-benefit balance for each control strategy considered. Poultry producers can adapt the model to real-life

conditions and decide if/when to use fly screens and/or vaccines selecting the most beneficial decision/s in every real-life situation.

MATERIALS AND METHODS

Probabilistic Graphical Models (PGMs)

Poultry producers need to make decisions related to the implementation of control strategies against *Campylobacter* in poultry flocks before they even know if the birds will be challenged or infected with Campylobacter. The probabilistic graphical model presented in this manuscript has been designed using the HUGIN tool which is a commercial software package (16). Briefly, PGMs include prior knowledge and uncertainties using a number of variables and probabilistic dependence relationships between the variables (14, 15). PGMs formed by a set of variables have two components: a qualitative and a quantitative part. The qualitative part includes diverse "nodes" such as variables, decision nodes and utility functions and arcs representing relationships between them (as exemplified in Figure 1). A chance node (an oval in Figure 1) represents a random variable, a decision node (a rectangle in Figure 1) defines decision alternatives at a specific point in time and a utility node (a diamond in Figure 1) represents a reward or cost function. The relationships of dependence or independence between the variables included in the models can be represented and the strength of the relationships can be defined using conditional probability distributions (17). The quantitative part of the models includes the mathematical expressions and probability distributions. The results from the models comprise posterior probability distributions (for every strategy) related to expected *Campylobacter* numbers in the flock (in logs) before and after the implementation of the decision/s and the expected cost-reward balance associated with each decision/s.

Prior knowledge related to poultry production management factors

A decision model has been developed (Figure 1) based on the results from an epidemiological study on risk factors potentially associated with *Campylobacter* infection in Danish broiler flocks (10). These authors analysed data from 2835 flocks originating from 187 farms from December 2009 to November 2010 (database: Quality Assurance System in Danish Broiler Production). The database included more than 1700 variables and the following risk factors were found significantly associated with *Campylobacter* positive flocks: season, increasing age of the birds, the age of the poultry houses, previous *Campylobacter* positive flocks in the same houses and the number of persons entering the poultry houses (10). Odds Ratio (OR) values were used to represent the concept of "increased risk" due to the factor/s considered in their research. To integrate this information into the PGM, the significant risk factors were included in the model (Figure 1) and the OR values were transformed to a probability infection with Campylobacter at varying states of the risk factor (P(s)) using formula 1 (Table 1). In addition, *Campylobacter* introduction in the flock due to the presence of risk factors was assumed to be conditional to a baseline *Campylobacter* flock prevalence (b_p: lowest level of *Campylobacter* in broilers close to slaughter time found in the literature) of 14 % (10).

Formula 1 Formula applied to calculate probabilities of the diverse states of risk factors (P(s)) based on the baseline Campylobacter flock prevalence (b_p) and odds ratios (OR_s)

$$P(s) = \frac{\exp(\ln(b_p/(1-b_p)) + \ln(OR_s))}{1+\exp(\ln(b_p/(1-b_p)) + \ln(OR_s))}$$

Table 1 Significant risk factors, frequency of occurrence and associated probability of Campylobacter introduction in broiler flocks in Denmark (Chowdhury et al., 2012)

Risk factor and frequency of occurrence (ref.)	**p-value**	**Odds Ratio (95% CI)**	**Probability Campylobacter positive***
Season	<0.001		
Summer (28.05%)		12.59 (6.79-23.36)	0.6721
Fall (22.15%)		5.27 (2.77-10.02)	0.4618
Spring (25%)		1.33 (0.66-2.67)	0.178
Winter (24.8%)[a]			
Number of persons entering the house	0.009		
>1 person (85.97%)		2.03 (1.19-3.84)	0.2482
1 person (14.03%) [a]			
Campylobacter infection status (previous flock) Positive (21.61%)	0.002	1.80 (1.22-2.63)	0.2266
Negative (78.39%)[a]			
Establishment year of the house	0.002		
Before or during 1990 (46.03%)		1.60 (1.17-2.18)	0.2066
After 1990 (53.97%)[a]			
Age of birds at slaughter	0.04		
>35.5 days (45.68%)		1.33 (1.02-1.76)	0.178
<=35.5 days (54.32%)[a]			
[a] Reference category.			
****Based on a basic level of Campylobacter of 14% (Chowdhury et al., 2012)***			

The "Commercial Broilers in Denmark" model design and assumptions

This model (Figure 1) was developed to assist poultry producers in Denmark to make decisions regarding *Campylobacter* controls in commercial broilers based on the following assumptions:

- A microbiological detection level of *Campylobacter* was considered to be 2 logs CFU/g or ml of sample while the maximum colonization level was assumed to be 10 logs CFU/g or ml of sample. Thus, in this model, a *Campylobacter* level of 0-2 logs will produce a negative result while a positive result suggests *Campylobacter* numbers present in the samples from 2 to 10

logs. Intervals for bacterial concentration with two log widths (e.g.0-2 logs, 2-4 logs, 4-6 logs, 6-8 logs and 8-10 logs) were considered.

- The contributions from different risk factors to the level of *Campylobacter* are independent.
- The "measured *Campylobacter* numbers at slaughter" will depend on the microbiological quantitative methods used and the "true numbers" of *Campylobacter* in chickens. In the model, a hypothetical nearly-perfect quantitative method has been considered.
- The *Campylobacter* controls included in the model are: the use of fly screens and hypothetical *Campylobacter* vaccines A and B. Fly screens are used during the summer but the costs associated with the use of fly screens are always considered (18).

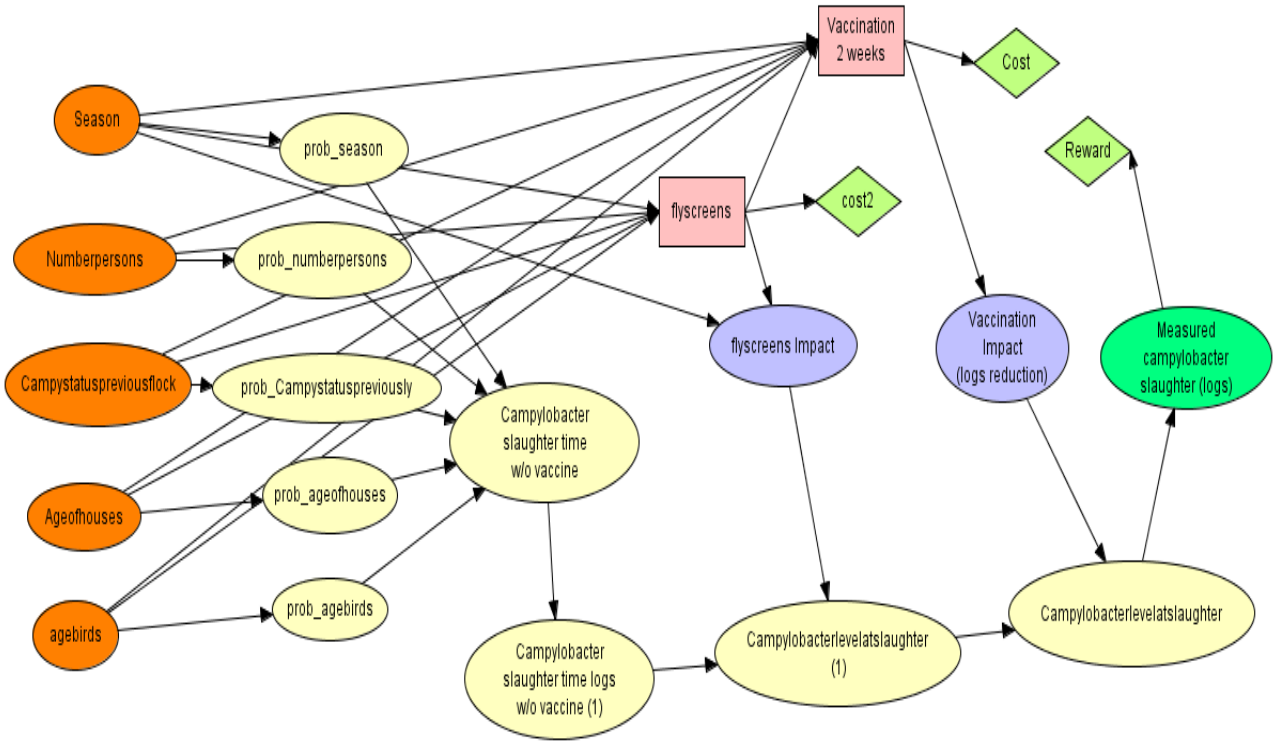

Figure 1 The Commercial Broilers in Denmark (ComBDK) model designed based on epidemiological and financial data from Denmark

Data related to the influence of the risk factors on the expected *Campylobacter* status of the flock are based on a positive (2-10 logs)/negative (0-2 logs) result while data related to the effect of

interventions are based on a log-scale because the objective is to develop a control strategy (e.g. vaccination strategy) able to reduce the numbers of *Campylobacter* in commercial broilers. Data must be on the same scale to obtain reliable results from the model. For this reason, a mathematical transformation from positive/negative to the diverse *Campylobacter* levels (in logs)-scale has been performed. Figure 2 illustrates the mathematical transformation used to translate results from a positive/negative format ("Campylobacter slaughter time w/o vaccine") to a log scale format ("Campylobacter slaughter time logs w/o vaccine"). A flat distribution has been selected to transform a general expected posterior probability of a Campylobacter flock (e.g. 71.60 %) into a distribution of equal probabilities (17.90 %) for the different levels of Campylobacter considered in the model (Figure 2).

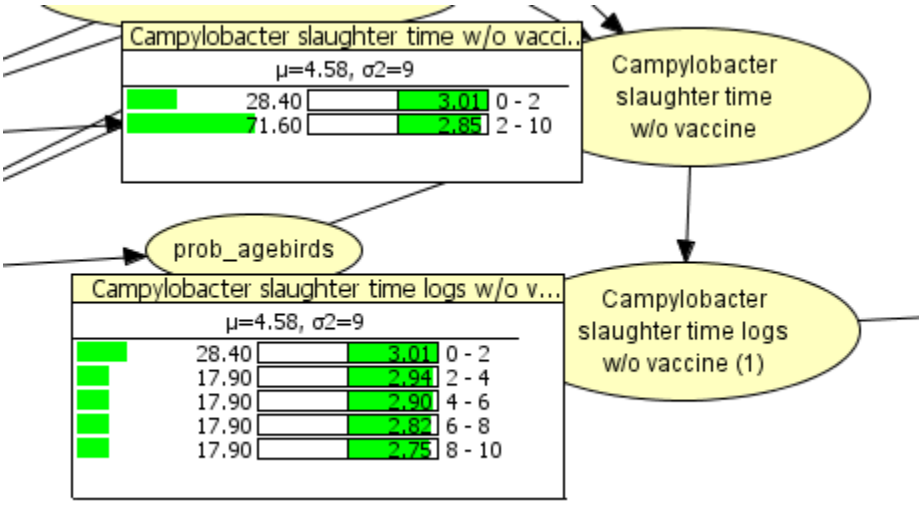

Figure 2 Illustration showing the part of the model where a mathematical transformation is performed to translate results from a positive/negative format ("Campylobacter slaughter time w/o vaccine") to a log scale format ("Campylobacter slaughter time logs w/o vaccine").

The effectiveness of Campylobacter controls and cost-reward functions included in the model

The use of a cost-effective vaccine against *Campylobacter* in chickens is desirable but no commercial vaccine (and price) is currently available. Based on prices of other vaccines used to

control diseases in poultry (19), a hypothetical price can be considered to be around 0.15-0.50 Danish Kroners (DKK). Two hypothetical vaccines (A and B) were incorporated in the model with different vaccine effectiveness and costs (Table 2).

The use of fly screens in poultry houses in order to control *Campylobacter* has been recommended (6, 12, 20). The average cost (including capital costs that cover long term investments and variable expenses) of implementing fly screens on broiler farms has been calculated to be 0.13 DKK/chicken (18) and it has been included in the model (Table 2). The impact of interventions and the cost-reward functions are based on a log-scale because the objective is to develop a control strategy that can reduce the numbers of *Campylobacter* in already infected chickens. The use of fly screens can reduce the prevalence of Campylobacter in broiler flocks significantly and therefore, in the model it was assumed that the effectiveness of fly screens was 90% (expected reduction of *Campylobacter* numbers in more than 2 logs).

Table 2 Costs and effectiveness related to control measures included in the model

Control measure	**Cost (DKK/chicken)**	**Effectiveness (expected reductions of Campylobacter numbers in more than 2 logs)**
Vaccine A	-0.19	40%
Vac B	-0.45	80%
Fly screens	-0.13	90%
Vac A + fly screens	-0.32	95%
Vac B + fly screens	-0.58	100%

The reward system (Table 3) has been designed based on an "average" gross profit (for Danish farmers producing chickens carrying an "average" number of *Campylobacter)* considered to be 2.92 (DKK/chicken) based on financial data from 2013 (21). It was assumed that an average broiler chicken from a positive flock in Denmark will carry *Campylobacter* in a concentration of 4 to 6 logs CFU/g or ml of sample (from the digestive tract). The reward (reward system 1) is based on a system implemented in Denmark where poultry producers get an extra payment when the flock is

identified as *Campylobacter* negative before slaughter (personal communication). In the model, an extra payment (around 2%) is given to farmers producing chickens carrying *Campylobacter* in numbers less than 4 logs and a reduced payment (about 4% less) is established for farmers producing poultry flocks that carry *Campylobacter* in numbers greater than 6 logs before slaughter (Table 3).

Table 3 Reward system 1

Campy level (logs)	0-2	2-4	4-6	6-8	8-10
Gross profit (DKK/chicken)	3.04	2.98	2.92	2.80	2.69

Sensitivity analyses

Sensitivity analyses were performed in order to determine the sensitivity of the decisions considered in the model under diverse evidence scenarios with respect to single parameters of the models. In the Commercial Broilers in Denmark (ComBDK) model, the following were included: a different reward system 2 (based on an extra payment of 2.5 times the normal price for chickens testing *Campylobacter* negative before slaughter) and a cost-effective hypothetical vaccine C (with an effectiveness level of 90% and a cost of 0.26 DKK/ chicken).

RESULTS

Interpretation of results obtained from the ComBDK model

A high number of the diverse risk factors' states and combinations or scenarios could be considered in the model and therefore it is up to the user to select the most adequate combination for the real life problem under investigation. Three combinations are described in Table 4 in order to illustrate the potential outputs of the models in terms of expected posterior probabilities related to *Campylobacter* numbers in chickens and associated expected cost-benefit analyses (Table 5).

Table 4 Risk factors and frequency of occurrence in every scenario

Best scenario	**Worst scenario**	**Real-life scenario (based on study data)**
Winter (100%)	Summer (100%)	Summer (28) Fall (22%) Spring (25%) Winter (25%)
Age of broilers ≤35.5 days (100%)	Age of broilers >35.5 days (100%)	Age of broilers >35.5 days (46%) ≤35.5 days (54%)
Number of persons 1 person (100%)	Number persons more than one person (100%)	Number of persons 1 person (14%) >1 person (86%)
Campylobacter status of previous flock Negative (100%)	Campylobacter status of previous flock Positive (100%)	Campylobacter status of previous flock Positive (22%) Negative (78%)
Age of poultry houses After 1990 (100%)	Age of poultry houses Before 1990 (100%)	Age of poultry houses After 1990 (54%) Before 1990 (46%)

Table 5 Results from "Commercial Broilers in Denmark" model

Scenarios	**Best**		**Worst**		**"most likely"**	
Decisions	***Posterior probabilities related to expected Campylobacter positive results (2-10 logs)***	**Expected cost-reward financial balance Gross profit (DKK/ chicken)**	***Posterior probabilities related to expected Campylobacter positive results (2-10 logs)***	**Expected cost-reward financial balance Gross profit (DKK/ chicken)**	***Posterior probabilities related to expected Campylobacter positive results (2-10 logs)***	**Expected cost-reward financial balance Gross profit (DKK/ chicken)**
None	55%	+2.94	87%	+2.87	72%	+2.90
Vac A	42%	+2.78	65%	+2.74	55%	+2.76
Vac B	30%	+2.55	46%	+2.53	39%	+2.54
Fly screens	55%	+2.81	13%	+2.89	52%	+2.81
Vac A + fly screens	42%	+2.65	11%	+2.71	40%	+2.66
Vac B + fly screens	30%	+2.42	9%	+2.45	29%	+2.43

Results obtained from the model based on the previously described assumptions suggested that the best solution in financial terms will be to use the fly screens alone in the worst case scenario and not to implement the controls under the assumed conditions in the "most likely" and best case scenarios. Nevertheless, the best solution from a public health point of view will be the use of fly screens and vaccine B synergistically. Based on the results from the model, this strategy (fly screens and vaccine B) can decrease significantly the posterior probability related to expected *Campylobacter* positive results in all scenarios (from 72% to 29% in the most likely scenario; from 55% to 30% in the best scenario and from 87% to 9% in the worst case-scenario) although the implementation of this strategy will translate on a decrease of expected gross profit (around 0.50 DKK/chicken). During the summer, the use of fly screens alone and/or synergistically with vaccines A or B may be able to reduce the posterior probability related to expected *Campylobacter* positive results below the considered "baseline level of 14%". In fact, using fly screens alone during the summer will reduce the posterior probability related to expected *Campylobacter* positive results from 87% to 13% resulting also in a small increase of the expected gross profit (based on the cost-reward balance).

3.2. Results from the sensitivity analyses

Results from the model when using the reward system 2 (based on an extra payment of 2.5 times the normal price for chickens testing *Campylobacter* negative before slaughter) and the diverse control options (including vaccine C) are presented in Table 6.

Table 6 Results from the model when considering reward system 2 and an additional vaccine C

Scenarios	**Best**		**Worst**		**"most likely"**	
Decisions	***Posterior probabilities related to expected Campylobacter positive results (2-10 logs)***	**Expected cost-reward financial balance Gross profit (DKK/ chicken)**	***Posterior probabilities related to expected Campylobacter positive results (2-10 logs)***	**Expected cost-reward financial balance Gross profit (DKK/ chicken)**	***Posterior probabilities related to expected Campylobacter positive results (2-10 logs)***	**Expected cost-reward financial balance Gross profit (DKK/ chicken)**
None	55%	+4.89	87%	+3.37	72%	+4.07
Vac A	42%	+5.47	65%	+4.46	55%	+4.92
Vac B	30%	+5.87	46%	+5.29	39%	+5.56
Fly screens	55%	+4.76	13%	+6.76	52%	+4.90
Vac A + fly screens	42%	+5.34	11%	+6.68	40%	+5.44
Vac B + fly screens	30%	+5.74	9%	+6.51	29%	+5.80
Vac C	29%	+6.18	44%	+5.69	37%	+5.91
Vac C + fly screens	29%	+6.05	9%	+6.72	28%	+6.10

Sensitivity analyses performed showed that the financial variables (cost/reward functions) and to a lesser extend the effectiveness of the control measures (e.g. vaccination impact) drive the model's results. The results presented in Table 5 (Reward system 1) and Table 6 (Reward system 2) indicate that the posterior probabilities related to expected *Campylobacter* positive results are the same but the financial results (cost-benefit balances) are very different. As a result of implementing reward system 2 the farmers will potentially obtain higher payments and the differences between implementing diverse controls are more pronounced.

The effectiveness of vaccines B and C are very similar but vaccine C is more cost-effective and desirable for this reason. From a public health perspective, the best *Campylobacter* control strategy will be the use fly screens together with vaccine B or C. However, from an economic point of view,

the best solution will depend on the scenario considered, for example, in the most likely scenario, the best solution will be using fly screens and vaccine C while in the worst case scenario (during the summer) the use of fly screens alone is most rewarding financially. The use of vaccine C alone in the best case scenario seems to be the most cost-effective strategy based on the results from the model.

Discussion

Poultry producers need to make decisions and sometimes expensive investments to control *Campylobacter* before they even know for sure if the flock will become infected or challenged with *Campylobacter*. The integration of increasing amounts of knowledge from diverse sources makes the decision process complex but if done properly, the accuracy of the results will increase and there will be less uncertainty surrounding the decision making process. Bayesian inference is used when decisions have to be made under conditions of uncertainty and statistical inference is required (22). Many potential risk factors for the introduction of *Campylobacter* in poultry flocks may be considered in predictive models increasing their complexity (15). The development of a generic global model would be almost utopic because risk factors may vary between geographical regions as well as between production systems (23-25). For this reason, it seems useful to include risk factors found to be statistically significant for the introduction of *Campylobacter* in poultry flocks under specified conditions. Results related to the control of *Campylobacter* in broiler flocks in Denmark obtained from the developed model depend on the qualitative and quantitative data considered. The risk factors incorporated in this model were those found significant for the presence of *Campylobacter* in Danish broilers in a recent study (10). Other studies have also found a clear correlation between biosecurity-related risk factors and flock infection with *Campylobacter* (26, 27).

More *Campylobacter* controls could have been incorporated in the model but the complexity will increase accordingly. For example, hygiene barriers have been suggested as the most important biosecurity measures to produce *Campylobacter*-free chickens (28-31). The use of fly screens has been recommended for the control of *Campylobacter* in Danish broiler systems (5, 6, 12, 20). The effectiveness of the use of fly screens against *Campylobacter* in poultry has been reported in terms of decreased *Campylobacter* prevalence in broiler flocks in Denmark. The experimental use of fly screens during the 2006 summer (June–October) produced a statistically significant decrease from 51.4% to 15.4% of *Campylobacter* positive flocks in comparison with control houses (6). A recently published study (20) reports data related to the long-term effect of the use of fly screens on the prevalence of *Campylobacter* in broiler flocks in Denmark collected over the years 2006-2009. These authors reported a statistically significant decrease in *Campylobacter* prevalence from 41.4% in 2003–2005 (before the use of fly screens) to 10.3% in 2006–2009. The use of fly screens was tested on poultry farms in Iceland during the summer of 2008 and significant reductions on *Campylobacter* prevalence were observed (32). Results from the experimental studies previously mentioned relate to average *Campylobacter* prevalence values obtained from diverse farms and flocks and therefore different farming conditions.Comparison of the results related to the use of fly screens on broiler farms previously reported (6, 20, 32) with the results from the ComBDK model presented here are not straight forward. Results from the ComBDK model are based on one flock level and the expected posterior probability related to a *Campylobacter* positive result for that flock under specific conditions. Even more, a “nearly perfect” quantitative microbiological test has been incorporated in the model and consequently results related to the expected probability of a *Campylobacter* positive result might be higher than the real result if a quantitative *Campylobacter* test was performed in that particular flock (the detection limit of most microbiological test are

around 2 logs). Nonetheless, the flexibility of the model allows the user to test diverse controls, different farming conditions, microbiological protocols and cost-reward systems.

Sensitivity analyses performed indicated that the factors that influenced the results to a greater extent were the financial variables (cost/reward functions) and the effectiveness of the *Campylobacter* control strategies. The assessment of the effectiveness of control strategies might prove challenging in some cases e.g. the assessment of vaccine effectiveness (13). The effectiveness of fly screens was assumed to be 90% but only used during the summer. The financial impact of the implementation of disease control strategies needs to be considered in disease control programs. The financial results obtained from the model seemed to be highly dependent on the reward systems and the scenarios considered. From a public health perspective, the best *Campylobacter* control strategy will be the use fly screens alone or synergistically with vaccine B or C. In some occasions, the financial results obtained from the model indicated that the expected financial gains might be too small to justify the implementation of controls. Nevertheless, this type of information might prove very valuable and it is likely that producers will find this decision making tool more beneficial at times when the consequences from implementing alternative decisions for the control of *Campylobacter* are not so obvious. In contrast, when considering the most cost-efficient choices (e.g. reward system 2 and the use of vaccine C) significant reductions in expected *Campylobacter* levels and substantial expected financial gains were obtained. Sensitivity analyses can be used to test diverse hypothetical vaccines and reward systems in order to compare them and their combinations. Clearly, cost-effective control measures will be preferred by poultry producers and a reward system that can translate on an attractive cost-reward balance will be a good incentive for producers to implement *Campylobacter* control strategies in poultry production. The implementation of cost-effective control strategies during primary poultry production will potentially translate into a decrease of *Campylobacter* prevalence in chickens, a reduction of

Campylobacter numbers in poultry products and in turn, a decrease of human campylobacteriosis cases. From a public health perspective, a *Campylobacter* prevalence level of 10% in broilers could potentially result in a reduction of human cases by nearly 50% (5, 33).

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9. GENERAL DISCUSSION AND CONCLUSIONS

A reduction of the incidence of human campylobacteriosis cases has been documented following temporary limitation on the consumption of chicken meat in the Netherlands and Belgium, after successful implementation of *Campylobacter* controls in poultry in Iceland, Denmark and New Zealand (Vellinga *et al.*, 2002; Stern *et al.*, 2003; Rosenquist *et al*., 2009; EFSA, 2010b; Sears *et al.*, 2011) and it has been forecasted by risk assessment models (Rosenquist *et al*., 2003; Nauta *et al*., 2009; EFSA, 2011b). Risk assessments conducted in Denmark (Rosenquist *et al*., 2003), the Netherlands (Nauta *et al*., 2005), United Kingdom (Hartnett *et al*., 2001, 2002) and New Zealand (Lake *et al.*, 2007) provide "farm-to-fork" estimations of the risk of human *Campylobacter* infection via the consumption of poultry meat. Risk assessments aim to assess the public health risks associated with the consumption of contaminated poultry meat but also to assess the effectiveness of potential control measures (Rosenquist *et al*., 2003; Nauta *et al.*, 2005). The evidence demonstrates that efforts directed towards the control of foodborne campylobacteriosis and in particular, successful implementation of effective control strategies against *Campylobacter* in poultry can produce additional public health benefits (Baker *et al*., 2012) because humans might become infected via other pathways apart from poultry meat. The production of *Campylobacter*-free flocks has been achieved experimentally although under commercial conditions can be challenging but not impossible. Currently there is no one single decontamination technology alone capable to eliminate *Campylobacter* or reduce it to negligible levels in foods without altering food characteristics. Consequently, an integrative approach must be followed in order to control food-borne *Campylobacter*, implementing several effective control measures throughout the food chain. An integrated approach to the control of *Campylobacter* in poultry has been adopted in Denmark where increased biosecurity, allocation of meat from positive flocks to the production of frozen foods and consumer education campaigns have led to a significant decrease in *Campylobacter* prevalence in broiler flocks (from 43% in 2002 to 27% in 2007), a reduction of *Campylobacter*-positive samples of fresh broiler meat and a decrease in registered human cases by 12% from 2002 to 2007 (Rosenquist *et al.*, 2009). The synergistic application of effective control measures against *Campylobacter* such as freezing, irradiation and proper cooking could achieve a human risk reduction of 90-100% assuming that no re-contamination occurs (Havelaar *et al*., 2007a/b; EFSA, 2011a). On the other hand, estimations of the effectiveness of controls against *Campylobacter* are uncertain, frequently based on limited data that might not be representative.

Extensive research has been conducted to identify the most effective *Campylobacter* control strategies that could be implemented during poultry production. For example, a considerable number of epidemiological studies have been conducted to identify significant risk factors for *Campylobacter* infections in poultry and to assess the effect of interventions for the control of *Campylobacter* in poultry from farm to fork. On-farm controls based on identified risk factors for *Campylobacter* infection of poultry may be effective. An extensive review of the potential control measures and interventions against *Campylobacter* in poultry production from "farm-to-fork" has been provided in Chapter 5 of this thesis.

Estimated *Campylobacter* prevalence in the environment around broiler houses from different farms seems to be quite similar independently of the biosecurity level (Hald *et al*., 2000; Hansson *et al*., 2007; Ridley *et al.*, 2011a). Consequently, *Campylobacter* must be carried from the environment into chicken houses. First of all, controls should be implemented to avoid *Campylobacter* introduction from the environment into poultry flocks, once a bird is colonized with *Campylobacter* the rest of the birds will be infected in a short time (Jacobs-Reitsma, 1997). Once *Campylobacter* is present in poultry flocks, control strategies should be implemented in order to reduce the numbers of *Campylobacter* in chickens and their environment. Some *Campylobacter* control strategies aim to reduce the numbers of *Campylobacter* in the digestive tract of chickens to achieve a reduction of *Campylobacter* in chicken meat. Additionally, controls should be implemented during transport, slaughter, further processing and retail in order to reduce the numbers of *Campylobacter* in already infected chickens, to avoid contamination of the food processing environment and to protect *Campylobacter*-free chickens and their products from being contaminated. Moreover, cleaning and disinfection of poultry houses, transport materials, food processing areas should be effective inactivating *Campylobacter*. The poultry house becomes contaminated with *Campylobacter* for a long time when a poultry flock becomes positive (Hiett *et al*., 2002; Herman *et al*., 2003; Johnsen *et al*., 2006). The presence of previous *Campylobacter* positive flocks in a house has been found as a significant risk factor for *Campylobacter* infection of new flocks (Refregier-Petton *et al.*, 2001; Chowdhury *et al*., 2012).

The implementation of effective biosecurity measures seems crucial to prevent *Campylobacter* introduction into the poultry houses (Ridley *et al*., 2008, 2011a, 2011b). A clear correlation between the level of biosecurity and poultry flock infection with *Campylobacter* has been observed in Norway and Senegal (Cardinale *et al*., 2004; Johnsen *et al*., 2006). Additionally, synergistic effects

are expected from the implementation of several biosecurity control measures. The implementation of hygienic measures and biosecurity barriers such as the control of rodents and insects produced significant reductions in *Campylobacter* prevalence of broiler flocks in the Netherlands (van de Giessen *et al.,* 1998). Although high levels of hygiene and biosecurity may not be sufficient to produce a *Campylobacter*-free flock, the risk of *Campylobacter* introduction into poultry flocks may decrease considerably (Gibbens *et al*., 2001). Increased biosecurity could be particularly important at times of the year when the risk of introducing *Campylobacter* in broiler flocks is considered high due in part to seasonality effects (Kapperud *et al*., 1993.; Jacobs-Reitsma *et al*., 1994; Berndtson et al., 1996; Evans and Sawyers, 2000; Refregier-Petton *et al*., 2001; Bouwknegt *et al*., 2004; Hofshagen and Kruse, 2005; Puterflam *et al*., 2005; Russa *et al*., 2005; Barrios *et al*., 2006; Johnsen *et al.*, 2006; McCrea *et al.*, 2006; Hansson *et al*., 2007; Huneau-Salaun *et al.*, 2007; McDowell *et al*., 2008). In Denmark, the use of fly screens during the summer to prevent the access of flies to poultry houses has been recommended (Hald et al., 2004, 2007a/b; Bahrndorff *et al*., 2013).

Field trials need to be conducted to examine the practical effects of the most promising *Campylobacter* control measures. The effectiveness of some control strategies such as phage therapy, vaccination and competitive exclusion products may be influenced by the genomic instability of *Campylobacter* (Ridley *et al*., 2008a). *Campylobacter* strains and the type of production system will influence the results of field trials. *Campylobacter* prevalence in free-range poultry flocks is usually higher than in poultry flocks produced in intensive conditions (Lund *et al*., 2003; Ring *et al*., 2005; McCrea *et al.*, 2006). Even more, controls that aim to avoid *Campylobacter* introduction in flocks produced in extensive conditions might not work because *Campylobacter* is highly prevalent in the environment and can resist environmental stresses (Garcia and Percival, 2011).

The poultry industry needs to be highly integrated in order to maintain profit margins which are usually very low and to meet consumer demands. *Campylobacter* control measures that can be applied at low cost are generally accepted by the poultry industry although the consistency with which the controls are implemented may vary. On the other hand, controls that require efforts and/or extra costs are not usually welcome by poultry producers. For this reason, proposed controls should be backed up with strong evidence of effectiveness and a satisfactory viable cost-benefit balance. Nevertheless, food producers need to comply with relevant legislation related to the

protection of public health. The poultry industry should implement and sustain best hygiene practices through adequate assessment, monitoring and staff education and motivation (Berndtson *et al*., 1996; van de Giessen *et al*., 1998).

Some controls against *Campylobacter* in poultry production can be relatively easy to implement at a low cost while other strategies might be more difficult to introduce, to maintain and/or might be expensive. Some controls could be easy to implement but the consumers' preferences and/or food market characteristics might impede the implementation of such controls. For example, reducing the slaughter age of poultry may be an effective control strategy that could be used synergistically with other *Campylobacter* controls (Newell *et al*., 2011). However, some consumers demand chickens of a particular weight when sold at retail and the production of smaller chickens might not be accepted by consumers. Consumers should also be properly informed about the safety of some food controls (Jordan and Stockley, 2010). For example, consumers seem to appreciate the benefits and safety of irradiation as a food safety control; as a result, several countries are implementing irradiation of chicken products for the control of pathogens such as *Campylobacter* (International Consultative Group on Food Irradiation [ICGFI], 1999).

Accurate and reliable *Campylobacter* quantitative data are crucial for *Campylobacter* risk assessments and for the assessment of *Campylobacter* control strategies. However, there seems to be no consensus regarding the most appropriate sampling protocol to obtain accurate *Campylobacter* quantitative data. Quantitative microbiological data and data analyses results will be influenced by the sampling protocol (including methods, sample size, sample origin, time of sampling and other aspects). For example, samples obtained on farm and/or at the slaughterhouse are usually pooled for practical reasons but quantitative data related to the concentration of *Campylobacter* in chickens might differ between individual and pooled samples. In this thesis, we present research conducted to share light in some of these aspects related to the production of accurate *Campylobacter* quantitative data. In our studies, high variability between chickens was observed related to the numbers of *C. jejuni* recovered from caecal samples and fecal samples at different time-points in agreement with other findings that showed that *Campylobacter* colonization levels differ between broiler chickens (Hansson *et al*., 2010). Even more, in our studies, results from mixed linear models indicated that the most of the variation can be attributed to individual chickens and to a lesser extent to the isolators suggesting that in commercial situations, differences might be observed between flocks but even greater differences might be expected due to individual chickens. This research finding suggests that individual factors such as chicken genetics may affect

Campylobacter dynamics in poultry flocks (Stern *et al*., 1990). What's more, in commercial farms chickens might be infected with *Campylobacter* at different times and diverse initial concentrations while in this study broilers were inoculated with the same dose of *C. jejuni* at the same time. In addition, poultry digestive physiology might influence the intermittent excretion of Campylobacter. As a result, concerns are raised in relation to limited sampling of poultry flocks not being representative of the real *Campylobacter* situation in large flocks. Moreover, faecal samples collected on farm might not be a good predictor of the caecal load of *Campylobacter* in individual chickens going for slaughter (Hansson *et al*., 2010). Additionally, results from this study suggest that pooling of samples will probably lead to an underestimation of the numbers of *Campylobacter* in the flock.

In our studies, *Campylobacter jejuni* concentrations in fecal samples were slightly lower than in caecum samples, nevertheless, a significant correlation was observed between faecal and caecal *C. jejuni* concentrations at slaughter (r = 0.7; C.I. = 0.5 – 0-8) suggesting that *Campylobacter* counts from fecal samples at slaughter might be a good indicator of *Campylobacter* concentration in the caecum at slaughter. This significant correlation is in agreement with other studies (Fluckey *et al*., 2003) supporting recommendations made related to the sampling of chickens closer to slaughter time (Hansson *et al*., 2005). Moreover, if there is a significant positive correlation between the numbers of *Campylobacter* in chickens at slaughter and the numbers of *Campylobacter* in carcasses as it has been suggested (Rosenquist *et al*., 2003; Lindblad *et al*., 2005; Reich *et al*., 2008), *Campylobacter* testing of fecal samples from chickens just before slaughter will aim on the implementation of *Campylobacter* control strategies such as logistic, schedule slaughter and others.

Vaccines against *Campylobacter* have been developed for humans (Baqar *et al*., 1995; Scott, 1997, Scott and Tribble 2000), chickens (Khuory and Meinersmann, 1995; Newell and Wagenaar, 2000; Noor *et al*., 1995; Rice *et al*., 1997; Widders *et al*., 1996) and other animals. However, a general cost-efficient vaccine for the control of *Campylobacter* in chickens and humans has not been developed despite years of research (Jagusztyn-Krynicka *et al*., 2009). A successful commercial vaccine should be safe, cost-effective and produced in large quantities. Conventional vaccines usually perform poorly when applied to chickens due to the interaction of *Campylobacter* with the intestinal niche in poultry (Ringoir and Korolik, 2003; Walker, 2005). Moreover, immunity against *Campylobacter* seems to be strain-specific. Hence, the development of a vaccine able to protect the host against all *Campylobacter* strains seems challenging. The use of genomics and proteomics for

the identification of genes that are essential for *Campylobacter* colonization of chickens and new antigens may be crucial for the development of an effective vaccine against all *Campylobacter* strains in poultry (Jagusztyn-Krynicka *et al.*, 2009).

In this thesis, we have presented clinical trials conducted for the assessment of the *Campylobacter* vaccine ACE393 in broilers under experimental conditions. Ideally, a statistically significant reduction of the numbers of Campylobacter in vaccinated chickens (by at least 2 logs) was expected. The potential effect of the vaccine was analyzed using diverse data analyses methods of increased complexity. Results from our vaccination experiments indicated that the apparent observed differences between vaccinated and placebo groups related to *Campylobacter* counts could be attributed to the variation between birds in the same group and between groups. Data analyses using methods that did not consider the experimental design indicated that some differences between vaccinated and placebo groups related to *Campylobacter* counts were statistically significant (based on a significance level of p-value<0.05) although the desired vaccine effect (reducing *Campylobacter* counts in vaccinated chickens by 2 logs) was never achieved. However, when taking on account the hierarchical design, the results from the mixed linear models indicated that the differences between vaccinated and placebo broilers in terms of the numbers of *C. jejuni* recovered were not statistically significant, based on a significance level of p-value<0.05. It was concluded that there was no statistically significant effect of the vaccine ACE 393 in this clinical trial in broilers under the experimental conditions applied. We demonstrated how crucial is to consider the "clustering effect" when analyzing quantitative data and also when designing multilevel clinical trials. Clustered designs are desirable in some cases although they can be more costly and require more individuals and more complex data analysis.

In conclusion, sampling protocols and microbiological techniques used for the detection and quantification of *Campylobacter* will influence the results of *Campylobacter* testing in poultry and/or research studies e.g. the effect of vaccines in clinical trials. Moreover, quantitative microbiological data need to be properly analyzed. Sample size calculations and forecasted group effects need to be carefully considered during the experimental design phase. Additionally, data analysis methodologies should be carefully selected based on the experimental design (Garcia *et al.*, 2012).

Reliable *Campylobacter* quantification is crucial to assess public health risks and to ensure food safety. Consequently, fast, sensitive and accurate methodologies and data analysis techniques need to be properly tested, improved or developed. Real-time PCR is considered a promising technique for the accurate quantification of *Campylobacter* on chicken carcasses (Josefsen *et al.*, 2010). Real-time PCR allows for the detection and quantification of viable but non-culturable (VBNC) microbial forms that might be of public health relevance in some cases (Postollec *et al*., 2011). On the other hand, accurate direct (without the use of enrichment) quantification of viable *Campylobacter* in complex biological samples such as chicken feces can be challenging (Rudi *et al.*, 2004; Leblanc-Maridor *et al*., 2011). In the studies presented in this thesis, several DNA extraction methods were assessed in their effectiveness for the direct quantification of *Campylobacter jejuni* present in spiked chicken fecal samples using real-time PCR. Moreover, two of the methods (Easy-DNA and MiniMAG extraction methods) were used to quantify *Campylobacter* (by real-time PCR) present in different naturally infected chicken fecal samples and the results were compared to *Campylobacter* quantitative data obtained from traditional culture. When using real-time PCR, amplified DNA from dead cells may lead to an overestimation of the numbers of the target organism or even false-positive results (Wolffs *et al.*, 2005). For that reason, it was expected that quantification results from real-time PCR were higher than those obtained by traditional culture. The results indicated that there were differences between *Campylobacter* quantification data obtained by culture and by real-time PCR when using two different extraction methods (Easy-DNA and MiniMAG extraction methods). However, there were no statistically significant differences between culture and real-time PCR in these studies. Several reasons can be hypothesised to try to explain the results obtained. The presence of inhibitors in chicken fecal samples can reduce the efficiency of real-time PCR assays significantly (Perch-Nielsen *et al.*, 2003; Guy *et al.*, 2003; Rådström *et al.*, 2004; Sunen *et al.*, 2004; Jiang *et al*., 2005; Stratagene, 2004). In theory, overestimation of the numbers of the target organism are expected when using real-time PCR but due to the presence of inhibitors, this effect might be suppressed which could explain the similar results obtained by real-time PCR and culture in this study. The fact that chicken fecal samples were used for the preparation of spiked samples and construction of standard curves could partly explain this agreement because we already accounted for the effect of inhibitors that may be present in chicken fecal samples when building the standard curves. It could also be possible that not a great amount of stressed *Campylobacter* cells, VBNC *Campylobacter* states or free *Campylobacter* DNA were present in the naturally infected samples because samples were fresh

and processed within 30 hours of collection. This observation could also explain the statistical agreement between *Campylobacter* quantification results obtained by culture and by real-time PCR. Novel and accurate methods able to discriminate between the different *Campylobacter* viable and non-viable states could share light in this matter.

The culture used for spiking in our studies was prepared with a particular strain of *C.jejuni* CCUG 11284 but the use of several *Campylobacter* strains has been recommended (Greer *et al*., 1992; Birk *et al.,* 2010; Boyse, 2012). Moreover, research data based on naturally infected birds and commercial production conditions are desirable. For these reasons, the selected two DNA extraction methods were tested using chicken fecal samples naturally infected with *Campylobacter* and more than one *Campylobacter* strain were likely to be present in these samples.

The assessment of *Campylobacter* controls in poultry will partly depend on the quantitative microbiological techniques used and hence the use of reliable and accurate techniques for *Campylobacter* quantification in diverse samples is crucial. A thorough investigation of the inhibitors present on particular sample matrices is desirable in order to design the best sample treatment and select the most appropriate DNA extraction methodology. Rigorous real-time PCR data analyses and accurate estimations of efficiencies of each real-time PCR reaction will be ideal. Though, this approach might be demanding in terms of time and other resources. On the other hand, experimental variability can be very high even when the best methodologies are used and experiments are performed under controlled conditions. Variability between different PCR plates or runs can be high; even when considering only one specific PCR plate, intra-plate variability can be significant (Karlen *et al.*, 2007). In fact, biological variability between samples and replicates can also be high and partly explain different real-time PCR efficiencies and therefore should be accounted for when analysing real-time PCR data. Novel mathematical methods to calculate PCR efficiencies and quantification of samples have been developed (Lalam *et al*., 2004). The use of nonlinear regression analysis has been proposed instead of relying on the cycle threshold method for absolute sample quantification (Goll *et al*., 2006). Stochastic models such as Bayesian models have also been designed to analyze real-time PCR experimental and simulated data (Lalam, 2007).

Accurate and reliable enumeration of viable pathogens present in foods and/or environmental samples will assist exposure assessment, risk assessment models and the evaluation of the effectiveness of food safety measures and public health protection programs. In particular, accurate and reliable quantification of *Campylobacter* in poultry will provide good quality data for the

probabilistic models presented in this thesis that have been designed to assist the poultry industry in making decisions related to the control of *Campylobacter* in poultry. Increased consumer concerns related to food safety are applying pressure on food producers to implement food safety assurance systems. Poultry producers need to make important and complex decisions and related investments (sometimes expensive) for the sustainability of their businesses. In particular, poultry producers should implement effective controls against *Campylobacter* in poultry to increase food safety and to reduce the burden of human campylobacteriosis. The use of decision support systems can aid poultry producers to make difficult decisions under conditions of uncertainty. Decisions on vaccination and other public health controls have to be generally made under conditions of uncertainty. Probabilistic graphical models (PGMs) are widely used to support knowledge management and decision making under conditions of uncertainty (Kjærulff and Madsen, 2008; Darwiche, 2009; Koller and Friedman, 2009; Madsen *et al.*, 2012). PGMs represent knowledge and probabilistic conditional relationships in structured models designed to represent real situations where uncertainty plays an important role. PGMs use Bayesian networks and other methods to deal with uncertainty and efficiently represent and integrate knowledge obtained from sources such as epidemiological data, scientific knowledge, research data and expert opinions in order to support decision processes made under conditions of uncertainty.

Several probabilistic graphical models have been designed and presented in this thesis to assist in decision making regarding *Campylobacter* vaccination of poultry and/or other *Campylobacter* control strategies. Considerations regarding the epidemiological and microbiological factors to be included in the models together with important challenges for the development of the quantitative part of the models were included in this thesis. Generic conceptual models for the control of *Campylobacter* in poultry that could be applied to many different real-life situations, poultry farming conditions and geographical areas were presented in this thesis. However, the quantitative part of generic models presents important challenges. For that reason, our recommendation is that specific PGMs may be constructed or adapted to address particular decision making processes under specific circumstances. Moreover, the conditions, model assumptions, selection of factors, different parts of the models, quantitative data should be clearly specified to add value and perspective to the decision support system designed in every case.

The selection of factors to include in the models and the use of reliable, good quality data will significantly influence the results of the models. In our models, we distinguished between the true

numbers of *Campylobacter* in birds and the detected or measured numbers. The latter depends on a number of factors such as sampling protocols and quantitative microbiological methods. Some quantitative microbiological techniques are being tested, improved or under development and the detection limit of most methodologies seems to be 100 CFU/g or ml (depending on sample type and sample preparation). Even more, as explained previously in this thesis, the quantification limit can be higher than the detection limit when using some microbiological methods. Consequently, we considered in our models that a negative result might actually indicate low numbers of *Campylobacter* in samples (1 to 100 CFU/ g or ml). It was also considered that a positive result will translate on a distribution of the numbers of *Campylobacter* in chickens in a flock between 2 and 10 logs because it seems difficult to obtain continuous accurate data on the numbers of *Campylobacter* in chickens in a flock during the rearing period. Even more, chickens might carry *Campylobacter* at different concentrations; as previously shown in this thesis, variability between chickens in a flock can be considerable.

Sensitivity analyses performed using specific models indicated that the factors that influenced the results to a greater extent were the financial variables (cost/reward functions) and the effectiveness of the control strategies considered, e.g. vaccination impact. The results from specific models indicated that the public health impact of the control strategies considered in the models will greatly depend on the effectiveness of the controls, costs of the controls and the reward systems. The assessment of the effectiveness of diverse control strategies might prove challenging especially when the control measure is innovative or not even commercially available e.g. the assessment of *Campylobacter* vaccines.

Campylobacter control strategies that can significantly reduce the probability of *Campylobacter* introduction into a flock and/or the numbers of *Campylobacter* in already infected chickens should be implemented from a public health perspective. On the other hand, poultry producers will usually base their strategic decisions on financial gains and consequently a reward system that can translate on an attractive cost-reward balance will be a good incentive for poultry producers to implement *Campylobacter* control strategies. The reward system might not be in place in most parts of the world, as a result it could be hypothesized for inclusion in the models. Reward systems could be designed based on the gross profit/per chicken for farmers in specific areas and/or production systems (e.g. organic farmers might obtain a higher gross profit/chicken than farmers producing commercial broilers). Financial gain will also depend on the effectiveness of the vaccine (and/or

other control strategies) and the costs associated with the controls. A cost-efficient vaccine against *Campylobacter* in chickens is not commercially available at present. We considered that the market price of a cost-effective vaccine against *Campylobacter* in chickens should be less than 10% of the gross profit per chicken to be competitive. However, the market price could be higher depending on the effectiveness of the vaccine and the reward system. The flexibility of PGMs allows for the inclusion and comparison of more than one *Campylobacter* vaccine and other control measures and more than one reward system. Tailor-made properly developed PGMs will assist poultry producers to make important decisions in order to solve complex problems such as the control of *Campylobacter*. Even more, engagement of different stakeholders in the PGMs development process is highly desirable. The use of sophisticated and complex computing interfaces and mathematical expressions and probabilities distributions needs to be reconciled with a simple and efficient tool that can be used by different stakeholders (Madsen *et al.*, 2012). On the other hand, newer technologies seem to be more flexible in relation to supporting individuals' creativity and innovation.

In conclusion, *Campylobacter* controls in poultry should be cost-effective, reliable, easy to implement, easy to maintain and accepted by consumers. Consumers' acceptance is highly relevant; for example, in the case that a cost-effective vaccine against *Campylobacter* in poultry was finally commercialized but not accepted by the consumers, significant efforts and investments made could be lost.

10. PERSPECTIVE AND FUTURE TRENDS

Ideal *Campylobacter* control strategies should be safe, cost-effective, cheap, easy to implement and maintain and accepted by the industry and consumers. Innovative controls and/or integration of effective controls should be investigated further and potential short-term and long-term consequences should be considered. For example, some antibiotics may reduce *C. jejuni* concentrations in chickens (Farnell *et al*., 2005; Hermans *et al*., 2010). However, there are huge concerns regarding antimicrobial resistance problems in humans and animals (CWF, 2011) and antibiotics may only be used therapeutically when prescribed by a veterinarian. Effective vaccines offer a good alternative to the use of antibiotics. Successful vaccines will probably be the most effective control against *Campylobacter* but the availability of a cost-effective commercial vaccine remains a major goal (Djenane and Roncalés, 2011: Garcia *et al*., 2012) mainly due to the absence of a strong immune response against *Campylobacter* in chickens (de Zoete *et al*., 2007). Genetic selection of poultry with superior immunological responses to *Campylobacter* could be explored further (Kapperud *et al*., 1993; Swaggerty *et al.*, 2009). What's more, the genetic diversity of *Campylobacter* might hamper controls based on immunization of chickens. The design of a cost-efficient vaccine that can decrease the numbers of all pathogenic strains of *Campylobacter* in poultry is desirable. In addition, the vaccine administration method might influence the results of clinical trials and/or the commercialization of the vaccine for use in poultry production systems. In the clinical trials presented in this thesis, the vaccine ACE 393 was administered intramuscularly following instructions from the manufacturer. Nevertheless, oral administration of vaccines against *Campylobacter* in poultry might be the preferred option.

Theoret *et al.*, (2012) performed oral vaccination of chickens (via oral gavage) with a recombinant attenuated *Salmonella enterica* strain synthesizing the *C. jejuni* Dps protein with promising results. The attenuated Salmonella vector achieved a reduction of 2.5 logs of *C. jejuni* numbers in vaccinated chickens. Experimental trials conducted at the University of Arizona as part of the CamVac project demonstrated that water vaccination in poultry is feasible. The vaccine utilized in these trials was a *Salmonella* vector expressing CjLAJ1 administered in the chickens' drinking water which resulted in a reduction of *Campylobacter* counts in vaccinated chickens by 2.5 logs CFU (personal communication). Greater variability related to the numbers of *Campylobacter* was observed in vaccinated groups possibly as a reflection of differences in protection levels related to the different vaccine doses ingested by chickens (via drinking water).

The use of antibodies against *Campylobacter* in poultry has been proposed. In fact, a strong protection against *C. jejuni* in chickens seemed to be induced by the oral administration of immunoglobulin preparations from milk or eggs (Tsubokura *et al*., 1997). In a recent preliminary study conducted by Heegaard *et al.* (2012) avian immunoglobulins (200 mg) were administered to chickens orally together with the challenge (at day 21 of age). Results showed that caecal and faecal counts of *Campylobacter* were between 0.5 and 1.0 log lower in birds treated with avian immunoglobulins. Immunoglobulins can be produced in great quantities from renewable sources but the price of the immunoglobulin products needs to be low to become a real alternative to antibiotics.

The implementation of *Campylobacter* controls "from farm-to-fork" may require great efforts and investments. For this reason, the development and application of mathematical models to estimate and compare potential effects of interventions "from farm-to-fork" can be very useful (Nauta *et al*., 2009; EFSA, 2011). The results from the models will partly depend on high quality quantitative microbiological data. In future studies, novel and thorough analytical and/or statistical methods could be applied to accurately quantify viable pathogens such as *Campylobacter* present in biological samples. Even more, innovative *Campylobacter* control strategies could be tested experimentally and/or included in mathematical models for the assessment of potential effectiveness. New technologies such as nanotechnology and reverse vaccinology can provide novel food safety controls (Malsch, 2005). Innovations in control strategies, quantitative microbiological and mathematical methods and the integration of advances related to high quality data collection and data management will assist producers in making important decisions related to *Campylobacter* controls in poultry production. From a public health perspective, results from mathematical models in terms of reduced risk and/or decreased numbers of *Campylobacter* in chickens after the implementation of controls could be translated into the expected reduction in human campylobacteriosis cases using mathematical models such as risk assessment models.

Socio-economic aspects related to the implementation of *Campylobacter* control strategies in poultry production should not be ignored. The poultry industry generally works within narrow profit margins and for that reason, poultry producers will usually make strategic decisions based on financial gains. On the other hand, food producers might be inclined to offer products that may increase their popularity, image, social recognition by consumers and/or the power of the brand. In agreement with the previous statement, food producers will feel reluctant to implement food safety

controls that might not be accepted by consumers. In general, food safety controls that change the smell, appearance and taste of chickens will not be accepted by consumers. For example, chickens that have been slightly cooked during food processing and/or the use of decontamination technologies such as steam ultrasound will not be purchased by consumers, even though they might be safer to eat than other chickens. Consumers drive the market and consumers' preferences will influence the type of products available. Consumers demand safe, natural, nutritious, high quality foods with extended shelf-life and natural flavor. Consumers prefer natural food preservatives such as plant extracts to the use of artificial compounds. Consumers might not accept foods that have been modified or altered in particular ways. For this reason, some potential *Campylobacter* controls in poultry might not be feasible. The use of bioengineered modified foods may not be accepted by the consumers. Interestingly, a study conducted by Jordan and Stockley (2010) related to consumers' decisions when buying chickens indicated that consumers trusted that there was adequate legislation to ensure food safety and in particular to protect them against *Campylobacter*. These authors recommended addressing consumers 'concerns related to the safety of new interventions and/or the effect of new interventions on the organoleptic characteristics and /or the price of chickens. Consumer education on kitchen hygiene practices and food safety is crucial to prevent human campylobacteriosis (Rosenquist *et al.*, 2003; Uyttendaele *et al.*, 2006) and it has been promoted by governments. Interestingly, consumers seem to trust the governments to ensure food safety and in this way elude their own responsibility. Nonetheless, food safety is everyone's responsibility.

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12. APPENDICES

12.1 Appendix 1

Tables 5 and 6 Descriptive statistics showing the results obtained in the clinical trial with commercial broilers.

Table 5 Results from descriptive statistics (data related to placebo chickens). Table shows mean ± standard deviation and maximum/minimum log CFU per gram fecal or cecal mass. ***R*=** rotation and ***I*=** isolator number

		Log CFU/g of fecal material from placebo chickens						**Log CFU/g of cecal content placebo chickens**	
R	***I***	***Day 35***		***Day 38***		***Day 42***		***Day 42***	
1	1	No individual samples taken						8.52±0.37	8.00/9.08
	2							8.20±0.70	6.63/8.80
	3							8.40±0.61	7.51/9.26
	4							8.12±0.27	7.63/8.43
2	1	7.72±0.54	7.15/8.76	7.42±1.28	4.90/8.84	7.19±0.62	6.45/8.08	7.91±0.84	6.72/8.82
	2	7.09±0.87	5.75/8.08	7.60±0.79	6.40/8,57	7.15±1.05	5.51/9.20	8.16±0.66	7.04/9.30
	3	7.13±1.00	5.26/8.26	7.07±0.92	5.57/8.28	6.61±0.63	5.96/8.00	7.47±0.55	6.86/8.34
	4	7.38±0.63	6.04/7.89	6.49±0.83	5.51/7.76	6.60±1.11	5.46/8.18	8.12±0.40	7.53/8.71
3	1	6.58±0.40	6.15/7.20	7.05±0.74	5.79/7.86	7.24±0.46	6.54/7.84	8.16±0.51	7.30/8.81
	2	7.06±0.81	6.20/8.15	6.94±0.43	6.26/7.32	7.46±0.52	6.81/8.15	8.41±0.32	8.00/8.87
	3	8.20±0.88	7.32/9.41	7.84±0.59	7.18/8.58	7.29±0.21	7.00/7.49	8.48±0.34	8.18/8.95
	4	*		*		*		*	
4	1	7.48±0.49	6.67/8.30	7.58±0.50	6.81/8.11	7.29±0.58	6.40/8.30	7.89±0.25	7.36/8.26
	2	7.28±0.70	5.64/8.00	7.15±0.95	4.94/8.53	7.43±0.59	6.43/8.34	8.61±0.28	8.18/9.04
	3	7.89±0.46	7.04/8.36	7.45±0.31	7.11/7.98	7.00±1.08	5.28/9.38	7.24±0.95	5.26/8.41
	4	7.42±0.38	6.80/8.20	6.86±0.69	5.96/8.18	5.671.13	4.04/7.38	6.27±1.07	4.81/8.30

* Birds from this isolator were not included due to a functional breakdown of the isolator

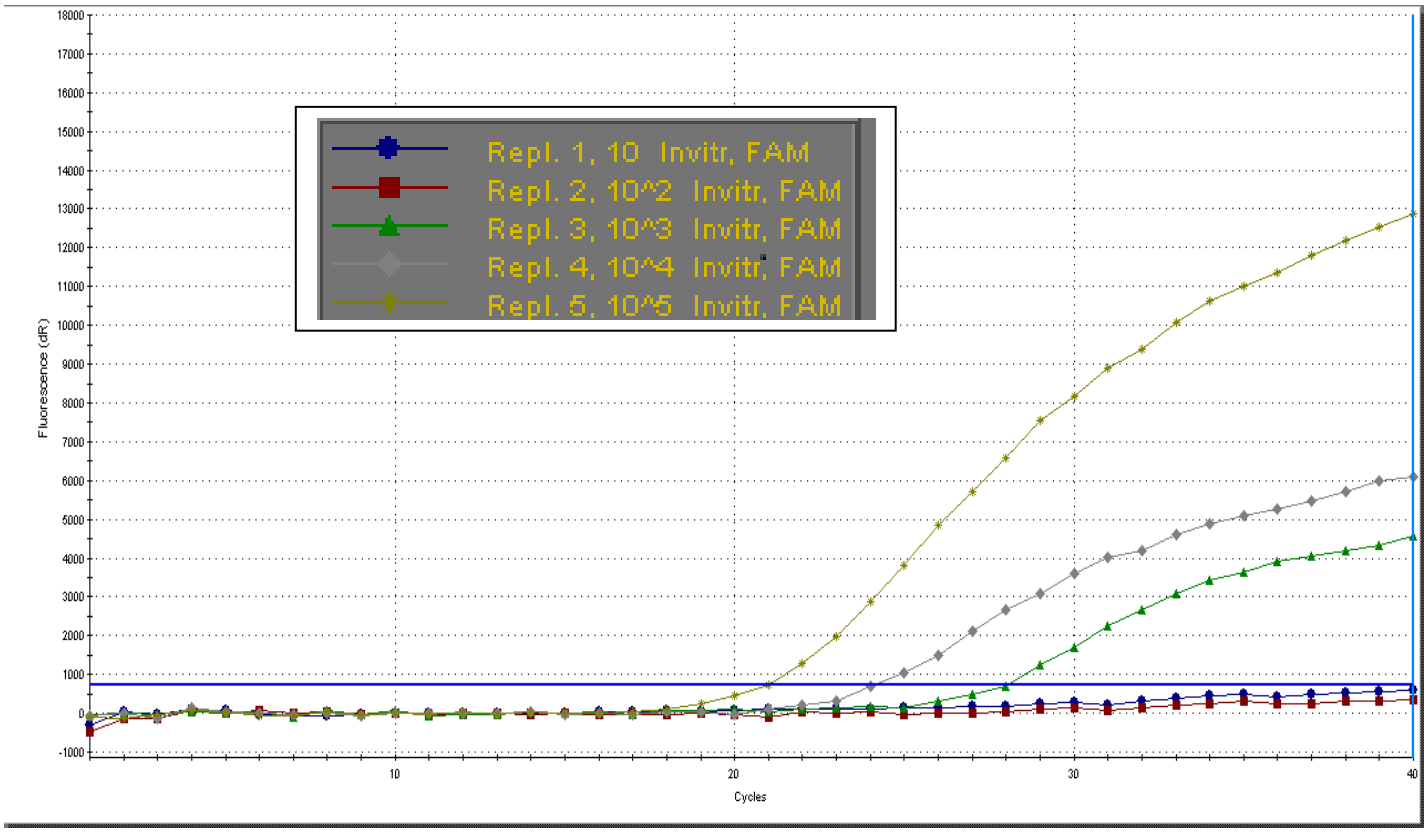
Table 6 Results from descriptive statistics (data related to vaccinated chickens). Table shows mean ± standard deviation and maximum/minimum log CFU per gram fecal or cecal mass. ***R*=** rotation and ***I*=** isolator number.

		Log CFU/g of fecal material from vaccinated chickens						Log CFU/g of cecal content from vaccinated chickens	
R	***I***	***Day 35***		***Day 38***		***Day 42***		***Day 42***	
1	1	No individual samples taken						7.94±0.50	7.28/8.73
	2							8.77±0.45	8.08/9.38
	3							8.66±0.64	7.30/9.41
	4							7.31±0.57	6.30/8.08
2	1	7.94±0.87	6.63/9.11	8.07±1.04	6.15/9.52	7.35±1.02	6.32/9.51	8.68±0.86	6.95/9.81
	2	7.17±0.58	6.28/7.88	7.51±0.55	6.79/8.32	7.31±0.93	6.08/8.38	8.16±0.36	7.77/8.72
	3	7.36±0.43	6.54/7.81	7.27±0.46	6.57/7.91	7.14±0.61	6.48/8.00	7.94±0.38	7.30/8.46
	4	7.12±0.92	6.08/9.08	6.65±0.73	5.32/7.71	6.72±0.58	5.77/7.52	8.33±0.40	7.69/9.04
3	1	7.46±0.34	7.04/7.90	7.98±0.83	7.08/9.32	8.37±0.62	7.41/9.18	8.79±0.32	8.26/9.30
	2	7.28±0.17	7.04/7.52	7.71±0.40	7.34/8.45	7.51±0.56	6.88/8.49	8.18±0.77	6.86/9.11
	3	6.81±0.65	5.69/7.78	6.92±0.90	6.08/8.65	7.12±0.88	5.45/8.32	7.78±0.83	5.48/8.46
	4	7.59±0.43	6.79/8.04	7.39±0.60	6.36/8.23	7.22±0.58	6.28/8.20	8.14±0.38	7.41/8.54
4	1	7.46±0.57	5.76/7.98	7.43±0.36	6.82/8.08	7.07±0.94	4.83/8.20	7.63±0.33	7.20/8.04
	2	7.17±0.40	6.34/7.58	7.62±0.62	6.15/8.30	7.45±0.66	6.26/8.36	8.08±0.56	6.95/8.85
	3	6.98±0.43	5.88/7.74	6.86±0.46	5.79/7.61	7.14±0.69	6.11/8.18	8.10±0.47	6.97/8.83
	4	7.60±0.76	6.52/9.30	7.02±0.60	5.97/7.71	6.97±0.78	5.34/7.92	7.86±0.61	7.11/8.97

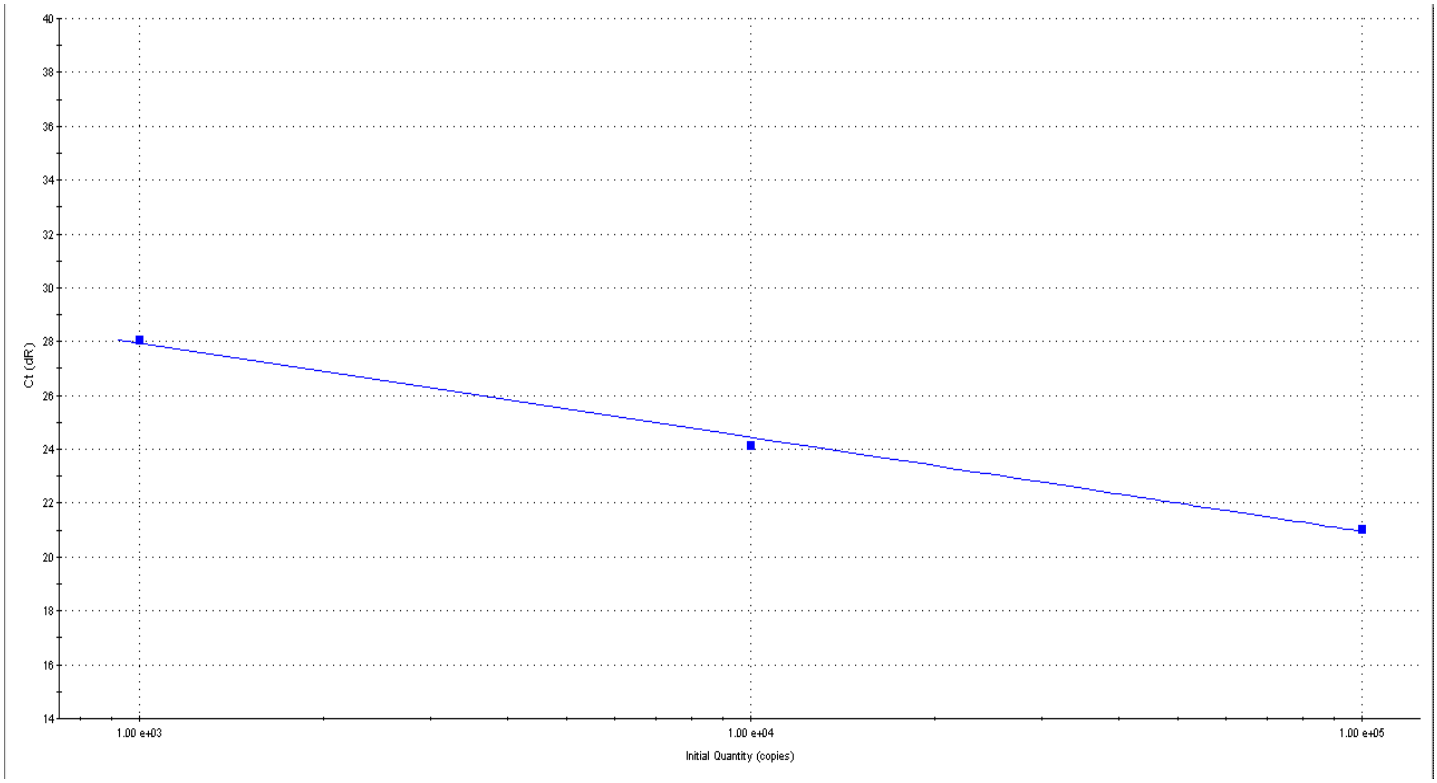
12.2 Appendix 2

Amplification plots and standard curves generated from all DNA extraction methods tested. Each amplification curve and each point in the standard curves represent the average of biological and PCR replicates per *Campylobacter* concentration level (five replicates when using Easy-DNA and four replicates when using miniMAG).

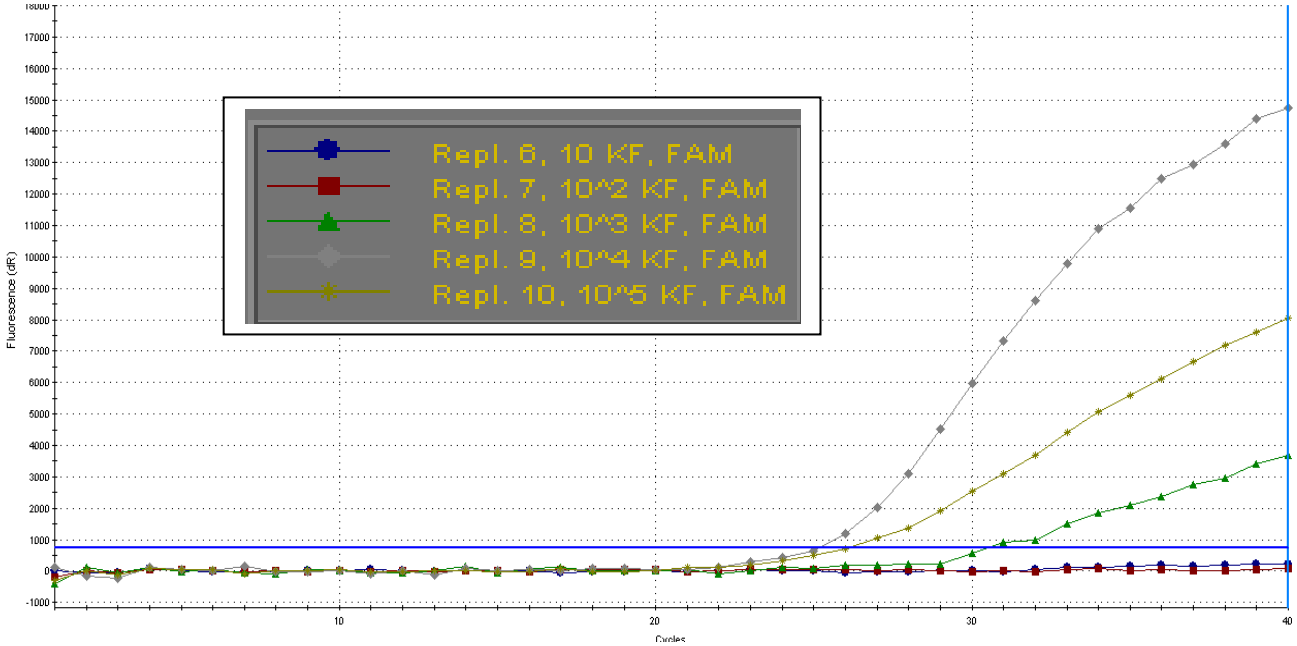
1) Easy-DNA Invitrogen

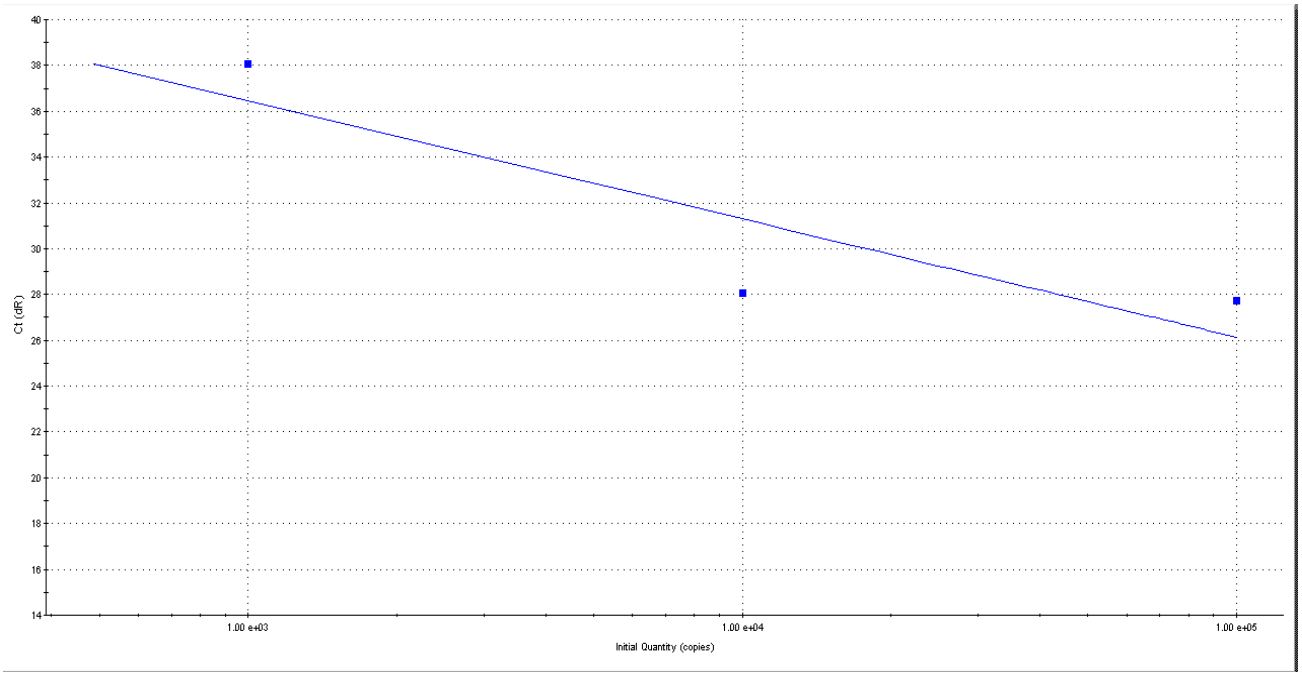


Easy-DNA Invitrogen (standard curve)

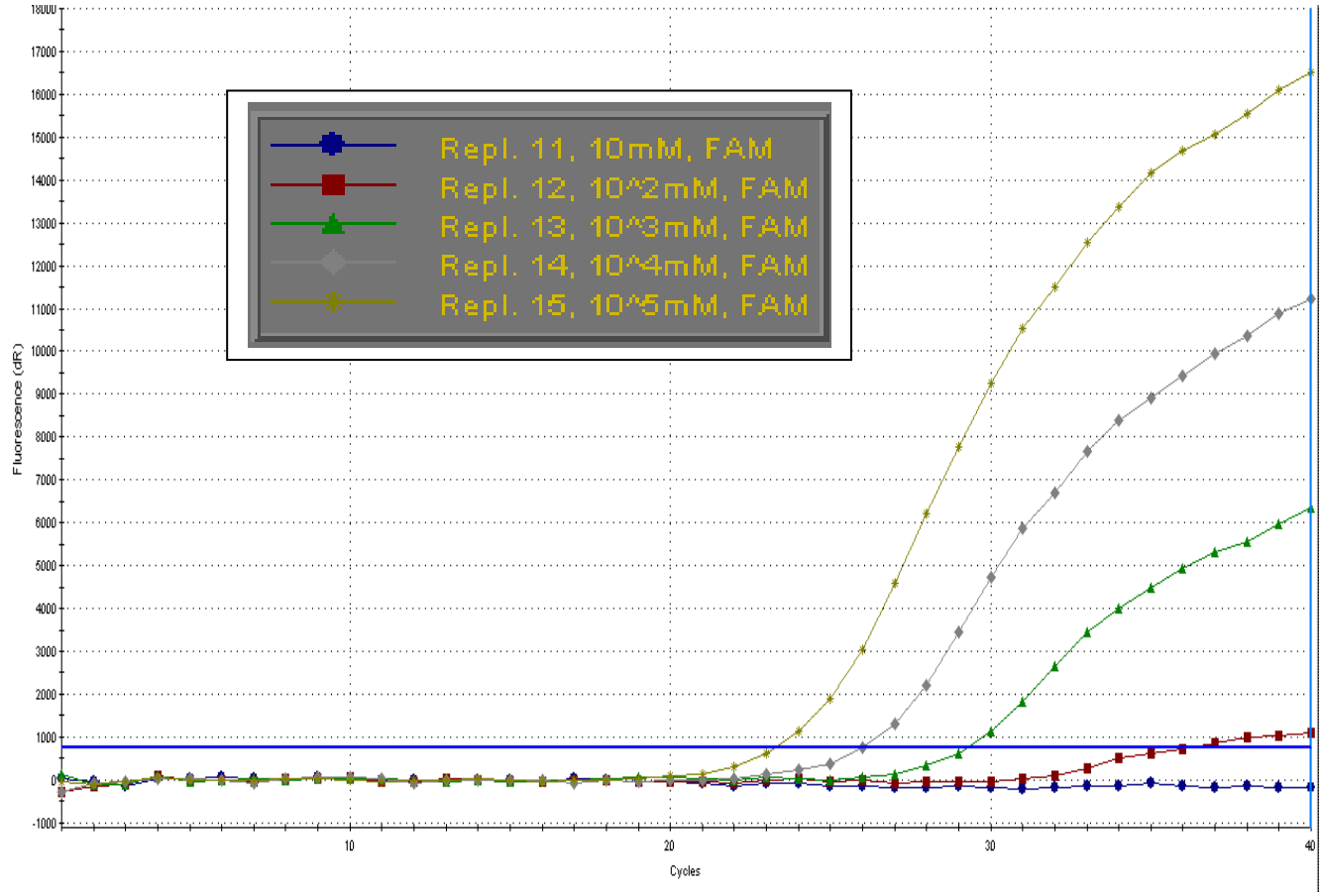


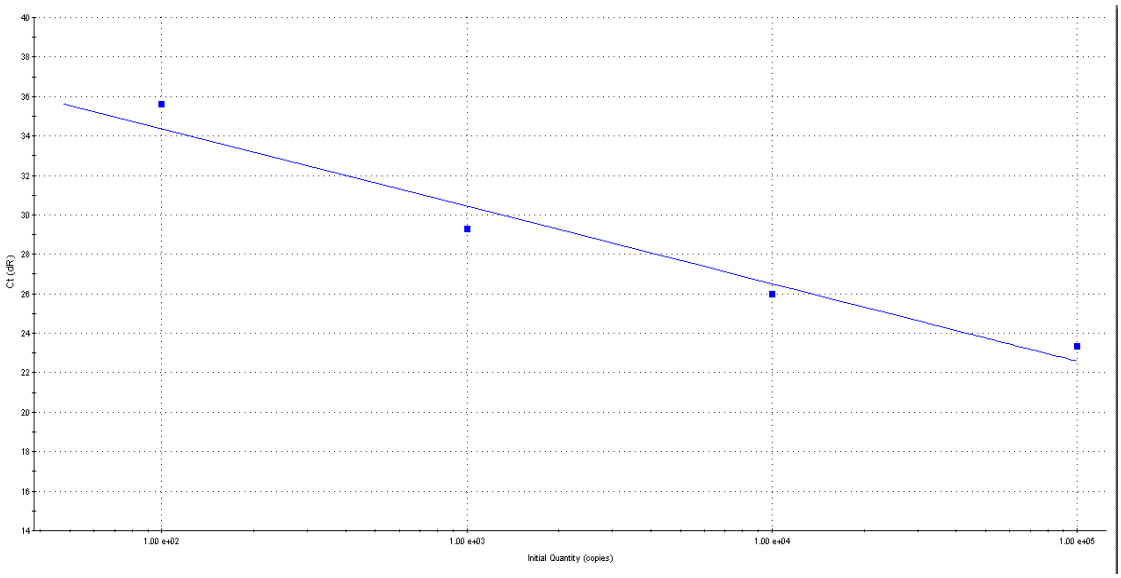
2) MagneSil® KingFisher



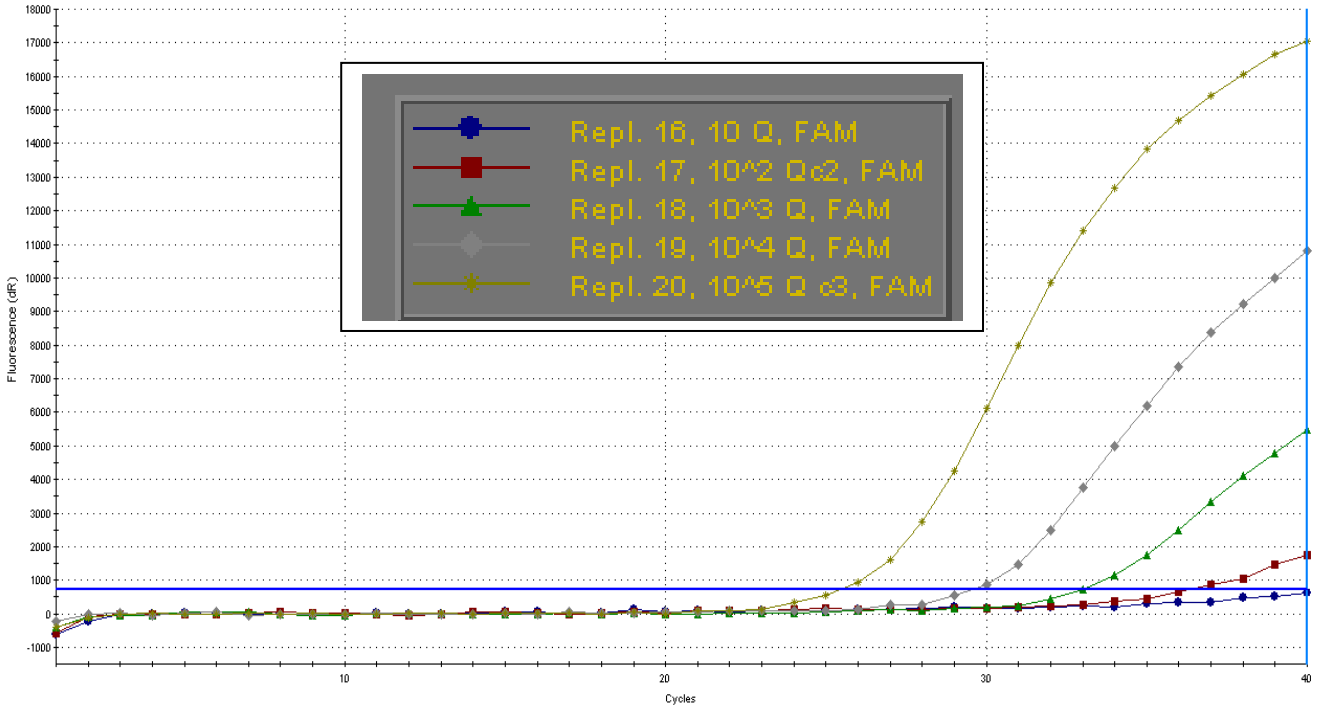


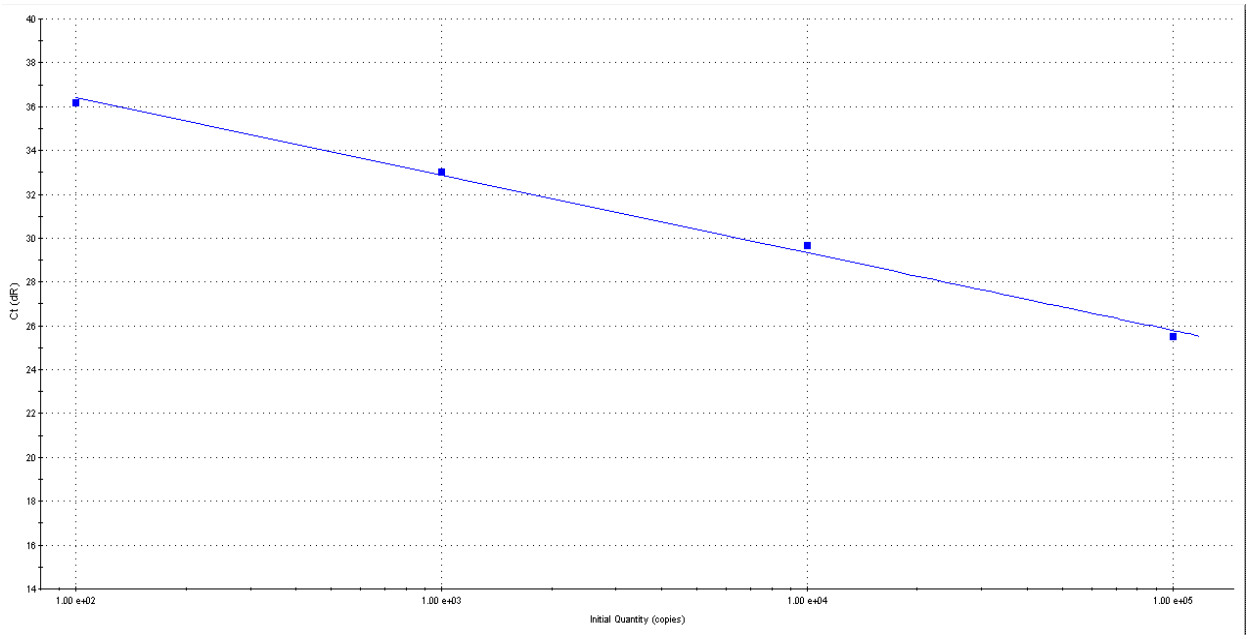
3) NucliSENS® miniMAG



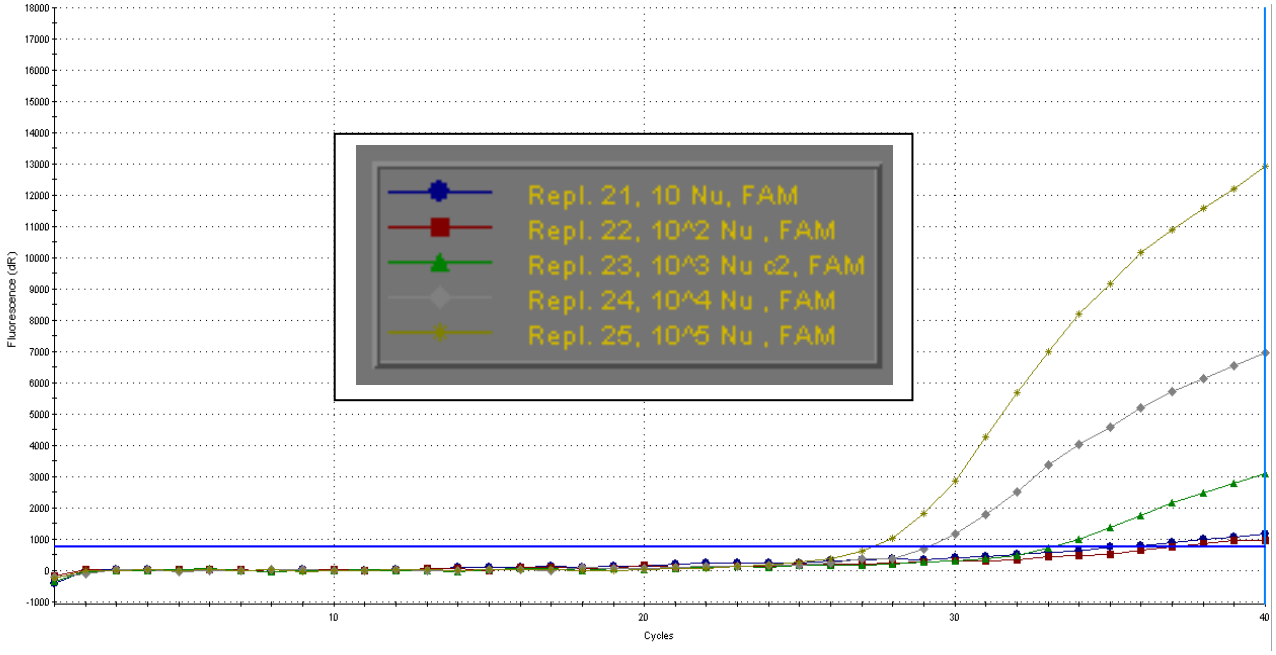


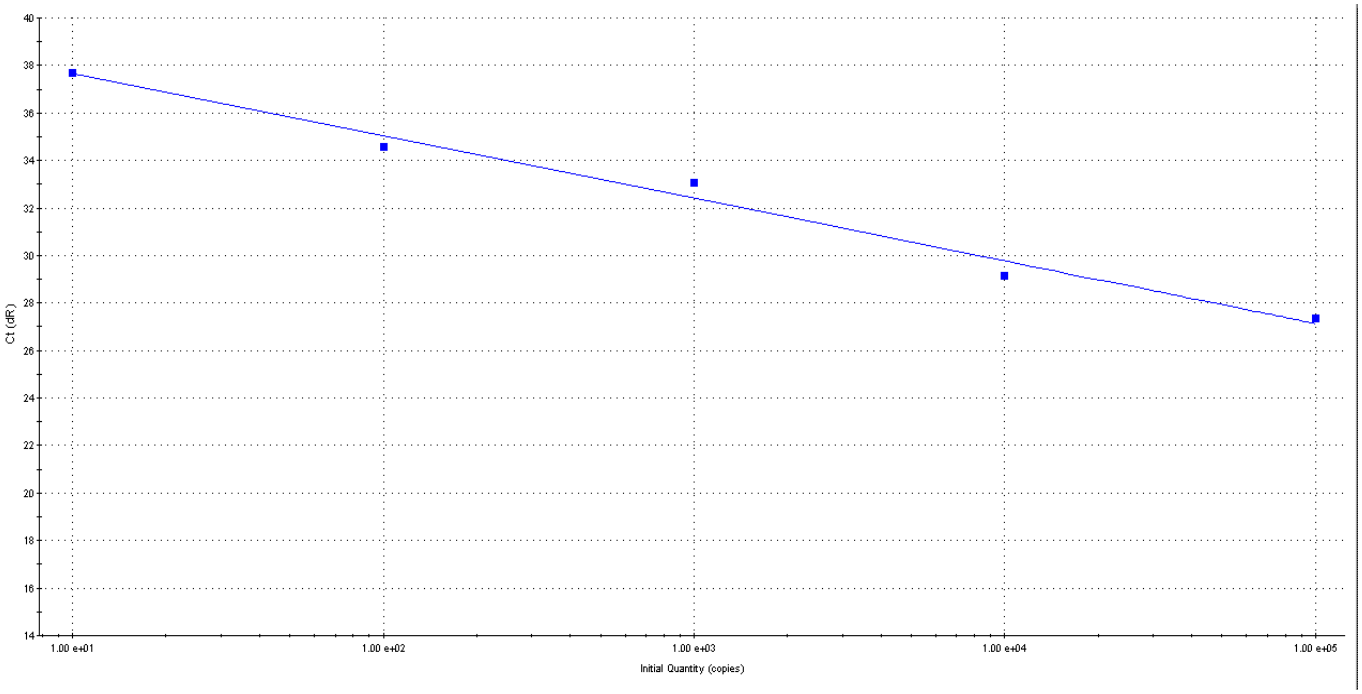
4) QIAamp Qiagen



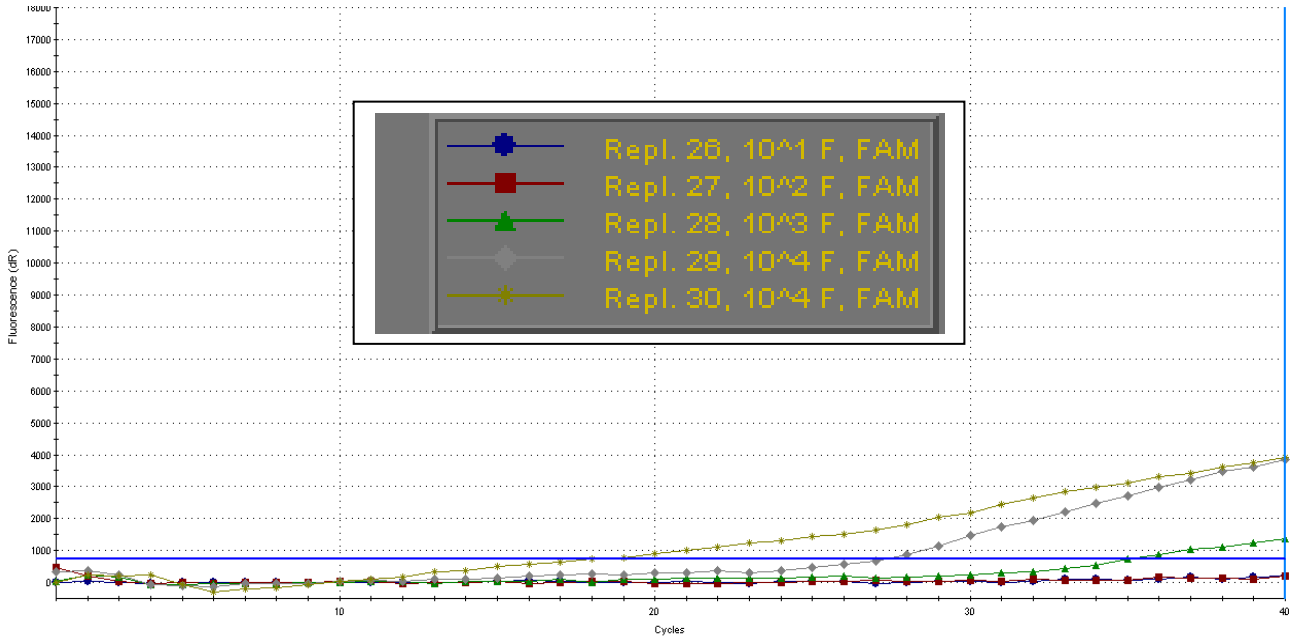


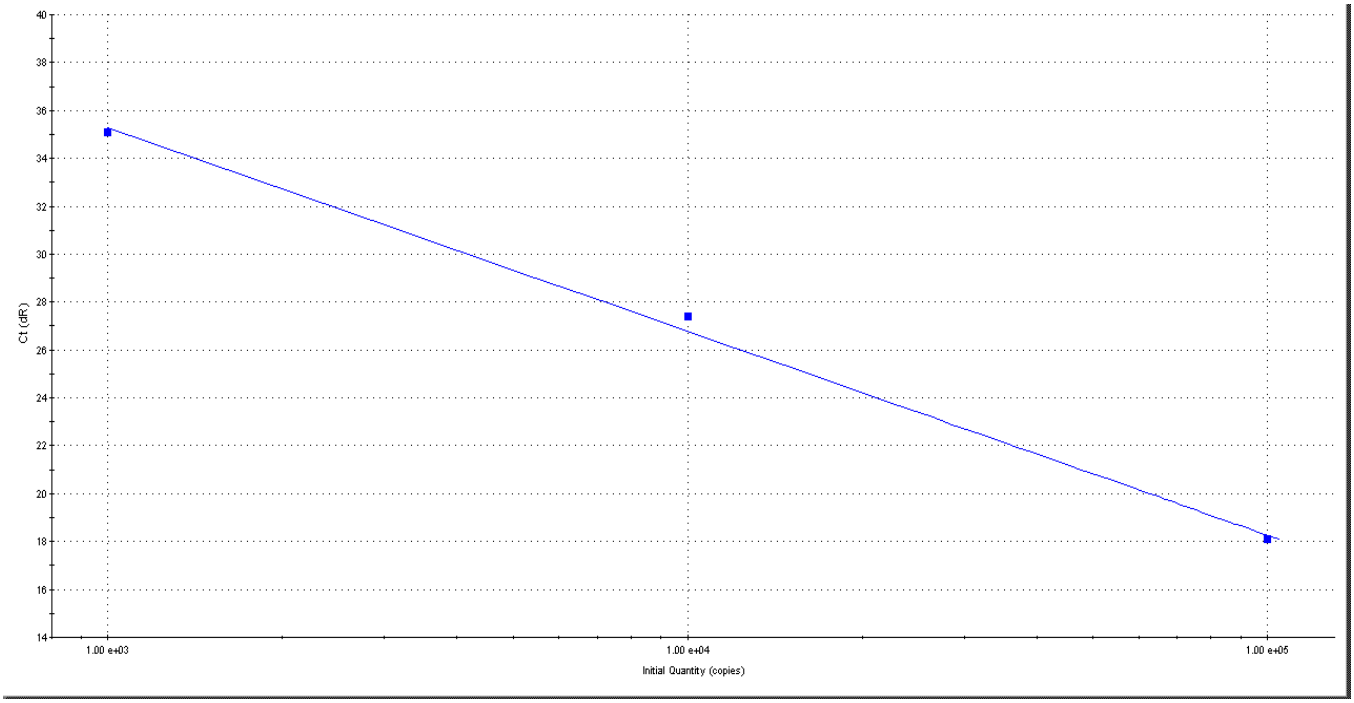
5) NucleoSpin® Tissue





6) SureFood® PREP





13. LIST OF ADDITIONAL MANUSCRIPTS

1. Madsen, A.L.; Karlsen, M.; Barker, G.C.; Garcia, A.B.; Hoorfar, J.; Jensen, F.; Vigre, H. *An Architecture For Web Deployment Of Decision Support Systems Based On Probabilistic Graphical Models With Applications.* Tech Report TR-12-001 Department of Computer Science, Aalborg University, 2012. ISBN: 1601-0590.

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