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**Predictive modelling of the growth of *Campylobacter jejuni* in chilled chicken during transportation for the purposes of 'biosensor' based consumer safety monitoring**

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## CONTENTS

FIGURES	5
ACKNOWLEDGEMENTS	8
ABSTRACT	9
INTRODUCTION	
1.0 Campylobacter Reduction Strategies	11
1.1 Chicken Produce Packaging	13
1.2 Vacuum and Modified Atmosphere Packaging	13
1.3 Hazard Analysis and Critical Control Points (HACCP)	14
1.4 Transport of Chilled Chicken Produce	15
1.5 Aims, Objectives and Limitations	16
LITERATURE REVIEW	
2.0 Introduction	18
2.1 The Genus <i>Campylobacter</i>	20
2.2 Campylobacter Environmental Adaptation	21
2.3 Limitations in Campylobacter Detection and Enumeration	22
2.4 Campylobacter Host Colonisation and Immunopathology	24
2.5 Broiler Flock Colonisation Vectors	27
2.6 Campylobacter Reduction Strategies	30
2.7 Conclusions	32
RESEARCH STRATEGY	35

## CONTENTS (Continued)

### METHODOLOGY

#### Preliminary Studies

3.0	<i>Campylobacter Spp</i> Experimental Strain	36
3.1	<i>C. jejuni</i> ATCC-33291 Population Dynamics in Subculture	36
3.2	Comparison of Bacterial Recovery According to Selective Media	37
3.3	<i>C. jejuni</i> ATCC-33291 Inoculant Generation	37
3.4	Determination of Experimental Temperature Parameters	38
3.5	Vacuum and Modified Atmosphere Packaging	39
3.6	Adjustments to Vacuum and MAP 30°C Protocols	39

#### Experimental Methodology

3.7	Sample Preparation	40
3.8	Inoculant Preparation, Verification, and Enumeration	41
3.9	Sample Inoculation, Packaging, and Incubation	41
3.10	Residual Gas Composition Measurement	42
3.11	<i>C. jejuni</i> ATCC-33291 Enumeration	43
3.12	Turbidity/Optical Density Measurement	43
3.13	RT-PCR (Real Time Polymerase Chain Reaction)	44
3.14	Compensation Factors for Data Comparison	46
3.15	Statistical Analysis	46

CONTENTS (Continued)

RESULTS AND DISCUSSION

Preliminary Studies

4.0	Comparative incubation	48
4.1	<i>jejuni</i> ATCC33291 Population Dynamics in Subculture	48
4.2	Comparison of Bacterial Recovery According to Selective Media	49
4.3	Determination of Incubation Temperatures	49

Experimental Results

4.4	<i>C. jejuni</i> ATCC-33291 Adhesion and Colonisation	51
4.5	Campylobacter Growth Under Vacuum Conditions	52
4.6	Proliferation of Campylobacter Under MAP Conditions	56
4.7	RT-PCR Analysis of <i>C. Jejuni</i> ATCC-33291 Proliferation	58
4.8	Residual Gas Composition	62
4.9	Digital Modelling of Bacterial Proliferation	65
4.10	<i>Vibrio Spp.</i> Cross-Contamination	67
4.11	Colonisation Potential in VBNC <i>C. jejuni</i> ATCC-33291	69

CONCLUSIONS

	Evaluation and Implications	70
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REFERENCES	74
------------	----

APPENDICES	87
------------	----

## FIGURES

Figure 1.1 – Campylobacter reduction targets for processed chicken carcasses (FSA, 2015)	12
Figure 4.1 – Illustrating increasing recorded temperatures for uncooked chicken pieces stored in a polystyrene shipping container at room temperature ~25°C. (n=2)	41
Figure 4.2 – Proliferation of <i>C. jejuni</i> ATCC-33291 vacuum packaged and incubated at 4°C	52
Figure 4.3 – Proliferation of <i>C. jejuni</i> ATCC-33291 vacuum packaged and incubated at 37°C	53
Figure 4.4 – Proliferation of <i>C. jejuni</i> ATCC-33291 vacuum packaged and incubated at 42°C	53
Figure 4.5 – Proliferation of <i>C. jejuni</i> ATCC-33291 vacuum packaged and incubated at 10°C	54
Figure 4.6 – Proliferation of <i>C. jejuni</i> ATCC-33291 vacuum packaged and incubated at 20°C	55
Figure 4.7 – Proliferation of <i>C. jejuni</i> ATCC-33291 vacuum packaged and incubated at 30°C	55
Figure 4.8 – Proliferation of <i>C. jejuni</i> ATCC-33291 MAP packaged and incubated at 4°C	56
Figure 4.9 – Proliferation of <i>C. jejuni</i> ATCC-33291 MAP packaged and incubated at 10°C	57
Figure 4.10 – Proliferation of <i>C. jejuni</i> ATCC-33291 MAP packaged and incubated at 20°C	57

FIGURES (Continued)

Figure 4.11 – Proliferation of <i>C. jejuni</i> ATCC-33291 MAP packaged and incubated at 30°C	58
Figure 4.12 – Proliferation of <i>C. jejuni</i> ATCC-33291 vacuum packaged and incubated at 20°C – PCR analysis	58
Figure 4.13 – Proliferation of <i>C. jejuni</i> ATCC-33291 vacuum packaged and incubated at 30°C – PCR analysis	59
Figure 4.14 – Proliferation of <i>C. jejuni</i> ATCC-33291 MAP packaged and incubated at 20°C – PCR analysis	60
Figure 4.15 – Proliferation of <i>C. jejuni</i> ATCC-33291 MAP packaged and incubated at 30°C – PCR analysis	60
Figure 4.16 – Proliferation of <i>C. jejuni</i> ATCC-33291 MAP packaged and incubated at 37°C – PCR analysis	61
Figure 4.17 – Gas analysis showing percentage CO <sub>2</sub> content for sample and control chicken portions incubated at 10°C under MAP conditions	62
Figure 4.18 – Gas analysis showing percentage CO <sub>2</sub> content for sample and control chicken portions; incubated at 20°C under MAP conditions	63
Figure 4.19 – Gas analysis showing percentage CO <sub>2</sub> content for sample and control chicken portions; incubated at 30°C under MAP conditions	64
Figure 4.20 – Proliferation of <i>Campylobacter jejuni</i> ATCC-33291 subject to vacuum packaging – protocols 4°C - 42°C, represented as a percentage relative to t <sub>0</sub>	66

FIGURES (Continued)

Figure 4.21 – Proliferation of *Campylobacter jejuni* ATCC-33291 67  
subject to vacuum packaging – PCR data for protocols 20°C - 37°C;  
generated through integration of inoculant based standard curve

Figure 4.22 – Proliferation of *Vibrio parahaemolyticus* in TSB determined 68  
by optical density measurement for temperatures from 4°C - 42°C  
over 48 hours; representing maximum growth rates determined for 5  
wild-type strains

## ACKNOWLEDGEMENTS

For the very best of advice, help and encouragement, an immeasurable debt of gratitude is owed to:

Dr Daniel Anang  
Kathy Lees  
Pam Flint  
Aly Goncharova  
Phoebe Apeagyei  
Dr Haruna Musa  
Mark Kelly  
Dr Andrew Plunkett  
Phil Evans  
Jane Barlow  
Karen Wright  
Siobhan Knight  
Darren Gamble

This project was funded through the Manchester Metropolitan University Knowledge Exchange Innovation Fund; Project No: M-80024.8.5.

## ABSTRACT

In the EU annually, an estimated 9 million people suffer from *Campylobacter Spp.* related food poisoning, with a loss of productivity in the region of 2.4 billion Euros. A Gram-negative bacteria about which little is known outside commercial broiler chicken rearing, the proliferation of campylobacter on fresh chilled chicken produce as a result of temperature abuse in the supply chain is considered to represent a significant risk to consumer health.

The current study employed both culture-based methodology and real time polymerase chain reaction (RT-PCR) analysis to determine the population dynamics of *Campylobacter jejuni* ATCC-33291 on raw chilled chicken produce when packaged under those vacuum and modified atmosphere packaging (MAP) conditions commonly employed in the control of bacterial spoilage. Bacterial growth or decline was determined at 4°C, 10°C, 20°C, 30°C, 37°C and 42°C; reflecting both the typical range of temperature abuse in transit and facilitating comparison with the published literature. Regression analysis of the experimentally derived data was employed to construct a digital model of campylobacter population dynamics; enabling the prediction of bacterial proliferation, and in turn consumer safety, for any given combination of packaged chilled chicken produce and temperature breach parameters.

Digital modelling of the RT-PCR analysis derived data indicates an increase in campylobacter DNA copy number at temperatures of 20°C and 30°C, not reflected in the equivalent enumeration by culture-based microbiology. This observed disparity may reflect a cycling bacteria population or the expression of exogenous bacterial DNA, and indicates a requirement to define the pathology of campylobacteriosis in relation to biofilm structural components. Modelling of *Vibrio Spp.* experimentally isolated from chicken produce highlights both a necessity to characterise bacterial proliferation within a range of campylobacter strains representative of those present in chilled

chicken produce, and indicates a role for RT-PCR based campylobacter identification within both poultry produce monitoring and clinical isolation. Although requiring characterisation, determination of residual gas in MAP packaging shows a significant difference in CO<sub>2</sub> content between control and samples inoculated with *C. jejuni* ATCC-33291 when incubated at 30°C. Further definition may indicate an application for gas analysis in the detection of campylobacter proliferation in retail packaging, in conjunction with digital proliferation modelling, or as a standalone system.

The feasibility of methodology for the generation, integration and digital modelling of bacterial proliferation is discussed, in the context of a temperature and humidity logging tracking system with empirical decision making capability in relation to consumer safety, biosecurity and product origination. Shortcomings in the understanding of *Campylobacter Spp.* lifecycle and host colonisation routes are discussed, serving to highlight an opportunity to both build on the current knowledge of food borne pathogens and form a direct scientific basis for the direction of consumer safety measures.

## INTRODUCTION

On the 3<sup>rd</sup> December 1988, a statement by the then Minister for Health, Edwina Currie MP, failed to differentiate between laying hens and eggs in regard to salmonella infection. While having a valid scientific basis, the ambiguity of the Minister's words resulted in a 60% fall in egg sales, the slaughter of 4 million laying hens due to lost revenue and the bankruptcy of many producers. A failure to successfully tackle the health risk posed by campylobacteriosis could all too easily have similar consequences for the broiler chicken industry today. Campylobacter cross contamination of chicken carcasses is considered an unavoidable consequence of the mechanised processing of broiler chickens; the carcasses of hand processed turkeys appear to suffer no detectable *campylobacter Spp.* colonisation, however there are currently no economically viable alternatives to automated techniques. The primary approach of the industry has therefore focused on implementing strategies to prevent the bacterial infection of the carcasses during processing; to investigate strategies by which to prevent the campylobacter colonisation of the birds' gastro-intestinal tract the micro biota in the first instance; and to utilise novel packaging solutions and consumer education to prevent cross contamination in food preparation. Unfortunately, despite significant research, investment and comprehensive implementation of biosecurity controls, the targets set for the reduction of campylobacter infection levels in broiler chickens by year ending December 2015 are unlikely to be met.

### 1.0 Campylobacter Reduction Strategies

With a target of reducing both the social and economic impacts of campylobacteriosis in the UK, the Food Standards Agency (FSA); the Department for Environment, Food and Rural Affairs (Defra); the British Retail Consortium (BRC), British Poultry Council (BPC) and the National Farmers Union (NFU) have formed the joint government-industry working group ACT

(Acting on Campylobacter Together). In conjunction with other UK research funders, the members of this group have agreed a comprehensive action plan, which includes the adoption of FSA targets to reduce campylobacter counts on processed chicken produce against reference broiler chicken colonisation levels determined in 2008.

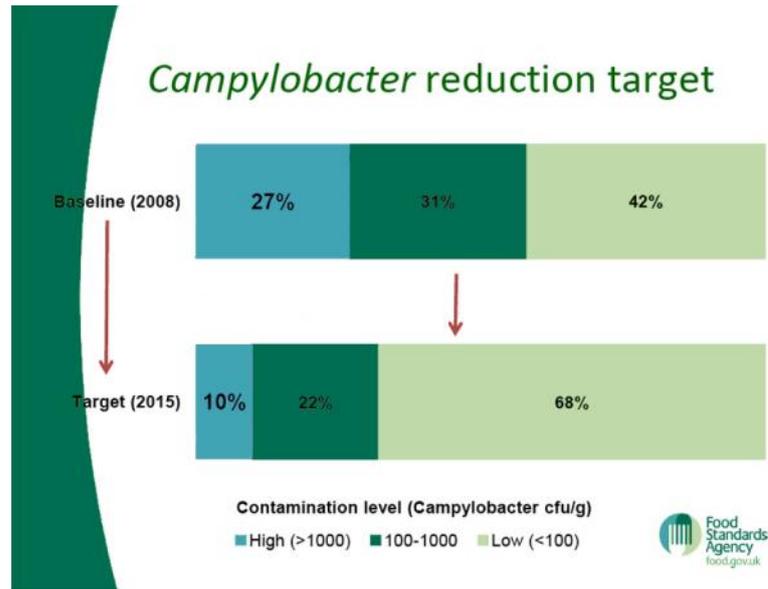


Figure 1.1 – Campylobacter reduction targets for processed chicken carcasses (FSA, 2015).

The FSA, and latterly ACT, have striven to achieve these aims through improvement of farm assurance and standards, and biosecurity measures - novel crate and module disinfection methods; prevention of colonisation during processing alongside implementation of decontamination technologies such as Sonosteam and rapid surface chilling; and a combination of consumer education and novel packaging solutions to reduce kitchen cross-contamination. However, despite significant industry investment and commitment, campylobacter levels in retail chicken have not met FSA 2014 targets, and are unlikely to meet the ACT target of 10% produce carrying less than 1000 campylobacter cfu/g for 2015 - initially set for June but since

extended to December. The most recently published data from the FSA provides a stark indication of the challenges facing the industry, with 73% of retail chicken produce in the year ending February 2015 testing positive for *C. jejuni* or *C. coli* with 25% at the highest level (FSA, 2015).

### 1.1 Chicken Produce Packaging

Until relatively recently, the majority of chicken produce was packed in stretched PVC film, hot tack sealed, and usually contained in a polystyrene tray with an absorbent pad to contain purge spillage. PVC film packaging however, does not adequately absorb moisture, and leads to problems with bacterial contamination of retail spaces through chicken seepage (Harrison *et al*, 2001). Consequently it has been largely replaced by stretch end seal (SES) and barrier display film (BDF). Suited to automated chicken packaging, SES and BDF packs include a leak resistant seal, and facilitate the inclusion of a modified gas atmosphere reducing microbial spoilage and extending shelf life, however care must be taken during packaging to avoid contamination of the external surfaces. Designed to wrap whole birds, BDF hermetically seals the carcass in an antimicrobial atmosphere and is then heat shrunk, ensuring complete gas mix ingress and extending the shelf life to 12 days or more. Most recently, and in response to the concerns of retailers, companies such as Cryovac and Sealed Air have developed 'oven-able' packaging; retaining the extended shelf life while supplying a pre-prepared product with no contamination risk to the consumer.

### 1.2 Vacuum and Modified Atmosphere Packaging

Developed primarily for the preservation of fruit during transport at sea, modified atmosphere packaging (MAP) - be it through the substitution of air for a protective gas mix or removal of the gas component in its entirety - has become a standard technique in prolonging the shelf life of fresh produce through the prevention of spoilage microorganism proliferation. When applied

in packaging of chilled chicken produce, both vacuum packing and carbon dioxide rich modified atmospheres inhibit the growth of Gram-negative aerobic spoilage bacteria such as *Pseudomonas* and *Achromobacter*. In vacuum packaging however, evacuation pressures in excess of 50millibar may result in crushing of the internal cavities when applied to a whole chicken carcass, and so the technique is usually applied in the packaging of portioned pieces, with pressures as high as 500millibar. *Campylobacter Spp.* population dynamics under both vacuum and MAP conditions are yet to be fully characterised, however, low oxygen and elevated CO<sub>2</sub> conditions favourable to *Campylobacter Sp.* colonisation may represent a health risk in the event of temperature abuse (Meredith *et al*, 2014).

### 1.3 Hazard Analysis and Critical Control Point (HACCP)

The effectiveness of MAP in both extending the shelf life of chicken produce and reducing the risk to consumer health is entirely dependent on maintenance of a low temperature to prevent the growth of spoilage and pathogenic microorganisms. EU legislation regarding the transport and storage of poultry produce states: 'Only the conditions fresh (-2°C until +4°C, frozen (-12°C), and quick-frozen (-18°C) are permitted. Fresh poultry must be conserved only at between -2°C and +4°C; frozen and quick frozen poultry meat cannot be sold in a defrosted state' (AVEC-poultry.eu, 2015).

Furthermore, the United Kingdom, EC regulation 852/2004 – The Food Hygiene regulation 2006, states 'this requirement is for the temperature of the food, not the surrounding air' (FSA, 2015). Production, transport and storage of chicken produce within these constraints is the responsibility of each stakeholder within the cold supply chain and, as detailed by the EFSA, FSA (UK) and the European Chilled Food Federation (ECFF), ensuring HACCP principals are applied in maintaining food safety and consumer protection.

While the entire cold supply chain from farm to fork is susceptible to temperature abuse, those critical control points identified within the retail

sector comprise chilled produce delivery and storage, refrigerated display cabinets, transportation after purchase and the storage of chilled produce after purchase (Hammond *et al*, 2011; Derens-Bertheau *et al*, 2014). The necessity to address temperature regulation in retail chill cabinets has coincided with EU targets to reduce carbon emissions through improved efficiencies and is the focus of considerable study (Ochieng *et al*, 2014), while consumer education programs relating food safety practices, including refrigeration, are on-going through the Food Standards Agency; with campylobacter receiving considerable attention. Those precautions implemented in relation to critical control points identified in the delivery, unloading and storage of chilled produce however, remain the responsibility of the specific supply chain stakeholders.

#### 1.4 Transport of Chilled Chicken Produce

In the instance of storage and refrigerated transport of chilled poultry meat, the maintenance, monitoring and recording of temperatures between  $-2^{\circ}\text{C}$  to  $+4^{\circ}\text{C}$  represents both a prerequisite of HACCP implementation and a legal requirement as stated in EC regulation 852/2004. While meeting the stipulations of such legislation represents a relatively minor challenge in the context of a temperature controlled storage facility, road-transport refrigeration equipment must operate reliably under much harsher conditions, in a wide range of operating environments and within constraints of space and weight (Tassou *et al*, 2009).

All such refrigerated transport must comply with the agreement on International Carriage of Perishable foodstuffs and on the special equipment to be used for such carriage (ATP agreement), defining standards, testing and certification. Trailer units however may vary significantly in terms of design, insulation level, refrigeration equipment and performance. In common with chilled retail display, the refrigerated transport industry has faced significant pressure to increase efficiencies and reduce carbon dioxide emissions and,

while the diesel powered tractor-trailer unit remains the most cost effective method of transporting chilled produce, electrical backup supplies are increasingly mandatory for powering stationary refrigeration units in ports, lorry parks, rail marshalling yards and at sea.

Given the extreme operating conditions and the maintenance demands of both a primary diesel power unit and a backup electrical supply, the road transport of chilled chicken produce must be considered a significant critical control point in terms of mandatory temperature maintenance. Both backup battery electrical systems and mains connection points on ports, lorry parks and ship freight decks present problems in terms of supply specification and reliability, and safety; as highlighted by a trailer fire aboard the *Commodore Clipper* (MAIB, 2010), attributed to problems with both the ship power supply and the trailer refrigeration control unit. Given the economic pressures inherent in the transport industry, the majority of minor incidents and associated temperature breaches are likely to both go unreported and to remain uncharacterised in terms of microbiological predictive modelling. In terms of *Campylobacter Spp.* proliferation, such incidents represent a health risk as yet un-quantified.

### 1.5 Aims, Objectives & Limitations

Utilising data generated from both selective media culture based enumeration and RT-PCR analysis (real time polymerase chain reaction), the following research aims to characterise the proliferation of *Campylobacter jejuni* on chilled chicken produce subject to packaging and temperature parameters common to transport between processing facilities and the consumer.

Statistical extrapolation of campylobacter population dynamics for each experimentally tested parameter set will form the basis of a digital model from which bacterial colonisation, and in turn consumer safety, may be predicted for fresh chilled chicken produce in transit according to monitored environmental conditions.

The accomplishment of the study aims were realised through the specific objectives:

- Evaluate the growth pattern of *C.jejuni* subject to both typical poultry-industry packaging regimes and temperature breach regimes.
- Enumeration of campylobacter proliferation on sample pieces at appropriate intervals over the duration of incubation, employing culture-based methodology, RT-PCR, and determination of residual gas composition for the associated packaging.
- Predictive modelling of the growth characteristics of *C.jejuni* according to experimentally tested poultry-industry packaging regimes and temperature breach regimes.
- Robust testing of the predictive digital modelling; interrogation over static and dynamic temperature profiles for comparison with experimentally derived data.

In consideration of both the research timescale constraints and the acknowledged fastidious nature of *Campylobacter Spp*, the aims of this study are limited to a validation of the predictive modelling methodology. As such, the experimental protocols will utilise a single *C. jejuni* subspecies: *Jejuni* (Jones *et al*) Véron and Chatelain (ATCC 33291), a focus of significant comparative published literature. Pivotal to predictive modelling for the purposes of determining consumer risk in this case is an accurate evaluation of the initial levels of bacterial loading on chicken pieces at processing. The investigation of routes of bacterial colonisation, adhesion and cell invasion key to determining such initial parameters within the poultry processing environment represents a feasible separate research project.

## LITERATURE REVIEW

### 2.0 Introduction

In reviewing the literature relating to *Campylobacter*, the initial impression is one of a genus of thermophilic, microaerophilic, Gram-negative bacteria, for the most part sharing pathology with similar enteric micro biota. Widely considered a commensal bacteria in birds and other wild animals and presenting a significant health risk in zoonotic transmission from food animals, the majority of associated research has sought to determine the aetiology of *Campylobacter* related enteric disease; in particular the transmission of bacterial infection from the primary reservoir in broiler chicken flocks to humans. This review will examine those mechanisms of survival and adaptation characterising the unique evolutionary position of the genus, discuss those challenges inherent to detection and enumeration of campylobacter, outline the current understanding of campylobacter host colonisation, and discuss both the ubiquitous nature of the bacteria in the broiler chicken industry and the strategies for its management.

First classified by Sebald and Véron in 1968, *Campylobacter Spp.* gained recognition as the major cause of bacterial enterocolitis by the mid-1980s (Butzler, 2004). *Campylobacter jejuni* and *C. coli* cause the majority of human campylobacteriosis, with infection following an incubation period of 1 to 7 days and characterised by typical enterocolitis symptoms of fever, abdominal cramps and diarrhoea (Schielke *et al*, 2014). The condition is usually self-limiting, but where symptoms do recur, the antibiotics erythromycin and ciproflaxin are particularly effective, although strains resistant to both are beginning to emerge (Jeon *et al*, 2009). In around 1 in 1000 cases, campylobacteriosis is a factor in the development of Guillain-Barré Syndrome (GBS); an autoimmune targeting of the peripheral nervous system which, untreated, may lead to respiratory failure and death in less than four hours from first symptoms. (Nachamkin *et al*, 1998). Recorded cases of

campylobacteriosis are typically those where systemic infection bacteraemia and hospitalisation take place, and number around 210,000 in the EU each year. The vast majority of incidence is thought to be self-resolving and thus unreported, but is estimated as high as 9 million cases annually in the EU (FSA, 2015).

Laboratory reported campylobacteriosis cases in the United Kingdom have risen from around 10,000 cases in 1981 to around 72,000 from 2014, and with the current cost to annual productivity estimated at around £900 million (FSA, 2015), measures aimed at the reduction of incidence have received considerable attention from the poultry industry, the public health and scientific communities. While both improvements in detection method technologies and EU prohibition of antibiotic administration within animal feeds bear consideration in relation to the observed incidence increase, the coinciding growth of hypersensitivity immune disease suggests a need to further define the role of the human immune system in campylobacteriosis. The zoonotic transmission of campylobacter is primarily attributed to the consumption of undercooked chicken, or of ready-to-eat foods that have been in contact with raw chicken. *Campylobacter* are typically established in the gastro intestinal tract (GIT) of broiler birds, and contamination of chicken meat products by contents of the GIT is a seemingly unavoidable consequence of the evisceration, de-feathering and rinsing measures central to poultry processing (Berrang *et al*, 2001; Rosenquist *et al*, 2006; Allen *et al*, 2007). Between 50% and 80% of human campylobacteriosis is currently attributed to chicken produce (FSA, 2015), and given the perceived health benefits to consumers when compared to beef and pork meat products, growth in the popularity of chilled chicken produce is likely to be reflected in an increased incidence of related illness.

An estimated annual cost to European Union productivity of 2.4 billion Euros (EFSA, 2011) has ensured a continued focus from within both the food industry and the research community; the elimination of *Campylobacter* from

broiler flocks considered the most practical approach to reducing the associated health and financial burden (BBSRC, 2014). Mucosal competitive exclusion (Stern *et al*, 2001), vaccination (de Zoete *et al*, 2007), and manipulation of gut micro-flora through dietary modulation (Yang *et al*, 2009), singly or in combination, are all considered to have potential applications in reducing or preventing *Campylobacter* colonisation of broiler flocks. Despite the identification of a number of possible virulence factors, in particular elements of the bacterial flagella structure, neither the nature of *Campylobacter Spp.* pathogenicity nor the mechanisms underlying host colonisation are clearly understood (Roux *et al*, 2013), and much of the data relating to bacteria-host interactions demonstrates significant variability, both within and between studies.

## 2.1 The Genus *Campylobacter*

The campylobacter genus comprises 34 species and 14 subspecies (LPSN Bacterio.net), all of which are oxidase positive and reduce nitrate. Up to 8µm in length, possessing single or bi-polar flagellum, and curved, spiral or gull-winged in appearance, the bacteria are highly motile in a characteristic corkscrew fashion and relatively easily identified by phase contrast microscopy. Most *Campylobacter* species are thermophilic, microaerophilic and capnophilic however, despite optimum growth temperatures ranging from 15°C to 42°C, are able to survive low temperatures and prolonged freezing with limited loss of viability (Membre *et al*, 2013). In transmission through the environment and food animal agriculture, *Campylobacter* must survive hostile environmental conditions and does so in part by forming biofilms in response to nutrient stress, desiccation, or oxygen tension. Under conditions such as prolonged planktonic suspension or low temperature, *Campylobacter* may also adopt a coccoid phenotype, which, although non-culturable, remains viable and maintains some degree of host colonisation capacity (Rollins and Colwell, 1986; Jones *et al*, 1991; Thomas *et al*, 2002; Chaisowwong *et al*, 2012). Relatively small at 1.6 mega bases, the *C. jejuni* genome is notable for

an almost complete lack of repeat, phage associated, or insertion sequences, and a skewed guanine/cytosine content of 30.6% (Parkhill *et al*, 2000). Also unusually in Gram-negative bacteria, *Campylobacter* utilises both N- and O-linked glycosylation in post-translational modification. With biosynthetic precursors and respective molecular pathways similar to those found in eukaryotes, such associated modified glycoproteins may fulfil comparable biological roles (Szymanski *et al*, 2003).

## 2.2 *Campylobacter* Environmental Adaptation

*Campylobacter Spp.* may be isolated from rivers, estuarine and coastal waters (Keener *et al*, 2004), but it is thought they exist largely as commensal gut bacteria in poultry, cattle and other animals (Hendrixson and DiRita, 2004). From a phylogeny comprising three separate clades or branches, all *Campylobacter* strains known to cause disease in humans, and the majority of those found in farm animals, belong to a single clade originating from a branching event around 2500 years ago. Coinciding with early agriculture and the housing of multiple food animals in close proximity, the colonisation of creatures with varying physiology by similar strains of bacteria may suggest adoption of a newly available niche by a specifically adapted bacterial lineage rather than one sharing a common gene pool with a pre-existing natural reservoir (Sheppard *et al*, 2010). Alternatively, for a lineage possessing adaptations facilitating the colonisation of multiple host types via water borne transmission, the relatively stable agricultural niche may simply represent a prime opportunity for specialisation. Contemporary *Campylobacter* strains however, are likely to reflect a niche specific population shaped by the selective influence of agricultural micro flora reduction strategies such as dietary modulation and routine antibiotic treatments, and may differ greatly from primary colonising and antecedent strains.

Multi locus sequence typing (MLST) of the housekeeping genes from *Campylobacter* strains in the broiler flocks of New Zealand, a country with a

relatively self-contained broiler chicken supply chain, demonstrates a correlation between distinct *Campylobacter* subtypes and particular poultry production facilities (Mullner *et al*, 2009). Furthermore, analysis of *Campylobacter* subtypes worldwide reveals a complex pattern of host-strain specificity and spatial distribution, indicating specificity between individual broiler production companies and bacterial subtypes, the propagation of which is likely to have stemmed from a relatively small number of independent introduction and expansion events (Sheppard *et al*, 2011). From this perspective, the specific bacteria-host interactions occurring in the broiler production facilities of companies like Aviagen, who supply almost half of the 50 billion broiler chickens produced annually worldwide, represent both a unique research opportunity and a key target area in the reduction of poultry production related campylobacteriosis.

### 2.3 Limitations in Campylobacter Detection and Enumeration

*Campylobacter Spp.* are generally understood to establish in the GIT of broiler chickens (Hermans *et al*, 2003) however, the mechanisms underlying the colonisation and pathogenicity characteristics are not clearly understood. Electron microscopy indicates predominant sites of localisation in the ceca, large intestine and the cloaca of the birds, colonising the lining mucus in numbers of the order  $10^7$  to  $10^9$  cfu/ml without attaching to the crypt microvilli (Beery *et al*, 1988). The overall impression therefore is one of an enteric bacterium displaying adaptations suited to asymptotically colonising the broiler chicken GIT and enduring as part of the commensal micro biota. This characterisation however, is not completely borne out in the published literature, and the high degree of variability evident in the experimentally derived data appears to confirm the complex bacteria-host interaction indicated by MLST analysis, an interaction not readily conforming to existing models of microbial survival and proliferation.

Those difficulties encountered in attempting to define the colonisation mechanisms of *Campylobacter* are compounded by a reliance on culture based methodology. Quite aside from limitations in terms of resolution, culture techniques are unable to detect *Campylobacter Spp.* of a viable but non-culturable phenotype (VBNC). On entering VBNC *Campylobacter* progressively adopt a coccoid shape similar to that observed in lag phase decline. The bacteria remain metabolically active, retaining cellular and genomic integrity (Besnard *et al*, 2002; Thomas *et al*, 2002; Cook and Bolster, 2007). Generally considered a survival strategy in response to stress, a variety of bacterial species, when facing challenges such as nutrient stress or low temperature, express a VBNC phenotype (Du *et al*, 2007; Ducret *et al*, 2014). Given that planktonic suspension and low temperatures prompt VBNC phenotype switching in *Campylobacter*, the distinction between a survival strategy and a water borne transmission route is not clearly defined.

Characterisation of the VBNC phenotype in *Escherichia coli* indicates modifications of the outer membrane to be specific to the nature of the challenge inducing the non-culturable state; the structure of the outer membrane expressed in response to light and seawater differing to that induced by low temperature and freshwater (Muela *et al*, 2008). In expressing components best suited to survival or transmission in the encountered environment, those outer membrane proteins specific to differing VBNC phenotypes may influence a range of factors including virulence determinants, antibiotic resistance, adhesion properties and colonisation potential (Li *et al*, 2014). While those genes principal to biofilm formation in the face of stressors such as pH, heat shock and oxidative stress are well defined, (Bronowski *et al*, 2014); VBNC phenotype specificity in *Campylobacter Spp.* is poorly understood. Considering the associated energetic cost of increased genomic 'programming' capacity and current understanding of bacterial polysaccharide usage (Cuccui and Wren, 2014), post-translational glycosylation pathways are likely to play a significant role in the expression of both VBNC and biofilm phenotypes. Understanding the molecular basis of VBNC cell encapsulation

and its co-ordination may both indicate control points for elimination of waterborne transmission vectors, and provide an insight as to the lifecycle of the bacteria beyond the agricultural clade.

Unquestionably, studies employing culture based isolation and enumeration technique must consider the non-detection or underestimation of viable cells in any sample thought to contain *Campylobacter Spp*, in a consumer safety or clinical context. In attempting to define *Campylobacter* transmission routes in broiler chicken flocks, widespread application of culture techniques promotes an understanding of colonisation and bacterial population dynamics differing to that gathered by real time polymerase chain reaction (RT-PCR). For instance, the failure to detect *Campylobacter* in chicks below 14 days of age by culture methods supports a role for maternal antibodies in preventing bacterial infection. Conversely, the detection of *Campylobacter* DNA in broiler chicken embryos by RT-PCR analysis supports a hypothesis of bacterial transmission inherent to the reproductive process (Hiatt *et al*, 2013). However, with PCR unable to differentiate between DNA from viable cells, from dead cells, or possibly from biofilm exogenous DNA expression, no definite conclusion may be drawn. Modification of the RT-PCR protocol to bind the those DNA fragments from compromised and dead cells with propidium monoazide before extracting and amplifying the DNA of viable cells in a sample (Josefson *et al*, 2010) is proposed to neatly circumvent this problem, however implementation of the methodology can exhibit incomplete extraneous DNA binding (Pacholewicz *et al*, 2013). At present, clinical diagnostics, food safety evaluation and microbiological research lack a quick and definitive method of *Campylobacter* detection and enumeration.

#### 2.4 *Campylobacter Spp*. Host Colonisation and Immunopathology

As acknowledged throughout the published literature, current understanding of those mechanisms underlying the pathogenicity characteristics of *Campylobacter Spp*. is relatively limited. In human campylobacteriosis, the

symptoms correlate with those of invasive inflammatory diarrhoea, possibly generated by a Th1 biased immune recognition of *Campylobacter* membrane glycoproteins rather than an inherent bacterial pathogenicity. In the progression of *C. jejuni* associated GBS, characterised in some detail, a Th1 polarised response generates antibodies specific to distinct non-self *C. jejuni* cell surface proteins. The antibody epitope is however also cross-reactive to ganglioside glycoproteins within the peripheral nervous system. This Th1 inflammatory response facilitates permeation of the blood-nerve barrier by opsonising antibodies, which recruit macrophages to the ganglioside antigen. The subsequent de-myelination of the nerve fibres results in a loss of signalling integrity and peripheral nervous system paralysis (Malik *et al*, 2014).

As the largest interface between the interior of an animal and its environment, the gut associated lymphoid tissue (GALT) plays a fundamental role in modulating and shaping specific host responses to gut-borne pathogens (Li *et al*, 2010). GALT acts as a reservoir of immune cells including T lymphocytes, the most abundant effector cells, classified as CD4<sup>+</sup> (cluster of differentiation) and CD8<sup>+</sup> according to the expression of surface molecules. CD4<sup>+</sup> or T helper cells generate the majority of cytokine hormonal messengers, mediating the immune response according to the nature of the pathogen. Macrophages, CD8<sup>+</sup> T cells, IFN- $\gamma$ , TNF- $\beta$  and inflammatory interleukins are associated with a pro-inflammatory, T helper 1 (Th1) immune response against intracellular pathogens; anti-inflammatory interleukins, B-cells, eosinophils and mast cells associated with a Th2 humoral response against extracellular parasites (Berger, 2000; Saito *et al*, 2010). A number of autoimmune diseases are acknowledged to have a basis in a disproportional response to commensal microflora and interactions of GIT populations as a whole are thought to modulate host responses and immunological tolerances to both commensal and pathogenic bacteria (Kelly *et al*, 2005).

The symptoms of campylobacteriosis in humans contrasts significantly with *Campylobacter* colonisation of commercial broiler chicken flocks, observed to be the generally tolerogenic (Hermans *et al*, 2012). Characterisation of the host-bacteria interaction in these birds however, is the subject of considerable debate. While investigation of the associated immunopathology reveals complex interactions, largely indicative of a Th1 biased immune response and appropriate to invasive bacterial infection, current understanding of the nature of colonisation, pathogenicity and tolerance mechanisms is relatively limited. The immune response to *Campylobacter* in broiler chickens is shown to stem from an initial recognition of bacterial membrane proteins by Toll-like receptor 4 (TLR4) and TLR21 (Humphrey *et al*, 2014), triggering an innate and in turn adaptive immune response. The pattern of TLR activation in humans differs to that of chickens, suggesting a role for the innate immune system in the differing pathology observed in each host, and possibly influenced by antigen exposure during early adaptive immune system plasticity.

Quantification of IL1 $\beta$  and IL-8 mRNA expression differed in response to *Campylobacter* infection, with a markedly smaller expression of cytokines in chicken macrophages than that of human MM6 monocytes (de Zoete *et al*, 2010). Interestingly, expression of mRNA for both cytokines was significantly higher when challenged with disrupted bacterial cells than with viable *Campylobacter* - 175 fold as opposed to 4 fold for IL1 $\beta$  and 125 fold compared to 16 fold for IL8. In both humans and chickens, only disrupted bacteria activate TLR2. Increased exposure to bacterial protein antigens may account for the increased immune response to lysed campylobacter cells, and may indicate immunomodulation on the part of campylobacter; and a role for lysed bacteria or exogenous proteins in the pathology of human campylobacteriosis. An up-regulation of the synthesis of human  $\beta$  defensins (epithelial antimicrobial peptides) is also observed in response to human *Campylobacter* infection, however in this host, loss of the polysaccharide capsule in disruption of the bacterial cell does not influence expression levels

of the protein (Zilbauer *et al*, 2005). In light of the role of TLRs in triggering adaptive immunity, the lysing of bacterial cells by  $\beta$  defensins may act as an amplifying feedback loop to the inflammatory response observed in human *Campylobacter* infection.

The experimental infection of birds by oral infection of *Campylobacter* has been shown to illicit an increase in circulating heterophils (human neutrophil equivalent) and intestinal inflammation appropriate to a bacterial infection (Smith *et al*, 2008; Humphreys *et al*, 2014), however this was not judged to lead to disease. Comparison of *Campylobacter* and *Salmonella* human infection *in vivo* noted no reduction of circulating heterophils in response to *Campylobacter*, while expression of antimicrobial peptides (AMP), shown to increase following *Salmonella* infection, was found to decrease in response to *Campylobacter* challenge, again suggesting a degree of immune-modulation potential (Shaughnessy *et al*, 2008). Caution must be exercised in evaluating data gathered through experimental colonisation however, as stress related variation in the blood corticosterone concentrations of subject birds may lead to increased expression of pro-inflammatory chemokines and a bias to the Th1/Th2 response (Shini and Kiaser, 2009) in both sample and control groups.

## 2.5 Broiler Flock Colonisation Vectors

Within the poultry industry, colonisation of broiler chickens is considered largely asymptomatic, and the bacteria forms part of the commensal microbiota in the great majority of farmed poultry. Broiler chicken houses appear to provide an ideal environment for the transmission of campylobacter between birds, and from the first detection of campylobacter infection, an entire flock may be colonised within a few days (Miflin *et al*, 2001). Those vectors leading to the initial colonisation of a campylobacter negative flock however, are at present unclear, and are likely to be both numerous and diverse in nature. All broiler-breeding flocks test positive for campylobacter and thus vertical

transmission; bacterial infection of the egg within the brood chicken, represents an obvious mechanism for broiler flock colonisation. RT-PCR analysis of broiler eggs may indicate the presence of campylobacter DNA (Hiett *et al*, 2013); however there is no evidence of viable bacterial colonisation, and such detection may be attributed to the DNA from lysed bacterial cells, or to sequences common to both 'housekeeping' genes and to exogenous biofilm derived DNA.

Naturally occurring polyclonal antibodies in chicken egg yolk are shown to afford a degree of protection against the vertical transmission of *Salmonella enteritidis* (Herrera *et al*, 2013), and the presence of campylobacter specific maternal antibodies in hatchling chicks (Shoaf-Sweeney *et al*, 2008) may be indicative of a similar protective immune mechanism. Considering campylobacter infection of a single egg sufficient to cause colonisation of an entire flock, it is unrealistic to dismiss the possibility of vertical transmission. However, plate culture analysis of day old chick cecal contents show no indications of campylobacter infection. In the absence of evidence to the contrary therefore, placed hatchling flocks are deemed campylobacter free and subsequently, initial bird colonisation is assumed to occur by means of on-farm environmental vectors, and once established in a member of the broiler flock, bird-to-bird transmission of campylobacter is thought to result from the contamination of drinking water through the shedding of viable bacteria in the faeces of infected birds. Between harvesting of a flock and placing of its replacement, campylobacter may exist as planktonic VBNC bacterial cells in pooled water or as biofilms on broiler house surfaces and in transmission through the food chain, campylobacter biofilm formation represents a strategy for survival through unfavourable environmental conditions. Adopted in the avoidance of oxidative stress, biofilm formation may protect the bacteria from ultraviolet radiation, anti-microbial agents, desiccation, and predation, effectively doubling the viable duration of *Campylobacter* under atmospheric and waste or drainage water system conditions (Reuter *et al*, 2010).

Although able to form mono-species biofilms, campylobacter appears more suited to a role as a secondary coloniser. It has limited biosynthesis capability and is to some degree dependent on the secondary metabolites of other bacterial species (Hanning *et al*, 2008). Multi-species biofilm structures are highly dynamic, with signalling networks acting to stabilise micro biomes through the switching of phenotypes in a precisely co-ordinated manner and regulating co-enzymes, siderophores, virulence factors and physical population structures (Kerenyi *et al*, 2013). Autolysis and the associated expression of extra-cellular DNA may act to trigger *Campylobacter* biofilm formation or dispersal and, interestingly, entire motile flagella are important factors in both biofilm formation and in host colonisation (Svensson *et al*, 2014).

From chick placement, campylobacter colonisation of a broiler flock usually takes around 2 to 3 weeks, with total flock infection usual within a further 48 hours. Broiler chicks are thought to gain some protection from bacterial colonisation in this early stage from maternal antibodies and comparison of IgG (antigen specific antibody generated by B cells in response to pathogen infection) expression in response to campylobacter colonisation reveals an elevated and faster response as the birds' age (Sahin *et al*, 2003). Whether from environmental reservoirs or faecal shedding, campylobacter isolates possess differing colonisation potentials depending on phenotype. The variation observed in the up-regulation of this process may be as complex as a sampling of the internal environment of a prospective host organism, modifying cell surface protein expression to maximise colonisation, or may simply reflect the percentage of the bacterial population expressing a spiral and motile as opposed to VBNC phenotype. Shown to exhibit a chemotactic response to mucin, flagellar motility is considered fundamental to the campylobacter colonisation process (Lertsethtakarn *et al*, 2011). While the duration of passage and the subsequent phenotypic switching may account for the lag phase observed in flock colonisation (Cawthraw *et al*, 1996), those

mechanisms underlying campylobacter host colonisation are poorly understood. Isogenic campylobacter strains are shown to exhibit variable and unpredictable population structures *in vivo*, and factors such as founder effect are thought to play an important role in subsequent bacterial colony organisation (Coward *et al*, 2008).

## 2.6 Campylobacter Reduction Strategies

A variety of biosecurity measures are shown effective in reducing the infection levels of birds raised in barns; the ubiquitous nature of *Campylobacter Spp.* in the natural environment precludes the possibility of protecting free-range birds from colonisation. In the first instance, sterilisation of equipment, storage, floor space and buildings before the placement of chicks is considered essential. Thereafter, the growing area is effectively a 'clean room', with controlled staff entry, barriers between all other areas and the exterior, and provision of contamination prevention measures such as boot washes and workspace specific equipment. The introduction of fly exclusion screens has shown some success (Hald *et al*, 2007), however, meticulous biosecurity practise constitutes the primary strategy in reducing colonisation of broiler chicken flocks.

Delivery of a campylobacter free flock to the processing facility is generally sufficient to ensure campylobacter free produce, however the transport of broiler flocks for harvesting presents its own challenges. The biosecurity of bird crates, modules - fork-lift friendly multiple crate racks - and trailer units presents potential hazards both in the transport of flocks, and in sterilisation of equipment between flocks. With the practice of thinning, partial harvesting of a broiler flock to meet the market demand for varying bird sizes, and harvesting broiler flocks at night, any delay in processing where birds are kept in inadequately sterilised crates at relatively high temperatures through the day may represent a high infection risk. Both broiler chicken campylobacter colonisation level and human campylobacteriosis incidence exhibit seasonal

variations correlating with increasing external temperatures and daylight hours (Meldrum *et al*, 2004). Interestingly however, the seasonal increase in human campylobacteriosis is shown to occur weeks before that of observed colonisation increases in housed broiler chickens, and may be indicative of a common, unidentified, environmental vector (Meldrum *et al*, 2005; Patrick *et al*, 2004).

Other than systemic bacterial infection due to stressors such as ill health, thinning, or prolonged transport confinement, campylobacter contamination of chicken produce is considered primarily a consequence of mechanised processing techniques (Berrang *et al*, 2001; Rosenquist *et al*, 2006; Allen *et al*, 2007). Progression through the processes to packaged whole or portioned birds highlights key areas where the risk of carcass contamination with bacterial contents of the gastrointestinal tract is especially high. Particularly true of mechanical intestine removal during the evisceration process; where equipment adjustment to the correct bird size for a specific flock is critical; water borne transmission during scalding and aerosol contamination during de-feathering represent equally critical control points. During scalding, water temperatures of 50°C are employed to loosen feathers without compromising the meat appearance rather than having any antibacterial role and with the addition of chemical additives strictly regulated, both water surface foam and lime scale deposits are acknowledged to act as a bacterial reservoir (Humphrey and Lanning, 1987). Around the plucking equipment itself, aerosol bacterial numbers are high enough for direct quantification by selective media and represent both a contamination risk and a significant campylobacteriosis hazard to the workforce. Retail packaging of poultry produce represents a further control point, and inadequate standards of hygiene, ineffectual process design, or poor equipment maintenance may result in detection of higher campylobacter numbers on portioned chicken meat than on a whole bird carcass (Corry and Atabay, 2001).

In reducing the future health and financial burden of campylobacteriosis, the complete exclusion of the bacteria from broiler flocks is considered the best possible solution within both the poultry industry and the scientific community, however while a number of approaches show promise, that 'silver bullet' solution has remained elusive. Considerable research has centred on the vaccination of broiler flocks, but implementation presents significant challenges in terms of the identification of novel non cross-reactive, protection inducing antigens; induction of a rapid, potent immune response; and development of novel adjuvants to enhance the immune response against the target bacteria (de Zeote *et al*, 2007). The passive immunisation of laying hens with both whole cell lysate and with hydrophobic protein fraction of *C. jejuni* is shown to achieve significant reductions in bacterial colonisation following experimental oral seeding of broiler chickens, and a variety of *C. jejuni* proteins associated with cell functions such as chemotaxis and adhesion are thought to represent promising targets for vaccine development and are also highly conserved between bacterial strains (Hermans *et al*, 2014). Mucosal exclusion is considered a possible means of achieving complete eradication, however prophylactic measures intended to clear flocks of *Campylobacter* under experimental conditions are not considered adequate to completely eradicate either *Salmonella enteritidis* or *Campylobacter* in fowl (Calenge and Beaumont, 2012).

## 2.7 Conclusions

In a large part due to the associated health risks, the great majority of associated research has centred on treatment of *Campylobacter Spp.* pathogenicity in humans and routes of transmission both to and between broiler chickens. The current understanding of *Campylobacter Spp.* routes of colonisation, mechanisms of pathogenicity and lifecycle outside of the agricultural niche however, remain poorly understood. *Campylobacter* colonisation is observed to illicit widely varying responses in even isotypical hosts, and formulating an appropriate response must seek to reconcile the

application of a rigid mechanistic understanding to what appears to be an inherently plastic process. Furthermore, while the emergence of Campylobacteriosis in the mid 1980's is commonly associated with the development of and dissemination of isolation and enumeration techniques; the quantification of an existing health problem made possible by technological advances, the categorisation of campylobacteriosis as a condition with a basis in allergic intolerance cannot be discounted. It is therefore essential to establish a differentiation between immunological pathogenicity, with symptoms of illness attributable to the host response, and inherent bacterial toxogenicity.

It is suggested that both immunisation programmes and a reduced exposure to microbiological antigens in childhood may account for the observed increased prevalence of atopy since the 1980's, and this may hold true to some degree for campylobacteriosis incidence. Interestingly, the current understanding of the protective effects of exposure to allergenic proteins during the immunological plasticity of human infancy are set to undergo a fundamental re-examination in light of seemingly contradictory findings relating to peanut allergies (Gruchalla and Sampson, 2015). Certainly, vaccination based immuno-tolerance or the tempering of the human immune response to render campylobacter infection asymptomatic may emerge as the most effective means to reduce both the health and financial burden.

The basis of any further actions aimed at the control of *Campylobacter Spp.* as a food-borne pathogen however, must reflect a complete understanding of the possible consequences. The collective study of campylobacter has served to highlight the complexity of this species rather than adding specific definition to our understanding, and the consequences of acting without at least an approximation of the full facts are, at best, uncertain. As surely as the effective loss of broad-spectrum antibiotics through misguided application is certain to have far-reaching consequences for both human and food animal health, injudicious implementation of campylobacter eradication techniques,

be it vaccination, mucosal exclusion or alternative programs, runs the risk of selecting in favour of treatment resistant bacteria and triggering a whole new set of challenges. In the interim, the current reduction in levels of 'on-farm' broiler flock campylobacter colonisation are not sufficient to reduce campylobacteriosis incidence to any significant degree (FSA, 2015).

Efforts to reduce the extent of human infection must therefore focus on post harvest reduction strategies such as supply chain biosecurity; novel 'bake-able' packaging; and consumer education relating to 'good kitchen practises'. Of these, bacterial proliferation resulting from temperature breach during the storage or refrigerated transport of chilled poultry produce represents a health risk yet to be quantified, and little is known in relation to the population dynamics of *Campylobacter Spp.* when subject to modified atmosphere or vacuum packaging conditions primarily employed to inhibit the growth of Gram-negative aerobic spoilage bacteria. Application of both selective media culture-based enumeration and RT-PCR in the determination of the effects of atmosphere on campylobacter proliferation may provide an insight into key survival processes such as survival triggered gene mutation up-regulation, and represents a significant objective in terms of both consumer safety and a contribution to the contemporary understanding of bacterial population dynamics.

## RESEARCH STRATEGY

- Sample inoculation with *Campylobacter jejuni*; employing, as closely as practical, a sample matrix and methodology reflecting chicken produce colonisation during processing and an inoculant representative of the bacterial population density of the chicken gastro-intestinal tract
- Enumeration of bacterial colonisation at time zero as a reference value for the comparison of sample matrix bacterial population dynamics
- Sample incubation according to experimental protocol parameters of temperature and gas packaging:
  - Temperatures 4°C, 10°C, 20°C, 30°C, 37°C, and 42°C
  - Vacuum packaging and industry standard poultry packing MAP
- Bacterial enumeration at time points over the experimental protocol duration:
  - t = 1, 2, 4, 6, 8, 12, 18, 24, 48, 72, 96, 120 and 144 hours as applicable to observed campylobacter population dynamics
- Data tabulation, statistical analysis and digital modelling; characterisation of bacterial population dynamics according to packaging and temperature parameters and utilising regression analysis for the purposes of interpolation and proliferation prediction

## METHODOLOGY

### Preliminary Studies

#### 3.0 *Campylobacter Spp.* Experimental Strain

Sourced as a freeze-dried colony (Oxoid UK, Cultiloop CL1400), *Campylobacter jejuni* ATCC 33291 was activated as per Oxoid recommendations and incubated in triptone soy broth (TSB (Oxoid UK, CM0876)) for 24 hours at 37°C. A limited gas headspace was utilised to limit oxidative stress; acknowledged to both inhibit bacterial growth and to promote expression of the VBNC phenotype in *Campylobacter Spp.* 24-hour incubation yielded a broth culture with a turbidity value of around 6 on the McFarlane scale indicating a cell density in the region of  $10^6$  to  $10^7$  colony-forming units per millilitre (cfu/ml) and. The viability of this working culture was confirmed by streaking on *Campylobacter* blood free selective agar (Oxoid UK, CM0739) mixed with a CCDA selective supplement (Oxoid UK, SR0155), followed by comparative incubation at 37°C under both a 10% CO<sub>2</sub> atmosphere regulated incubator and the CampyGen micro-aerophilic atmosphere sachet system; generating an atmosphere of approximately 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85%N<sub>2</sub>.

#### 3.1 *C. jejuni* ATCC33291 Population Dynamics in Subculture

A second-generation *C. jejuni* ATCC 33291 culture, incubated from a single positively identified plate colony was plated and incubated as above with comparative incubation at 37°C utilising a 20% CO<sub>2</sub> atmosphere regulated incubator and the CampyGen micro-aerophilic atmosphere generating sachet system. Gram staining, oxidase testing, and API-Campy analysis (API-biomerieux SA, marcy l'Etoile, France) served to validate aseptic technique and confirm the culture strain as *C. jejuni* ATCC 33291 prior to further subculture and enumeration. Bacterial colony numbers were recorded for serial subculture and incubation, repeated through to the expression of the VBNC (viable but non-culturable) phenotype.

### 3.2 Comparison of Bacterial Recovery According to Selective Media

Serial dilutions of bacterial culture were prepared in ¼ strength Ringers solution pH7 (Oxoid UK, BR0052) and 100ul of dilution factors  $10^{-2}$  to  $10^{-6}$  plated on media with a variety of selective supplements: Blaser-Wong (Oxoid UK, SR0098); Butzler (Oxoid UK, SR0085); CCDA; Karmali (Oxoid UK, CM0935); Preston (Oxoid UK, CM0689); and Skirrow (Oxoid UK, SR0069). All plates were incubated under a micro-aerophilic atmosphere for 48 hours; at 37°C for CCDA selective media and at 42°C for all others as per the supplier specification. Bacterial recovery for those plates numbering between 30 and 300 colonies were recorded for comparison. Gram staining, oxidase testing and API-Campy analysis served to confirm culture strain.

### 3.3 *C. jejuni* ATCC-33291 inoculant Generation

Whatever the transmission route; aerosol dispersal of cecal mucus, colonisation of rinsing agents or biofilm formation on equipment surfaces, *C. jejuni* and *C. coli* are detected on the majority of chicken produce currently sold in the UK. Given the high degree of sensitivity observed in the human immune response to campylobacter therefore, proliferation of even relatively small numbers of bacterial cells may represent a significant risk to human health when subject to a temperature breach.

Detection of such relatively low cell numbers however, is at the limits of resolution for both plate culture and PCR techniques, while the enumeration of *Campylobacter Spp.* proliferation at this scale is neither suited to modelling purposes nor practical or sufficient to generate statistically reliable data. The experimental design therefore, must balance the generation of usable data with an accurate representation of the sample matrix bacterial inoculation and packaging conditions *in transit*. As a component of chicken cecal mucosal flora, *Campylobacter Spp.* populations typically number between  $10^5$  and  $10^7$  cfu/ml. Consequently the number of bacterial cells contaminating chicken

produce by either aerosol infection or by cell sloughing from surface biofilms is unlikely to exceed this concentration. For the purpose of sample inoculation therefore, a bacterial culture of this magnitude is representative of wild-type campylobacter colonisation of the chicken sample matrix and statistically valid when applied to digital modelling of both positive and negative population trends. In generating inoculation cultures from pathogenic bacteria such as *E. coli* or *Salmonella*, it is both effective and convenient to maintain a working culture by a serially doubling of the inoculant in line with known bacterial proliferation at a given temperature, in campylobacter however, oxidative stress inherent to the methodology induces a VBNC phenotype.

Building on those techniques effective in maintaining a bacterial culture, the transfer of 100µl of  $10^6$ cfu/ml culture to a 500ml Duran bottle in which the headspace was limited by filling TSB to the 600ml level proved the most reliable process for generating a viable inoculant. By this method, a 72-hour incubation at 37°C generates an inoculant in the order of  $10^7$ cfu/ml, while doing so in multiples of 600ml allows for experimental flexibility. Hanging drop microscopy to evaluate bacterial activity and morphology was applied in evaluating the inoculant phenotype while Gram staining, oxidase testing, and API-Campy analysis served to validate aseptic technique and to confirm culture strain. Aeration of the bacterial inoculant during both decanting and sample inoculation was minimised to prevent oxidative stress and in turn bacterial expression of the VBNC phenotype.

### 3.4 Determination of Experimental Temperature Parameters

Temperature and humidity data was collected on the freight cargo deck of *Stena Mersey* for the day sailing on September 11<sup>th</sup> 2014 and the 13/14<sup>th</sup> overnight crossing for the longest duration sea route from Ireland to the United Kingdom; Belfast to Liverpool (DT-172 Temperature and Humidity Data logger, CEM Instruments UK). The thaw characteristics of chicken produce subject to temperature breach in transport were determined

experimentally based on an initial internal temperature of +4°C and an ambient temperature of +25°C. Once surrounded by Ice Brix gel packs and sealed in a polystyrene shipping container, the internal temperature of chicken pieces was logged (Tec-troniks UK) through to parity with room temperature.

### 3.5 Vacuum and Modified Atmosphere Packaging

Packaging chilled uncooked chicken products in a carbon dioxide rich atmosphere inhibits the growth of spoilage bacteria, and in particular Gram-negative aerobic species. Percentages of CO<sub>2</sub> in excess of 25% however cause discolouration of the meat (Dairyscience.info, 2015), and thus a ratio of 20% carbon dioxide to 80% nitrogen has become industry standard for the MAP of chilled raw poultry. Vacuum packing is increasingly applied in the packaging of portioned chicken pieces, but less so to whole bird carcasses. The experimental method will therefore quantify the proliferation of *C. jejuni* ATC 33291 under MAP conditions of 20% CO<sub>2</sub>; 80% N<sub>2</sub>, and vacuum packaging at 500millibar when incubated at temperatures of 4°C, 10°C, 20°C, 30°C, 37°C and 42°C. Sampling for enumeration and tabulation will retain a degree of flexibility, with intervals adjusted to best describe both growth and decline trends observed in *C. jejuni* ATC 33291 for given experimental protocol temperature and packaging parameters.

### 3.6 Adjustments to Vacuum and MAP 30°C Protocol

Swabs from contaminated control Butzler selective media plates were TSB incubated at 42°C under microaerophilic conditions for streak-plate isolation and identification. Review of both the aseptic technique and the methodology employed to sterilise the chicken portions prior to inoculation indicated no deviation from the methodology. Determination of the *Campylobacter* ATCC-33291 proliferation characteristics under conditions of MAP at 30°C was resolved through inoculation of complete chicken portion as per the methodology, but with the immediate excision of the 25g section usually

removed following incubation; for homogenation, serial dilution and enumeration. The excised section was packed and sealed along with the remaining portion on separate sterilised weighing boats within the same packaging pouch and incubated as per the experimental protocol.

## Experimental Methodology

### 3.7 Sample Preparation

Oak Farms of Yorkshire supplied the sample chicken in 5kg MAP batches consisting ~22 pieces between 200g and 250g in weight. The opened packaging was stored for up to 72 hours at 4°C to allow dissipation of MAP gases from fat and tissue of the meat. *Campylobacter* cross-contamination occurs during scalding (~50°C), plucking, evisceration and portioning, and sample chicken pieces were allowed to warm to the laboratory ambient temperature (~25°C); considered representative of the later and higher risk processes. Under aseptic conditions, the chicken pieces were sterilised by immersion in alcohol for approximately 60 seconds.

Following air-drying on a sterilised wire rack for five minutes, each chicken piece was passed through a Bunsen flame to remove residual alcohol and placed in one of two sterile 10 litre borosilicate glass beakers; one each for sample and control. Other than the reference time-point (t0), where three sample pieces were prepared against a single control, a single sample and control were prepared for each experimental time-point (t4, t18, t24 etc.) according to experimental protocol requirements. In consideration of both equipment availability and laboratory access, each experimental protocol followed a 7-day schedule with a maximum incubation period of 168 hours.

### 3.8 Inoculant Preparation, Verification and Enumeration

As with the chicken samples, the campylobacter inoculant was allowed to cool to ambient temperature (~25°C), and then mixed thoroughly before use. In consideration of the fastidious nature of *C. jejuni* ATCC 33291 and the difficulties experienced in generating a reliable bacterial culture, hanging drop microscopy was employed to evaluate bacterial cell morphology, motility and turbidity. A viable bacterial inoculant of the order  $10^6$  to  $10^8$  cfu/ml will display turbidity in the upper range of the McFarlane scale, and highly motile bacterial cells of a rod or spiral morphology. Observation of coccoidal morphology or reduced motility indicates a viable but non-culturable (VBNC) inoculant applicable only to determination of the colonisation potential for this phenotype. Immediately prior to use, 2 x 1ml samples of inoculant were removed for enumeration by polymerase chain reaction (PCR) analysis and by plating on Butzler and Karmali selective media.

### 3.9 Sample Inoculation, Packaging, and Incubation

Sample and control chicken pieces were inoculated with *C. jejuni* ATCC 33291 culture and a sterile TSB broth respectively; at a volume twice that by weight of the prepared chicken pieces for a given experimental protocol. In consideration of the sensitivity of campylobacter to oxidative stress, the inoculant was decanted by pouring from the Duran bottle onto the inside glass surface of the tilted 10 litre glass beaker to limit aeration. The chicken pieces remained immersed for 30 minutes to ensure sufficient bacterial attachment, with a gentle stirring every ten minutes to ensure complete coverage. On draining of the inoculant, the samples were promptly homogenised or packed in a modified atmosphere to minimise bacterial oxidative stress.

All sample and control packaging was carried out using a MultiVac C200 (MultiVac UK) countertop gas packer in combination with multilayer polyethylene/polyamide vacuum pouches. Where MAP was applied, gas

balances were regulated through the Witt Gasetechnik KM20-5 (MulitVac UK) with the gas mix calibrated using a Dansensor CheckMate 3 (Dansensor UK). Inoculated sample and control chicken pieces, in a medium size vacuum bag, were placed in the C200 evacuation chamber with the bag opening located on the heat-sealing bar. Application of a pressure of -500millibar acts to remove all air from the chamber and, in the case of vacuum packaging, the pouch is sealed by application of the heat-sealing bar. The chamber is equalised to atmospheric pressure by the controlled introduction of air whereby atmospheric pressure forms the multilayer construction of the pouch around the packaged chicken portion.

In MAP, the packaging pouch was not sealed following evacuation of the air in the chamber, but rather filled by a gas mix of 20% CO<sub>2</sub> and 80% N<sub>2</sub>, regulated by the Witt Gasetechnik CM20-5. Equalisation of the MultiVac chamber by the controlled introduction of the modified atmosphere gas mix ensures total control over the gas composition of the produce packaging. Activating the heat-sealing bar at ambient pressure minus 50millibar pre-seals the opening of the vacuum pouch, and prevents entry of atmospheric gases.

Where practical and appropriate to characterisation of the bacterial growth curve, incubation and subsequent bacterial enumeration was carried out at 1, 2, 4, 6, 8, 16 & 18 hours from inoculation. Incubation of vacuum packaged sample and control portions for the purpose of culture based enumeration and PCR analysis was carried out at 4°C, 10°C, 20°C, 30°C, 37°C and 42°C, and MAP packaged at 4°C, 10°C, 20°C and 30°C.

### 3.10 Residual Gas Composition Measurement

For each experimental sampling time-point, and prior to sampling, the gas content of the MAP pouch was determined, and a mean of three measurements tabulated. As in calibration of gas mixtures supplied by the Witt Gasetechnik unit, the residual gas generated through the bacterial

spoilage in sample and control gas packaged chicken pieces was determined using the Dansensor CheckMate 3. The needle of the Dansensor Smart Pen was introduced to the vacuum bag through an adhesive, self-sealing spot, preventing both leakage and contamination of the gas contents, and the tip placed in the approximate midpoint of the gas content. On activation of a 'Manual Spot' measurement, values for the O<sub>2</sub> and CO<sub>2</sub> content of the modified atmosphere was quantified from mean measurement over a period of 15 seconds.

### 3.11 *C. jejuni* ATCC-33291 Enumeration

Standard methodology for sample preparation in food microbiology stipulates the aseptic excision of a 25g sample for homogenisation and enumeration. In this instance however, *C. jejuni* ATCC-33291 colonisation of the chicken pieces is present only on the sample surface as opposed to being distributed throughout the sample matrix. Therefore, the 25g portion removed was no more than 1mm in depth and removed from the surface area. This sample tissue was placed in a stomacher bag with Ringers 1/4 strength solution at a ratio of 1:9 and homogenised for 60 seconds (Stomacher 400 Laboratory Blender). On completion, 2000µl of homogenate sample was frozen for PCR analysis, while 1000µl was further serially diluted to a range considered representative of sample colonisation range; typically a 10<sup>-3</sup> to 10<sup>-6</sup> dilution factor. 100µl of the appropriate diluent was plated on both Butzler and Karmali selective media. Following 48 hours microaerophilic incubation at 42°C, those plates numbering 30 to 300 colonies were counted, and the data tabulated.

### 3.12 Turbidity/Optical Density Measurement

For the purposes of proliferation of measurement of *Vibrio Spp.* bacteria, the optical density of bacterial colonies in TSB was compared over time against a control sterile TSB sample using a scanning spectrometer at 660nm (Genysys 10uv). An average of four measurements was tabulated for samples

incubated in TSB at time-points 1, 2, 4, 6, 8, 24 and 48 hours for temperatures 10°C, 20°C, 30°C, 37°C and 42°C, and facilitating digital modelling of wild-type bacteria isolated from each batch of processed chicken portions.

### 3.13 RT-PCR (Real Time Polymerase Chain Reaction)

As per supplied directions, Congen Kit F1008 was used to isolate *Campylobacter Spp.* DNA from both homogenised sample/control diluent and inoculant cultures. 1000µl of the sample homogenate was placed in an Eppendorf tube and centrifuged for 5 minutes at 12,000 rpm to remove the bacterial cells from suspension. After pouring off the supernatant, 400µl of the Congen kit lysis buffer was mixed with the pelleted campylobacter cells, and incubated at 99°C on an Eppendorf heated agitating plate for 10 minutes, denaturing cell membranes and releasing the bacterial genomic DNA.

After a 1-minute centrifuge at 12,000rpm removed the larger cellular debris, the remaining lysate supernatant was pipetted onto a clear spin filter, again supplied with the Congen kit. After a 1-minute incubation to allow the lysate to permeate, the spin filter was centrifuged for a further minute at 12,000rpm, separating the remaining large cellular debris from the bacterial DNA. The resulting filtrate was mixed with 200µl of Congen binding buffer and added to a Congen yellow spin filter. After a short incubation, the yellow filter and receiver tube were centrifuged, again for a minute at 12,000rpm, to spread the bound DNA through the filter media.

On discarding this filtrate, any remaining debris associated with the filter-bound DNA was washed out using 550µl of wash buffer, and centrifuging at 12,000rpm for 1 minute. This step was repeated, and on the second occasion, the filter was centrifuged for a further 2 minutes at 12,000rpm to remove any remaining wash buffer ethanol. In order to remove the bound DNA from the spin filter, 100µl of preheated elution buffer at 60°C was added to the filter. Allowed to incubate for 3 minutes, the filter was placed in a final

receiver tube and centrifuged for 1 minute, releasing the bacterial DNA and culminating in 100ul DNA sample suited to PCR analysis.

For the purposes of *Campylobacter Spp.* DNA quantification, the Campylobacter Plus kit (SureFast) was used in conjunction with a Lightcycler 96 PCR unit (Roche). This kit detects the housekeeping genes of *C. coli*, *C. jejuni*, and *C. lari*, with a lower limit of five copies or less dependent on the sample matrix. Each PCR reaction well comprises 19.9µl of reaction mix, 0.1µl Taq polymerase, and 5ul of the bacterial DNA test sample. When used with the Lightcycler unit, the setup comprises an initial denaturation step of 60sec at 95°C followed by 45 amplification cycles of 15sec at 60°C and 10sec at 95°C, with amplification of both sample and the reaction mix internal control represented visually in real time.

Pre-programmed computational algorithms are employed by the Lightcycler 96 to generate all correlation coefficients, y-intercept, and relative quantification data from the fluorescence curves in accordance with MIQE guidelines. Other than the assignment of standard curve values, this functionality is not user-modifiable. The MIQE acronym relates to the Minimum Information for Publication of Quantitative real-time PCR Experiments, and refers to the minimum standards for the evaluation and comparison of research data generated by qRT-PCR. For the purposes of quantitative comparison, a standard curve was formulated from *C. jejuni* ATCC-33291 inoculant; comprising 100%, 50%, 25%, 10% and 5%. Although theoretically inclusion of the standard curve facilitates quantitative analysis, error may arise through the inability to differentiate between viable bacterial cells DNA, dead cell DNA and exogenous DNA expression. Therefore a relative value was calculated to enable comparison with the selective media data tabulation, and yielding comparable data in terms of inoculation efficiencies and bacterial population growth or decline over the experimental protocol.

### 3.14 Compensation factors for Data Comparison

Bacterial numbers were calculated relative to a reference value: the enumeration of bacterial attachment from samples inoculated alongside those of the selected experimental protocol, but homogenised and plated on selective media at time-point zero. In tabulating the results of each experimental protocol, the mean bacterial enumeration for t0 was assigned a value of 100%, to which any increase or decline in bacterial numbers was referenced; likewise as a percentage. In application, this approach compensates for the experimental variance inherent to both culture preparation and sample inoculation, and enables comparison of experimental data derived from both culture method enumeration and PCR analysis.

### 3.15 Statistical Analysis

Regression analysis was performed for the selective media data and, where applicable, RT-PCR data determined in each experimental protocol. Utilising Microsoft Excel, 2<sup>nd</sup> order polynomial, linear or exponential regression was applied to generate a trend-line; best fitting the experimental data while consistent with recognised *Campylobacter Spp.* population dynamics. Inherent experimental variance is presented as a standard deviation calculated from all t0 sample values, and extrapolated as a percentage of relative sampling-point values. For the purposes of both statistical comparison and the digital modelling of bacterial population dynamics, a time dependent data set was calculated from the trendline equation generated for each experimental protocol.

The resulting datasets represent neither normal distribution nor form paired or independent samples, rendering the application of the Mann-Whitney U test or dependent t test inappropriate for the purpose of statistical analysis. The Wilcoxon signed rank test, being suited to the differentiation of non-parametric but related data sets was applied, assuming a null hypothesis of the

compared samples belonging to the same dataset. By pooled ranking for all observed differences between two samples, the Wilcoxon signed rank test generates a standard score and 'p' value denoting the confidence level for the comparison. In consisting of one measurement variable and two nominal variables, comparative analysis of those data relating changes in the gas composition of MAP packaged samples and controls through incubation at temperatures 10°C, 20°C and 30°C were compared utilising a paired t-test. The null hypothesis that both datasets are from the same sample was employed as a test premise.

## RESULTS & DISCUSSION

### Preliminary Studies

#### 4.0 Comparative Incubation

Comparative incubation of *C. jejuni* ATCC 33291 plated on CCDA selective media using the Campygen sachet/gas jar system and the Thermo Scientific incubator set at 10% CO<sub>2</sub> shows a reduced growth rate for the Incubator plates. Incubating at 20% CO<sub>2</sub> showed no significant differences in terms of colony morphology or enumeration when compared with those incubated with the Campygen system. As such, it follows that the increased campylobacter proliferation observed at 20% CO<sub>2</sub> may be attributable to the corresponding decrease in incubator O<sub>2</sub> levels and in turn reduced oxidative stress. Should this be the case, it serves to underline the high degree of oxygen sensitivity inherent to the *C. jejuni* strain ATCC 33291.

#### 4.1 *C. jejuni* ATCC33291 Population Dynamics in Subculture

Cultiloop strain *C. jejuni* ATCC 33291 typically produces a culture of density 10<sup>6</sup>cfu/ml when incubated at 37°C for 48 hours in TSB with a minimal headspace to reduce oxygen stress. Repeated 72-hour sub-culture generates a population in the order of 10<sup>5</sup>cfu/ml until a further decimal reduction precedes expression of a VBNC phenotype. Interestingly, continued 72-hour sub-culture of VBNC *C. jejuni* ATCC33291 generates broth cultures with an approximate turbidity of 6 on the McFarlane scale, indicating bacterial metabolism and cell division in this phenotype. Maintaining a working culture therefore necessitates alternating the growth of *C. jejuni* ATCC 33291 cultures between TSB media and selective media. For the purposes of experimental development, *C. jejuni* strain ATCC 33291 when incubated in TSB broth at 37°C, exhibits a stationary phase 72 to 96 hours after subculture, with viable cell counts between 10<sup>5</sup> and 10<sup>6</sup>cfu/ml. During decline phase, recoverable cell

counts fall exponentially to zero at around 144 hours, and during which time campylobacter may express a VBNC phenotype. Unfortunately, neither polymerase chain reaction (PCR) nor standard culture methods are sufficient to differentiate between VBNC bacteria, non-viable cells and exogenous DNA. In addition to nutrient depletion, expression of the campylobacter VBNC phenotype may be induced by long-term planktonic suspension, and represents those challenges central to maintaining a viable working TSB culture.

#### 4.2 Comparison of Bacterial Recovery According to Selective Media

Butzler selective media performed best for the purposes of bacterial enumeration over the course of the experimental duration, with Karmali media providing a secondary count for comparison. Furthermore, the high degree of uniformity observed in Butzler and Karmali selective media constituted a homogenous background in terms of growth rates and colony morphology, ideally suited to the differentiation and identification of related wild-type bacterial strains recovered from experimental sample. CCDA media failed to reliably isolate *C. jejuni* strain ATCC 33291 and may indicate a higher sensitivity to oxidative stress in this strain as all other selective media contain oxygen quenching laked blood requiring incubation at 42°C.

#### 4.3 Determination of Incubation Temperatures

From a hazard and critical point analysis (HACCP) perspective, un-scheduled or prolonged tractor-unit stoppages represent a significant temperature abuse risk, and in particular the transport of chilled shipping containers by sea. As the longest duration crossing for chilled chicken produce in transit from Ireland to the United Kingdom and Europe, refrigerated containers on the Belfast – Liverpool route will run on battery backup or shipboard power for more than 14 hours. Other than the trailer coefficient of heat transfer, the extent of any temperature increase resulting from an unrecorded refrigeration failure (the vehicle deck is off limits at sea) is therefore dependent on ambient temperature and humidity. Data gathered on the cargo deck of the *Stena*

*Mersey* for the day sailing on September 11<sup>th</sup> 2014 and the overnight crossing on 13/14<sup>th</sup> shows an average temperature of 19.86°C with a relative humidity of 65% despite a relatively consistent external temperature of 10°C (unpublished). Air temperature on the freight deck rose during loading and unloading to a maximum of 26.7°C, possibly attributable to Ro-Ro arc lighting, radiating tractor-unit engine heat and the restarting of diesel refrigeration units. Based on a probable temperature breach range of 10°C to 30°C therefore, experimental protocols will quantify *C. jejuni* ATC 33291 population dynamics at 10°C, 20°C and 30°C. By way of a control enabling comparison with the published literature, *Campylobacter* dynamics will be characterised at 4°C - static or slow decline in bacterial cell numbers, at 37°C - threshold growth, and at 42°C - optimum growth rate (Membre *et al*, 2013).

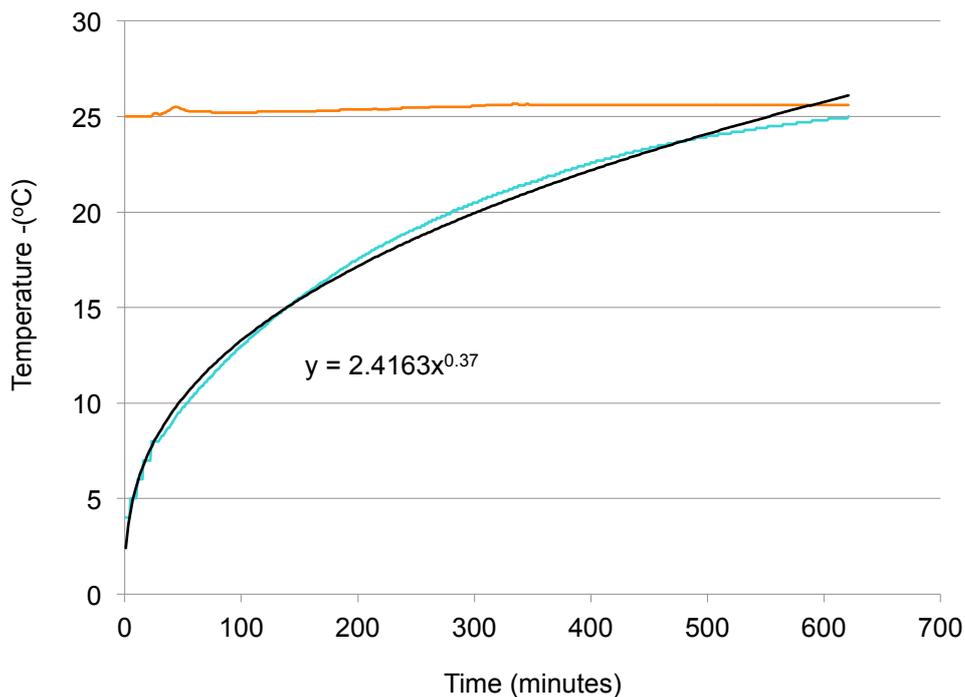


Figure 4.1 – Illustrating increasing recorded temperatures for uncooked chicken pieces stored in a polystyrene shipping container at room temperature ~25°C, (n=2).

In the absence of figures relating the thaw characteristics of a refrigerated trailer unit, the data presented in figure 4.1 describes the internal temperature

of chicken pieces sealed in a polystyrene shipping container, surrounded by Ice Brix gel packs, and allowed to warm from +4°C to a room temperature of approximately 25°C (Tec-troniks UK). Characterisation of *C. jejuni* ATC 33291 proliferation on inoculated chicken samples under both MAP and vacuum packaged conditions when subject to this temperature profile will enable the generation of campylobacter growth curves for dynamic temperature change and enable comparisons with digital modelling derived from enumeration of bacterial growth under static temperature protocols.

## Experimental Results

Through the course of each experimental protocol, the generated data were tabulated for the purposes of regression analysis, population growth/decline comparison, and the generation of digital modelling. As anticipated, the fastidious nature of *Campylobacter jejuni* gave rise to difficulties in both the maintenance of a working culture; in generation of bacterial inoculant; and in execution of the experimental protocol. Expression of a viable but non-culturable phenotype (VBNC) generated no culture based bacterial proliferation data in 9 experiments of 40. Previous systemic colonisation of the sample matrix by *Vibrio spp.* bacteria impervious to surface layer sterilisation resulted in unreliable observations in a further 5 experiments prior to successful modification of the experimental method. Those data contributing to digital modelling of *C. jejuni* ATC-33291 constitute values determined over 26 experiments for 10 experimental protocols and, although minimal in terms of statistical analysis, may be considered representative of the effects of temperature and atmospheric conditions on bacterial population dynamics.

### 4.4 *C. jejuni* ATCC-33291 Adhesion and Colonisation

Over the course of those 26 experimental protocols in which *C. jejuni* adhesion was observed, an average inoculation efficiency of 2.25% calculated for a mean inoculant density of  $8.1 \times 10^7$  cfu/ml. In consideration of the low

experimental repeat numbers, an estimate of the variability inherent to the inoculation methodology was calculated from the variance observed in t0 samples. A standard deviation of 24.4% was calculated from the percentage deviation from the mean for each inoculation protocol, and applied to those of subsequent sampling time-points.

#### 4.5 Campylobacter Growth Under Vacuum Conditions

As illustrated in figures 4.2, 4.3, and 4.4, those temperature protocols providing a comparison with the published literature (4°C, 37°C and 42°C) indicate the proliferation of *Campylobacter jejuni* ATCC-33291 under conditions of vacuum packaging to correspond to those characterised in the published literature (Membre, 2013). At 4°C, bacterial populations are maintained over the entire 168-hour duration of the experiment, reflecting the limited loss of viability documented in the published literature. Visualisation of proliferation at both 37°C and 42°C under conditions of vacuum packaging show typical bacterial lag-log-decline characteristics again mirroring the acknowledged *C. jejuni* population dynamics over this temperatures range.

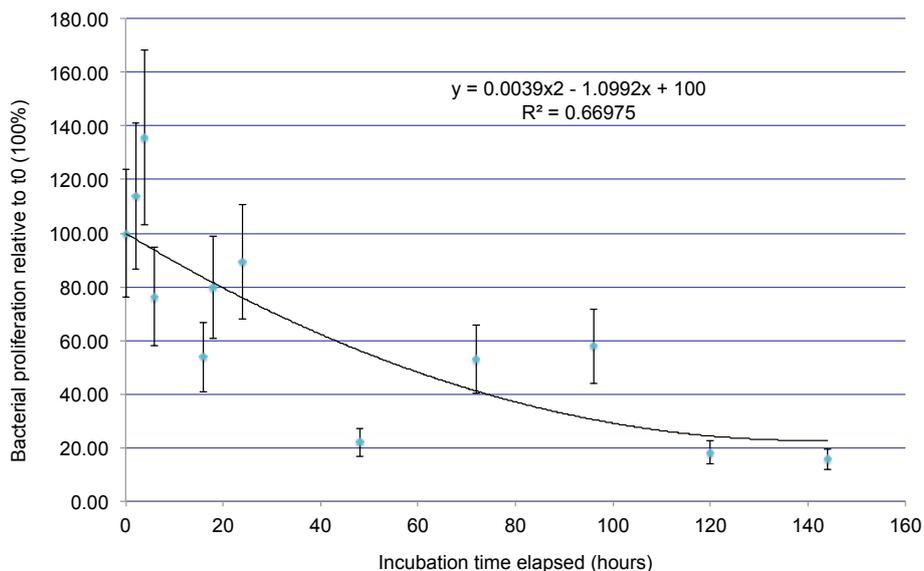


Figure 4.2 – Proliferation of *C. jejuni* ATCC-33291 vacuum packaged and Incubated at 4°C

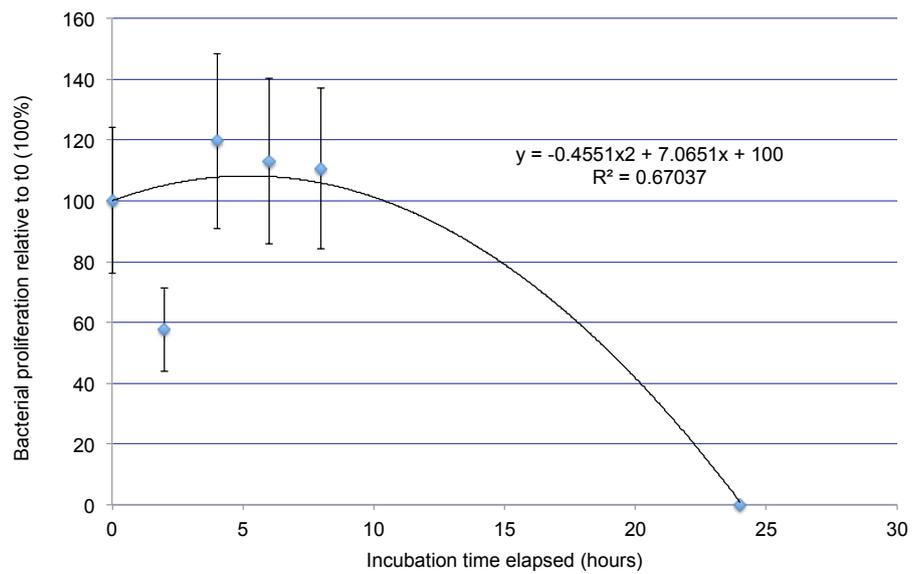


Figure 4.3 – Proliferation of *C. jejuni* ATCC-33291 vacuum packaged and Incubated at 37°C

Wilcoxon signed rank analysis comparison of bacterial populations dynamics at 37°C and 42°C under conditions of vacuum packaging highlights no significant differences between these protocols, while both differ significantly to that at 4°C. It is worth considering however, both the contained nature of

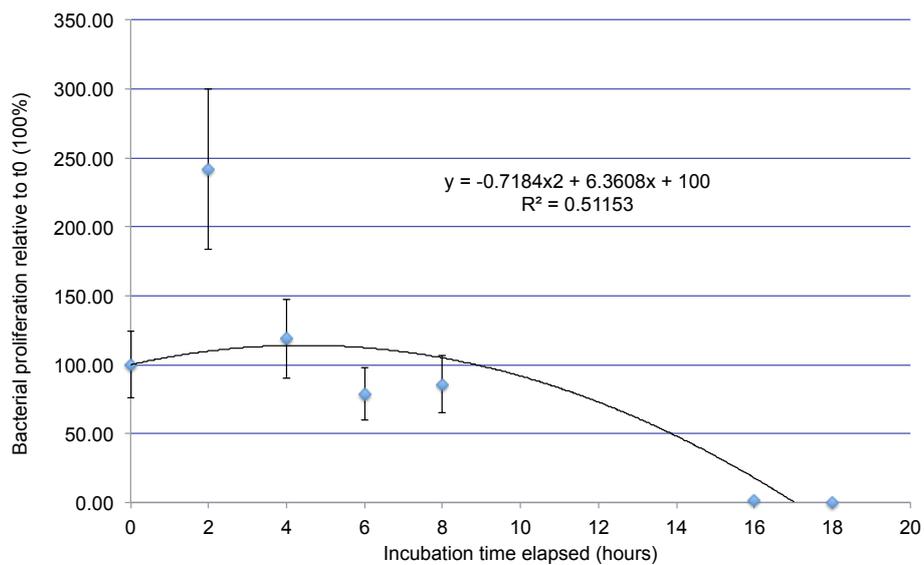


Figure 4.4 – Proliferation of *C. jejuni* ATCC-33291 vacuum packaged and Incubated at 42°C

the modified-atmosphere packaging pouch and the considerably higher bacterial colonisation levels likely to arise from experimental inoculation when compared to bacterial contamination during mechanised poultry processing. Higher campylobacter numbers may result in a significantly increased rate of proliferation, and subsequent decline, than that observed in the supply chain and representing a possible factor in the predictive modelling process. Wilcoxon analysis testing indicates a significant difference not only between the population dynamics of *C. jejuni* ATCC-33291 incubated under conditions of vacuum packaging at 10°C, 20°C and 30°C when compared with those at 4°C. Significant variation is also indicated between protocols, suggesting a high degree of variability within campylobacter population dynamics over the temperature range 10°C to 30°C.

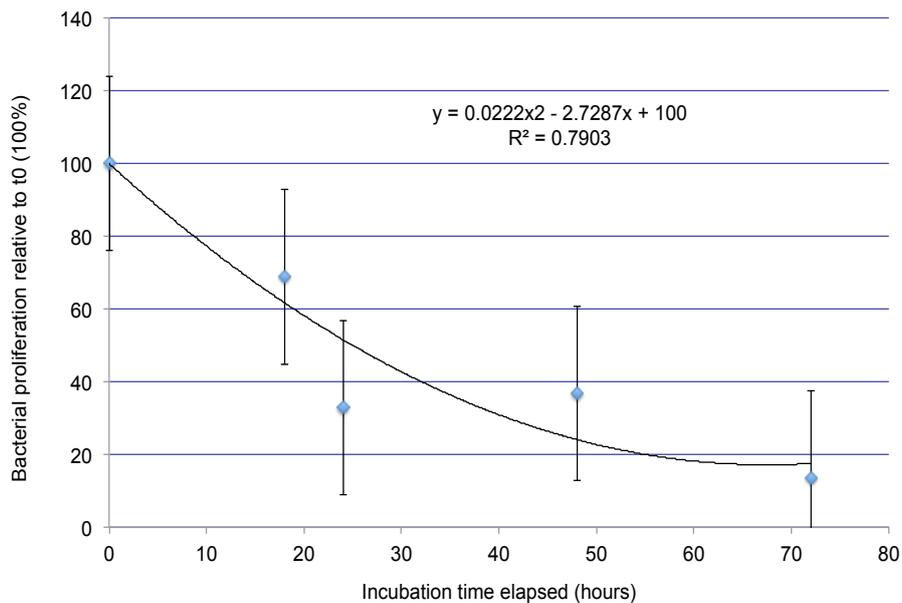


Figure 4.5 – Proliferation of *C. jejuni* ATCC-33291 vacuum packaged and Incubated at 10°C

This statistical difference between rates of population decline observed in vacuum protocols 10°C, 20°C and 30°C is evident in graphical representation of the data (Figures 4.5, 4.6 and 4.7). Interestingly, bacterial survival is shown not to be temperature dependent through this range, and while the highest

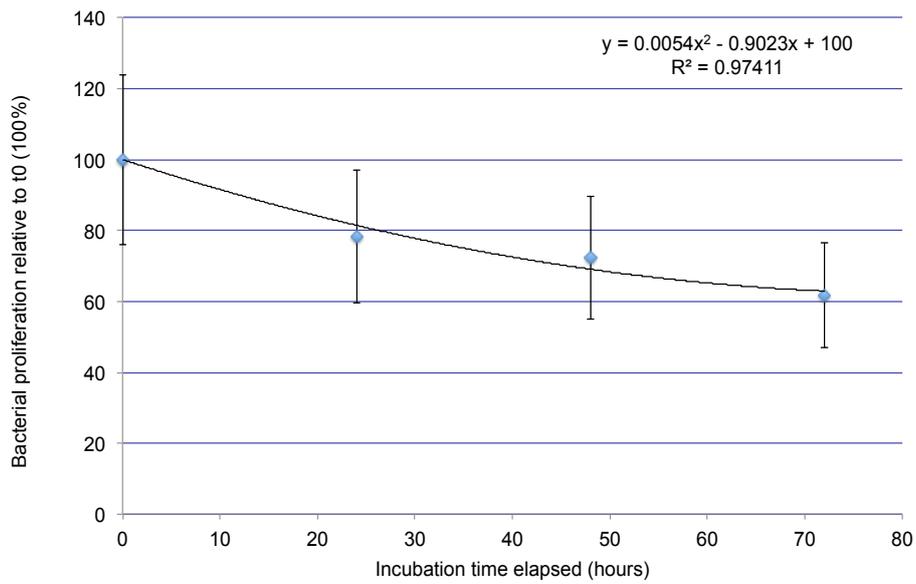


Figure 4.6 – Proliferation of *C. jejuni* ATCC-33291 vacuum packaged and Incubated at 20°C

levels of bacterial survival observed at 20°C might represent an artefact of regression analysis of a limited dataset, such correlation may relate to expression of temperature-specific genes specific to biofilm formation; up-regulation of genetic mutation or uncharacterised, survival strategies.

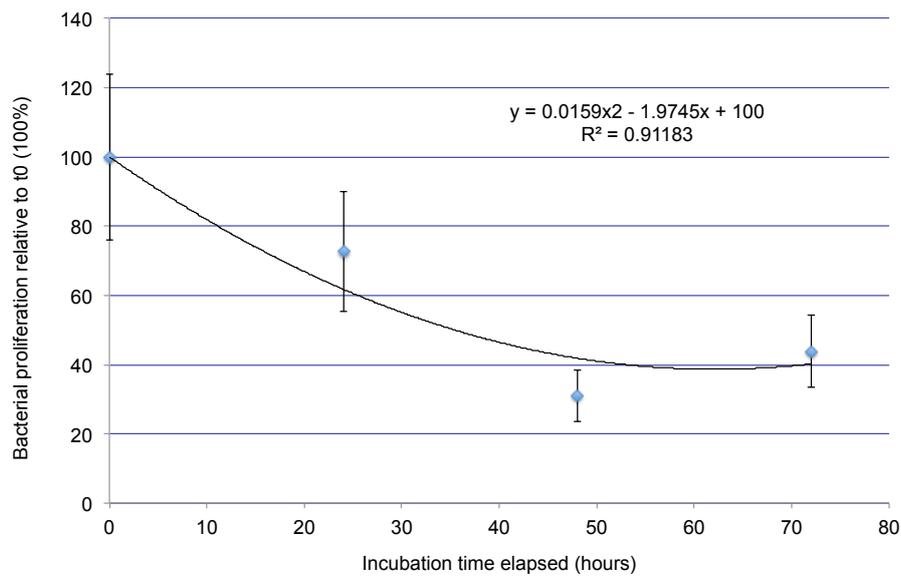


Figure 4.7 – Proliferation of *C. jejuni* ATCC-33291 vacuum packaged and Incubated at 30°C

#### 4.6 Proliferation of Campylobacter Under MAP Conditions

Wilcoxon signed rank comparison in the comparison of those data generated by incubation of vacuum and MAP packaged samples at 4°C highlighted a significant difference attributable to the difference in packaging conditions. Likewise, comparison of data generated for experimental protocols 10°C and 30°C incubated under MAP conditions show significant difference to that of the 4°C protocol, while the 20°C dataset again shows correlation with that of 4°C highlighting possible temperature specific gene expression (Figures 4.8 to 4.11). Comparison of campylobacter population dynamics when subject to incubation under conditions of vacuum packaging with those of

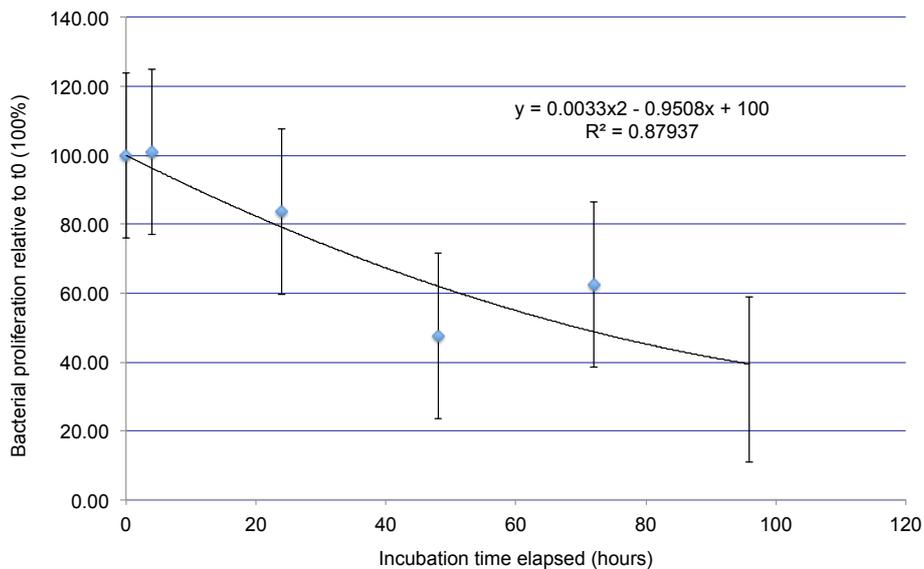


Figure 4.8 – Proliferation of *C. jejuni* ATCC-33291 MAP packaged and incubated at 4°C

MAP exhibit statistically significant dissimilarity at 10°C and 20°C, however no notable difference is evident at 30°C. Whether elevated CO<sub>2</sub> and the removal of O<sub>2</sub>, and thus oxidative stress, represents an environment suited to the maintenance of campylobacter populations or adoption of a temperature specific response at

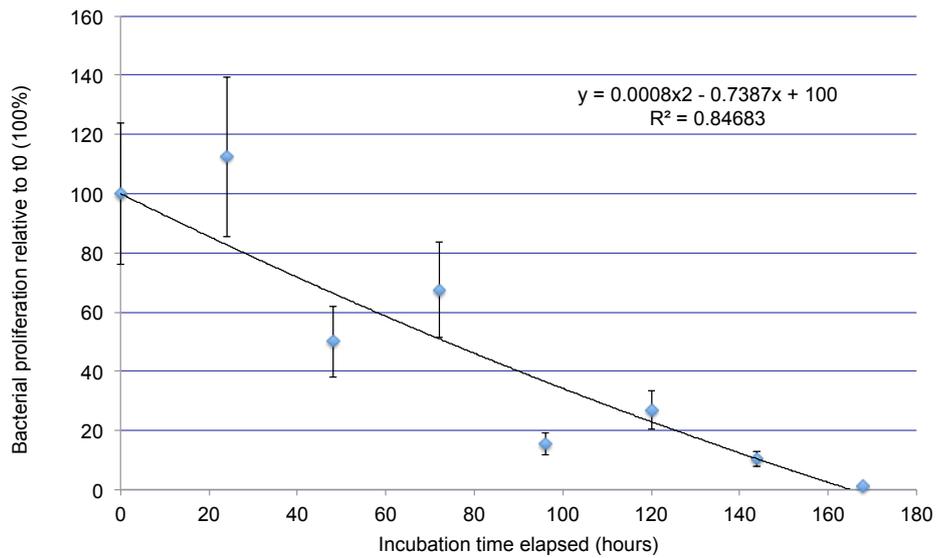


Figure 4.9 – Proliferation of *C. jejuni* ATCC-33291 MAP packaged and incubated at 10°C

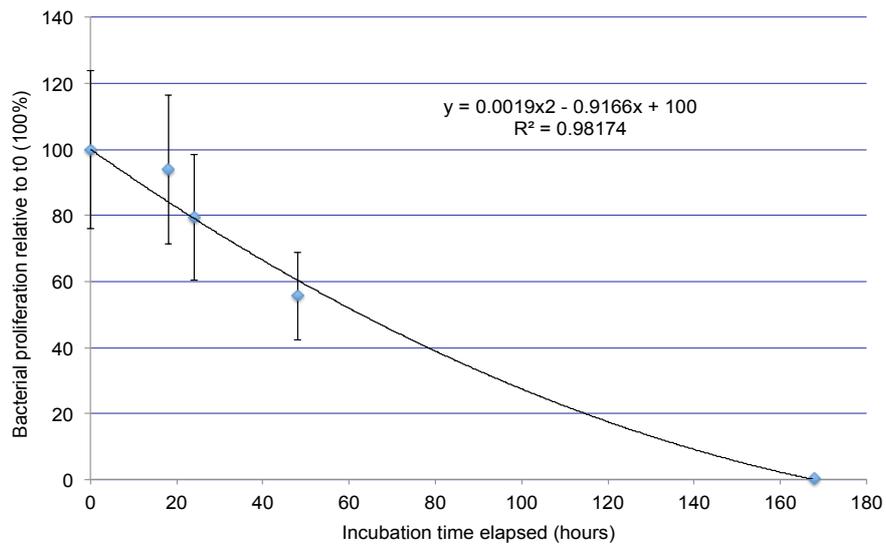


Figure 4.10 – Proliferation of *C. jejuni* ATCC-33291 MAP packaged and incubated at 20°C

30°C regardless of atmospheric conditions is unclear, however removal of outlying data points served to accentuate rather than reduce the Z associated p-value, indicating the statistical evaluation to be valid.

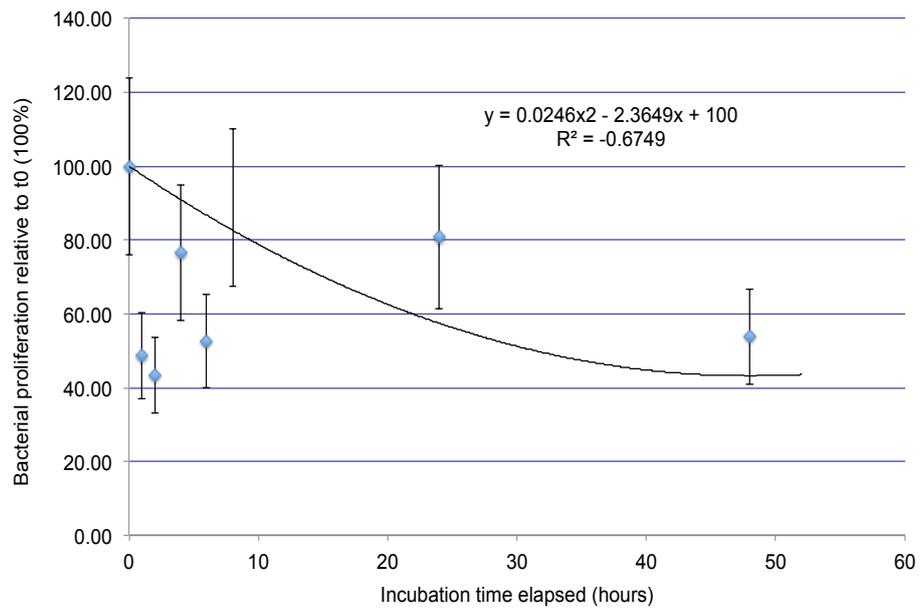


Figure 4.11 – Proliferation of *C. jejuni* ATCC-33291 MAP packaged and incubated at 30°C

#### 4.7 RT-PCR analysis of *C. jejuni* ATCC-33291 Proliferation

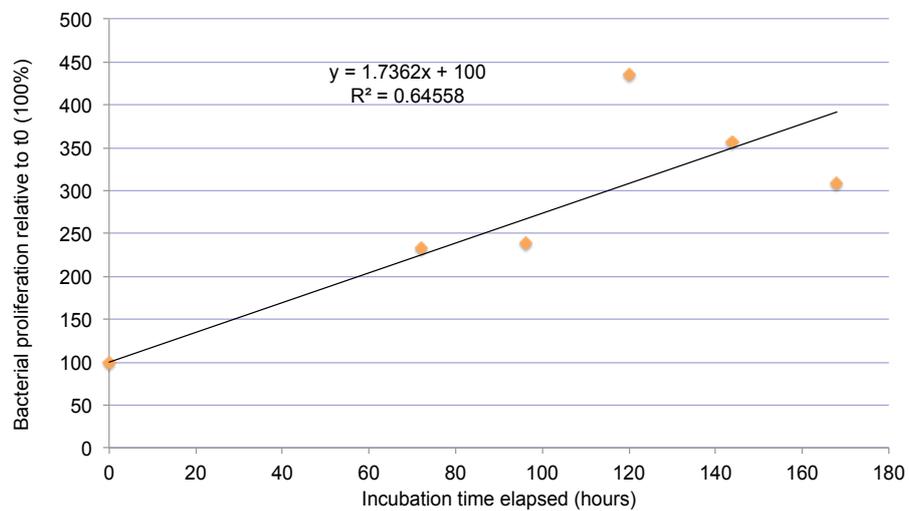


Figure 4.12 – Proliferation of *C. jejuni* ATCC-33291 vacuum packaged and incubated at 20°C – PCR analysis

Interestingly, those values generated through PCR analysis of homogenate and inoculant samples from vacuum and MAP experimental protocols

incubated at 20°C and 30°C (Figures 4.12 to 4.15). show a significant difference to those determined by standard plate culture technique. Those data derived by culture methods, when considered in the context of published growth characteristics for *C. jejuni* ATCC-33291, represent a population of non-replicating bacterial cells exhibiting decline phase characteristics. PCR analysis of these homogenate samples however, shows an increase in DNA copy numbers over the duration of experimental incubation. The lower threshold for bacterial replication in *C. jejuni* ATCC-33291 is considered 37°C, however interpretation of the 20°C and 30°C RT-PCR and culture media derived datasets in combination data suggest either a bacterial population showing a decline in viable cell numbers despite cell replication, or expression of exogenous DNA in formation of biofilm structures.

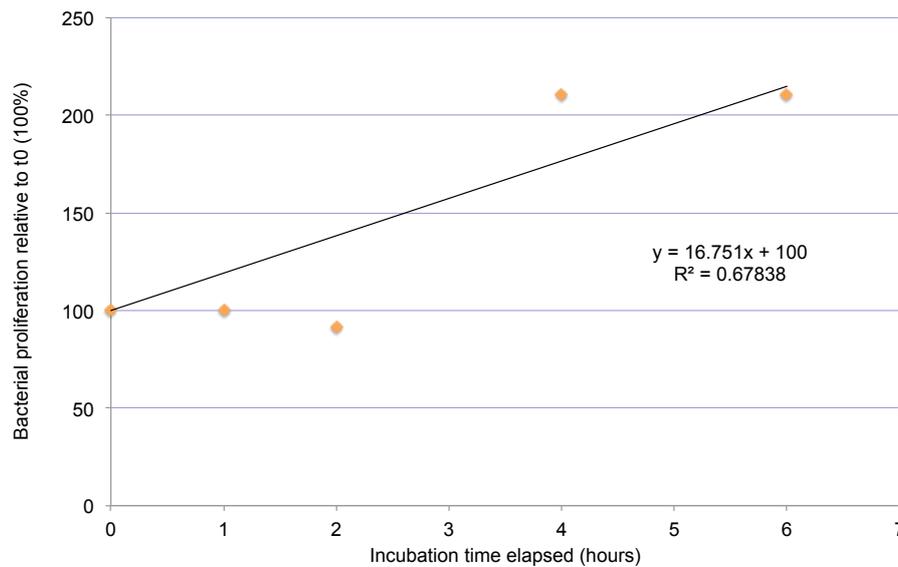


Figure 4.13 – Proliferation of *C. jejuni* ATCC-33291 vacuum packaged and incubated at 30°C – PCR analysis

While Congen, the supplier of the PCR analysis kit, do not publish the specific primer base-pair sequences, it is likely however that these are specific to the coding of the 16s ribosomal RNA gene in campylobacter. The 16s rRNA gene codes for a structural element of ribosome subunit 30s, and is particularly

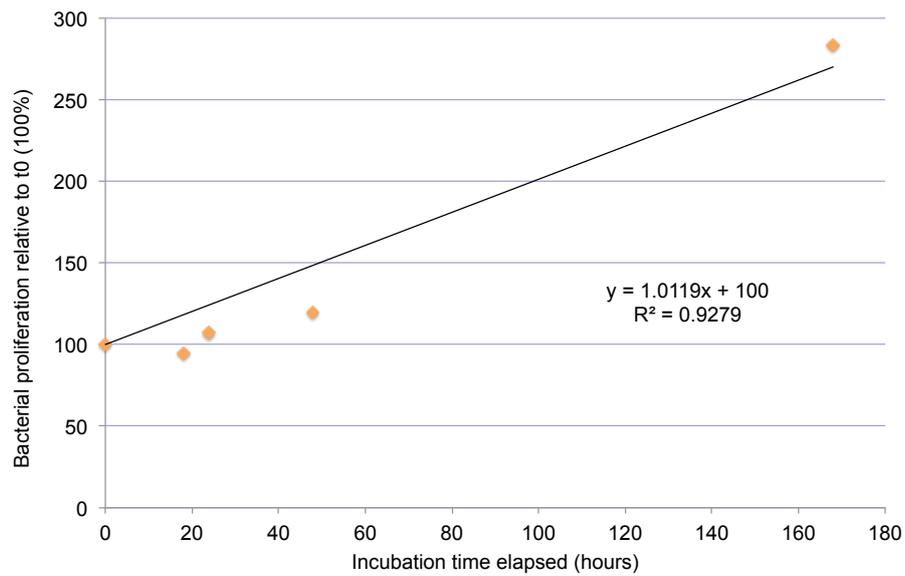


Figure 4.14 – Proliferation of *C. jejuni* ATCC-33291 over time –MAP packaged at 20°C – PCR analysis

conserved in the prokaryotic genome, with a distinct homologue acting as a highly specific RT-PCR identification marker for *C. jejuni* (Linton *et al*, 1994). The distribution of prokaryotic ribosomal activity however, is such that is not possible to relate the increase of 16s sequence copy number to biofilm

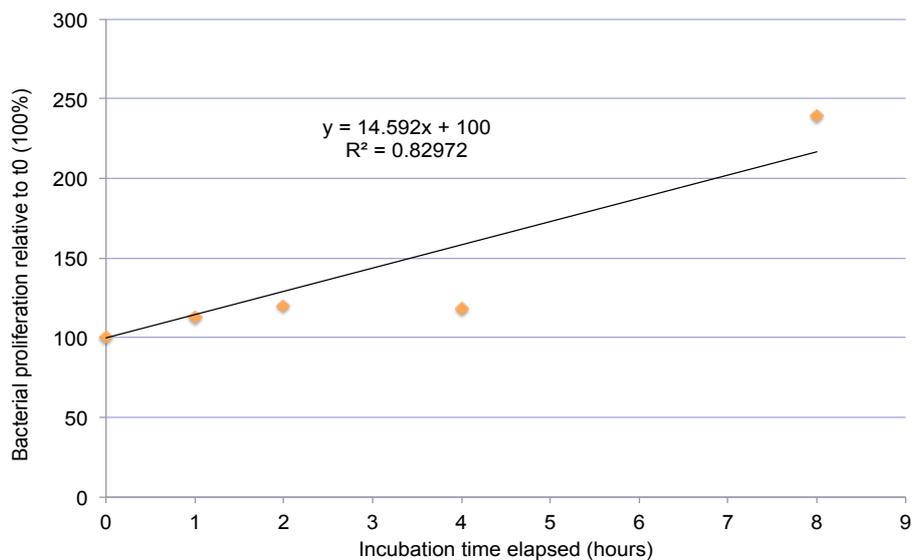


Figure 4.15 – Proliferation of *C. jejuni* ATCC-33291 MAP packaged and incubated at 30°C – PCR analysis

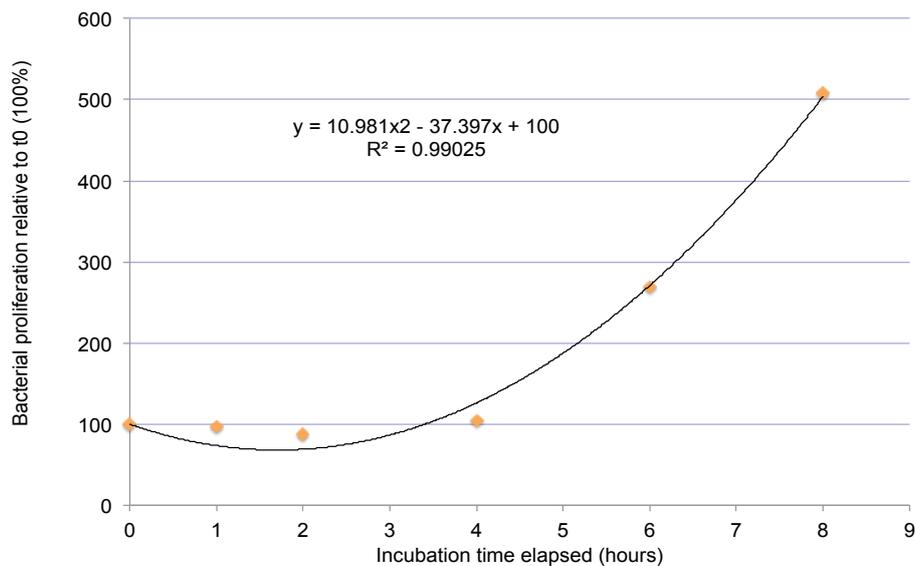


Figure 4.16 – Proliferation of *C. jejuni* ATCC-33291 MAP packaged and incubated at 37°C – PCR analysis

formation or to cell division. Cell lysis and the expression of exogenous bacterial DNA are considered key factors in the formation of biofilms (Svensson *et al*, 2014); therefore any subsequent translation and synthesis of those proteins fundamental to biofilm formation, structure, or signalling may utilise the 30s ribosome of which the 16s protein is a subunit. Although not documented in the published literature in relation to *Campylobacter* Spp. the observed increase in 16s rRNA copy numbers may be indicative of genetic mutation up-regulation observed in bacterial responses to antibiotic challenge or heat-shock (Foster, 2005; Martinez and Baquero, 2007).

In the absence of culture based and RT-PCR characterisation of campylobacter proliferation under the both discussed experimental packaging conditions and sample matrix, it is not possible to rule out cell division at temperatures below 37°C. In describing *C. jejuni* ATCC-33291 proliferation when subject to vacuum packaging and incubation at 37°C (Figure 4.16), the correlation of both PCR and culture media generated data is more easily related to the published characteristics of campylobacter during log phase growth stage of bacterial growth. Such agreement may be considered to both

confirm the validity of RT-PCR protocol, and support an interpretation of the available data in describing sample incubation under experimental conditions of vacuum and MAP packaged incubation at lower temperatures.

#### 4.8 Residual Gas Composition

Due to equipment availability, the determination of changes in gas composition within the packaging for both sample and control chicken portions was possible only over those 10°C, 20°C and 30°C MAP experimental protocols. The results as presented in figures 4.17-4.19 represent changes in the ratio of CO<sub>2</sub> over the course of incubation. Gas analysis values for both modified and non-modified 30°C incubation protocols show similar values, the mean of which was plotted. Statistical analysis indicated no significant difference between the CO<sub>2</sub> content measured for sample and control packaging 10°C or 20°C over 168 hours incubation, however the removal of

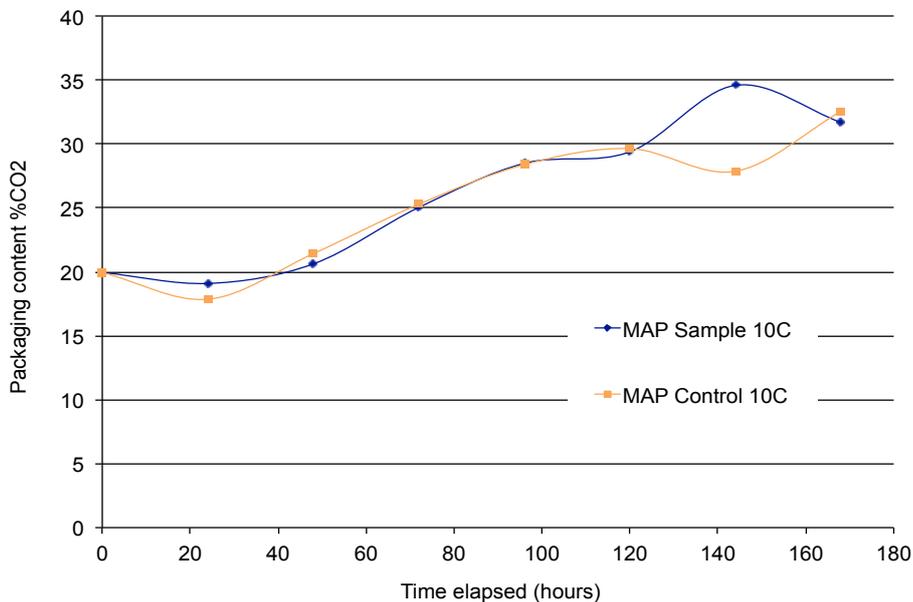


Figure 4.17 – Gas analysis showing percentage CO<sub>2</sub> content for sample and control chicken portions; incubated at 10°C under MAP conditions

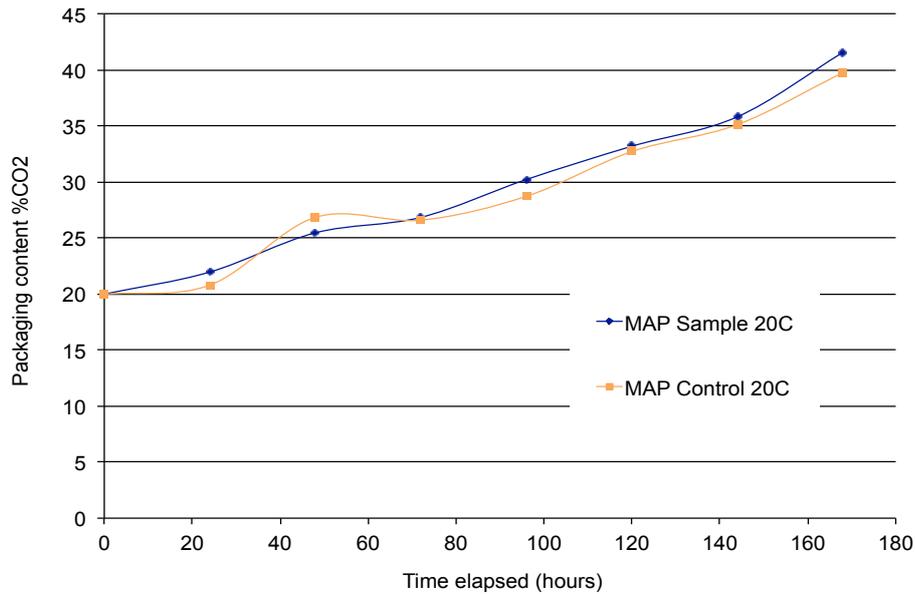


Figure 4.18 – Gas analysis showing percentage CO<sub>2</sub> content for sample and control chicken portions; incubated at 20°C under MAP conditions

data. Through incubation of 148 hours at 30°C however, the percentage of residual CO<sub>2</sub> present in the packaging of *C. jejuni* ATCC-33291 inoculated chicken samples is significantly higher than that recorded for the control. Considering this difference in the context of those similarities present in the graphic representation of the 20°C and 30°C datasets, it seems likely that the generation of further data sets, facilitating the valid removal of outlying points in the dataset, may show a significant difference between the inoculated sample residual gas composition and that of the control when incubated at 20°C. The observed change in gas composition attributable to *C. jejuni* ATCC-33291 appears to correlate with the observed increase in DNA copy number over the same temperature range and, similarly, may relate to biofilm formation, up-regulation of mutation, or further uncharacterised survival strategies.

The level of CO<sub>2</sub> observed in the packaging of campylobacter inoculated chicken pieces incubated at 30°C changes only over the initial 24 hours and remains relatively stable, 3.3% higher than that measured for the control,

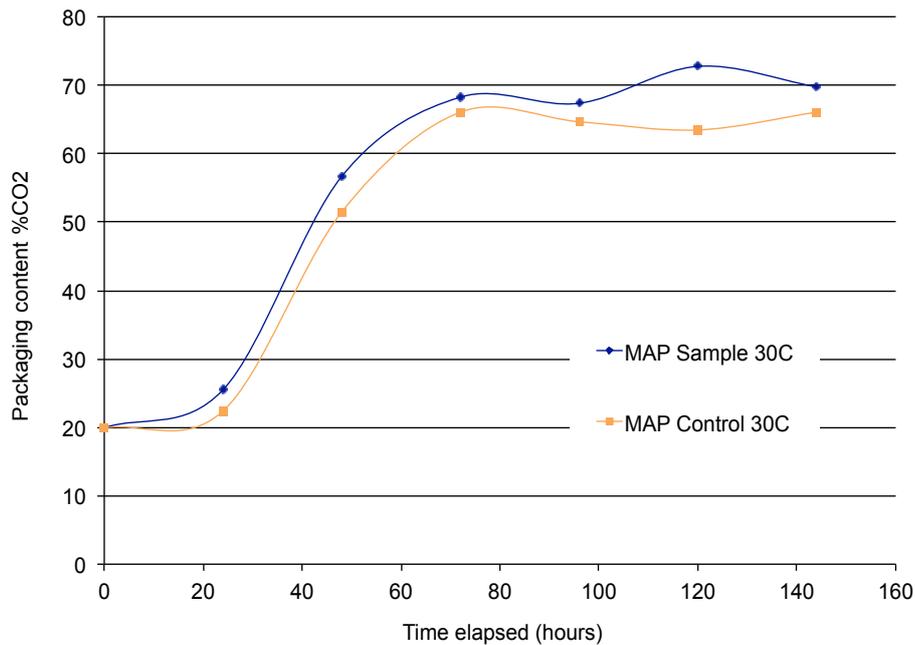


Figure 4.19 – Gas analysis showing percentage CO<sub>2</sub> content for sample and control chicken portions; incubated at 30°C under MAP conditions

through exponential growth and into the stationary phase. Again, the generation of further datasets would enable both the removal of outlying data points, and further define the characteristics of the campylobacter influenced change in residual gas composition, and may indicate the variance observed at 120 hours to be an experimental artefact. However, both RT-PCR and culture method derived data suggest a campylobacter response specific to 30°C, and any CO<sub>2</sub> portion of residual gas corresponding to biofilm formation or an up-regulation of gene mutation rates might be expected to increase over the period of bacterial growth.

Variation in the metabolism of campylobacters are suggested as indicative of changes in bacterial cell physiology and although not comprehensively categorised, an initial 'burst' is followed by significantly more moderate production of CO<sub>2</sub> in *C. jejuni* metabolism of many substrates (Westfall *et al*, 1986). Characterisation of CO<sub>2</sub> production on a sample matrix of raw chilled chicken may provide a basis for the identification of increased DNA copy number, be it through biofilm formation or other survival strategies, within

retail packaging. In the instance of a correlation between DNA copy number and increased immunogenicity, the removal of such product from the supply chain prior to retail display represents a practical measure in the prevention of food-borne campylobacteriosis.

#### 4.9 Digital Modelling of Campylobacter Proliferation

Figure 4.20 represents *Campylobacter jejuni* ATCC-33291 population dynamics when incubated under conditions of vacuum packaging between 4°C and 42°C; digitally modelled from regression analysis of proliferation data gathered to date by means of culture media. The model describes a decline in bacterial numbers for the temperature range 4°C to 30°C, with proliferation evident only during incubation at control temperatures of 37°C and 42°C. Likewise, digital modelling based on culture media enumeration of modified atmosphere packaging protocols between 4°C and 30°C show only a decline in bacterial numbers and therefore has no practical application in the prediction of food borne campylobacter proliferation for the purposes of consumer safety.

Regression modelling of *C. jejuni* ATCC-33291 DNA copy number for both vacuum and MAP protocols however, describes the observed increase in 16s rRNA coding DNA copy number, attributable to biofilm formation, a cycling population, or uncharacterised bacterial survival strategies. Culture media derived data may be presented in colony-forming units per millimetre (cfu/ml) through interpolation of a known inoculation value, for example FSA data relating cfu/ml counts for a specific processing plant or broiler chicken house.

This relationship is equally applicable to PCR derived data through integration of a reference standard within the PCR protocol, as illustrated in figure 4.21, once referenced to an experimental inoculant culture log cfu/ml. As with the generation of statistically robust digital modelling, larger sample sets and increased experimental 'n' numbers will result in a higher degree of precision

both when preparing a reference standard applicable to both evaluation of consumer health risks and determining the margin of error inherent to digital modelling.

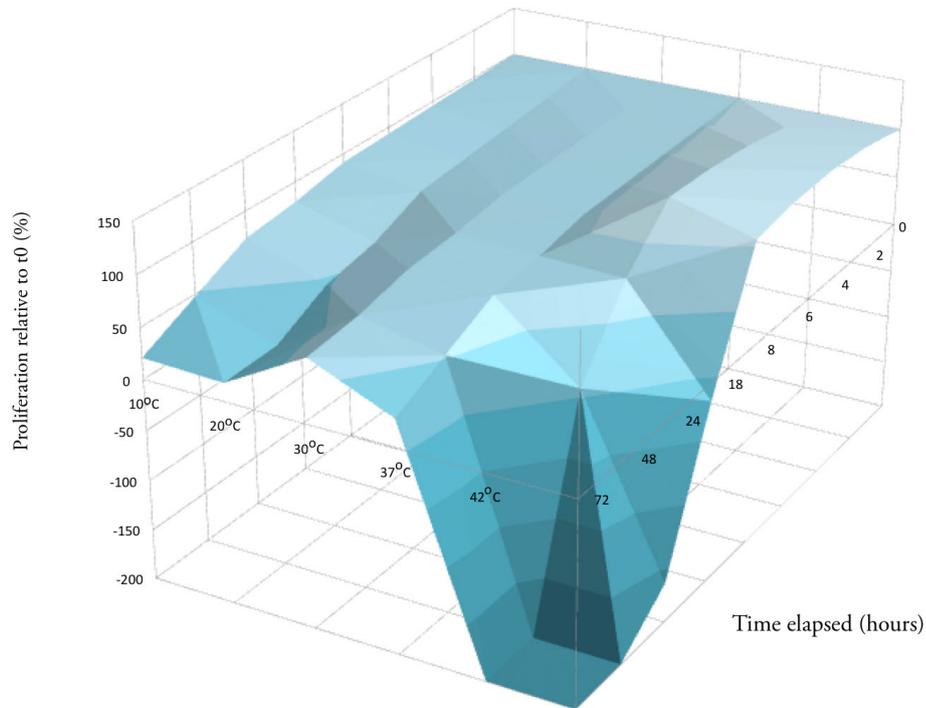


Figure 4.20 – Proliferation of *Campylobacter jejuni* ATCC-33291 subject to vacuum packaging – experimental protocols 4°C - 42°C, represented as a percentage relative to t0

In representing bacterial growth in this manner, it is also critical to consider sampling resolution when evaluating the validity of a value calculated for any point between experimental values: the robustness of such predictive function is directly proportional to the interval frequency and interpolation dynamics of experimentally generated proliferation data. While the increased 16s rRNA copy number at both 20oC and 30oC highlighted by the digital model is likely to reflect biofilm formation as opposed to bacterial proliferation, neither the formation of campylobacter biofilms on chicken produce nor the immunogenic properties of the related protein structures have received significant coverage within the published literature. This omission represents a significant gap in

scientific understanding of the most economically significant contemporary food borne pathogen.

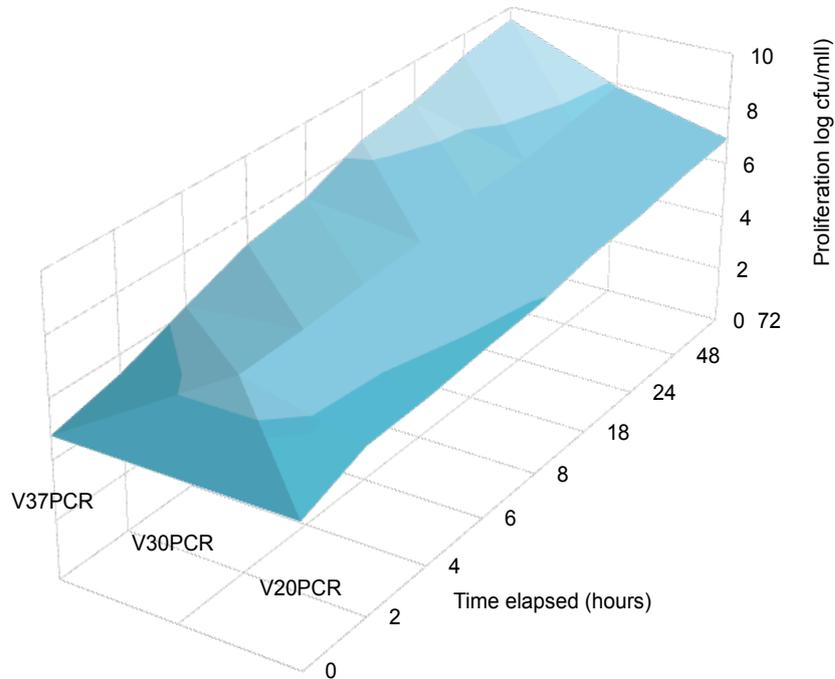


Figure 4.21 – Proliferation of *Campylobacter jejuni* ATCC-33291 subject to vacuum packaging – PCR data for protocols 20°C - 37°C; generated through integration of inoculant based standard curve

#### 4.10 *Vibrio Spp.* Cross-Contamination

The appearance of *Vibrio* like bacteria in the execution of 30°C protocols for both vacuum and MAP incubation is discussed in the preliminary study methodology. Subsequent plating and microaerophilic incubation of the isolated *Vibrio Spp.* bacteria on plate count agar generated 5 monocultures similar in appearance both to each other and to *Campylobacter Spp.* Testing with API 20NE strip ((API-biomerieux SA, marcy l'Etoile, France) yielded an identical positive identification of *Vibrio parahaemolyticus* for each culture, while PCR analysis of incubated broth culture samples showed no detectable campylobacter DNA. The growth rates of those *Vibrio* strains isolated from each of the five batches of chicken portions determined by optical density

measurement however, showed widely varying growth characteristics once tabulated. Cfu/ml counts from accompanying plate count agar serial dilutions for each strain enabled a compensation factor to be applied and the resulting comparable proliferation rates to be represented graphically for comparison. Maximum proliferation for each sample point is represented in figure 4.22.

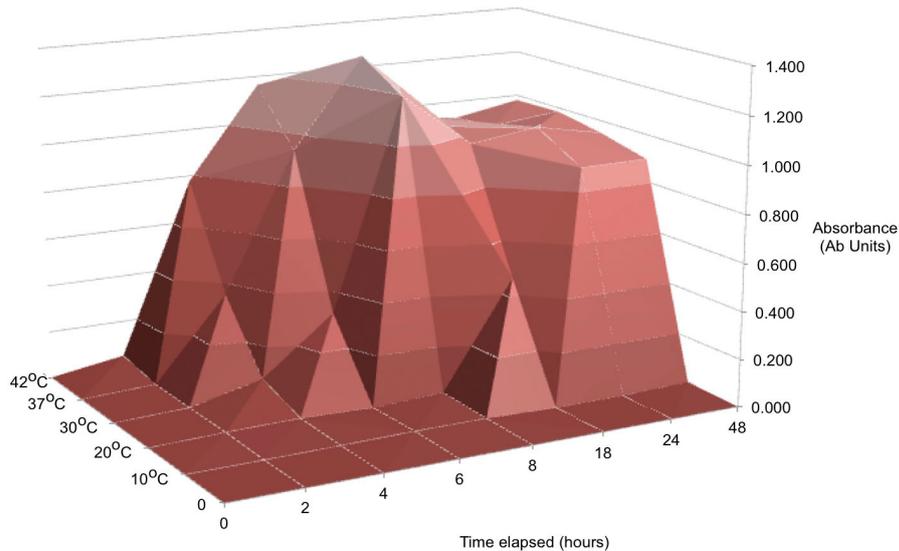


Figure 4.22 – Proliferation of *Vibrio parahaemolyticus* in TSB determined by optical density measurement for temperatures from 4oC - 42oC over 48 hours; representing maximum growth rates determined for 5 wild-type strains

Given the genetic variation shown in *Campylobacter Spp.* strains between highlighted by MLST studies examining broiler chicken rearing facilities, the possibility of widely varying growth characteristics must be considered in the modelling of campylobacter for the determination of consumer health risks. Additionally, since plating of *Vibrio parahaemolyticus* on both CCDA and Butler agar as formulated using Oxoid selective supplements, resulting in colonies sharing a morphology and appearance with campylobacter, the possibility of *Vibrio parahaemolyticus* related food poisoning in the case of non-laboratory confirmation being wrongly ascribed to campylobacter cannot be dismissed. Little is known in relation to *Vibrio Spp.* in the poultry industry,

and it is unlikely that the full extent the contribution to human enterocolitis and related illness will be understood without the application of RT-PCR for FSA monitoring of bacterial pathogens.

#### 4.9 Colonisation Potential in VBNC *Campylobacter Jejuni* ATCC-33291

RT-PCR analysis detected no campylobacter in t0 homogenate samples for chicken portions where the initial inoculation was performed with a VBNC inoculant, indicating a loss of colonisation potential in this phenotype for the experimental sample matrix. Considering the documented mucin driven chemotaxis observed in *Campylobacter Spp.* (Hong *et al*, 2014; Beery *et al*, 1988) physiology specific cues are likely to play a role in phenotypic switching of VBNC cells to facilitate host colonisation, and in particular expression of flagella; considered essential to both motility and adhesion (Svensson *et al*, 2014).

## CONCLUSIONS

### Evaluation and Implications

There can be no doubt that every practical measure to reduce the health and social impact of campylobacteriosis is being implemented by the poultry industry, and with the NVQ system of food preparation training and the public awareness programmes streamed by the FSA, the effective monitoring of bacterial produce in supply chain represents an essential requirement in assuring the safety of chicken produce from 'farm to fork'. Between producer and consumer, the European Food Safety Authority legislative framework stipulates a legally binding requirement for all chilled produce to be stored and transported within a temperature range of -2°C to +4°C.

In identifying an un-quantified hazard and critical point analysis control point (HACCP) in terms of chilled chicken produce temperature abuse in transit as a risk to human health, the objectives of the preceding research demonstrate the viability of developing a biosensor based tracking system; monitoring produce temperature *in transit* for the purposes of predicting a risk to consumer safety from bacterial colonisation. The characterisation of growth dynamics for pathogenic bacteria under conditions typical of chilled produce transport is key to the functionality of any predictive modelling system from which empirical decisions relating to consumer safety may be conveyed to the end user. Association with such 'biosensor' tracked produce would assure the consumer of produce safety and quality, while acting to increase market share for affiliated producers and retailers.

The research presented represents a methodology suited to the enumeration and digital modelling of spoilage bacteria population dynamics, and outlines how this may be integrated with existing technology to form the foundation of a tracking and temperature monitoring 'biosensor' system. While both the fastidious nature of *Campylobacter Spp.* and its ready expression of a VBNC phenotype have contributed to a dataset where statistical robustness could be improved by further experimentation, the findings may be considered broadly

representative of campylobacter proliferation on chilled chicken produce in the supply chain, under both vacuum and MAP packaging conditions.

Data derived from culture media and relating campylobacter growth according to temperature correlate with the published literature and the characterisation of *C. jejuni* for temperatures in the range 4°C to 42°C. From the experimental results and subsequent modelling, a slow decline in bacterial number is evident at 4°C, with an increase in temperature having the effect of accelerating this decline. From the growth threshold temperature 37°C however, the rate of campylobacter population increase rises to the acknowledged optimum growth rate at 42°C. The modelling also reflects the contained environment of the retail packaging, and where bacterial growth is present; typical log, lag, and decline phases are observed. Such result may be considered to validate the experimental methodology, and substantiate the reduced rate of campylobacter decline observed under MAP conditions; in turn suggesting the elevated CO<sub>2</sub> packing environment more suited to the capnophilic bacteria than that of atmospheric O<sub>2</sub> or vacuum packaging.

Those data determined from the residual gas analysis demonstrate a statistically significant initial 'burst' of bacterial CO<sub>2</sub> production in samples incubated at 30°C and, when represented graphically, the data relating residual gas content at 20°C suggests similar characteristics. While both the composition and extent of bacterial cross contamination are likely to differ significantly to the experimental inoculation protocol, further characterisation of campylobacter substrate metabolism may have application in the detection of *C. jejuni* and *C. coli* related spoilage in retail packaging.

Evaluation of the experimental findings in combination with those determined through RT-PCR analysis indicates a need to further characterise campylobacter gene expression at lower temperatures. Regardless of whether it is attributable to biofilm formation; a survival strategy based on the up-regulation of genetic mutation; or further uncharacterised survival strategies, the observed increasing DNA copy number may represent a previously unrecognised and thus un-quantified risk to consumer health.

Considering the elevated human immune response to lysed campylobacter cells over live bacterial cells (de Zoete *et al*, 2010), an understanding of the human immune response in relation to exogenous bacterial DNA and associated proteins represents the basis of further research key to both understanding the epidemiology of campylobacteriosis and to and determining any associated threshold level for pathogenesis.

Current monitoring of campylobacter levels both in broiler flocks and on chilled chicken produce for the purposes of consumer safety, executed using approved Oxoid culture based media only, may not be sufficient to fully quantify the risk to human health. When considered in conjunction with similarities in both cellular and colony morphology, the possible misidentification of *Vibrio Spp.* on Oxoid selective media specific to campylobacter, must be considered in the aetiology of campylobacteriosis. Additionally, in light of the growth rate variation observed in those *Vibrio Spp* strains characterised by optical density measurement, bacterial proliferation modelling for the purposes of consumer safety must consider the entire gamut of wild type strains isolated from the retail product in order to be fully representative.

The integration of technologies in the construction of a tracking and temperature monitoring 'biosensor' represents a relatively straightforward task in comparison to that of generating multiple factor characterisation of *Campylobacter Spp.* proliferation. Many refrigerated transport companies operate a tracking and temperature monitoring service within their business, while many more rely on one of the many commercially available temperature monitoring data-logger units with mobile telephone network connectivity. In addition to real time broadcast of temperature or humidity values, the widespread coverage of the GPRS telephone network enables the tracking of the unit by signal triangulation, and represents a biosecurity tracking measure within the transport supply chain. In terms of sensor data processing, the use of a .csv file format is widespread, and facilitates compatibility with software such as Excel (Microsoft Corporation) and Matlab (Mathworks) for the

purposes of digital model interrogation. As such, datasets might easily be compared or integrated within a given database for the determination of the proliferation of particular pathogens. Centralisation of both temperature and location monitoring would enable data from multiple trailer units to be processed through a single easily updateable and amended product specific spoilage database, and facilitate alarm features and perhaps real time remote command of refrigerated trailer chilling unit control systems in transit.

To date, the experimental results and subsequent modelling illustrate the viability of the methodology in achieving the objectives of the research. The findings reflect the complexity inherent to characterising bacterial proliferation in relation to consumer safety, and highlight a necessity to combine the application of microbiological identification and enumeration tools in generating both robust datasets and representative digital modelling. Rather than have similarities with existing microbiological predictive modelling databases such as Combase, the methodology described through the preceding study represents a basis from which extrapolations may be generated from data relating actual bacterial population dynamics when subject to a characterised set of on-going environmental conditions.

By quantifying the proliferation of *Campylobacter Spp.* in isolation, the protocol does not account for the influence of those enteric bacteria sharing the biome of the chicken GIT, in turn cross-contamination vectors during processing, and which may factor in campylobacter proliferation and biofilm formation. Similarly, replication of the sample matrix represented by chicken carcasses in processing is not feasible experimentally, but may have considerable bearing on bacterial virulence factors, adhesion and cell invasion. Despite these shortcomings, the research highlights the necessity of combining the application of technologies in understanding and quantifying the risk to human health posed by *Campylobacter Spp.* in chilled chicken produce, and forms the basis of a valid and robust methodology applicable to the achievement of this objective.

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APPENDICES

Appendix 1

**4C Vacuum Protocol**

02/09/2014

			<i>Mean</i>
Inoculant cfu/ml	1.77E+08	1.56E+08	1.34E+08
Inoculation efficiency			1.32%

				<i>Percentage relative to t0</i>
<i>Sampling timepoint (hours)</i>		<i>Mean cfu/ml</i>		
0	1.70E+06	2.41E+06	2.06E+06	100.00
2	2.50E+06	2.18E+06	2.34E+06	113.87
4	3.20E+06	2.91E+06	3.06E+06	148.66
6	1.50E+06	1.64E+06	1.57E+06	76.40

22/08/2014

Inoculant cfu/ml		2.92E+08
Inoculation efficiency		1.55%

				<i>Percentage relative to t0</i>
<i>Sampling timepoint (hours)</i>		<i>Mean cfu/ml</i>		
0		4.53E+06		100.00
2				
4		5.56E+06		122.74
6				
8				
12				
16				
18	3.62E+06		3.62E+06	79.91
24	4.05E+06		4.05E+06	89.40
48	1.00E+06		1.00E+06	22.08
72	2.70E+06	2.10E+06	2.40E+06	52.98
96	2.62E+06		2.62E+06	57.84

06/09/2014

Inoculant cfu/ml		5.60E+06
Inoculation efficiency		2.30%

				<i>Percentage relative to t0</i>
<i>Sampling timepoint (hours)</i>		<i>Mean cfu/ml</i>		
0		1.29E+05		100.00
2				
4				
6				
8				
12				
16	5.17E+04	8.70E+04	6.94E+04	53.76
18				
24				
48				
72				
96				
120			2.35E+04	18.22
144			2.02E+04	15.66

## Appendix 2

**10C Vacuum Protocol**

23/09/2014

Inoculant cfu/ml	1.20E+06	1.91E+06	<i>Mean</i>	1.56E+06
Inoculation efficiency				1.62%

Sampling timepoint (hours)				<i>Mean cfu/ml</i>	<i>Percentage relative to t0</i>
0	1.51E+04	2.50E+04	1.81E+04	1.94E+04	100.00
2					
4					
6					
8					
12					
16					
18	1.50E+04	1.17E+04		1.34E+04	68.81
24	4.40E+03	4.00E+03		4.20E+03	21.65
48	8.30E+03	6.00E+03		7.15E+03	36.86
72	2.30E+03	3.00E+03		2.65E+03	13.66

## Appendix 3

**20C Vacuum Protocol**

23/09/2014

Inoculant cfu/ml	1.20E+06	1.91E+06	<i>Mean</i>	1.56E+06
Inoculation efficiency				1.62%

Sampling timepoint (hours)				<i>Mean cfu/ml</i>	<i>Percentage relative to t0</i>
0	1.51E+04	2.50E+04	1.81E+04	1.94E+04	100.00
2					
4					
6					
8					
12					
16					
18					
24	1.24E+04	1.80E+04		1.52E+04	78.35
48	1.40E+04	1.33E+03		7.67E+03	39.51
72	1.20E+04	9.80E+03		1.09E+04	56.19

## Appendix 4

**30C Vacuum protocol**

23/09/2014

Inoculant cfu/ml 1.20E+06 1.91E+06  
 Inoculation efficiency Mean 1.56E+06  
 1.62%

Sampling timepoint (hours)	Mean cfu/ml				Percentage relative to t0
0	1.51E+04	2.50E+04	1.81E+04	1.94E+04	100.00
2					
4					
6					
8					
12					
16					
18					
24	1.80E+04	1.02E+04		1.41E+04	72.68
48	6.10E+03	5.90E+03		6.00E+03	30.93
72	8.22E+03	8.80E+03		8.51E+03	43.87

## Appendix 5

**37C Vacuum Protocol**

09/09/2014

Inoculant cfu/ml 8.65E+07  
 Inoculation efficiency 0.77%

Sampling timepoint (hours)	Mean cfu/ml				Percentage relative to t0
0	6.69E+05			6.69E+05	100.00
2	3.85E+05			3.85E+05	57.55
4	8.00E+05			8.00E+05	119.58
6	7.56E+05			7.56E+05	113.00
8	7.40E+05			7.40E+05	110.61
12	1.08E+06			1.08E+06	161.43
16					
18					
24	3.10E+01	3.00E+00		1.70E+01	0.00
48	0			0	0

Appendix 6

**42C Vacuum Protocol**

22/08/2014

Inoculant cfu/ml 1.96E+08  
 Inoculation efficiency 0.43%

Sampling timepoint (hours)	Mean cfu/ml			Percentage relative to t0
	0	8.40E+05	8.40E+05	8.40E+05
2	2.03E+06	2.03E+06	2.03E+06	241.67
4	1.26E+06	1.26E+06	1.26E+06	150.00
6	6.60E+05	6.60E+05	6.60E+05	78.57
8	7.20E+05	7.20E+05	7.20E+05	85.71
12				
16				
18				
24	0	0	0	0

21/08/2014

Inoculant cfu/ml 1.85E+08  
 Inoculation efficiency 3.99%

Sampling timepoint (hours)	Mean cfu/ml			Percentage relative to t0
	0	6.94E+06	7.80E+06	7.37E+06
2				
4	6.44E+06		6.44E+06	87.38
6				
8				
12				
16				
18	1.67E+04		1.67E+04	0.23
24	0		0	0

08/09/2014

Inoculant cfu/ml 5.60E+06  
 Inoculation efficiency 2.30%

Sampling timepoint (hours)	Mean cfu/ml			Percentage relative to t0
	0			1.29E+05
2				
4				
6				
8				
12				
16	2.20E+03	2.00E+03	2.10E+03	1.63
18				
24	0		0	0

Appendix 7

**4C MAP Protocol**

02/09/2018

Inoculant cfu/ml	1.18E+05	8.90E+05	<i>Mean</i>	5.04E+05
Inoculation efficiency				2.59%

<i>Sampling timepoint (hours)</i>				<i>Mean cfu/ml</i>	<i>Percentage relative to t0</i>
0	1.35E+04	1.58E+04	9.80E+03	1.30E+04	100.00
1					
2					
4	1.48E+04	1.56E+04	9.10E+03	1.32E+04	101.02
6					
8					
12					
16					
18					
24	1.18E+04	6.90E+03	1.40E+04	1.09E+04	83.63
48	7.00E+03	5.40E+03		6.20E+03	47.57
72	8.00E+03	8.30E+03		8.15E+03	62.53
168	0	0			0

Appendix 8

**10C MAP Protocol**

13/12/2014

**Inoculant cfu/ml** 3.70E+07  
**Inoculation efficiency** 2.28%

Sampling timepoint (hours)	Mean cfu/ml				Percentage relative to t0	
0	5.00E+05	4.40E+05	1.36E+06	1.07E+06	8.43E+05	100.00%
2						
4						
6						
8						
12						
16						
18						
24						
48						
72	5.18E+05	6.20E+05			5.69E+05	67.54%
96	1.31E+05				1.31E+05	15.55%
120	1.40E+05	3.14E+05			2.27E+05	26.94%
144	1.57E+05	1.70E+04			8.70E+04	10.33%
168	1.20E+04	1.01E+04			1.11E+04	1.31%

16/12/2014

**Inoculant cfu/ml** 2.01E+07  
**Inoculation efficiency** 3.98%

Sampling timepoint (hours)	Mean cfu/ml			Percentage relative to t0	
0	5.00E+05	9.00E+05	1.00E+06	8.00E+05	100.00%
2					
4					
6					
8					
12					
16					
18					
24	9.00E+05			9.00E+05	112.50%
48	4.00E+05			4.00E+05	50.00%

Appendix 9

**20C MAP Protocol**

28/11/2014

**Inoculant cfu/ml**  
**Inoculation efficiency**

1.20E+07  
6.00%

Sampling timepoint (hours)	Mean cfu/ml					Percentage relative to $t_0$
	7.20E+05	7.90E+05	6.30E+05	7.13E+05	7.13E+05	
0	7.20E+05	7.90E+05	6.30E+05	7.13E+05	7.13E+05	100.00%
2						
4						
6						
8						
12						
16						
18	6.20E+05	7.20E+05		6.70E+05	6.70E+05	93.93%
24	5.35E+05	6.30E+05	5.03E+05	6.00E+05	5.67E+05	79.49%
48	3.77E+05	4.80E+05	2.59E+05	4.70E+05	3.97E+05	55.58%
72						
96						
120						
144						
168	3.40E+03	3.10E+01		1.72E+03	1.72E+03	0.24%

Appendix 10

**30C MAP Protocol**

22/01/2015

**Inoculant cfu/ml** 2.02E+06  
**Inoculation efficiency** 1.47%

Sampling timepoint (hours)	Mean cfu/ml				Percentage relative to t0
0	3.22E+04	2.55E+04	3.12E+04	2.96E+04	100.00%
1	1.48E+04	1.40E+04		1.44E+04	48.59%
2	1.57E+04	1.00E+04		1.29E+04	43.36%
4	2.43E+04	2.10E+04		2.27E+04	76.43%
6	1.72E+04	1.40E+04		1.56E+04	52.64%
8	2.56E+04	2.70E+04		2.63E+04	88.75%

20/01/2015

**Inoculant cfu/ml** 1.81E+05  
**Inoculation efficiency** 2.87%

Sampling timepoint (hours)	Mean cfu/ml				Percentage relative to t0
0	3.70E+03	2.90E+03	9.00E+03	5.20E+03	100.00%
1					
2					
4					
6					
8					
12					
16					
18					
24	4.20E+03			4.20E+03	80.77%
48	2.80E+03			2.80E+03	53.85%



Appendix 13

**37C Vacuum Protocol PCR Analysis**

09/09/2014

Inoculant cfu/ml  
Inoculation efficiency

0.77%

Sampling timepoint (hours)					Mean cfu/ml	Percentage relative to t0
					Mean CFU count	Percentage relative to t0
0	2.46E-01	2.60E-01	1.60E-01	2.00E-01	2.17E-01	100.00
1	2.15E-01	2.01E-01			2.08E-01	96.10
2	1.66E-01	2.15E-01			1.91E-01	87.99
4	2.20E-01	2.30E-01			2.25E-01	103.93
6	6.03E-01	5.60E-01			5.81E-01	268.55
8	1.14E+00	1.06E+00			1.10E+00	509.01

Appendix 14

**20C MAP Protocol PCR Analysis**

12/03/2015

Inoculant cfu/ml  
Inoculation efficiency

1.80E+02 1.68E+02

Mean  
1.74E+02  
0.14%

Sampling timepoint (hours)					Mean CFU count	Percentage relative to t0
0	2.46E-01	2.01E-01	2.20E-01	2.34E-01	2.27E-01	100.00%
2						
4						
6						
8						
12						
16						
18	2.18E-01	2.13E-01			2.16E-01	94.99%
24	1.96E-01	2.91E-01	1.96E-01	2.91E-01	2.44E-01	107.35%
48	2.57E-01	2.85E-01	2.57E-01	2.85E-01	2.71E-01	119.48%
72						
96						
120						
144						
168	6.63E-01	6.23E-01			6.43E-01	283.21%

Appendix 15

**30C MAP Protocol PCR Analysis**

12/03/2015

**Inoculant cfu/ml**  
**Inoculation efficiency**

Mean  
0.00E+00  
1.47%

<i>Sampling timepoint (hours)</i>				<i>Mean CFU count</i>	<i>Percentage relative to t0</i>
	2.46E-01	1.14E-01	1.60E-01		
	2.60E-01	9.48E-02	2.00E-01		
0	2.53E-01	1.04E-01	1.20E-01	1.59E-01	100.00%
1	1.88E-01	1.72E-01		1.80E-01	113.11%
2	1.66E-01	2.15E-01		1.91E-01	119.71%
4	2.20E-01	2.30E-01		2.25E-01	118.11%
6	1.23E-01	1.24E-01		1.24E-01	77.61%
8	3.85E-01	3.77E-01		3.81E-01	239.42%

Appendix 16

**Wilcoxon Signed Ranks Test**

		Ranks		
		N	Mean Rank	Sum of Ranks
V37C - V4C	Negative Ranks	4 <sup>a</sup>	5.50	22.00
	Positive Ranks	4 <sup>b</sup>	3.50	14.00
	Ties	1 <sup>c</sup>		
	Total	9		
V42C - V4C	Negative Ranks	4 <sup>d</sup>	6.50	26.00
	Positive Ranks	4 <sup>e</sup>	2.50	10.00
	Ties	1 <sup>f</sup>		
	Total	9		
V42C - V37C	Negative Ranks	8 <sup>g</sup>	4.50	36.00
	Positive Ranks	0 <sup>h</sup>	.00	.00
	Ties	1 <sup>i</sup>		
	Total	9		

a. V37C < V4C

b. V37C > V4C

c. V37C = V4C

d. V42C < V4C

e. V42C > V4C

f. V42C = V4C

g. V42C < V37C

h. V42C > V37C

i. V42C = V37C

Test Statistics <sup>a</sup>			
	V37C - V4C	V42C - V4C	V42C - V37C
Z	-.560 <sup>b</sup>	-1.120 <sup>b</sup>	-2.521 <sup>b</sup>
Asymp. Sig. (2-tailed)	.575	.263	.012

a. Wilcoxon Signed Ranks Test

b. Based on positive ranks.

Appendix 17

**Wilcoxon Signed Ranks Test**

		Ranks		
		N	Mean Rank	Sum of Ranks
V10C - V4C	Negative Ranks	8 <sup>a</sup>	4.50	36.00
	Positive Ranks	0 <sup>b</sup>	.00	.00
	Ties	1 <sup>c</sup>		
	Total	9		
V20C - V4C	Negative Ranks	0 <sup>d</sup>	.00	.00
	Positive Ranks	8 <sup>e</sup>	4.50	36.00
	Ties	1 <sup>f</sup>		
	Total	9		
V30C - V4C	Negative Ranks	0 <sup>g</sup>	.00	.00
	Positive Ranks	8 <sup>h</sup>	4.50	36.00
	Ties	1 <sup>i</sup>		
	Total	9		

a. V10C < V4C

b. V10C > V4C

c. V10C = V4C

d. V20C < V4C

e. V20C > V4C

f. V20C = V4C

g. V30C < V4C

h. V30C > V4C

i. V30C = V4C

Test Statistics <sup>a</sup>			
	V10C - V4C	V20C - V4C	V30C - V4C
Z	-2.521 <sup>b</sup>	-2.521 <sup>c</sup>	-2.521 <sup>c</sup>
Asymp. Sig. (2-tailed)	.012	.012	.012

a. Wilcoxon Signed Ranks Test

b. Based on positive ranks.

c. Based on negative ranks.

Appendix 18

**Wilcoxon Signed Ranks Test**

		Ranks		
		N	Mean Rank	Sum of Ranks
V20C - V10C	Negative Ranks	0 <sup>a</sup>	.00	.00
	Positive Ranks	8 <sup>b</sup>	4.50	36.00
	Ties	1 <sup>c</sup>		
	Total	9		
V30C - V10C	Negative Ranks	0 <sup>d</sup>	.00	.00
	Positive Ranks	8 <sup>e</sup>	4.50	36.00
	Ties	1 <sup>f</sup>		
	Total	9		
V30C - V20C	Negative Ranks	8 <sup>g</sup>	4.50	36.00
	Positive Ranks	0 <sup>h</sup>	.00	.00
	Ties	1 <sup>i</sup>		
	Total	9		

- a. V20C < V10C
- b. V20C > V10C
- c. V20C = V10C
- d. V30C < V10C
- e. V30C > V10C
- f. V30C = V10C
- g. V30C < V20C
- h. V30C > V20C
- i. V30C = V20C

Test Statistics <sup>a</sup>			
	V20C - V10C	V30C - V10C	V30C - V20C
Z	-2.521 <sup>b</sup>	-2.521 <sup>b</sup>	-2.521 <sup>c</sup>
Asymp. Sig. (2-tailed)	.012	.012	.012

- a. Wilcoxon Signed Ranks Test
- b. Based on negative ranks.
- c. Based on positive ranks.

Appendix 19

**Wilcoxon Signed Ranks Test**

		Ranks		
		N	Mean Rank	Sum of Ranks
MAP4C - V4C	Negative Ranks	0 <sup>a</sup>	.00	.00
	Positive Ranks	8 <sup>b</sup>	4.50	36.00
	Ties	1 <sup>c</sup>		
	Total	9		

- a. MAP4C < V4C
- b. MAP4C > V4C
- c. MAP4C = V4C

Test Statistics <sup>a</sup>	
	MAP4C - V4C
Z	-2.521 <sup>b</sup>
Asymp. Sig. (2-tailed)	.012

- a. Wilcoxon Signed Ranks Test
- b. Based on negative ranks.

Appendix 20

**Wilcoxon Signed Ranks Test**

		Ranks		
		N	Mean Rank	Sum of Ranks
MAP10C - MAP4C	Negative Ranks	0 <sup>a</sup>	.00	.00
	Positive Ranks	8 <sup>b</sup>	4.50	36.00
	Ties	1 <sup>c</sup>		
	Total	9		
MAP20C - MAP4C	Negative Ranks	2 <sup>d</sup>	7.50	15.00
	Positive Ranks	6 <sup>e</sup>	3.50	21.00
	Ties	1 <sup>f</sup>		
	Total	9		
MAP30C - MAP4C	Negative Ranks	7 <sup>g</sup>	4.57	32.00
	Positive Ranks	1 <sup>h</sup>	4.00	4.00
	Ties	1 <sup>i</sup>		
	Total	9		

a. MAP10C < MAP4C

b. MAP10C > MAP4C

c. MAP10C = MAP4C

d. MAP20C < MAP4C

e. MAP20C > MAP4C

f. MAP20C = MAP4C

g. MAP30C < MAP4C

h. MAP30C > MAP4C

i. MAP30C = MAP4C

Test Statistics <sup>a</sup>			
	MAP10C - MAP4C	MAP20C - MAP4C	MAP30C - MAP4C
Z	-2.521 <sup>b</sup>	-.420 <sup>b</sup>	-1.960 <sup>c</sup>
Asymp. Sig. (2-tailed)	.012	.674	.050

a. Wilcoxon Signed Ranks Test

b. Based on negative ranks.

c. Based on positive ranks.

Appendix 21

**Wilcoxon Signed Ranks Test**

		Ranks		
		N	Mean Rank	Sum of Ranks
MAP10C - V10C	Negative Ranks	0 <sup>a</sup>	.00	.00
	Positive Ranks	8 <sup>b</sup>	4.50	36.00
	Ties	1 <sup>c</sup>		
	Total	9		
MAP20C - V20C	Negative Ranks	8 <sup>d</sup>	4.50	36.00
	Positive Ranks	0 <sup>e</sup>	.00	.00
	Ties	1 <sup>f</sup>		
	Total	9		
MAP30C - V30C	Negative Ranks	7 <sup>g</sup>	4.00	28.00
	Positive Ranks	1 <sup>h</sup>	8.00	8.00
	Ties	1 <sup>i</sup>		
	Total	9		

- a. MAP10C < V10C
- b. MAP10C > V10C
- c. MAP10C = V10C
- d. MAP20C < V20C
- e. MAP20C > V20C
- f. MAP20C = V20C
- g. MAP30C < V30C
- h. MAP30C > V30C
- i. MAP30C = V30C

Test Statistics <sup>a</sup>			
	MAP10C - V10C	MAP20C - V20C	MAP30C - V30C
Z	-2.521 <sup>b</sup>	-2.521 <sup>c</sup>	-1.400 <sup>c</sup>
Asymp. Sig. (2-tailed)	.012	.012	.161

- a. Wilcoxon Signed Ranks Test
- b. Based on negative ranks.
- c. Based on positive ranks.

Appendix 22

**Wilcoxon Signed Ranks Test**

		Ranks		
		N	Mean Rank	Sum of Ranks
V20CPCR - V20C	Negative Ranks	0 <sup>a</sup>	.00	.00
	Positive Ranks	8 <sup>b</sup>	4.50	36.00
	Ties	1 <sup>c</sup>		
	Total	9		
V30CPCR - V30C	Negative Ranks	0 <sup>d</sup>	.00	.00
	Positive Ranks	8 <sup>e</sup>	4.50	36.00
	Ties	1 <sup>f</sup>		
	Total	9		
V37CPCR - V37C	Negative Ranks	4 <sup>g</sup>	2.50	10.00
	Positive Ranks	4 <sup>h</sup>	6.50	26.00
	Ties	1 <sup>i</sup>		
	Total	9		

- a. V20CPCR < V20C
- b. V20CPCR > V20C
- c. V20CPCR = V20C
- d. V30CPCR < V30C
- e. V30CPCR > V30C
- f. V30CPCR = V30C
- g. V37CPCR < V37C
- h. V37CPCR > V37C
- i. V37CPCR = V37C

Test Statistics <sup>a</sup>			
	V20CPCR - V20C	V30CPCR - V30C	V37CPCR - V37C
Z	-2.521 <sup>b</sup>	-2.521 <sup>b</sup>	-1.120 <sup>b</sup>
Asymp. Sig. (2-tailed)	.012	.012	.263

- a. Wilcoxon Signed Ranks Test
- b. Based on negative ranks.

Appendix 23

**Wilcoxon Signed Ranks Test**

		Ranks		
		N	Mean Rank	Sum of Ranks
MAP20PCR - MAP20C	Negative Ranks	0 <sup>a</sup>	.00	.00
	Positive Ranks	8 <sup>b</sup>	4.50	36.00
	Ties	1 <sup>c</sup>		
	Total	9		
MAP30PCR - MAP30C	Negative Ranks	0 <sup>d</sup>	.00	.00
	Positive Ranks	8 <sup>e</sup>	4.50	36.00
	Ties	1 <sup>f</sup>		
	Total	9		

- a. MAP20PCR < MAP20C
- b. MAP20PCR > MAP20C
- c. MAP20PCR = MAP20C
- d. MAP30PCR < MAP30C
- e. MAP30PCR > MAP30C
- f. MAP30PCR = MAP30C

Test Statistics <sup>a</sup>		
	MAP20PCR - MAP20C	MAP30PCR - MAP30C
Z	-2.521 <sup>b</sup>	-2.521 <sup>b</sup>
Asymp. Sig. (2-tailed)	.012	.012

- a. Wilcoxon Signed Ranks Test
- b. Based on negative ranks.

Appendix 24

**Wilcoxon Signed Ranks Test**

		Ranks		
		N	Mean Rank	Sum of Ranks
MAP20PCR - V20CPCR	Negative Ranks	8 <sup>a</sup>	4.50	36.00
	Positive Ranks	0 <sup>b</sup>	.00	.00
	Ties	1 <sup>c</sup>		
	Total	9		
MAP30PCR - V30CPCR	Negative Ranks	8 <sup>d</sup>	4.50	36.00
	Positive Ranks	0 <sup>e</sup>	.00	.00
	Ties	1 <sup>f</sup>		
	Total	9		

a. MAP20PCR < V20CPCR

b. MAP20PCR > V20CPCR

c. MAP20PCR = V20CPCR

d. MAP30PCR < V30CPCR

e. MAP30PCR > V30CPCR

f. MAP30PCR = V30CPCR

Test Statistics <sup>a</sup>		
	MAP20PCR - V20CPCR	MAP30PCR - V30CPCR
Z	-2.521 <sup>b</sup>	-2.521 <sup>b</sup>
Asymp. Sig. (2-tailed)	.012	.012

a. Wilcoxon Signed Ranks Test

b. Based on positive ranks.

Appendix 25

**Wilcoxon Signed Ranks Test**

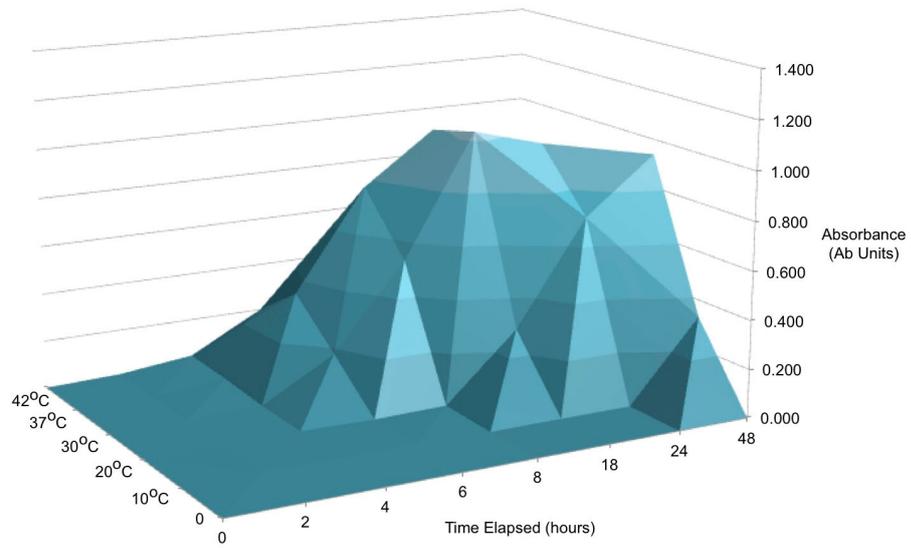
		Ranks		
		N	Mean Rank	Sum of Ranks
VAR00004 - VAR00003	Negative Ranks	3 <sup>a</sup>	4.67	14.00
	Positive Ranks	4 <sup>b</sup>	3.50	14.00
	Ties	1 <sup>c</sup>		
	Total	8		
VAR00006 - VAR00005	Negative Ranks	6 <sup>d</sup>	3.83	23.00
	Positive Ranks	1 <sup>e</sup>	5.00	5.00
	Ties	1 <sup>f</sup>		
	Total	8		
VAR00008 - VAR00007	Negative Ranks	6 <sup>g</sup>	3.50	21.00
	Positive Ranks	0 <sup>h</sup>	.00	.00
	Ties	1 <sup>i</sup>		
	Total	7		

- a. VAR00004 < VAR00003
- b. VAR00004 > VAR00003
- c. VAR00004 = VAR00003
- d. VAR00006 < VAR00005
- e. VAR00006 > VAR00005
- f. VAR00006 = VAR00005
- g. VAR00008 < VAR00007
- h. VAR00008 > VAR00007
- i. VAR00008 = VAR00007

Test Statistics <sup>a</sup>			
	VAR00004 - VAR00003	VAR00006 - VAR00005	VAR00008 - VAR00007
Z	.000 <sup>b</sup>	-1.521 <sup>c</sup>	-2.201 <sup>c</sup>
Asymp. Sig. (2-tailed)	1.000	.128	.028

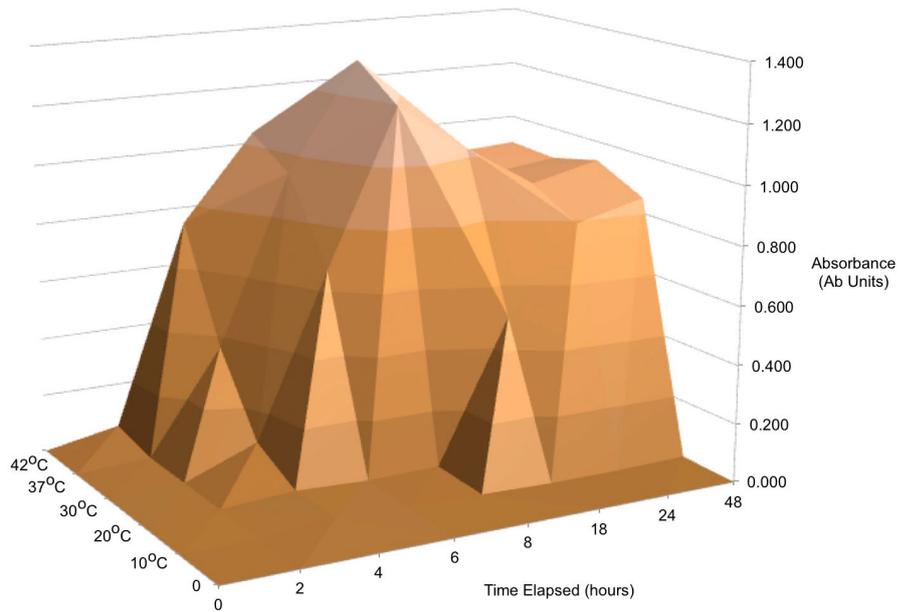
- a. Wilcoxon Signed Ranks Test
- b. The sum of negative ranks equals the sum of positive ranks.
- c. Based on positive ranks.

Appendix 26



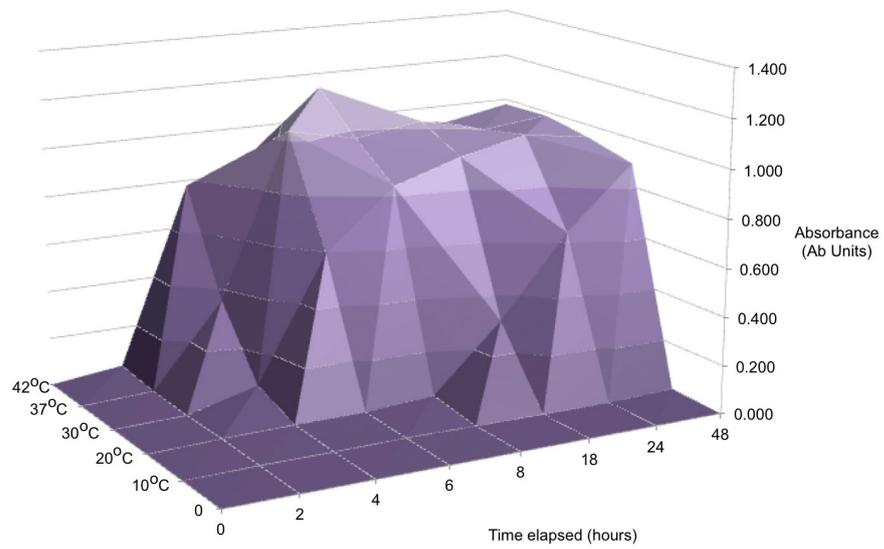
Proliferation characteristics subject to temperature over time - Vibrio Strain 259

Appendix 27



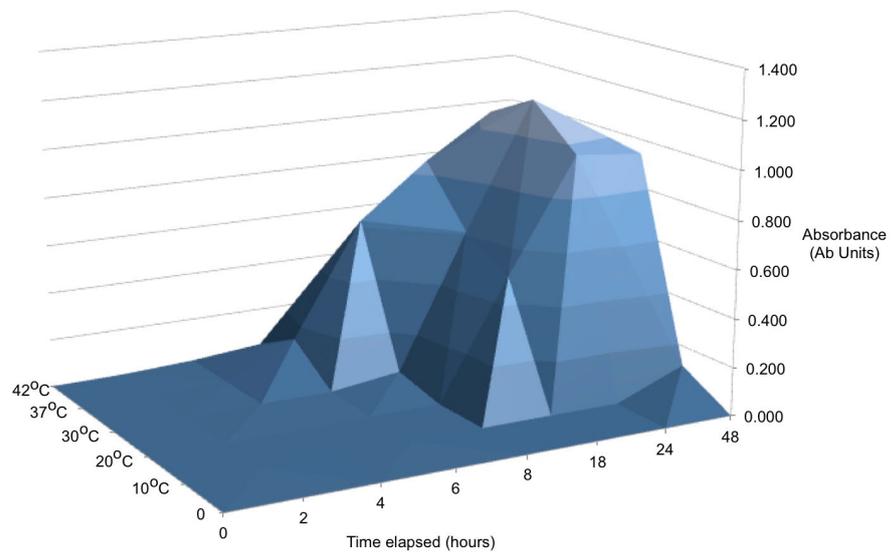
Proliferation characteristics subject to temperature over time - Vibrio Strain 2310

Appendix 28



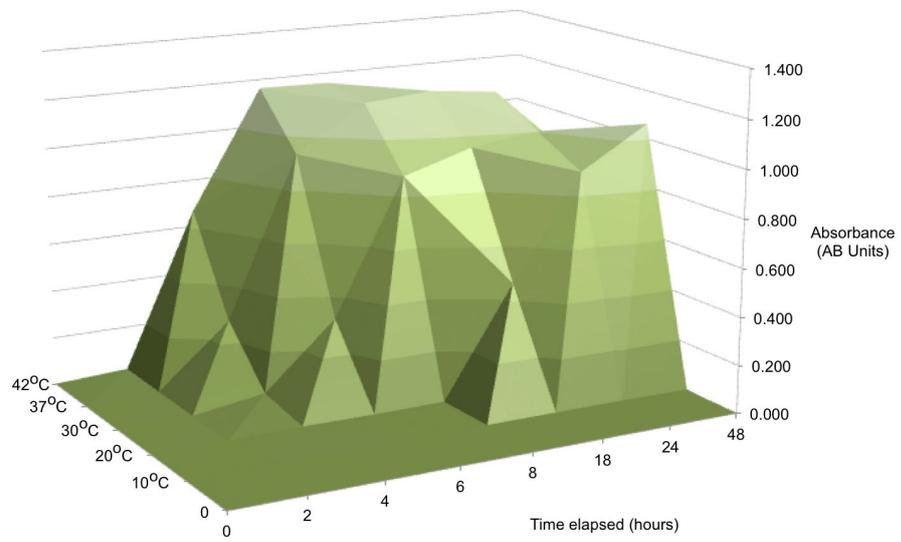
Proliferation characteristics subject to temperature over time - Vibrio Strain 2411

Appendix 29



Proliferation characteristics subject to temperature over time - Vibrio Strain 211

Appendix 30



Proliferation characteristics subject to temperature over time - Vibrio Strain 2411