出國報告（出國類別：進修）

雞隻產氣莢膜芽胞梭菌致病機轉及其與公共衛生關聯性之研究

服務機關：行政院農業委員會動植物防疫檢疫局
姓名職稱：楊文淵 技正
派赴國家：美國
出國期間：**104** 年 **8** 月 **5** 日至 **107** 年 **12** 月 **27** 日
報告日期：**108** 年 **1** 月 **30** 日

摘要

本報告以博士論文內容為主，包含目的及研究主題、研究過程及發現、心得及建議。產氣莢膜芽胞梭菌（*Clostridium perfringens*）為全美前五名食媒性病原之一，對人及動物均可造成腸道性疾病。感染人主症為腸道不適與食物中毒，於雞隻則引發壞死性腸炎，造成全球肉雞產業每年 6 百億美元的重大損失。鑑於該菌致病的多樣性，以及對產業經濟與公共衛生的重要性，符合進修目的，爰研究該病致病機轉及其與公共衛生之關聯性。結果顯示本研究所採之雞源性產氣莢膜芽胞梭菌未攜有腸毒素（*cpe*）基因，對人類無致病風險；致病機轉方面，過去研究強調的關鍵毒素基因 *netB*，無論是病雞或正常雞隻來源的菌株，大部分均攜有該基因，且兩個來源彼此之間攜帶比率及帶有基因數均無統計學上差異性，表示 *netB* 基因的存否及帶有數量不適合作為評估產氣莢膜芽胞梭菌致病力的指標，應有其他因子共同參與疾病發展過程，透過交互作用後產生疾病。進一步探討疾病發展過程發現，單獨使用帶有 *netB* 基因的產氣莢膜芽胞梭菌攻毒雞隻無法產生壞死性腸炎，僅於加入誘發因子（如餵飼高蛋白飼料或球蟲先期感染等）併同致病菌株的給予，才會誘發疾病，而參與的誘發因子越多，疾病發生率及嚴重性顯著地增加（$p < 0.05$），此結果代表著誘發因子亦為雞隻壞死性腸炎發展的關鍵。另以總體 16S rRNA 基因體學（Metagenomics）研究致病機轉結果，雞隻空腸內 *Clostridium sensu stricto 1* 屬（Genus）細菌（包含產氣莢膜芽胞梭菌）的過度增殖與壞死性腸炎的發生具有直接相關性（$p < 0.05$），除了產氣莢膜芽胞梭菌，*Clostridium sensu stricto 1* 屬內的其他細菌亦參與了壞死性腸炎發展的過程；而單以球蟲感染雞隻後無法引發疾病的結果顯示，需

外源性給予一定量 *Clostridium sensu stricto 1* 屬細菌配合誘發因子的作用，使該屬細菌於空腸內大量增殖，以繼發壞死性腸炎。本研究透過收集分析一定數量的田間分離菌株中腸毒素基因的表現情形探討雞源性產氣莢膜芽胞梭菌與公共衛生的關連性，未見有致人類食物中毒的公共衛生風險。另成功地建立雞隻壞死性腸炎疾病發展模式，並透過實驗設計、總體基因體學及進階統計分析釐出疾病發展的重要因子、主要菌相及參與菌原，結果交互證明雞隻接觸一定量 *Clostridium sensu stricto 1* 屬細菌及誘發因子後於空腸內過度增殖是壞死性腸炎發生的關鍵，此結果可作為防治策略發展應用之參考，並使用本研究已建立的疾病發展模式驗證其有效性。

目次

表次

圖次

壹、 目的及研究主題

一、 目的

多年服務於獸醫公務領域，參與多項重要動物傳染病防治策略的擬定及執行，發現臺灣獸醫領域面對食媒性疾病的控制，與公共衛生上的連結應用仍有強化空間，鑑於國外已有健全研究及管理機制，透過實地赴美進修學習並參與研究，希望能習得相關技術及知能導入行政管理系統，以期逐步強化動物生產及其產品之安全。

二、 研究主題

產氣莢膜芽胞梭菌（*Clostridium perfringens*）為全美前五名食媒性病原（Foodborne pathogen）之一，感染人主症為腸道不適（Illness）與食物中毒（Food poisoning），於美國每年約造成 1 百萬人腸道下痢問題（Batz et al., 2012; Centers for Disease Control and Prevention, 2017; Scallan et al., 2011），在雞隻則引發壞死性腸炎（Necrotic enteritis；NE），對全球肉雞產業造成每年 6 百億美元的重大損失。由於該菌致病的多樣性，以及對公共衛生與產業經濟的重要性，使得先進國家紛紛投入經費進行研究，以期找出有效策略予以防治。經與指導教授多次研討研究方向及應用價值後，商定針對雞隻來源之產氣莢膜芽胞梭菌探討其致病機轉（Pathogenesis）及其與公共衛生之關聯性。

三、 研究背景說明

（一）食物中毒及毒素指標基因

A 型產氣莢膜芽胞梭菌被公認為全球重要的食媒性病原之一（Petit et al., 1999），其原因在於此型梭菌可攜有腸毒素基因（*cpe*），於芽胞化（Sporulation）過程中產生腸毒素（Enterotoxin；簡稱 CPE），造成人類食物中毒跟下痢。依據美國相關研究及疾病管制局（Centers for Disease Control and Prevention；CDC）資料統計，攜有 *cpe* 基因的 A 型產氣莢膜芽胞梭菌每年可於美國造成約 1 百萬人的腸道下痢，而最常見的感染來源為牛肉、雞肉及豬肉（Grass et al., 2013），且每次疫情的爆發均與不適當的冷卻或再加熱已經污染的前述肉品有關（Schlundt 2015），但最重要保有 *cpe* 基因產氣莢膜芽胞梭菌的動物，以及帶有該菌的感染環境至今仍無法被確認（McClane, 2007）。至今研究瞭解，*cpe* 基因一旦表現，會產生腸毒素（CPE），進一步造成人類臨床上可見的食物中毒或非中毒性的腸道不適（Sarker et al., 1999）。因此，*cpe* 基因已被認定為人類食物中毒與腸道不適的主要篩選基因，爰本研究將其列為與公共衛生連結的重要指標，並進行調查。

為釐清帶有 *cpe* 基因產氣莢膜芽胞梭菌可能的帶菌來源，一項公共衛生研究針對 2,659 個來自於動物、人類以及食物中的 A 型產氣莢膜芽胞梭菌分離株進行調查，結果僅有低於 5%的分離株帶有 *cpe* 基因（Daube et al., 1996），顯示非常少數的產氣莢膜芽胞梭菌族群帶有食物中毒基因。美國雖將禽肉列為 A 型產氣莢膜芽胞梭菌食物中毒可能來源之一，但自 2011 年後，未有禽肉直接引致人類食物中毒的正式報告。過去曾有幾個食物中毒案例與食用遭受 A 型產氣莢膜芽胞梭菌污染之雞肉

具有流行病學上的關聯性，調查顯示與禽肉經未符衛生的操作遭受污染有關，但污染來源不明，無法證實由雞源的 A 型產氣莢膜芽胞梭菌所導致（Hook et al., 1996; Schiemann, 1977）。鑒此，本研究多方收集田間雞場、肉雞屠宰場及實驗雞隻等雞源 A 型產氣莢膜芽胞梭菌，以期藉由 *cpe* 基因及相關毒素基因的篩選，連結其與公共衛生之關聯性。

（二）產氣莢膜芽胞梭菌

產氣莢膜芽胞梭菌屬革蘭氏陽性（Gram positive）厭氧菌，可產生芽胞（Spore）長時間抵抗外在不良的生存環境（Novak et al., 2003），已知廣泛存在於環境及脊椎動物腸道內，屬於常在菌（Timbermont et al., 2009）。命名分類歸屬於 *Firmicutes* 門（Phylum）、*Clostridia* 綱（Class）、*Clostridiales* 目（Order）、*Clostridiaceae* 科（Family）、*Clostridium* 屬（Genus）及 *perfringens* 種（Keto-Timonen et al., 2006），可引發人及動物多種胃腸道疾病（Uzal, 2016）。此菌特性在於擁有大量毒素基因（Toxin genes），表現後可以產生至少 17 種毒素（Toxins），但由於其缺乏合成 13 種必須胺基酸所需的基因，因此需要前述毒素配合所生產的細胞外酵素（Extracellular enzymes），協助獲取增殖所需的養分（Myers et al., 2006; Shimizu et al., 2002），並幫助該菌於宿主體內發展疾病（Freedman et al., 2015; Rood, 1998; Uzal et al., 2014）。由於這些毒素及酵素可以快速破壞組織、引發下痢（Diarrhea）及腸毒血症（Enterotoxemia），近年來已被歸類為毒力因子（Virulent factors）（Shimizu et al., 2002; Songer, 1996）。

（三）毒力型及毒素基因

產氣莢膜芽胞梭菌產生的多種毒素中，α（alpha）、β（beta）、ε（epsilon）與 ι（iota）屬於主要毒素（Major toxins），其他為附屬毒素（Minor toxins）。依據四種主要毒素的產生或其相對基因的存在與否，此菌可分為 A、B、C、D 與 E 五種毒力型（Toxinotypes）（Van Immerseel et al., 2004b），每個毒力型另有可能攜帶其他附屬毒素基因，以決定毒力，造成疾病及症狀，例如：*cpe* 基因表現所產的 CPE 毒素可以引致人類食物中毒，*netB* 基因表現產生的 Necrotic enteritis B-like（NetB）毒素會造成雞隻壞死性腸炎，產氣莢膜芽胞梭菌的毒力型及所攜帶的毒素基因詳見 Table 1.1。於主要毒素的產生上，A 型僅有 *cpa* 基因，故只產生 alpha 毒素，但 B 型有 *cpa*、*cpb* 及 *etx* 基因，分別產生 alpha、beta 及 epsilon 毒素。同理，C 型可生成 alpha 與 beta 毒素；D 型生產 alpha 與 epsilon 毒素；E 型產生 alpha 及 iota 毒素。而同一毒力型梭菌可能會帶有不同種類之附屬毒素，例如：同為 A 型菌，有的菌會帶有 *cpe* 及 *cpb2* 基因產生 CPE 與 CPB2 毒素（Gibert et al., 1997; Songer, 1996），有的菌僅帶有 *netB* 基因生產 NetB 毒素。目前，*netB* 基因及其 NetB 毒素只發現於 A 型產氣莢膜芽胞梭菌，雞隻壞死性腸炎的發生被證明與 *netB* 具有直接相關性（Keyburn et al., 2008; Keyburn et al., 2010）。

Table 1.1　產氣莢膜芽胞梭菌毒力型及其毒素基因

	毒素（Toxins）	基因（Genes）	毒力型（Toxinotypes）				
			A	B	C	D	E
主要毒素	Alpha	*cpa*	+	+	+	+	+
	Beta	*cpb*	-	+	+	-	-
	Epsilon	*etx*	-	+	-	+	-
	Iota	*iap*	-	-	-	-	+
附屬毒素	CPE	*cpe*	+/-	+/-	+/-	+/-	+/-
	Beta2	*cpb2*	+/-	+/-	+/-	+/-	+/-
	NetB	*netB*	+/-	-	-	-	-
	TpeL	*tpeL*	+/-	+	+/-	-	-

（四）毒力因子

梭菌性的疾病，毒素被認為是主要的毒力因子，例如：氣性壞疽（Gas gangrene）的症狀係由 A 型產氣莢膜芽胞梭菌所產的 alpha 毒素所導致；羊隻腸毒血症起因於 D 型產氣莢膜芽胞梭菌所產的 epsilon 毒素；仔牛仔豬梭菌性壞死性腸炎肇因於 C 型產氣莢膜芽胞梭菌生產的 beta 毒素等（Awad et al., 1995；Uzal et al., 2014；Sayeed et al., 2008）。然而雞隻壞死性腸炎的發生，不僅如前述梭菌性疾病只需要毒素即可導致，研究陸續指出，除有合適的腸道環境外，需有毒素、酵素、附著因子（Attachment factors）及細菌素（Bacteriocins）等共同作用，才能發展壞死性腸炎（Petit et al., 1999; Prescott et al., 2016a; Uzal et al., 2014）。所有細菌本身所產生助於疾病發展之物質，現統稱為毒力因子（Virulent factors），目前除了毒素以外，其餘毒力因子與雞隻壞死性腸炎疾病發展之關聯性，仍無一致性的研究發現及結果。

（五）壞死性腸炎

壞死性腸炎主為 A 型（極少數為 C 型）產氣莢膜芽胞梭菌所引起的雞隻腸道性疾病，並於空腸（Jejunum）產生壞死性病灶為疾病特徵（Paiva and McElroy, 2014），2 週齡至 6 月齡雞隻均具有感受性，但 2 至 6 週齡雞隻最易感染發病，此現象與雞隻體內母源抗體的消退有關（La Ragione and Woodward, 2003）。此病一年四季均可能發生，爆發後多呈散發（Cooper et al., 2016），通常持續 5-10 天（Merck Veterinary Manual, 1998），急性可見死亡情形，死亡率介於 2-10%，亦有高至 50%的報告（Paiva and McElroy, 2014），典型臨床症狀為嚴重沈鬱、不願移動相互集聚（Huddling）、羽毛粗亂（Ruffled feathers）、厭食、下痢及出現死亡。此病於田間呈現急性（Acute）與次臨床（Subclinical）兩種型態，急性型病程快，通常於出現症狀 1-2 小時候無預警死亡，次臨床型則無明顯症狀，但生產效率及飼料換肉率顯著降低（Cooper et al., 2013; Martin and Smyth, 2009），是造成家禽產業經濟損失的主要原因。除肉雞外，3 到 6 月齡商業蛋雞與各種禽類均可遭受感染，包括火雞、鴕鳥、鵪鶉、松雞（Capercaillies）、鵝、藍知更鳥（Bluebirds）、澳洲小鸚鵡（Lorikeets）及烏鴉（Cooper et al., 2013）。流行病學調查顯示此病於禽群（Flock）疾病盛行率為 12.3%（Hermans and Morgan, 2007），商業肉雞場可達 37%的發生率，導致全球家禽產業每年 6 百億美元的損失（Van Der Sluis, 2000; Wade and Keyburn., 2015）。若場內存有其他疾病問題、生物安全執行不佳、給予不良的飼料劑型與成分，以及未有使用預防性治療方式防治壞死性腸炎，均會提升罹病的風險（Hermans and Morgan, 2007）。

家禽產業過去仰賴低劑量的抗生素，將其添加入飼料中作為生長促進劑（Antimicrobial Growth Promoters；AGPs），有效地預防及控制壞死性腸炎，促進家禽生長效率。但基於長期使用有可能會產生抗藥性菌株，而且無法排除該菌株自家禽傳給人類的可能風險，歐洲國家明令禁止 AGPs 的使用，並帶動無抗生素飼養家禽意識的興起（Liu et al., 2010）。由於此病未有菌苗可以有效預防，於禁止使用 AGPs 的情形下，家禽產業陸續爆發疫情，成為近年來再浮現（Re-emerging）於家禽的重要細菌傳染病，造成產業重大損失（Casewell et al., 2003; Gaucher et al., 2015; Timbermont et al., 2011; Van Immerseel et al., 2009）。現今，家禽產業積極透過產學研究及合作尋求另項防治策略，以期預防及控制此高經濟衝擊的細菌性傳染疾病。

（六）致病機轉

大部分梭菌性腸炎係仰賴菌體的增殖伴隨產生大量的毒素，於宿主體內發展疾病（Theoret et al., 2016）。但雞隻壞死性腸炎的致病機轉複雜，已知無法藉由單一毒素的作用而引發疾病，多項研究指出除有 A 型產氣莢膜芽胞梭菌參與外，並需其他誘發因子（Predisposing factors）營造合適的腸內環境與提供細菌增殖所需營養來源，配合與毒力因子共同作用後產生壞死性腸炎；至於雞源產氣莢膜芽胞梭菌與人類食物中毒或腸道不適之關聯性部分，目前仍未建立。

（七）誘發因子

產氣莢膜芽胞梭菌屬於環境及腸道內的常在菌，依據此特性可得知，該菌無法單獨地誘發疾病（Craven, 2000; Van Immerseel et al., 2004b），經動物試驗研究顯示，雞隻壞死性腸炎疾病發展過程中，誘發因子可以協助產氣莢膜芽胞梭菌的增殖

及附著，以達有效感染，其後由細菌所產的毒素及其他毒力因子交互作用發展疾病。誘發因子包括球蟲感染、於飼料中添加高蛋白或高比例的非澱粉多醣類（Non-starch polysaccharides，NSP）、感染免疫抑制性疾病（如傳染性華氏囊病、雞傳染性貧血及馬立克病）、黴菌毒素（Mycotoxin）、密飼及低溫等緊迫（Lee et al., 2011; Park et al., 2008; Prescott et al., 2016b; Thompson et al., 2006; Williams, 2005; Williams et al., 2003）。

1. 球蟲感染

雞隻球蟲感染係由艾美球蟲屬（*Eimeria* spp）之寄生蟲寄生於宿主小腸及盲腸，藉由生活史寄生過程造成腸黏膜廣泛的損害 (Williams, 2005)；至今，球蟲感染具有多種機制誘使雞隻發展壞死性腸炎，如：直接造成小腸上皮損傷促進產氣莢膜芽胞梭菌的附著（Van Immerseel et al., 2009; Williams, 2005; Williams et al., 2003），以及藉由產生血漿蛋白提供產氣莢膜芽胞梭菌營養來源予以增殖（Van Immerseel et al., 2004b）。此外，球蟲感染亦會降低小腸消化率，誘發 T 細胞免疫反應增加黏蛋白（Mucin）產生，來增加產氣莢膜芽胞梭菌獲取這些營養進行增殖的機會（Collier et al., 2008）。

2. 飲食因子

給雞隻食用富含小麥、黑麥、大麥或燕麥等穀類（Cereals）或高動物蛋白（如魚粉）的飼料容易誘發壞死性腸炎（Cooper and Songer, 2009; McDevitt et al., 2007; Williams, 2005）。原因為穀類含有高含量不可消化且水溶性的非澱粉多醣類（NSP），增加腸內容物的黏稠性，減緩其通過腸道的時間，進而提高產氣莢膜芽胞梭菌獲取

增殖所需養分的機會(Annett et al., 2002; Langhout et al., 1999)。此外，這類物質可與上皮細胞上的醣蛋白作用增產黏蛋白（Kleessen et al., 2003），促進產氣莢膜芽胞梭菌過度增生（Shojadoost et al., 2012）；而 NSP 不可消化部分，另可作為微生物增殖所需養分(Choct and Annison, 2007)。至於高動物蛋白的飼料(尤其是添加魚粉者)，其可提供產氣莢膜芽胞梭菌無法自行合成的必須胺基酸，促進大量增殖(Drew et al., 2004; Titball et al., 1999)。飼料中高含量的魚粉同時會增加腸道 pH 值（McDevitt et al., 2007）與改變腸內菌叢（Stanley et al., 2012; Stanley et al., 2014b），利於產氣莢膜芽胞梭菌的生長與增生。

3. 免疫抑制

雞隻自身免疫狀態的改變亦會增加壞死性腸炎的發生率（Moore, 2016）。目前已知感染雞傳染性華氏囊炎（Infectious bursal disease；IBD）、雞傳染性貧血（Chicken infectious anemia；CIA）及馬立克病（Marek's disease；MD）均可造成家禽免疫抑制（Hoerr, 2010），於產氣莢膜芽胞梭菌存在下，進一步誘發壞死性腸炎的發生並增加感染的嚴重性(Gholamiandehkordi et al., 2007; Stringfellow et al., 2009; Timbermont et al., 2009; Williams et al., 2003）。另外，促使禽隻緊迫的原因也可能誘發壞死性腸炎及增加該病嚴重性，例如高密度飼養、低溫、環境中氨氣濃度過高等等（Hoerr, 2010; Tsiouris et al., 2015a, b）。

4. 黴菌毒素

黴菌毒素是黴菌於穀物或飼料中自然產生的二級代謝物（Secondary fungal metabolites)，其中黃麴毒素(Aflatoxins；AF)、玉米赤黴烯酮(Zearalenone；ZEN)、

棕麴黴毒素（Ochratoxin A；OTA）、伏馬毒素（Fumonisins；FUM）、菌毒素（Trichothecenes），如：嘔吐毒素（Deoxynivalenol；DON）以及 T-2 毒素，為已知會影響家禽健康及生產的黴菌毒素（Murugesan et al., 2015）。近來，嘔吐毒素被指出可透過對家禽上皮黏膜的傷害及增加腸內營養物質的獲取，幫助產氣莢膜芽胞梭菌的增生（Antonissen et al., 2014），此外，研究亦發現伏馬毒素可以改變家禽迴腸菌叢並影響小腸絨毛長度及腺窩深度（Crypt depth），促使雞隻發生壞死性腸炎（Antonissen et al., 2015）。雖然黴菌毒素曾有造成宿主免疫抑制的報告（Prescott et al., 2016b），但其是否可藉由免疫抑制的方式誘發雞隻壞死性腸炎的部分，需要進一步調查研究。

5. 其他因子

另有研究報告指出，某肉雞種別（Jang et al., 2013）及特殊基因的組成（Siegel et al., 1993）可以增加罹患壞死性腸炎的敏感性或抵抗性。

（八）小結

此研究目的除探究雞源性產氣莢膜芽胞梭菌與公共衛生之關聯性，並進一步研討雞隻壞死性腸炎的致病機轉，以期提供後續現場防治之應用。惟該病複雜的致病機轉使得防治研究具有高度挑戰性，雖各國/界投入相關經費積極研究至今，掌握誘發及毒力因子，並嘗試多項防治方法，例如：使用疫苗增加宿主免疫力、於飼料中添加益菌生（Prebiotics）、益生菌（Probiotics）或其他物質來競爭排除腸道內產氣莢膜芽胞梭菌、改善腸道菌叢避免產氣莢膜芽胞梭菌的增殖或附著或/及產生抑菌物質降低細菌數量等等，但是結果並不具一致性，即無法提供可接受程度的保護力

或防治效果。因此，必須深入探討產氣莢膜芽胞梭菌與相關因子的交互作用，並比較不同因子加入後腸內環境的差異，以期掌握致病關鍵，發展有效策略予以防治。

貳、 研究過程及發現

為探討雞隻壞死性腸炎的致病機轉及其與公共衛生關聯性，第一階段自田間及屠宰場等地收集病雞及正常雞隻腸道樣材並同步分離其內之產氣莢膜芽胞梭菌，匯集後應用核酸檢測相關技術檢驗病雞與正常雞來源菌株毒素基因之盛行比率及特定毒素基因帶有數，並將結果進行統計方法分析，以研析毒素基因的存否與致病之必須性，並依腸毒素（*cpe*）基因攜帶情形探討雞源性產氣莢膜芽胞梭菌與公共衛生之關聯性。

第二階段利用田間致病菌株於實驗室內攻毒雞隻建立壞死性腸炎發展模式，同時搭配不同誘發因子進行評估，探討產氣莢膜芽胞梭菌與誘發因子的交互作用，及其對臨床疾病發生之影響。

第三階段應用已建立之壞死性腸炎發展模式，分析不同誘發因子與產氣莢膜芽胞梭菌參與下腸內菌相的變化，評估有否其他細菌或因子參與雞隻壞死性腸炎的發展，並歸納可能之致病機轉，以供後續發展現場可行之防治措施。

一、 探討產氣莢膜芽胞梭菌毒素基因存否與壞死性腸炎致病之必須性及與公共衛生關聯性

毒素一向被認為是產氣莢膜芽胞梭菌引發疾病的重要肇因（Awad et al., 1995; Keyburn et al., 2008; Sarker et al., 1999; Sayeed et al., 2008; Uzal et al., 2014），於雞隻壞死性腸炎研究中，*netB* 毒素基因及其表現產生的 NetB 毒素因被證明符合分子學柯霍法則（Molecular Koch's postulate），而被視為致病的關鍵（Keyburn et al., 2006），即以具該毒素的菌株攻毒雞隻才能發展疾病，不具該毒素的菌株攻毒雞隻不會致病；另有不少研究指出，惟有使用帶有 *netB* 基因菌株可於實驗室內穩定地誘發雞隻壞死性腸炎（Cooper and Songer, 2010; Keyburn et al., 2010; Smyth and Martin, 2010; Timbermont et al., 2009）。然而，多起流行病學調查結果與前述實驗室結果存有落差，不少田間發病雞隻所分離的菌株不具有 *netB* 基因（Abildgaard et al., 2010; Bailey et al., 2015; Llanco et al., 2015; Martin and Smyth, 2009; Smyth and Martin, 2010），正常雞隻分離菌株卻有 *netB* 基因的存在，而非僅存於致病菌株（Martin and Smyth, 2009）。此外，單獨使用帶有 *netB* 基因的菌株進行攻毒，於沒有其他誘發因子的共同參與下，無法每次成功地誘發壞死性腸炎（Zhou et al., 2017）。因此，*netB* 基因的存否於致病機轉中所扮演的角色或重要性須進一步研究，予以釐清。

（一）方法

於田間雞場、肉雞屠宰場及實驗雞隻採集 15 隻不同場病雞及 15 隻不同來源正常雞隻的空腸樣材，以血液培養基分離出細菌，再以生化試驗鑑定（Biochemical tests）及聚合酶連鎖反應（Polymerase chain reaction；PCR）確認所選取的細菌為產

氣莢膜芽胞梭菌後，萃取每個菌株 DNA，並以針對目標毒素基因的引子對（Primer sets，詳見 Table 1.2）進行 PCR 檢測；針對 *netB* 基因，另使用高靈敏度的即時聚合酶連鎖反應(Real-time or quantitative PCR; qPCR)確定該基因攜帶情形及數量。qPCR 標準效能曲線詳見 Figure 1.1，相關結果以統計方法分析病雞與正常雞隻來源菌群彼此間有無顯著差異（$p < 0.05$）。

Table 1.2　毒素基因 PCR 及 qPCR 引子對及其增幅產物長度

基因	基因庫號碼	引子對	引子序列（5′-3′）	T_m（℃）	增幅產物（bp）	參考文獻
cpa	L43545	CPA5L CPA5R	AGTCTACGCTTGGGATGGAA TTTCCTGGGTTGTCCATTTC	55	900	（Fan et al., 2016）
cpb	X83275	CPBL CPBR	TCCTTTCTTGAGGGAGGATAAA TGAACCTCCTATTTTGTATCCCA	56	611	（Fan et al., 2016）
cpb2	L77965	CPB2L CPB2R	AGATTTTAAATATGATCCTAACC CAATACCCTTCACCAAATACTC	53	567	（Garmory et al., 2000）
etx	M95206	CPETXL CPETXR	TGGGAACTTCGATACAAGCA TTAACTCATCTCCCATAACTGCAC	56	396	（Fan et al., 2016）
iap	X73562	CPIL CPIR	AAACGCATTAAAGCTCACACC CTGCATAACCTGGAATGGCT	57	293	（Fan et al., 2016）
cpe	X81849	CPEL CPER	GGGGAACCCTCAGTAGTTTCA ACCAGCTGGATTTGAGTTTAATG	57	506	（Fan et al., 2016）
tpeL	EU848493	TPELF TPELR	ATATAGAGGCAAGCAGTGGAG GGAATACCACTTGATATACCTG	55	466	（Coursodon et al., 2012）
netB	GU433338	NETBL NETBR	TGATACCGCTTCACATAAAGGTTGG ATAAGTTTCAGGCCATTTCATTTTTCCG	61	169	本研究設計
16S rRNA	Y12669	16SL 16SR	CATCATTCAACCAAAGGAGCAATCC CATTATCTTCCCCAAAGACAGAGC	60	262	本研究設計

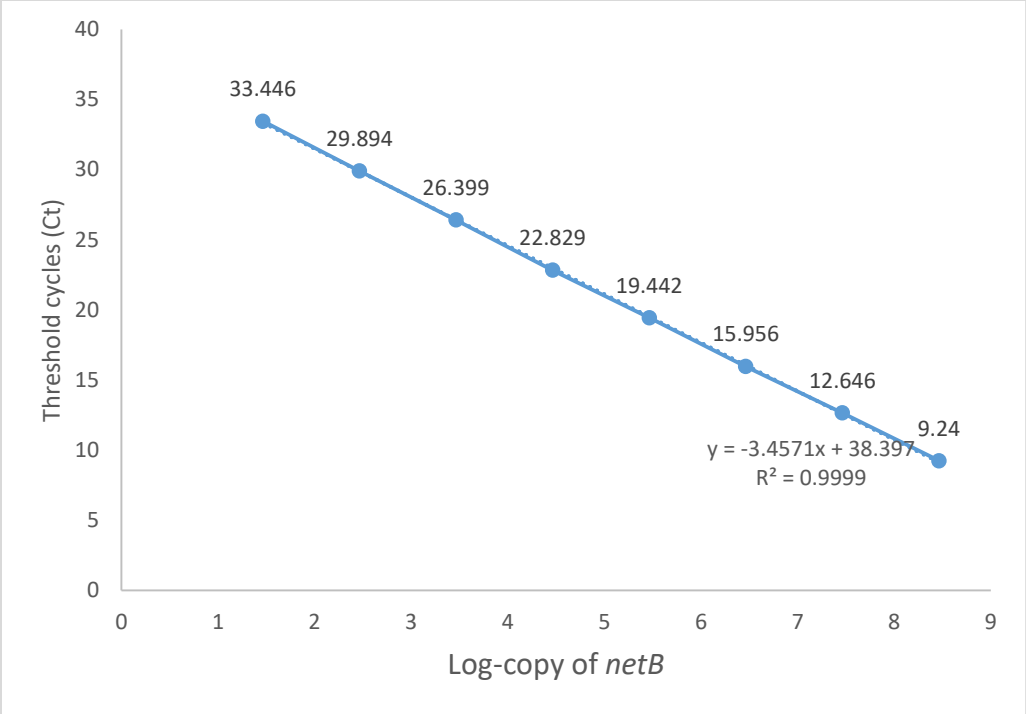

Figure 1.1　　*netB* 毒素基因 qPCR 標準效能曲線.

將 *netB* 毒素基因增幅產物透過基因選殖進入質體，萃取質體 DNA 並進行 10 倍稀釋（從 1 ng 到 100 ag）後以特殊引子對進行 qPCR，以所得 Ct 數值與基因數對數建立標準曲線，基因數範圍為 2.92×10^8 至 29.2。線性公式為 $y = -3.4571x + 38.397$（$R^2 = 0.9999$; 增幅效率 AE = 95%）。

（二）結果

以 PCR 檢測此 30 株產氣莢膜芽胞梭菌的毒素基因結果，所有受檢菌株（n=30）均為 A 型菌，且均未攜帶 *cpe* 基因，顯示本研究所分離之雞源性產氣莢膜芽胞梭菌無論來自壞死性腸炎患病雞隻（n=15）或正常雞隻（n=15），皆無公共衛生風險疑慮。針對附屬毒素偵測結果，基因 66.7%菌株帶有 *cpb2* 基因，6.7%菌株帶有 *netB* 基因，6.7%菌株帶有 *tpeL* 基因，致病菌株群與正常菌株群攜帶 *cpb2*、*netB* 及 *tpeL* 基因比率彼此間均無統計學上差異性。30 株產氣莢膜芽胞梭菌毒素基因檢驗結果詳如 Table 1.3。

Table 1.3　產氣莢膜芽胞梭菌致病菌株及正常菌株毒素基因檢測結果及 *netB* 基因數攜帶情形

分離株	來源物種	樣材來源	健康狀態	型別	*cpa*	*cpb*	*etx*	*iap*	*cpe*	*cpb2*	*tpeL*	*netB*		
												PCR	qPCR	Copies/100 ng DNA
JP17	陽性對照菌株	–	–	A	+	–	–	–	–	+	+	+	+	$>2.92\times10^8$
1N	Broiler	F1	NE	A	+	–	–	–	–	+	+	+	+	$>2.92\times10^8$
2N	Broiler	F2	NE	A	+	–	–	–	–	–	–	–	–	–
3N	Broiler	F3	NE	A	+	–	–	–	–	+	–	–	+	7.26×10^3
4N	Broiler	F4	NE	A	+	–	–	–	–	+	–	–	+	4.19×10
5N	Broiler	F5	NE	A	+	–	–	–	–	–	–	–	+	$<2.92\times10$
6N	Broiler	F6	NE	A	+	–	–	–	–	–	–	–	+	1.35×10^3
7N	Broiler	F7	NE	A	+	–	–	–	–	+	+	+	+	$>2.92\times10^8$
8N	Broiler	F8	NE	A	+	–	–	–	–	+	–	–	+	$<2.92\times10$
9N	Broiler	F9	NE	A	+	–	–	–	–	+	–	–	+	1.76×10^3
10N	Broiler	F10	NE	A	+	–	–	–	–	+	–	–	+	<2.92×10
11N	Broiler	F11	NE	A	+	–	–	–	–	+	–	–	+	<2.92×10
12N	Broiler	F12	NE	A	+	–	–	–	–	+	–	–	+	<2.92×10
13N	Broiler	F13; H2	NE	A	+	–	–	–	–	+	–	–	–	–
14N	Broiler	F13; H2	NE	A	+	–	–	–	–	–	–	–	–	–
15N	Broiler	F13; H2	NE	A	+	–	–	–	–	+	–	–	–	–
1C	Broiler	P1	non-NE	A	+	–	–	–	–	+	–	–	+	3.11×10
2C	Broiler	P1	non-NE	A	+	–	–	–	–	+	–	–	+	7.49×10
3C	Broiler	P1	non-NE	A	+	–	–	–	–	+	–	–	–	–
4C	Broiler	P1	non-NE	A	+	–	–	–	–	+	–	–	–	–
5C	Broiler	F14; H1	non-NE	A	+	–	–	–	–	+	–	–	–	–
6C	Broiler	F14; H2	non-NE	A	+	–	–	–	–	+	–	–	+	2.96×10
7C	Broiler	F14; H3	non-NE	A	+	–	–	–	–	+	–	–	+	4.40×10
8C	Broiler	F15; H1	non-NE	A	+	–	–	–	–	+	–	–	+	<2.92×10
9C	Broiler	F15; H2	non-NE	A	+	–	–	–	–	+	–	–	+	<2.92×10
10C	Layer	EC1	non-NE	A	+	–	–	–	–	–	–	–	–	–
11C	Layer	EC1	non-NE	A	+	–	–	–	–	–	–	–	+	7.23×10
12C	Layer	EC1	non-NE	A	+	–	–	–	–	–	–	–	+	$<2.92\times10$
13C	Broiler	EC2	non-NE	A	+	–	–	–	–	–	–	–	–	–
14C	Broiler	EC2	non-NE	A	+	–	–	–	–	–	–	–	+	6.45×10
15C	Broiler	EC2	non-NE	A	+	–	–	–	–	–	–	–	–	–

縮寫：F：雞場；H：雞舍；P：屠宰場；EC：實驗室內對照組雞隻；NE：壞死性腸炎；non-NE：未有染病或正常雞隻。
致病菌株群與正常菌株群所攜帶 netB 基因數無統計學上差異性（Wilcoxon Rank-Sum Test; $p > 0.05$）。

以 qPCR 檢測 *netB* 基因結果（Table 1.3），致病菌株帶有 *netB* 的基因數較高，但與正常菌株相比，無統計學上差異性（*Fisher's exact test; $p > 0.05$*）。*netB* 基因攜帶情形比較部分，73%致病菌株及 60%正常菌株帶有 *netB* 基因，兩菌群間比較如同

PCR 結果，無統計學上差異性（$p > 0.05$）。qPCR 檢出率為 66.7%，遠高於 PCR 檢出率 6.7%。

綜上，*cpb2*、*netB* 及 *tpeL* 等毒素基因的存否以及 *netB* 基因數量無法與壞死性腸炎相連結（意即僅有致病菌株攜帶，正常菌株不攜帶；或致病菌株攜帶比率或數量有統計意義地高於正常菌株）；依此研究結果，若需評估 *netB* 基因的存否，建議應使用高檢測敏感度的 qPCR 進行，以利適切評估。

（三）討論

本項研究之雞源性產氣莢膜芽胞梭菌未攜有腸毒素基因，無人類食物中毒的公共衛生風險，此結果與過去研究結果一致（Keyburn et al., 2006; Van Immerseel et al., 2009）。致病機轉方面，過去研究強調 *netB* 基因為雞隻壞死性腸炎關鍵毒力因子，臨床致病的菌株多為帶有 *netB* 基因菌株（Johansson et al., 2010; Keyburn et al., 2010a; Martin and Smyth, 2009），但本研究利用 qPCR 進行檢測發現，無論病雞或正常雞隻來源的產氣莢膜芽胞梭菌，大部分均攜有 *netB* 基因，且彼此間攜帶比率及基因數未有統計學上明顯差異，表示 *netB* 基因的存否及帶有數量並不適合作為評估產氣莢膜芽胞梭菌致病力的指標。高比率（66.7%）*netB* 基因攜帶結果暗示著，該基因應存於絕大部分的產氣莢膜芽胞梭菌族群裡，而該菌環境及腸道常在的特性使其無法輕易造成感染及發病，推測應有其他因子參與疾病過程，促使 *netB* 及相關基因的表現，由其產物與參與因子交互作用發展疾病。此推論已有研究提供部分支持證據，如 Zhou 等人於 2017 年研究發現，單帶有 *netB* 基因無法於動物試驗過程中成

功誘發疾病，需有其他位於細菌質體的 NELoc-1 基因位內基因的參與，才能出現臨床上所見之完全毒力 （Zhou et al., 2017）。

由於雞隻壞死性腸炎可歸為多因子共同參與之疾病（Multi-factorial disease），後續研究建議應進一步調查正常及發病雞隻腸道菌相、觸發環境，毒力及相關基因的調節機制，以利瞭解掌握產氣莢膜芽胞梭菌動態致病機轉。

二、 建立雞隻壞死性腸炎攻毒模式並評估帶有 *netB* 基因之產氣莢膜芽胞梭菌與誘發因子交互作用對臨床疾病發生的影響

於正常健康雞隻檢出帶有 *netB* 基因的產氣莢膜芽胞梭菌暗示著產氣莢膜芽胞梭菌即使帶有 *netB* 基因，也無法單獨地自行感染雞隻，引發壞死性腸炎（Martin and Smyth, 2009）；另有研究指出，缺乏誘發因子參與下，單以產氣莢膜芽胞梭菌攻毒雞隻經常無法成功地產生壞死性腸炎（Craven, 2000; Van Immerseel et al., 2004）。為深入瞭解該病致病機轉，首先需建立雞隻壞死性腸炎發展/攻毒模式，然後研析帶有 *netB* 基因產氣莢膜芽胞梭菌與誘發因子交互作用對臨床疾病發生之影響。因此，第二部分研究嘗試建立雞隻壞死性腸炎攻毒模式，以單一致病菌株、致病菌株併同高蛋白飼料及/或球蟲感染等方式進行，其後彙整相關實驗結果進行比較，並進行後續評估。

（一）方法

本項研究進行 2 場次雞隻試驗予以評估。第 1 場試驗未納入誘發因子，包括 2 個處理組，每組 21 隻，分別為致病菌株攻毒組（CP1-T1）及不處理的對照組（CTL-T1）。第 2 場試驗納入高蛋白飼料或/及球蟲感染等誘發因子，分為 3 個處理組，每組同為 21 隻，依次為餵以高蛋白飼料及致病菌株攻毒（CP1-T2）、餵以高蛋白飼料及致病菌株與球蟲共同感染組（CP1+*Eimeria*-T2）以及餵以高蛋白飼料（CTL-T2）的對照組。2 次試驗期間分別為期 21-23 天，每天由專人進行 2 次臨床檢查並記錄死亡率。以球蟲感染者，分別於 9-10 天以商用球蟲疫苗進行口餵誘發球蟲症（Coccidiosis），經產氣莢膜芽胞梭菌攻毒者，分別於第 14-15 天時以濃度 $2.5x10^8$

colony-forming units（CFU）/ml 的 3 ml 菌液進行口餵攻毒，分別連續 3-4 天。其後於特定時間點犧牲雞隻，進行剖檢確定壞死性腸炎病灶及發病情形。動物試驗相關執行及步驟經美國密西西比州立大學（Mississippi State University）實驗動物照護及使用委員會審核通，並於符合動物福利規範下進行。

壞死性腸炎之判定採用 Keyburn 等人於 2006 年所建立標準（Keyburn et al., 2006）進行，經剖檢後，小腸黏膜無肉眼病灶者判為 0 分，小腸黏膜充出血者為 1 分，小腸黏膜具有 1-5 處局部壞死或潰瘍病灶者為 2 分，小腸黏膜具有 6-15 處局部壞死或潰瘍病灶者為 3 分，小腸黏膜具有 16 處（含）以上局部壞死或潰瘍病灶者為 4 分。小腸黏膜病灶分數判為 2 分（含）以上者，認定為壞死性腸炎案例。

（二）結果

於試驗 1 中，單獨使用帶有 *netB* 基因之產氣莢膜芽胞梭菌攻毒雞隻（CP1-T1）無法誘發壞死性腸炎（如 Table 1.4）。於試驗 2 中加入含有魚粉的高蛋白飼料餵飼雞隻並給予帶有 *netB* 基因之產氣莢膜芽胞梭菌（CP1-T2），開始引發壞死性腸炎案例，但發生率低（2/19；10.5%）。另於餵飼高蛋白飼料及先期感染球蟲情形下，以帶有 *netB* 基因之產氣莢膜芽胞梭菌攻毒雞隻（CP1+*Eimeria*-T2）誘發最多壞死性腸炎案例，並有較高發生率（5/19；26.3%）。剖檢計分後，誘發因子參與數越多，所誘發的病灶分數越高，與對照組雞隻結果相比具有統計學上差異性（$p < 0.05$）。相關結果代表著誘發因子於雞隻壞死性腸炎疾病的發展具有重要性；於誘發因子參與下，壞死性腸炎的發生數及嚴重性（病灶分數）均顯著地上升，越多誘發因子參與，差異性越大（Figure 1.2）。壞死性腸炎肉眼病灶如 Figure 1.3。

Table 1.4　試驗 1 及試驗 2 攻毒雞隻壞死性腸炎案例數及病灶分數

試驗	組別	飼料性誘發因子[1]	病灶分數					小計	壞死性腸炎案例數	病灶分數（Mean±SD）
			0	1	2	3	4			
1	CP1-T1	-	1	20	0	0	0	21	0	0.95±0.2
	CTL[2]-T1	-	12	9	0	0	0	21	0	0.43±0.49
2	CP1-T2	+	0	17	1	1	0	19	2	1.16 ± 0.50*
	CP1+*Eimeria*[2]-T2	+	0	14	0	4	1	19	5	1.58 ± 1.02**
	CTL[2]-T2	+	6	15	0	0	0	21	0	0.71±0.46

[1] *飼料性誘發因子：餵飼含魚粉及小麥之飼料。*
[2] *Eimeria：球蟲；CTL：對照組；Mean：平均值；SD：標準差。*
代表具有統計差異性（$p < 0.05$）；代表具有高度差異性（$p < 0.01$）。*

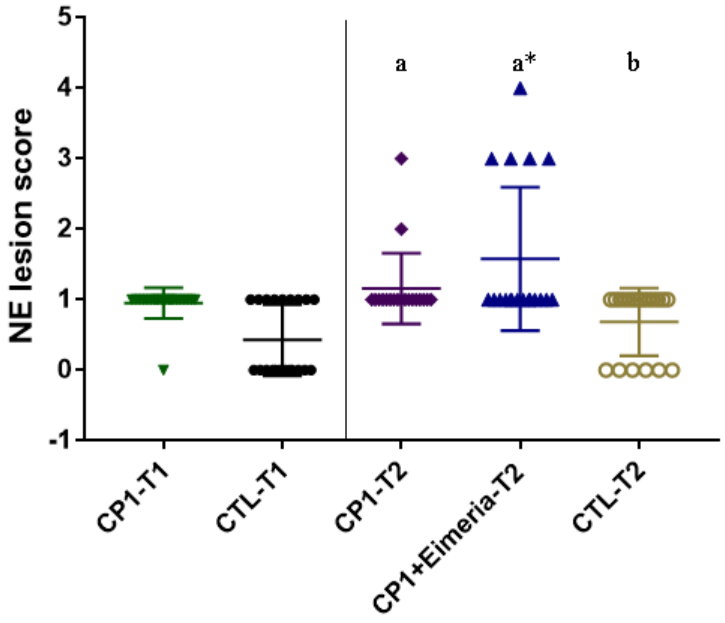

Figure 1.2　試驗 1 及試驗 2 各組病灶分數分布

此圖表資料呈現採取平均病灶分數 ± 標準差對應壞死性腸炎案例數。壞死性腸炎（NE）案例：病灶分數大於等於 2 者。不同英文字顯示試驗 2 各組相比具統計學上差異性（*Tukey's test*；$p < 0.05$）；星號（*）代表高度差異性（$p < 0.01$）。

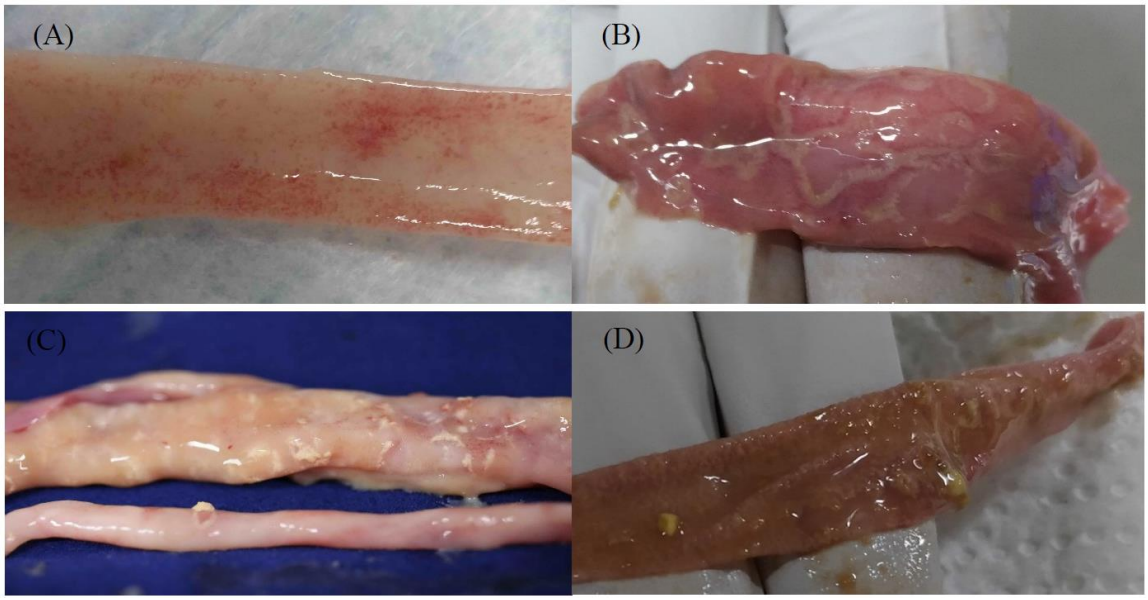

Figure 1.3　攻毒試驗所見壞死性腸炎肉眼病灶

（A）試驗 1；空腸黏膜充（鬱）血；病灶分數 1。（B）試驗 2：十二指腸黏膜多發性壞死灶；病灶分數 3。（C）試驗 2：空腸黏膜多發性壞死灶；病灶分數 3。（D）試驗 2：空腸黏膜廣泛性壞死病灶伴隨偽膜形成；病灶分數 4。

（三）討論

依過去研究結果顯示，產氣莢膜芽胞梭菌的菌株對於壞死性腸炎的誘發具有重要影響（Uzal et al., 2015），尤其是可以產生 NetB 毒素的菌株，一直被認為是造成雞隻壞死性腸炎的必要因子（Keyburn et al., 2008）。然而，這些研究鮮少提及他們所使用的攻毒試驗模式，有誘發因子的參與，例如：高蛋白飼料（Cooper and Songer, 2010; Keyburn et al., 2010; Timbermont et al., 2009）與/或球蟲等（Smyth and Martin, 2010; Timbermont et al., 2009），進而忽略其重要性而未討論。然而，田間雞隻壞死性腸炎疫情的發生，經調查常有餵飼不適當的飼料或與球蟲感染的情形（Broussard et al., 1986; Gazdzinski and Julian, 1992; Long, 1973; Porter, 1998），本項研究針對產

氣莢膜芽胞梭菌與誘發因子的交互作用進行研究，結果反映出單獨使用帶有 *netB* 基因的產氣莢膜芽胞梭菌確實無法產生壞死性腸炎，只有加入高蛋白飼料或先期感染球蟲後，併同致病菌株的給予，才會誘發疾病。而參與的誘發因子越多，壞死性腸炎的發生率及嚴重性顯著性地增加，此等結果顯示帶有 *netB* 基因的產氣莢膜芽胞梭菌以及誘發因子對雞隻壞死性腸炎的發展同等必須，也呼應過去研究的觀察結果，正常雞隻雖帶有 *netB* 基因的產氣莢膜芽胞梭菌卻不發病（Martin and Smyth, 2009），未帶有 *netB* 基因的產氣莢膜芽胞梭菌於誘發因子的共存下則可引發壞死性腸炎（Li et al., 2017）。

有無攜帶 *netB* 基因的產氣莢膜芽胞梭菌於誘發因子的參與下均可產生疾病的矛盾結果可歸因於基因檢測方法的敏感度，對於產氣莢膜芽胞梭菌中 *netB* 基因攜帶的有無，現今大部分研究均使用敏感度較低的 PCR 方式偵測後，進行研析及討論。然而，由第一部分研究結果可得知，*netB* 基因數須達 10^8，PCR 方可檢出，所以大部分攜帶少量 *netB* 基因數的菌株經 PCR 檢測會被判為 *netB* 基因陰性。而實際上，絕大部分產氣莢膜芽胞梭菌若以 qPCR 檢測，可見均帶有 *netB* 基因，因此，誘發因子的有無將主導著雞隻壞死性腸炎的發展與否，進一步地反證帶有 *netB* 基因的產氣莢膜芽胞梭菌以及誘發因子對雞隻壞死性腸炎的發展均為必須。

三、 以總體 16S rRNA 基因體學研究評估產氣莢膜芽胞梭菌與誘發因子單一及並存之腸道菌相與雞隻壞死性腸炎發展之關聯性

雞隻壞死性腸炎的發生除了產氣莢膜芽胞梭菌的存在及與誘發因子相互作用外，近來多項研究指出，腸道內的微生物菌叢因疾病所導致之失衡也會影響宿主免疫功能（Pan and Yu, 2014）及/或促進產氣莢膜芽胞梭菌的增殖與附著（Rehman et al., 2007），使雞隻容易感染產氣莢膜芽胞梭菌而發生疾病（Feng et al., 2010; Li et al., 2017b; Stanley et al., 2012b; Stanley et al., 2014）。此外，部分腸道菌相研究顯示，患有壞死性腸炎雞隻腸道內 *Escherichia-Shigella* 屬細菌有伴隨增殖的現象（Li et al., 2017b; Liu et al., 2010），推論該屬細菌可能參與雞隻壞死性腸炎的發展與致病，並建議進一步研究釐清（Moore, 2016; Prescott et al., 2016a）。為探討有否特定微生物或菌相參與致病機轉來誘發雞隻壞死性腸炎，本項研究針對產氣莢膜芽胞梭菌主要感染之空腸部位進行腸內菌相分析，評估腸道菌相與雞隻壞死性腸炎發展之關聯性及可能致病的關鍵菌叢。

（一）方法

依據第二部分研究結果，以帶有 *netB* 基因的產氣莢膜芽胞梭菌併同兩個誘發因子（高蛋白飼料及球蟲感染）成功誘發最多雞隻案例的方式作為壞死性腸炎陽性實驗組。本試驗（試驗 3）使用 50 隻 1 日齡雞隻，分為 5 組，每組均餵飼高蛋白飼料。分別為 A 組：產氣莢膜芽胞梭菌攻毒組（CP1-T3），B 組：產氣莢膜芽胞梭菌與球蟲混和感染組（CP1+*Eimeria*-T3），C 組：產氣莢膜芽胞梭菌與球蟲混和感染後使用月桂酸（lauric acid）防治組（CP1+*Eimeria*+Lauric acid-T3），D 組：單獨球

蟲感染組（*Eimeria*-T3）及 E 組：對照組（CTL-T3）。試驗期為 19 日，經球蟲處理者，於 10 日以商用球蟲疫苗進行口餵誘發球蟲症，而產氣莢膜芽胞梭菌處置者，於第 15 日時以濃度 $2.5x10^8$ CFU/ml 的 3 ml 菌液進行口餵攻毒，連續 4 天。於最後 1 日犧牲所有雞隻並執行剖檢，採取雞隻空腸及盲腸內容物進行腸道菌相分析，同時記錄壞死性腸炎案例數及病灶分數。

其後，每組選取 3 隻雞隻腸內容物萃取其內 DNA，樣本的選擇以壞死性腸炎案例（病灶分數大於等於 2 者）為優先，不足 3 隻的組別補入其餘病灶分數為 1 的雞隻，對照組則選用病灶分數為 0 分的雞隻腸內容物樣本。完成萃取的 DNA 以針對 16S rRNA V3-V4 區段的引子對增幅該區段序列產物，而後加入含有條碼連結序列的引子對製作序列文庫（Library），完成純化後送 MiSeq® System 產出總體 16S rRNA 基因序列資料。序列資料再經品質篩選（$Q \geq 20$）後，以軟體 UPARSE algorithm（Edgar, 2010）、RDP Classifier v2.11（Wang et al., 2007）、Qiime v1.9.1（Caporaso et al., 2010）及 R package v.3.3.1（http://www.R-project.org/）進行細菌操作分類單元 Operational taxonomic unit（OUT）歸類及分析，另使用 MetagenomeSeq、STAMP（Parks et al., 2014）及 LEfSe（Segata et al., 2011）等工具找出具有統計學上代表性的菌種。

（二）結果

如同第二部分研究結果，使用產氣莢膜芽胞梭菌併同給予高蛋白飼料及球蟲感染的方式成功地誘發最多壞死性腸炎案例，詳如 Table 1.5。

針對腸內容物總體 16S rRNA 序列分析結果，空腸內正常菌相以 *Lactobacillus* 屬（相對豐度佔 41.2%）及 *Clostridium sensu stricto 1* 屬（39.1%）為主，其次為無法分類菌屬（8.7%）、*Weissella*（3.6%）、 *Enterococcus* 屬（1.9%）、*Escherichia Shigella* 屬（1.8%）及 *Staphylococcus* 屬（1.6%）。 盲腸（cecum）內正常菌相以 *Bacteroides 屬*（相對豐度為 75.5%）為主，其次為無法分類菌屬（17.2%）, *Escherichia Shigella* 屬（3.1%）、*Eisenbergiella* 屬（1.7%）及 *Anaerotruncus* 屬（1.5%）（Figure 1.4）。

給予產氣莢膜芽胞梭菌會增加空腸內 *Clostridium sensu stricto 1*（54.75%）、*Escherichia Shigella*（9.57%）及 *Weissella*（4.99%）屬的相對豐度，但是顯著地減少 *Lactobacillus*（25.44%）屬的數量（Figure 1.5）。單獨球蟲感染顯著地增加空腸內 *Weissella*（16.01%）與 *Staphylococcus*（6.51%）屬的相對豐度，但減少 *Lactobacillus*（30.66%）與 *Clostridium sensu stricto 1*（27.69%）屬的細菌數量。產氣莢膜芽胞梭菌及球蟲混和感染所誘發的壞死性腸炎可見空腸內 *Clostridium sensu stricto 1*（71.89%）屬相對豐度顯著地增加（$p < 0.05$），*Escherichia Shigella*（4.68%）數量上升，但減少 *Lactobacillus*（16.99%）、*Weissella*（0.44%）及 *Staphylococcus*（0.40%）屬的相對豐度。比較不同組別之雞隻盲腸樣本則未見有相關菌相顯著性增加或減少。

Table 1.5　試驗 3 壞死性腸炎案例數及病灶分數

組別	處置	病灶分數分布					小計	平均病灶分數	NE[1] 案例數
		0	1	2	3	4			
A	CP1-T3	0	9	1	0	0	10	1.11 ± 0.31^{a2}	1
B	CP1+*Eimeria*-T3	0	8	0	1	1	10	$1.50 \pm 1.02^{a*2}$	2
C	CP1+*Eimeria*+LA[1]-T3	0	7	1	1	1	10	$1.60 \pm 1.02^{a*2}$	3
D	*Eimeria*-T3	-	-	-	-	-	10	-	-
E	CTL[1]-T3	5	4	0	0	0	9	0.44 ± 0.50^{b2}	0

[1]LA：月桂酸；NE：壞死性腸炎；CTL：對照組。

[2] 不同英文字顯示組別比較具有統計學上差異性（Tukey's test；$p < 0.05$）；星號（）代表具有高度差異性（$p < 0.01$）。*

對照組內有一隻雞隻因錯放組別而遭淘汰棄置，不列入研究範圍。

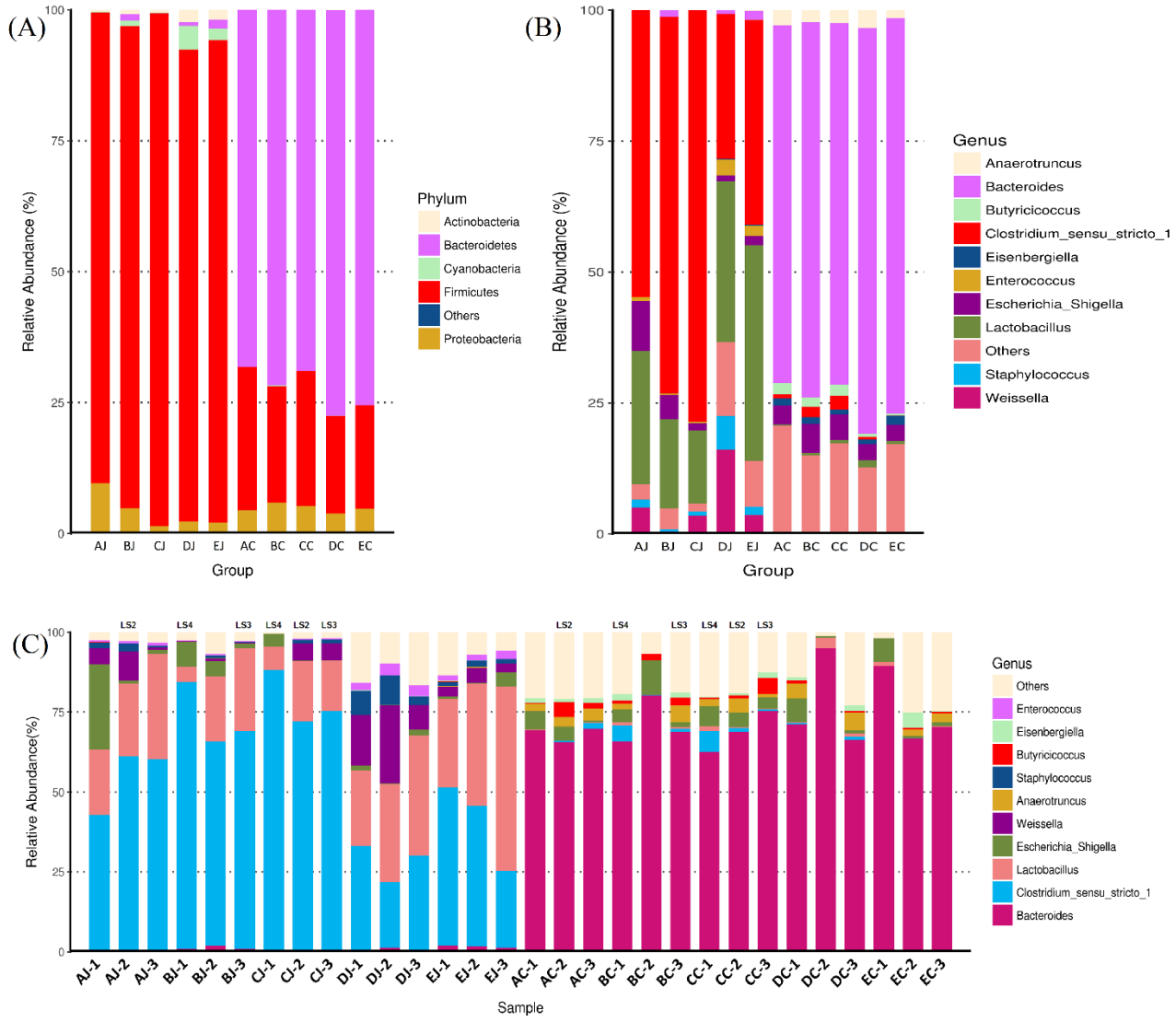

Figure 1.4　試驗 3 不同處置組別與雞隻之空腸及盲腸微生物菌相組成

（A）與（B）圖中每個直條代表每個組空腸或盲腸內的細菌組成，並顯示前五名相對豐度高的細菌種類。（A）、（B）圖內的細菌種類分別以菌門（Phylum）及菌屬（Genus）表示。（C）圖中每個直條代表每隻雞空腸或盲腸內的細菌組成，並以屬名顯示前 10 名相對豐度高的細菌種類。

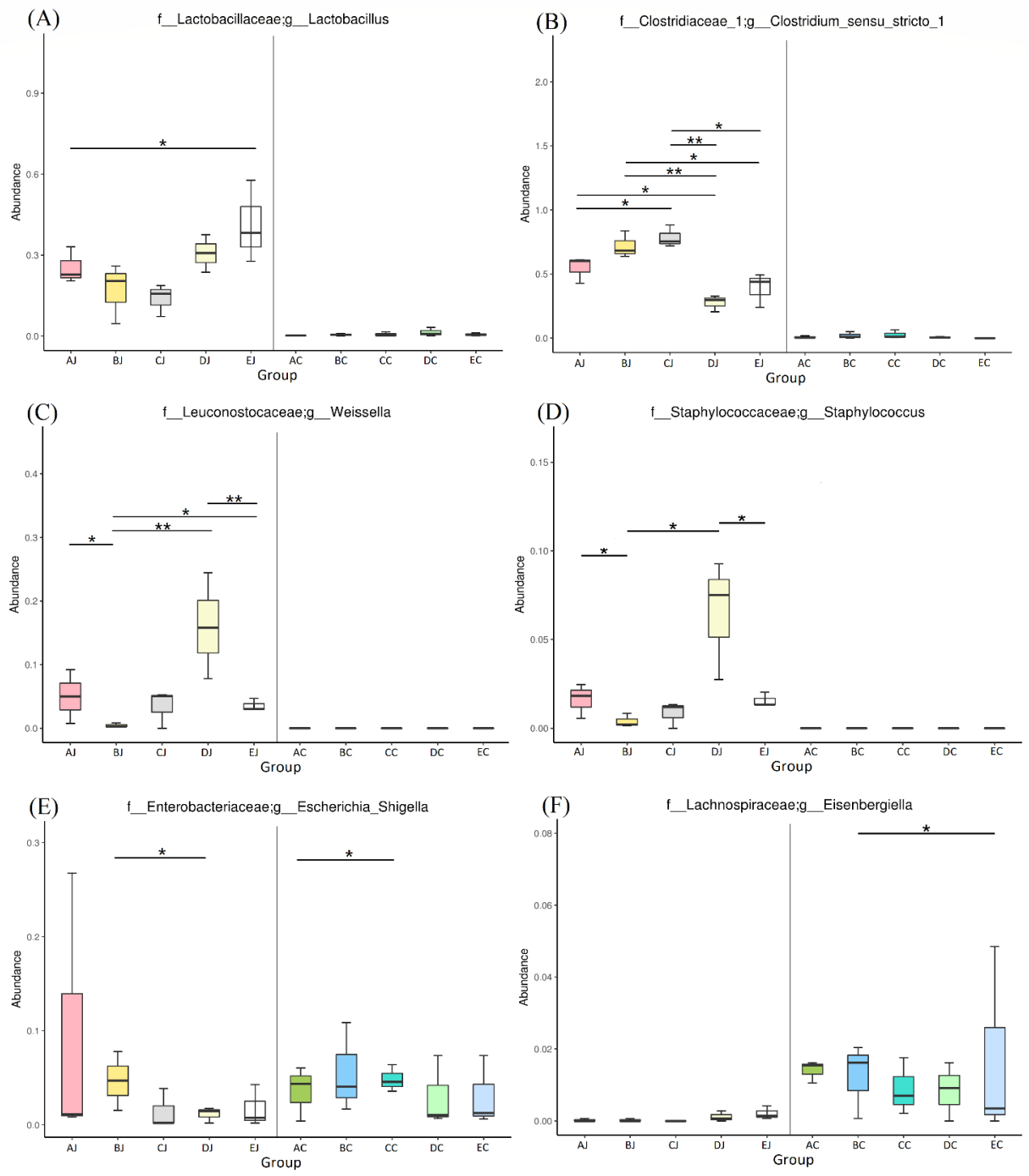

Figure 1.5　試驗 3 各組雞隻空腸及盲腸內菌屬豐度差異分析(以 metagenomeSeq 進行)

(A) *Lactobacillus* 屬；(B) *Clostridium sensu stricto 1* 屬；(C) *Weissella* 屬；(D) *Staphylococcus* 屬；(E) *Escherichia shigella* 屬；(F) *Eisenbergiella* 屬；* 表示 $p \leq 0.05$；** 表示 $p \leq 0.01$。

進一步分析壞死性腸炎的關鍵菌屬，發現以 *Clostridium sensu stricto 1* 屬細菌（其下含有產氣莢膜芽胞梭菌）為優勢菌種，病灶分數越高，相對豐度越高。例如：病灶分數 4 分雞隻腸內的 *Clostridium sensu stricto 1* 屬相對豐度高於 75%；2 分與 3 分雞隻相對豐度為 50-75%（Figure 1.4）。聚類熱圖分析（Heat map analysis）顯示，隨病灶分數的增加，壞死性腸炎案例空腸內 *Clostridium sensu stricto 1* 屬及產氣莢膜芽胞梭菌亦一致性地增加（Figure 1.6）。以主成分分析（Principal component analysis；PCA）結果，*Clostridium sensu stricto 1* 屬為壞死性腸炎案例群中主要貢獻菌屬（Figure 1.7）。另以 STAMP 分析，產氣莢膜芽胞梭菌及球蟲混和感染所誘發的壞死性腸炎，空腸內 *Clostridium sensu stricto 1* 屬與產氣莢膜芽胞梭菌均顯著性地增加（*Welch's t-test*；$p < 0.05$）；LEfSe 分析呈現與 STAMP 相同的結果，空腸內 *Clostridium sensu stricto 1* 屬與產氣莢膜芽胞梭菌均有意義地增加（Figure 1.8）

Figure 1.6　試驗 3 各組及其所含雞隻腸內容菌相聚類熱圖分析

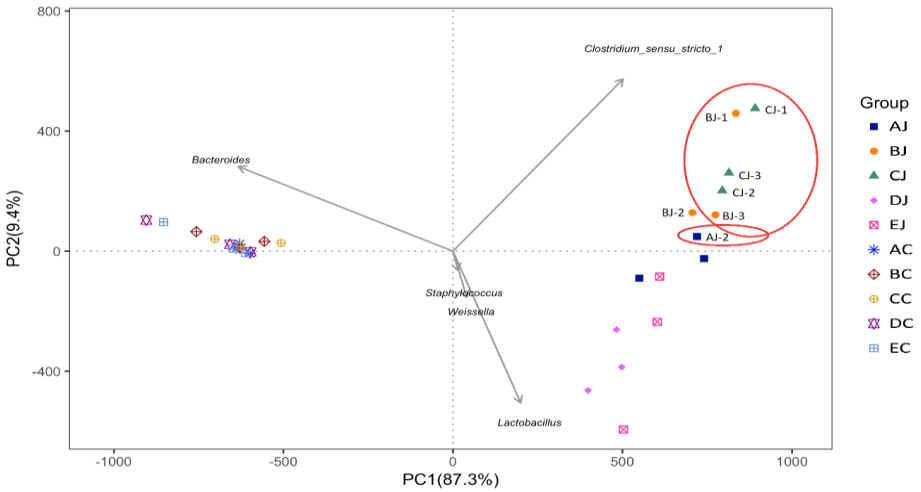

Figure 1.7　試驗 3 各組空腸及盲腸腸內菌相主成分分析（以階層分群法進行）

以階層分群法進行各組腸內菌相主成分分析（PCA with hierarchical clustering），空腸菌相集聚於右側象限，盲腸菌相集聚於左側象限。壞死性腸炎所呈現之腸道菌相聚集於右上區塊（紅色圈圈）呈現一致性，並以 *Clostridium sensu stricto 1* 屬為主要貢獻菌群（$p < 0.05$）。

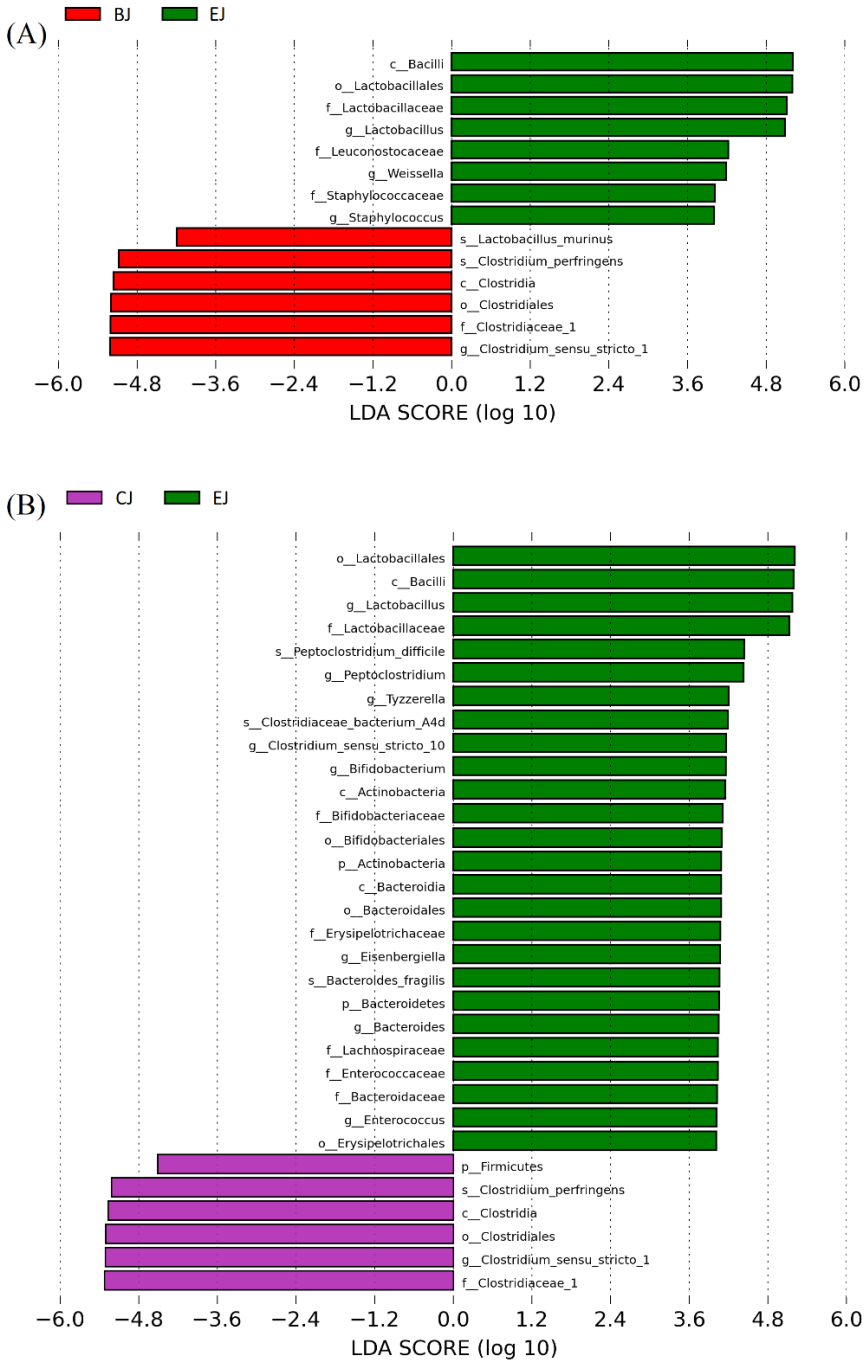

Figure 1.8　以 LEfSe 分析試驗 3 壞死性腸炎組及對照組空腸內差異菌群

圖（A）及（B）所示為兩組比較後具有統計學差異性的菌種（LDA 分數≥4），並列出其分類名。產氣莢膜芽胞梭菌單一感染或與球蟲混合感染雞隻空腸內 *Clostridium sensu stricto 1* 屬與產氣莢膜芽胞梭菌均顯著地增加。BJ：B 組雞隻空腸；CJ：C 組雞隻空腸；EJ：對照組雞隻空腸。

（三）討論

經比較壞死性腸炎陽性組（B 組）及對照組（E 組）雞隻空腸內菌相組成，壞死性腸炎組雞隻空腸內 *Clostridium sensu stricto 1* 屬菌顯著地增加（39.1%→71.89%），*Escherichia Shigella* 屬菌（1.8%→4.68%）數量亦上升，但 *Lactobacillus* 屬菌大量減少（41.2%→16.99%），*Weissella*（3.6%→0.44%）及 *Staphylococcus*（1.6%→0.40%）屬菌相對豐度減少。所有壞死性腸炎空腸內菌相一致性地呈現 *Clostridium sensu stricto 1* 屬菌顯著地增加，伴隨 *Lactobacillus* 屬菌大量減少。部分研究指出壞死性腸炎所伴隨 *Escherichia-Shigella* 屬細菌的增殖可能與該病的發展有關，然而研析使用月桂酸防治壞死性腸炎的 C 組，該組 3 雞隻均為壞死性腸炎案例，但其空腸內 *Escherichia Shigella* 屬菌的平均相對豐度，結果卻低於對照組（<1.8%）。此結果顯示，壞死性腸炎毋需伴隨 *Escherichia-Shigella* 屬細菌的增殖仍可產生，該屬細菌參與雞隻壞死性腸炎疾病發展的可能性低。

使用 MetagenomeSeq、PCA、STAMP 及 LEfSe 等各項統計分析空腸內代表性菌叢或菌相變化的結果，一致性地指出雞隻空腸內 *Clostridium sensu stricto 1* 屬細菌的過度增殖與壞死性腸炎的發生具有直接相關性（$p < 0.05$），而產氣莢膜芽胞梭菌原即屬於 *Clostridium sensu stricto 1* 屬細菌裡的一員，僅於 STAMP 與 LEfSe 分析中出現與壞死性腸炎的統計相關性，代表著除了產氣莢膜芽胞梭菌以外，*Clostridium sensu stricto 1* 屬內的其他細菌亦參與了疾病發展過程，此新發現助於相關研究進一步釐清 *Clostridium sensu stricto 1* 屬其他細菌成員於雞隻壞死性腸炎致病所扮演的角色。

此外，單以球蟲感染雞隻無法引發壞死性腸炎，反而致使感染雞隻空腸內 *Clostridium sensu stricto 1* 屬細菌數量比正常雞隻更少，說明腸內常在的 *Clostridium sensu stricto 1* 屬細菌或產氣莢膜芽胞梭菌無法透過球蟲感染而大量增殖，進而誘發疾病；但自外給予一定量 *Clostridium sensu stricto 1* 屬細菌（本研究給予產氣莢膜芽胞梭菌）混合球蟲感染後，卻可有效地誘發疾病，指出壞死性腸炎致病機轉需有外源性 *Clostridium sensu stricto 1* 屬細菌進入雞隻體內至一定量後，於空腸內產生該屬細菌的大量增殖，繼而發展雞隻壞死性腸炎。因此，該病的防治建議避免或減少接觸污染存有致病菌的物體、環境或罹病雞隻（糞便帶有高量病菌）來降低發病機率；另外，發展可以抑制小腸內 *Clostridium sensu stricto 1* 屬細菌或產氣莢膜芽胞梭菌大量增殖的方法，也可作為壞死性腸炎防治的有效策略之一。

月桂酸屬中鏈脂肪酸，具有很強的抑菌能力（Bertevello et al., 2012; Dierick et al., 2004; Zentek et al., 2012），曾有研究使用月桂酸防治壞死性腸炎，降低其發生率（Timbermont et al., 2010），因此，本研究嘗試應用月桂酸做為雞隻飼料添加物，評估其防治效果及分析其腸內菌相變化。結果顯示使用月桂酸並未減少壞死性腸炎的發生率及病灶嚴重程度，反而增加空腸內產氣莢膜芽胞梭菌的相對數量，說明月桂酸依本研究設計方式給予下，無法有效防治雞隻壞死性腸炎。

參、 心得

美國食品安全是由食品藥物管理局（Food and Drug Administration；FDA）及農業部（United States Department of Agriculture；USDA）分別透過聯邦食品藥物及化粧品管理法（Federal Food Drug and Cosmetic Act）、聯邦肉品檢查法（Federal Meat Inspection Act）、家禽及家禽產品檢查法（Poultry and Poultry Product Inspection Act）及蛋品檢查法（Egg Products Inspection Act）等法規連結相關檢查及行政措施，基本精神在於提供消費者購買到價金相符且安全無虞的食（產）品，透過管理「摻假（Adulteration）」跟「標示不實（Misbranding）」的行為，來確保前述基本精神。原則上，FDA 管理含肉率 2%以下的所有食品；USDA 管理肉品、家禽產品及蛋品（分工詳如圖 1）。兩個機關均有上市前驗證（Pre-market approvals）及市場端抽驗（Post-market surveillance）的措施及配套，FDA 會對原料來源進行審核，並著重於食品售後的監測，USDA 則主於生產屠宰端進行驗證，讓通過檢查者給予驗證樣章（如圖 2）後上市。對於有缺失者，亦均可對應相關規定給予限期立即改善、生產線停工至改善完畢、廠區停止運作、產品稽留（Suspension）及產品召回（Recall）等管理措施。為確保食品不會因摻假的問題影響消費者食安權益及公共衛生，FDA 每四年會定期發行 FDA 食品法典（FDA Food Code；如圖 3），提供食品零售或經銷相關體系一套執行食品安全及保護標準的參考，屬於自發性可依循的規定及建議。其內容包括食物中毒的案例評估數、人員及操作的管理、食物安全操作、烹煮冷藏及儲存建議時間、相關器具的管理、各種食品設施的定義、污水病媒等衛生管理措施、應注意的有毒物質與添加物等。

This table summarizes information concerning jurisdiction overlap for commercial products regulated by either or both FDA and USDA. It does not cover products made for on-site consumption such as pizza parlors, delicatessens, fast food sites, etc.

FDA JURISDICTION	USDA JURISDICTION		
21 USC 392(b) Meats and meat food products shall be exempt from the provisions of this Act to the extent of the application or the extension thereto of the Meat Inspection Act. FDA responsible for all non-specified red meats (bison, rabbits, game animals, zoo animals and all members of the deer family including elk (wapiti) and moose)). FDA responsible for all non-specified birds including wild turkeys, wild ducks, and wild geese.	The Federal Meat Inspection Act regulates the inspection of the following amenable species: cattle, sheep, swine, goats, horses, mules or other equines, including their carcasses and parts. It also covers any additional species of livestock that the Secretary of Agriculture considers appropriate. Mandatory Inspection of Ratites and Squab (including emu) announced by USDA/FSIS April 2001	The Poultry Products Inspection Act (PPIA) defines the term poultry as any domesticated bird. USDA has interpreted this to include domestic chickens, turkeys, ducks, geese and guineas. The Poultry Products Inspection Act states poultry and poultry products shall be exempt from the provisions of the FD&C Act to the extent they are covered by the PPIA. Mandatory Inspection of Ratites and Squab announced by USDA/FSIS April 2001	The Egg Products Inspection Act defines egg to mean the shell egg of domesticated chicken, turkey, duck, goose or guinea. Voluntary grading of shell eggs is done under USDA supervision. (FDA enforces labels/labeling of shell eggs.)
Products with 3% or less raw meat; less than 2% cooked meat or other portions of the carcass; or less than 30% fat, tallow or meat extract, alone or in combination. Products containing less than 2% cooked poultry meat; less than 10% cooked poultry skins, giblets, fat and poultry meat (limited to less than 2%) in any combination.* Closed-face sandwiches.	Products containing greater than 3% raw meat; 2% or more cooked meat or other portions of the carcass; or 30% or more fat, tallow or meat extract, alone or in combination.* Open-face sandwiches.	Products containing 2% or more cooked poultry; more than 10% cooked poultry skins, giblets, fat and poultry meat in any combination.*	Egg products processing plants (egg breaking and pasteurizing operations) are under USDA jurisdiction.
FDA is responsible for shell eggs and egg containing products that do not meet USDA's definition of "egg product." FDA also has jurisdiction in establishments not covered by USDA; e.g. restaurants, bakeries, cake mix plants, etc. Egg processing plants (egg washing, sorting, packing) are under FDA jurisdiction.			Products that meet USDA's definition of "egg product" are under USDA jurisdiction. The definition includes dried, frozen, or liquid eggs, with or without added ingredients, but mentions many exceptions. The following products, among others, are exempted as not being egg products: freeze-dried products, imitation egg products, egg substitutes, dietary foods, dried no-bake custard mixes, egg nog mixes, acidic dressings, noodles, milk and egg dip, cake mixes, French toast, sandwiches containing eggs or egg products, and balut and other similar ethnic delicacies. Products that do not fall under the definition, such as egg substitutes and cooked products, are under FDA jurisdiction.
Cheese pizza, onion and mushroom pizza, meat flavored spaghetti sauce (less than 3% red meat), meat flavored spaghetti sauce with mushrooms, (2% meat), pork and beans, sliced egg sandwich (closed-face), frozen fish dinner, rabbit stew, shrimp-flavored instant noodles, venison jerky, buffalo burgers, alligator nuggets, noodle soup chicken flavor	Pepperoni pizza, meat-lovers stuffed crust pizza, meat sauces (3% red meat or more), spaghetti sauce with meat balls, open-faced roast beef sandwich, hot dogs, corn dogs, beef/vegetable pot pie	Chicken sandwich (open face), chicken noodle soup	

Jurisdiction for products produced under the School Lunch Program, for military use, etc. is determined via the same algorithm although the purchases are made under strict specifications so that the burden of compliance falls on the contractor. Compliance Policy Guide 565.100, 567.200 and 567.300 provide additional examples of jurisdiction. IOM 3.2.1 and 2.7.1 provide more information on our interactions with USDA and Detention Authority.

圖 1、FDA 及 USDA 食品管理範圍一覽表

圖 2、USDA 食品安全檢查署 Food Safety and Inspection Service（FSIS）屠宰檢查合格章

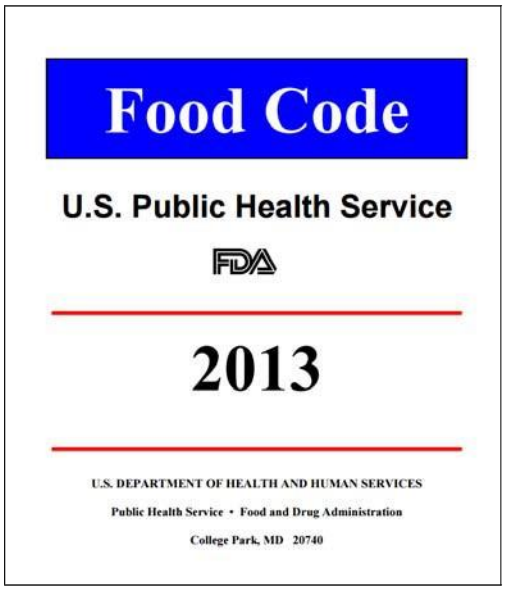

圖 3、FDA 食品法典

依美國法規管理體系的架構，維護食品安全係產業及業主本身應負責任，政府建立制度進行系統性管理，並輔導其瞭解可能的風險管控點，然後透過隨機監測與即時矯正等行政措施，降低食物中毒發生風險。另有通報調查機制，讓食安案件連結行政管理，一旦食安問題發生，政府會公布涉及產品及廠商以預警民眾，而美國廠商均會自發性召回產品以維商譽（若不自行召回，則由政府依法強制召回），民眾聚焦抵制是發生問題的食品及廠商，追究企業責任，鮮有問責政府。權責分明及合理的問責讓我對美國留下深刻印象，值得我們學習。

細菌性食物中毒係因污染病原菌或毒素的食品經人食用後，發病所導致，污染來源可能來自水源、空氣、土壤、生產端（如農場）、食品製程端（如食品加工廠）或供食用端（如餐廳），於加工過程或處理中造成污染。食物中毒事件發生時，美國是以摻假問題對涉案者進行處置，並針對召回的案例公布調查結果及可能污染的病原菌（可透過下列連結查詢：https://www.cdc.gov/foodsafety/outbreaks/index.html；https://www.fsis.usda.gov/wps/portal/fsis/topics/recalls-and-public-health-alerts/current-recalls-and-alerts），相關調查均以實證科學原則進行處理，並依據證據對外說明，不會擴大推論造成民眾臆測或恐慌，這是美國針對類案處理的重要原則，可以作為業務機關或單位執行相關業務的參考。

對於生產端食媒性疾病的預防，美國針對可以管理的部分，由美國農業部制訂輔導計畫與產業相互合作，由產業自主地強化生物安全及疾病管理，並配合定期監測後，由政府給予認證，使配合畜牧場間接提高收益；相關監測及認證由政府負擔，若監測過程中檢出疾病需淘汰所飼養家畜或家禽時，會與產業共同給予補助，降低農民損失，藉以提高畜牧場參加意願。以家禽場沙門氏桿菌預防部分為例，美國訂有國家家禽改善計畫（National Poultry Improvement Plan；NPIP），由家禽產業自主申請參加沙門氏桿菌監測及減除工作，對於監測場及清淨場發給認證標章（詳如圖 4），促進場內雞禽健康品質提升，禽隻生長效率增加，同時減少食品加工端可能遭受污染的機率，是防治食媒性疾病的良好推動策略。

圖 4、美國國家家禽改善計畫沙門氏桿菌監測與清淨標章

求學過程中，美國學制與台灣類似，一學年分為秋季（Fall）及春季（Spring）2 個學期，秋季學期由每年 8 月開始至 12 月，春季學期則為 1 月到 5 月，夏季有暑期課程（Summer Session）供學生自由選擇修課。美國博士學位是為了要培養研究學者和未來大學院校的教職成員所設立，因此，其要求畢業生應有所學領域厚實的基礎教育，並具邏輯性思考、解決問題及合理論述的能力，以符專業研究學者所需。美國畢業生必須修滿 60 個學分，包含高等課程、專題研討和論文撰寫，強調原創性的研究，並於指導教授監督下完成。密西西比州立大學依美國研究所教育規定，要求博士生通過 60 個學分修習始得畢業，其中 20 個為必修專業學分，包括研究所需專業課程、2 門統計學程及 3 門專題研討會（Seminar），專題研討會的目的在於獸醫學院師生均可參與的情形下，公開研究進展及問答。完成必修學分後，即可擬定博士研究計畫（Ph.D. Research Proposal），並邀集相關領域教授籌組審查評核委員小組，進行博士班資格考（Qualification exam）。這個考試為綜合性試驗，包含筆試與口試兩部分，筆試測試博士生專業領域知識程度，口試就考試學科進行廣泛及深入詢問，並挑戰所提博士研究計畫的合理性、邏輯性、原創性及應用價值，評量

受測學生是否能運用所學，並對所學領域有全面性的了解，以獨立及創意的方式來做研究。通過後，即獲得博士候選人（Ph.D. candidate）資格，專心衝刺於研究上。完成研究工作及畢業論文的撰寫後，可向審查評核委員小組提出畢業考試的要求，該考試係由博士候選人就論文內容進行公開簡報及答辯後，然後進入審查評核小組委員口試，審查論文內容。博士候選人於取得前述所需學分及通過畢業考試後，即獲得博士學位。本人參與進修的實驗室秉持大多數美國研究室的原則，須有 3 個主題研究並產出可供發表期刊論文的內容後，才能提交畢業考試的申請。在美國攻讀博士過程雖然辛苦，但基礎教育訓練紮實，不怕先天技不如人，只要肯學，整體環境會幫助進修人員慢慢地將所需知能培養起來，讓非科班出身的博士生也能逐步地厚實科學研究技能，體驗實證科學的魅力，成為一個科學人。

本研究透過收集分析一定數量的田間分離菌株中腸毒素基因的表現情形探討雞源性產氣莢膜芽胞梭菌與公共衛生的關連性，未見有致人類食物中毒的公共衛生風險。另成功地建立雞隻壞死性腸炎疾病發展模式，並透過實驗設計、總體基因體學及進階統計分析釐出疾病發展的重要因子、主要菌相及參與菌原，結果交互證明雞隻接觸一定量 *Clostridium sensu stricto 1* 屬細菌及誘發因子後於空腸內過度增殖是壞死性腸炎發生的關鍵，此結果可作為防治策略發展應用之參考，並使用本研究已建立的疾病發展模式驗證其有效性。

肆、 建議

食物於種植或飼養、採收儲存或上市、運送、製造加工、包裝烹調及盛裝等過程中都有可能遭受汙染，風險管控點眾多不易防堵，美國主要透過 FDA、USDA 及 CDC 等機關相互合作，執行上市前驗證及市場端抽驗的措施及配套，共同把關並調查感染來源，但每年仍有多起食物中毒案例，顯示食媒性病原於環境或宿主常在的特性使其不易防治，建議可考量美國近年作法，透過科技研發找出可應用的飼料添加物，日常給予產食動物，降低其腸道內食媒性病原數量，減少環境或後端可能污染的發生。此外，美國食物中毒案例經調查後，多與食物（材）經不當製程或衛生操作污染有關，應持續加強相關業者及消費者宣導，以及安全操作的輔導工作。

美國於學術研究領域具有很多重量級的專家學者，分別帶領著不同領域（如食品安全、流行病學、致病機轉等）的學術發展及專才訓練。於美國進修期間發現，各校（尤其是名校）時常邀請此類專家學者進行專題演講，分享其專業知能，並安排半日或以上時間面對面進行問答及研究指導，令參加人員獲益良多。為擴大此類效益，建議可應用培訓人員於進修國家所建立之專家人脈資源，計畫性地邀請專家學者來台進行專業知識分享或業務指導，一方面提高我國國際能見度，另一方面培育國內人才擴展國際視野，並輔助相關業務與國際接軌。另外，美國學制及教育體系著重基礎教育的養成，按步就班地培育人才，惟必修學分的修習需至少 2 年，不容易於三年期間完成研究及論文評核，取得博士學位，未來若有培育美系人才的需求，建議可研議延長赴該國修業補助年限的可行性。

伍、 參考文獻

Abildgaard, L., Sondergaard, T.E., Engberg, R.M., Schramm, A., Hojberg, O., 2010. In vitro production of necrotic enteritis toxin B, NetB, by *netB*-positive and *netB*-negative *Clostridium perfringens* originating from healthy and diseased broiler chickens. Veterinary Microbiology 144, 231-235.

Alimolaei, M., Golchin, M., Abshenas, J., Ezatkhah, M., Bafti, M.S., 2018. A Recombinant Probiotic, Lactobacillus casei, Expressing the *Clostridium perfringens* alpha-toxoid, as an Orally Vaccine Candidate Against Gas Gangrene and Necrotic Enteritis. Probiotics Antimicrob Proteins 10, 251-257.

Allaart, J.G., de Bruijn, N.D., van Asten, A.J., Fabri, T.H., Grone, A., 2012. NetB-producing and beta2-producing *Clostridium perfringens* associated with subclinical necrotic enteritis in laying hens in the Netherlands. Avian Pathology 41, 541-546.

Annett, C.B., Viste, J.R., Chirino-Trejo, M., Classen, H.L., Middleton, D.M., Simko, E., 2002. Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of *Clostridium perfringens* type A. Avian Pathology 31, 598-601.

Antonissen, G., Croubels, S., Pasmans, F., Ducatelle, R., Eeckhaut, V., Devreese, M., Verlinden, M., Haesebrouck, F., Eeckhout, M., De Saeger, S., Antlinger, B., Novak, B., Martel, A., Van Immerseel, F., 2015. Fumonisins affect the intestinal microbial homeostasis in broiler chickens, predisposing to necrotic enteritis. Veterinary Research 46, 98.

Antonissen, G., Van Immerseel, F., Pasmans, F., Ducatelle, R., Haesebrouck, F., Timbermont, L., Verlinden, M., Janssens, G.P., Eeckhaut, V., Eeckhout, M., De Saeger, S., Hessenberger, S., Martel, A., Croubels, S., 2014. The mycotoxin deoxynivalenol predisposes for the development of *Clostridium perfringens*-induced necrotic enteritis in broiler chickens. PloS One 9, e108775.

Awad, M.M., Bryant, A.E., Stevens, D.L., Rood, J.I., 1995. Virulence studies on chromosomal alpha-toxin and theta-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of alpha-toxin in *Clostridium perfringens*-mediated gas gangrene. Molecular Microbiology 15, 191-202.

Bailey, M.A., Macklin, K.S., Krehling, J.T., 2015. Low Prevalence of *netB* and *tpeL* in Historical *Clostridium perfringens* Isolates from Broiler Farms in Alabama. Avian Diseases 59, 46-51.

Barekatain, M.R., Antipatis, C., Rodgers, N., Walkden-Brown, S.W., Iji, P.A., Choct, M., 2013. Evaluation of high dietary inclusion of distillers dried grains with solubles and supplementation of protease and xylanase in the diets of broiler chickens

under necrotic enteritis challenge. Poultry Science 92, 1579-1594.

Batz, M.B., Hoffmann, S., Morris, J.G., Jr., 2012. Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. Journal of Food Protection 75, 1278-1291.

Beckmann, L., Simon, O., Vahjen, W., 2006. Isolation and identification of mixed linked beta -glucan degrading bacteria in the intestine of broiler chickens and partial characterization of respective 1,3-1,4-beta -glucanase activities. Journal of Basic Microbiology 46, 175-185.

Brennan, J., Bagg, R., Barnum, D., Wilson, J., Dick, P., 2001a. Efficacy of narasin in the prevention of necrotic enteritis in broiler chickens. Avian Diseases 45, 210-214.

Brennan, J., Moore, G., Poe, S.E., Zimmermann, A., Vessie, G., Barnum, D.A., Wilson, J., 2001b. Efficacy of in-feed tylosin phosphate for the treatment of necrotic enteritis in broiler chickens. Poultry Science 80, 1451-1454.

Caly, D.L., D'Inca, R., Auclair, E., Drider, D., 2015. Alternatives to Antibiotics to Prevent Necrotic Enteritis in Broiler Chickens: A Microbiologist's Perspective. Frontiers in Microbiology 6, 1336.

Candan, F., Unlu, M., Tepe, B., Daferera, D., Polissiou, M., Sokmen, A., Akpulat, H.A., 2003. Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium subsp. millefolium Afan*.（Asteraceae）. Journal of Ethnopharmacology 87, 215-220.

Carman, R.J., 1997. *Clostridium perfringens* in spontaneous and antibiotic-associated diarrhoea of man and other animals. Reviews in Medical Microbiology 8, S46.

Casewell, M., Friis, C., Marco, E., McMullin, P., Phillips, I., 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. Journal of Antimicrobial Chemotherapy 52, 159-161.

Centers for Disease Control and Prevention, 2017. Prevent Illness from C. perfringens. Retrieved from https://www.cdc.gov/features/clostridiumperfringens/index.html.

Chalmers, G., Bruce, H.L., Hunter, D.B., Parreira, V.R., Kulkarni, R.R., Jiang, Y.F., Prescott, J.F., Boerlin, P., 2008. Multilocus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. Journal of Clinical Microbiology 46, 3957-3964.

Chambers, J., Gong, J., 2011. The intestinal microbiota and its modulation for *Salmonella* control in chickens, Vol 44, 3149-3159 pp.

Chaveerach, P., Keuzenkamp, D.A., Lipman, L.J., Van Knapen, F., 2004. Effect of organic acids in drinking water for young broilers on *Campylobacter* infection, volatile fatty acid production, gut microflora and histological cell changes. Poultry Science 83, 330-334.

Chen, J., McClane, B.A., 2015. Characterization of *Clostridium perfringens* TpeL toxin gene carriage, production, cytotoxic contributions, and trypsin sensitivity. Infection and Immunity 83, 2369-2381.

Choct, M., Annison, G., 2007. The inhibition of nutrient digestion by wheat pentosans. British Journal of Nutrition 67, 123.

Choct, M., Hughes, R.J., Bedford, M.R., 1999. Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. British Poultry Science 40, 419-422.

Clavijo, V., Florez, M.J.V., 2018. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. Poultry Science 97, 1006-1021.

Collado, M.C., Gueimonde, M., Hernandez, M., Sanz, Y., Salminen, S., 2005. Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. Journal of Food Protection 68, 2672-2678.

Collier, C.T., Hofacre, C.L., Payne, A.M., Anderson, D.B., Kaiser, P., Mackie, R.I., Gaskins, H.R., 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Veterinary Immunology and Immunopathology 122, 104-115.

Cooper, K.K., Songer, J.G., 2009. Necrotic enteritis in chickens: a paradigm of enteric infection by *Clostridium perfringens* type A. Anaerobe 15, 55-60.

Cooper, K.K., Songer, J.G., Uzal, F.A., 2013. Diagnosing clostridial enteric disease in poultry. Journal of Veterinary Diagnostic Investigation 25, 314-327.

Cooper, K.K., Songer, J.G., Uzal, F.A., Songer, J.G., Prescott, J.F., Popoff, M.R. 2016. Necrotic Enteritis of Poultry, In: Clostridial Diseases of Animals. John Wiley & Sons, Inc, 123-137.

Coursodon, C.F., Glock, R.D., Moore, K.L., Cooper, K.K., Songer, J.G., 2012. TpeL-producing strains of *Clostridium perfringens* type A are highly virulent for broiler chicks. Anaerobe 18, 117-121.

Craven, S.E., 2000. Colonization of the intestinal tract by *Clostridium perfringens* and

fecal shedding in diet-stressed and unstressed broiler chickens. Poultry Science 79, 843-849.

Crespo, R., Fisher, D.J., Shivaprasad, H.L., Fernandez-Miyakawa, M.E., Uzal, F.A., 2007. Toxinotypes of *Clostridium perfringens* isolated from sick and healthy avian species. Journal of Veterinary Diagnostic Investigation 19, 329-333.

Crhanova, M., Hradecka, H., Faldynova, M., Matulova, M., Havlickova, H., Sisak, F., Rychlik, I., 2011. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica serovar enteritidis* infection. Infection and Immunity 79, 2755-2763.

Cross, D.E., McDevitt, R.M., Hillman, K., Acamovic, T., 2007. The effect of herbs and their associated essential oils on performance, dietary digestibility and gut microflora in chickens from 7 to 28 days of age. British Poultry Science 48, 496-506.

Dahiya, J.P., Wilkie, D.C., Van Kessel, A.G., Drew, M.D., 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Animal Feed Science and Technology 129, 60-88.

Daube, G., Simon, P., Limbourg, B., Manteca, C., Mainil, J., Kaeckenbeeck, A., 1996. Hybridization of 2,659 *Clostridium perfringens* isolates with gene probes for seven toxins （alpha, beta, epsilon, iota, theta, mu, and enterotoxin） and for sialidase. American Journal of Veterinary Research 57, 496-501.

Diaz-Sanchez, S., D'Souza, D., Biswas, D., Hanning, I., 2015. Botanical alternatives to antibiotics for use in organic poultry production. Poultry Science 94, 1419-1430.

Dorman, H.J., Deans, S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88, 308-316.

Drew, M.D., Syed, N.A., Goldade, B.G., Laarveld, B., Van Kessel, A.G., 2004. Effects of dietary protein source and level on intestinal populations of *Clostridium perfringens* in broiler chickens. Poultry Science 83, 414-420.

Forder, R.E., Howarth, G.S., Tivey, D.R., Hughes, R.J., 2007. Bacterial modulation of small intestinal goblet cells and mucin composition during early posthatch development of poultry. Poultry Science 86, 2396-2403.

Freedman, J.C., Shrestha, A., McClane, B.A., 2016. *Clostridium perfringens* Enterotoxin: Action, Genetics, and Translational Applications. Toxins 8.

Freedman, J.C., Theoret, J.R., Wisniewski, J.A., Uzal, F.A., Rood, J.I., McClane, B.A., 2015. *Clostridium perfringens* type A-E toxin plasmids. Research in

Microbiology 166, 264-279.

Gabriel, I., Lessire, M., Mallet, S., Guillot, J.F., 2006. Microflora of the digestive tract: critical factors and consequences for poultry. World's Poultry Science Journal 62, 499-511.

Gaucher, M.L., Quessy, S., Letellier, A., Arsenault, J., Boulianne, M., 2015. Impact of a drug-free program on broiler chicken growth performances, gut health, *Clostridium perfringens* and *Campylobacter jejuni* occurrences at the farm level. Poultry Science 94, 1791-1801.

Gholamiandehkordi, A.R., Timbermont, L., Lanckriet, A., Van Den Broeck, W., Pedersen, K., Dewulf, J., Pasmans, F., Haesebrouck, F., Ducatelle, R., Van Immerseel, F., 2007. Quantification of gut lesions in a subclinical necrotic enteritis model. Avian Pathology 36, 375-382.

Gholamiandekhordi, A.R., Ducatelle, R., Heyndrickx, M., Haesebrouck, F., Van Immerseel, F., 2006. Molecular and phenotypical characterization of *Clostridium perfringens* isolates from poultry flocks with different disease status. Veterinary Microbiology 113, 143-152.

Giannenas, I., Florou-Paneri, P., Papazahariadou, M., Christaki, E., Botsoglou, N.A., Spais, A.B., 2003. Effect of dietary supplementation with oregano essential oil on performance of broilers after experimental infection with *Eimeria tenella*. Archiv für Tierernahrung 57, 99-106.

Gibert, M., Jolivet-Reynaud, C., Popoff, M.R., 1997. Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. Gene 203, 65-73.

Gibson, G.R., Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. Journal of Nutrition 125, 1401-1412.

Gong, J., Forster, R.J., Yu, H., Chambers, J.R., Sabour, P.M., Wheatcroft, R., Chen, S., 2002. Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen. FEMS Microbiology Letters 208, 1-7.

Gong, J., Si, W., Forster, R.J., Huang, R., Yu, H., Yin, Y., Yang, C., Han, Y., 2007. 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. FEMS Microbiology Ecology 59, 147-157.

Gonzalez-Gil, F., Diaz-Sanchez, S., Pendleton, S., Andino, A., Zhang, N., Yard, C., Crilly, N., Harte, F., Hanning, I., 2014. Yerba mate enhances probiotic bacteria growth in

vitro but as a feed additive does not reduce *Salmonella Enteritidis* colonization in vivo. Poultry Science 93, 434-440.

Grant, K.A., Kenyon, S., Nwafor, I., Plowman, J., Ohai, C., Halford-Maw, R., Peck, M.W., McLauchlin, J., 2008. The identification and characterization of *Clostridium perfringens* by real-time PCR, location of enterotoxin gene, and heat resistance. Foodborne Pathogens and Disease 5, 629-639.

Grass, J.E., Gould, L.H., Mahon, B.E., 2013. Epidemiology of foodborne disease outbreaks caused by *Clostridium perfringens*, United States, 1998-2010. Foodborne Pathogens and Disease 10, 131-136.

Guo, S., Liu, D., Zhao, X., Li, C., Guo, Y., 2014. Xylanase supplementation of a wheat-based diet improved nutrient digestion and mRNA expression of intestinal nutrient transporters in broiler chickens infected with *Clostridium perfringens*. Poultry Science 93, 94-103.

Hamdy, A.H., Thomas, R.W., Kratzer, D.D., Davis, R.B., 1983. Lincomycin dose response for treatment of necrotic enteritis in broilers. Poultry Science 62, 585-588.

Hassan, H.M.A., Mohamed, M.A., Youssef, A.W., Hassan, E.R., 2010. Effect of Using Organic Acids to Substitute Antibiotic Growth Promoters on Performance and Intestinal Microflora of Broilers. Asian-Australas J Anim Sci 23, 1348-1353.

Hermans, D., Martel, A., Garmyn, A., Verlinden, M., Heyndrickx, M., Gantois, I., Haesebrouck, F., Pasmans, F., 2012. Application of medium-chain fatty acids in drinking water increases *Campylobacter jejuni* colonization threshold in broiler chicks. Poultry Science 91, 1733-1738.

Hermans, P.G., Morgan, K.L., 2007. Prevalence and associated risk factors of necrotic enteritis on broiler farms in the United Kingdom; a cross-sectional survey. Avian Pathology 36, 43-51.

Hoerr, F.J., 2010. Clinical aspects of immunosuppression in poultry. Avian Diseases 54, 2-15.

Hook, D., Jalaludin, B., Fitzsimmons, G., 1996. *Clostridium perfringens* food-borne outbreak: an epidemiological investigation. Australian and New Zealand Journal of Public Health 20, 119-122.

Izat, A.L., Tidwell, N.M., Thomas, R.A., Reiber, M.A., Adams, M.H., Colberg, M., Waldroup, P.W., 1990. Effects of a buffered propionic acid in diets on the performance of broiler chickens and on microflora of the intestine and carcass. Poultry Science 69, 818-826.

Jang, S.I., Lillehoj, H.S., Lee, S.H., Lee, K.W., Lillehoj, E.P., Hong, Y.H., An, D.J., Jeoung, D.H., Chun, J.E., 2013. Relative disease susceptibility and clostridial toxin antibody responses in three commercial broiler lines coinfected with *Clostridium perfringens* and *Eimeria maxima* using an experimental model of necrotic enteritis. Avian Diseases 57, 684-687.

Jerzsele, A., Szeker, K., Csizinszky, R., Gere, E., Jakab, C., Mallo, J.J., Galfi, P., 2012. Efficacy of protected sodium butyrate, a protected blend of essential oils, their combination, and Bacillus amyloliquefaciens spore suspension against artificially induced necrotic enteritis in broilers. Poultry Science 91, 837-843.

Jiang, Y., Mo, H., Willingham, C., Wang, S., Park, J.Y., Kong, W., Roland, K.L., Curtiss, R., 3rd, 2015. Protection Against Necrotic Enteritis in Broiler Chickens by Regulated Delayed Lysis *Salmonella* Vaccines. Avian Diseases 59, 475-485.

Juneja, V.K., Friedman, M., 2007. Carvacrol, cinnamaldehyde, oregano oil, and thymol inhibit *Clostridium perfringens* spore germination and outgrowth in ground turkey during chilling. Journal of Food Protection 70, 218-222.

Keto-Timonen, R., Heikinheimo, A., Eerola, E., Korkeala, H., 2006. Identification of *Clostridium* species and DNA fingerprinting of *Clostridium perfringens* by amplified fragment length polymorphism analysis. Journal of Clinical Microbiology 44, 4057-4065.

Keyburn, A.L., Boyce, J.D., Vaz, P., Bannam, T.L., Ford, M.E., Parker, D., Di Rubbo, A., Rood, J.I., Moore, R.J., 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathogens 4, e26.

Keyburn, A.L., Sheedy, S.A., Ford, M.E., Williamson, M.M., Awad, M.M., Rood, J.I., Moore, R.J., 2006. Alpha-toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens. Infection and Immunity 74, 6496-6500.

Keyburn, A.L., Yan, X.X., Bannam, T.L., Van Immerseel, F., Rood, J.I., Moore, R.J., 2010. Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing NetB toxin. Veterinary Research 41, 21.

Kien, C.L., Blauwiekel, R., Bunn, J.Y., Jetton, T.L., Frankel, W.L., Holst, J.J., 2007. Cecal infusion of butyrate increases intestinal cell proliferation in piglets. Journal of Nutrition 137, 916-922.

Kim, D.K., Lillehoj, H.S., Jang, S.I., Lee, S.H., Hong, Y.H., Cheng, H.H., 2014. Transcriptional profiles of host-pathogen responses to necrotic enteritis and differential regulation of immune genes in two inbreed chicken lines showing disparate disease susceptibility. PloS One 9, e114960.

Kim, G.B., Seo, Y.M., Kim, C.H., Paik, I.K., 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poultry Science 90, 75-82.

Kleessen, B., Hartmann, L., Blaut, M., 2003. Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. British Journal of Nutrition 89, 597-606.

Kum, S., Eren, U., Onol, A.G., Sandikci, M., 2010. Effects of dietary organic acid supplementation on the intestinal mucosa in broilers, Vol 161, 463-468 pp.

L. Hofacre, C., Beacorn, T., Collett, S., Mathis, G., 2003. Using Competitive Exclusion, Mannan-Oligosaccharide and Other Intestinal Products to Control Necrotic Enteritis, Vol 12.

La Ragione, R.M., Woodward, M.J., 2003. Competitive exclusion by Bacillus subtilis spores of *Salmonella enterica serotype Enteritidis* and *Clostridium perfringens* in young chickens. Veterinary Microbiology 94, 245-256.

Langhout, D.J., Schutte, J.B., Van Leeuwen, P., Wiebenga, J., Tamminga, S., 1999. Effect of dietary high- and low-methylated citrus pectin on the activity of the ileal microflora and morphology of the small intestinal wall of broiler chicks. British Poultry Science 40, 340-347.

Lee, K.W., Lillehoj, H.S., Jeong, W., Jeoung, H.Y., An, D.J., 2011. Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development. Poultry Science 90, 1381-1390.

Lepp, D., Roxas, B., Parreira, V.R., Marri, P.R., Rosey, E.L., Gong, J., Songer, J.G., Vedantam, G., Prescott, J.F., 2010. Identification of novel pathogenicity loci in *Clostridium perfringens* strains that cause avian necrotic enteritis. PloS One 5, e10795.

Li, G.Q., Kanu, S., Xiang, F.Y., Xiao, S.M., Zhang, L., Chen, H.W., Ye, H.J., 2004. Isolation and selection of ionophore-tolerant *Eimeria precocious* lines: *E. tenella*, *E. maxima* and *E. acervulina*. Veterinary Parasitology 119, 261-276.

Li, J., Adams, V., Bannam, T.L., Miyamoto, K., Garcia, J.P., Uzal, F.A., Rood, J.I., McClane, B.A., 2013. Toxin plasmids of *Clostridium perfringens*. Microbiology and Molecular Biology Reviews 77, 208-233.

Li, J., McClane, B.A., 2014. Contributions of NanI sialidase to Caco-2 cell adherence by *Clostridium perfringens* type A and C strains causing human intestinal disease. Infection and Immunity 82, 4620-4630.

Li, J., Sayeed, S., Robertson, S., Chen, J., McClane, B.A., 2011. Sialidases affect the host cell adherence and epsilon toxin-induced cytotoxicity of *Clostridium perfringens* type D strain CN3718. PLoS Pathogens 7, e1002429.

Lillehoj, H.S., Jang, S.I., Panebra, A., Lillehoj, E.P., Dupuis, L., Ben Arous, J., Lee, S.K., Oh, S.T., 2017. In ovo vaccination using *Eimeria* profilin and *Clostridium perfringens* NetB proteins in Montanide IMS adjuvant increases protective immunity against experimentally-induced necrotic enteritis. Asian-Australas J Anim Sci 30, 1478-1485.

Liu, D., Guo, S., Guo, Y., 2012. Xylanase supplementation to a wheat-based diet alleviated the intestinal mucosal barrier impairment of broiler chickens challenged by *Clostridium perfringens*. Avian Pathology 41, 291-298.

Liu, D., Guo, Y., Wang, Z., Yuan, J., 2010. Exogenous lysozyme influences *Clostridium perfringens* colonization and intestinal barrier function in broiler chickens. Avian Pathology 39, 17-24.

Llanco, L.A., Nakano, V., Avila-Campos, M.J., 2015. Sialidase production and genetic diversity in *Clostridium perfringens* type A isolated from chicken with necrotic enteritis in Brazil. Current Microbiology 70, 330-337.

Marshall, B.M., Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. Clinical Microbiology Reviews 24, 718-733.

Martin, T.G., Smyth, J.A., 2009. Prevalence of *netB* among some clinical isolates of *Clostridium perfringens* from animals in the United States. Veterinary Microbiology 136, 202-205.

McClane, B.A., 2007. *Clostridium perfringens*. In: Doyle, M.P. and Beuchat, L.R.（Eds.）, Food Microbiology: Fundamentals and Frontiers. Washington, DC: ASM Press; p. 423-444.

McDevitt, R.M., Brooker, J.D., Acamovic, T., Sparks, N.H.C., 2007. Necrotic enteritis; a continuing challenge for the poultry industry. World's Poultry Science Journal 62, 221-247.

Meimandipour, A., Shuhaimi, M., Hair-Bejo, M., Azhar, K., Kabeir, B.M., Rasti, B., Yazid, A.M., 2009. In vitro fermentation of broiler cecal content: the role of lactobacilli and pH value on the composition of microbiota and end products fermentation. Letters in Applied Microbiology 49, 415-420.

Mitsch, P., Zitterl-Eglseer, K., Kohler, B., Gabler, C., Losa, R., Zimpernik, I., 2004. The effect of two different blends of essential oil components on the proliferation of

Clostridium perfringens in the intestines of broiler chickens. Poultry Science 83, 669-675.

Mohamed, M., S Abdel Hafez, M., 2011. The Effect of a Mannanoligosaccharides （Biomos ） on ® Necrotic Enteritis Infection in Broiler Chickens, Vol 10.

Moore, R.J., 2016. Necrotic enteritis predisposing factors in broiler chickens. Avian Pathology 45, 275-281.

Mot, D., Timbermont, L., Haesebrouck, F., Ducatelle, R., Van Immerseel, F., 2014. Progress and problems in vaccination against necrotic enteritis in broiler chickens. Avian Pathology 43, 290-300.

Murugesan, G.R., Ledoux, D.R., Naehrer, K., Berthiller, F., Applegate, T.J., Grenier, B., Phillips, T.D., Schatzmayr, G., 2015. Prevalence and effects of mycotoxins on poultry health and performance, and recent development in mycotoxin counteracting strategies. Poultry Science 94, 1298-1315.

Mwangi, W.N., Beal, R.K., Powers, C., Wu, X., Humphrey, T., Watson, M., Bailey, M., Friedman, A., Smith, A.L., 2010. Regional and global changes in TCRalphabeta T cell repertoires in the gut are dependent upon the complexity of the enteric microflora. Developmental & Comparative Immunology 34, 406-417.

Myers, G.S., Rasko, D.A., Cheung, J.K., Ravel, J., Seshadri, R., DeBoy, R.T., Ren, Q., Varga, J., Awad, M.M., Brinkac, L.M., Daugherty, S.C., Haft, D.H., Dodson, R.J., Madupu, R., Nelson, W.C., Rosovitz, M.J., Sullivan, S.A., Khouri, H., Dimitrov, G.I., Watkins, K.L., Mulligan, S., Benton, J., Radune, D., Fisher, D.J., Atkins, H.S., Hiscox, T., Jost, B.H., Billington, S.J., Songer, J.G., McClane, B.A., Titball, R.W., Rood, J.I., Melville, S.B., Paulsen, I.T., 2006. Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. Genome Research 16, 1031-1040.

Nakano, V., Ignacio, A., Llanco, L., Bueris, V., Sircili, M.P., Avila-Campos, M.J., 2017. Multilocus sequence typing analyses of *Clostridium perfringens* type A strains harboring *tpeL* and *netB* genes. Anaerobe 44, 99-105.

Navarro, M.A., McClane, B.A., Uzal, F.A., 2018. Mechanisms of Action and Cell Death Associated with *Clostridium perfringens* Toxins. Toxins 10.

Nieman, C., 1954. Influence of trace amounts of fatty acids on the growth of microorganisms. Bacteriological Reviews 18, 147-163.

Novak, J.S., Juneja, V.K., McClane, B.A., 2003. An ultrastructural comparison of spores from various strains of *Clostridium perfringens* and correlations with heat resistance parameters. International Journal of Food Microbiology 86, 239-247.

Olkowski, A.A., Wojnarowicz, C., Chirino-Trejo, M., Laarveld, B., Sawicki, G., 2008. Sub-clinical necrotic enteritis in broiler chickens: novel etiological consideration based on ultra-structural and molecular changes in the intestinal tissue. Research in Veterinary Science 85, 543-553.

Paiva, D., McElroy, A., 2014. Necrotic enteritis: Applications for the poultry industry. The Journal of Applied Poultry Research 23, 557-566.

Pan, D., Yu, Z., 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5, 108-119.

Park, S.S., Lillehoj, H.S., Allen, P.C., Park, D.W., FitzCoy, S., Bautista, D.A., Lillehoje, E.P., 2008. Immunopathology and cytokine responses in broiler chickens coinfected with *Eimeria maxima* and *Clostridium perfringens* with the use of an animal model of necrotic enteritis. Avian Diseases 52, 14-22.

Patel, S., Goyal, A., 2012. The current trends and future perspectives of prebiotics research: a review. 3 Biotech 2, 115-125.

Petit, L., Gibert, M., Popoff, M.R., 1999. *Clostridium perfringens*: toxinotype and genotype. Trends in Microbiology 7, 104-110.

Prescott, J.F., Parreira, V.R., Mehdizadeh Gohari, I., Lepp, D., Gong, J., 2016a. The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know: a review. Avian Pathology 45, 288-294.

Prescott, J.F., Smyth, J.A., Shojadoost, B., Vince, A., 2016b. Experimental reproduction of necrotic enteritis in chickens: a review. Avian Pathology 45, 317-322.

Qu, A., Brulc, J.M., Wilson, M.K., Law, B.F., Theoret, J.R., Joens, L.A., Konkel, M.E., Angly, F., Dinsdale, E.A., Edwards, R.A., Nelson, K.E., White, B.A., 2008. Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. PloS One 3, e2945.

Rehman, H.U., Vahjen, W., Awad, W.A., Zentek, J., 2007. Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. Archives of Animal Nutrition 61, 319-335.

Riddell, C., Kong, X.M., 1992. The influence of diet on necrotic enteritis in broiler chickens. Avian Diseases 36, 499-503.

Rood, J.I., 1998. Virulence genes of *Clostridium perfringens*. Annual Review of Microbiology 52, 333-360.

Saengkerdsub, S., Anderson, R.C., Wilkinson, H.H., Kim, W.K., Nisbet, D.J., Ricke, S.C.,

2007a. Identification and quantification of methanogenic Archaea in adult chicken ceca. Applied and Environmental Microbiology 73, 353-356.

Saengkerdsub, S., Herrera, P., Woodward, C.L., Anderson, R.C., Nisbet, D.J., Ricke, S.C., 2007b. Detection of methane and quantification of methanogenic archaea in faeces from young broiler chickens using real-time PCR. Letters in Applied Microbiology 45, 629-634.

Sarker, M.R., Carman, R.J., McClane, B.A., 1999. Inactivation of the gene （cpe） encoding *Clostridium perfringens* enterotoxin eliminates the ability of two cpe-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. Molecular Microbiology 33, 946-958.

Sayeed, S., Uzal, F.A., Fisher, D.J., Saputo, J., Vidal, J.E., Chen, Y., Gupta, P., Rood, J.I., McClane, B.A., 2008. Beta toxin is essential for the intestinal virulence of *Clostridium perfringens* type C disease isolate CN3685 in a rabbit ileal loop model. Molecular Microbiology 67, 15-30.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States--major pathogens. Emerging Infectious Diseases 17, 7-15.

Schiemann, D.A., 1977. Laboratory confirmation of an outbreak of *Clostridium perfringens* food poisoning. Health Laboratory Science 14, 35-38.

Schlundt J., 2015. Foodborne intoxications. In: Heymann, D.L. （Eds.）, Control of communicable diseases manual, Washington, DC: American Public Health Association.

Sekirov, I., Finlay, B.B., 2009. The role of the intestinal microbiota in enteric infection. Journal of Physiology 587, 4159-4167.

Servin, A.L., Coconnier, M.H., 2003. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. Best Practice & Research: Clinical Gastroenterology 17, 741-754.

Shawkat A. M'Sadeq, S.W., Robert A. Swick, Mingan Choct, 2015. Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. Animal Nutrition 1, 1-11.

Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S., Hayashi, H., 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proceedings of the National Academy of Sciences of the United States of America 99, 996-1001.

Shojadoost, B., Vince, A.R., Prescott, J.F., 2012. The successful experimental induction

of necrotic enteritis in chickens by *Clostridium perfringens*: a critical review. Veterinary Research 43, 74.

Siegel, P.B., Larsen, A.S., Larsen, C.T., Dunnington, E.A., 1993. Research note: resistance of chickens to an outbreak of necrotic enteritis as influenced by major histocompatibility genotype and background genome. Poultry Science 72, 1189-1191.

Skrivanova, E., Marounek, M., Dlouha, G., Kanka, J., 2005. Susceptibility of *Clostridium perfringens* to C-C fatty acids. Letters in Applied Microbiology 41, 77-81.

Smyth, J.A., Martin, T.G., 2010. Disease producing capability of *netB* positive isolates of *C. perfringens* recovered from normal chickens and a cow, and *netB* positive and negative isolates from chickens with necrotic enteritis. Veterinary Microbiology 146, 76-84.

Songer, J.G., 1996. Clostridial enteric diseases of domestic animals. Clinical Microbiology Reviews 9, 216-234.

Stanley, D., Hughes, R.J., Moore, R.J., 2014a. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Applied Microbiology and Biotechnology 98, 4301-4310.

Stanley, D., Keyburn, A.L., Denman, S.E., Moore, R.J., 2012. Changes in the caecal microflora of chickens following *Clostridium perfringens* challenge to induce necrotic enteritis. Veterinary Microbiology 159, 155-162.

Stanley, D., Wu, S.B., Rodgers, N., Swick, R.A., Moore, R.J., 2014b. Differential responses of cecal microbiota to fishmeal, *Eimeria* and *Clostridium perfringens* in a necrotic enteritis challenge model in chickens. PloS One 9, e104739.

Stringfellow, K., McReynolds, J., Lee, J., Byrd, J., Nisbet, D., Farnell, M., 2009. Effect of bismuth citrate, lactose, and organic acid on necrotic enteritis in broilers. Poultry Science 88, 2280-2284.

Theoret, J.R., McClane, B.A., Uzal, F.A., Songer, J.G., Prescott, J.F., Popoff, M.R. 2016. Toxins of *Clostridium perfringens*, In: Clostridial Diseases of Animals. John Wiley & Sons, Inc, 45-60.

Thompson, D.R., Parreira, V.R., Kulkarni, R.R., Prescott, J.F., 2006. Live attenuated vaccine-based control of necrotic enteritis of broiler chickens. Veterinary Microbiology 113, 25-34.

Timbermont, L., De Smet, L., Van Nieuwerburgh, F., Parreira, V.R., Van Driessche, G., Haesebrouck, F., Ducatelle, R., Prescott, J., Deforce, D., Devreese, B., Van

Immerseel, F., 2014. Perfrin, a novel bacteriocin associated with *netB* positive *Clostridium perfringens* strains from broilers with necrotic enteritis. Veterinary Research 45, 40.

Timbermont, L., Haesebrouck, F., Ducatelle, R., Van Immerseel, F., 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathology 40, 341-347.

Timbermont, L., Lanckriet, A., Dewulf, J., Nollet, N., Schwarzer, K., Haesebrouck, F., Ducatelle, R., Van Immerseel, F., 2010. Control of *Clostridium perfringens*-induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils. Avian Pathology 39, 117-121.

Timbermont, L., Lanckriet, A., Gholamiandehkordi, A.R., Pasmans, F., Martel, A., Haesebrouck, F., Ducatelle, R., Van Immerseel, F., 2009. Origin of *Clostridium perfringens* isolates determines the ability to induce necrotic enteritis in broilers. Comparative Immunology, Microbiology and Infectious Diseases 32, 503-512.

Titball, R.W., Naylor, C.E., Basak, A.K., 1999. The *Clostridium perfringens* alpha-toxin. Anaerobe 5, 51-64.

Tsiouris, V., Georgopoulou, I., Batzios, C., Pappaioannou, N., Ducatelle, R., Fortomaris, P., 2015a. The effect of cold stress on the pathogenesis of necrotic enteritis in broiler chicks. Avian Pathology 44, 430-435.

Tsiouris, V., Georgopoulou, I., Batzios, C., Pappaioannou, N., Ducatelle, R., Fortomaris, P., 2015b. High stocking density as a predisposing factor for necrotic enteritis in broiler chicks. Avian Pathology 44, 59-66.

Uzal, F.A. 2016. Diseases Produced by *Clostridium perfringens* Type A in Mammalian Species, In: Francisco A. Uzal , J.G.S., John F. Prescott, Michel R. Popoff （Ed.） Clostridial Diseases of Animals. John Wiley & Sons, Inc.

Uzal, F.A., Freedman, J.C., Shrestha, A., Theoret, J.R., Garcia, J., Awad, M.M., Adams, V., Moore, R.J., Rood, J.I., McClane, B.A., 2014. Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. Future Microbiology 9, 361-377.

Van Der Sluis, W., 2000. Clostridial enteritis is an often underestimated problem. World poultry 16, 42-43.

Van Immerseel, F., De Buck, J., Boyen, F., Bohez, L., Pasmans, F., Volf, J., Sevcik, M., Rychlik, I., Haesebrouck, F., Ducatelle, R., 2004a. Medium-chain fatty acids decrease colonization and invasion through hilA suppression shortly after infection of chickens with *Salmonella enterica serovar Enteritidis*. Applied and

Environmental Microbiology 70, 3582-3587.

Van Immerseel, F., De Buck, J., Pasmans, F., Huyghebaert, G., Haesebrouck, F., Ducatelle, R., 2004b. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathology 33, 537-549.

Van Immerseel, F., Rood, J.I., Moore, R.J., Titball, R.W., 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in Microbiology 17, 32-36.

Wade, B., Keyburn., A., 2015. The true cost of necrotic enteritis. World Poultry 31, 16–17.

Williams, R.B., 2002. Anticoccidial vaccines for broiler chickens: pathways to success. Avian Pathology 31, 317-353.

Williams, R.B., 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology 34, 159-180.

Williams, R.B., Marshall, R.N., La Ragione, R.M., Catchpole, J., 2003. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis, coccidiosis and anticoccidial vaccination of chickens. Parasitology Research 90, 19-26.

Yang, Y., Iji, P.A., Kocher, A., Mikkelsen, L.L., Choct, M., 2008. Effects of dietary mannanoligosaccharide on growth performance, nutrient digestibility and gut development of broilers given different cereal-based diets. Journal of Animal Physiology and Animal Nutrition 92, 650-659.

Yeoman, C.J., Chia, N., Jeraldo, P., Sipos, M., Goldenfeld, N.D., White, B.A., 2012. The microbiome of the chicken gastrointestinal tract. Animal Health Research Reviews 13, 89-99.

Yin, Y., Lei, F., Zhu, L., Li, S., Wu, Z., Zhang, R., Gao, G.F., Zhu, B., Wang, X., 2010. Exposure of different bacterial inocula to newborn chicken affects gut microbiota development and ileum gene expression. ISME J 4, 367-376.

Zeiger, K., Popp, J., Becker, A., Hankel, J., Visscher, C., Klein, G., Meemken, D., 2017. Lauric acid as feed additive - An approach to reducing *Campylobacter* spp. in broiler meat. PloS One 12, e0175693.

Zhou, H., Lepp, D., Pei, Y., Liu, M., Yin, X., Ma, R., Prescott, J.F., Gong, J., 2017. Influence of pCP1NetB ancillary genes on the virulence of *Clostridium perfringens* poultry necrotic enteritis strain CP1. Gut Pathogens 9, 6.

陸、 附錄

附錄一、投稿 Anaerobe 期刊獲其刊登之第一部分研究成果

Anaerobe 54 (2018) 115–120

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Anaerobes in animal disease

Characterization of toxin genes and quantitative analysis of *netB* in necrotic enteritis (NE)-producing and non-NE-producing *Clostridium perfringens* isolated from chickens

Wen-Yuan Yang [a], Chung-Hsi Chou [b], Chinling Wang [a, *]

[a] Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi, Mississippi State, USA
[b] Zoonoses Research Center and School of Veterinary Medicine, National Taiwan University, Taipei City, Taiwan

ARTICLE INFO

Article history:
Received 12 January 2018
Received in revised form
6 August 2018
Accepted 21 August 2018
Available online 28 August 2018

Handling Editor. Dr. S.L.I. Chan

Keywords:
Clostridium perfringens
Necrotic enteritis
Toxins
netB
tpeL
cpb2
qPCR

ABSTRACT

Necrotic enteritis (NE) in chickens, a *Clostridium perfringens* infection, has re-emerged due to the removal of antibiotic growth promoters in feeds in recent years, thus contributing to significant economic losses for the industry. Toxins produced by *C. perfringens* in conjunction with predisposing factors are responsible for the onset and development of NE. Recently, several lines of evidence indicated the potential role of plasmid-encoded toxins in the virulence of NE, particularly necrotic enteritis B-like (NetB) toxin. However, the association of NetB, beta2 toxin (CPB2), and *C. perfringens* large cytotoxin (TpeL) in clinical NE isolates are not well-established. Therefore, we characterized the toxinotype and the presence of *netB*, *cpb2*, and *tpeL* genes in 15 NE-producing and 15 non-NE-producing *C. perfringens* isolates using conventional PCR and quantified *netB* among those isolates by quantitative PCR (qPCR). All isolates were characterized as toxinotype A and were negative for *cpe*, which is associated with human food poisoning. The *netB* was detected in 6.7% and 70% of NE-producing isolates by PCR and qPCR, respectively. In 15 non-NE-producing isolates, *netB* was not detected by conventional PCR, but was detected in 60% of isolates by qPCR. The presence of and the copy number of *netB* were not significantly different between NE- and non-NE-producing isolates ($p > 0.05$). No difference was observed between NE- and non-NE-producing isolates in the presence of *cpb2* or *tpeL* ($p > 0.05$). These results suggest that the presence of *netB*, *cpb2*, and *tpeL*, as well as the copy number of *netB* in *C. perfringens* is not correlated with clinical NE. In addition, we suggest that qPCR, but not conventional PCR, be used to detect *netB*.

1. Introduction

Necrotic enteritis (NE) is an important enteric disease in broiler chickens [1] characterized by necrosis and inflammation of the small intestine. NE is caused by *Clostridium perfringens*, an anaerobic, endospore-forming, and a Gram-positive bacterium, which results in significant levels of mortality in the clinical phase and loss of productivity and poor feed conversion in the subclinical form. Up to 37.3% of commercial broiler flocks could be affected by NE, contributing to an annual loss of 6.25 cents per bird and 6 billion dollars to the global poultry industry [2,3]. In the past, in-feed antimicrobial growth promoters (AGPs) were the most effective strategy used to control *C. perfringens*-associated NE in poultry [4]. However, the phase-out of AGPs due to concerns regarding the spread of antibiotic resistance has resulted in a spike in NE incidence, and hence considered as a re-emerging disease by the poultry industry [5–8].

C. perfringens is classified into A, B, C, D, and E toxinotypes based on the production of four major toxins, alpha (α), beta (β), epsilon (ε), and iota (ι) toxins [9]. NetB, a pore-forming toxin from NE strains *of C. perfringens*, has limited protein sequence identity to the beta-toxin of *C. perfringens*, which causes mucosal necrosis of the small intestine in humans and animals. It plays a major role in the virulence of NE based on an *in vivo* study demonstrating that the wild-type and complemented *netB C. perfringens* strain produced pathognomonic lesions in the small intestine, but a null mutant could not. In contrast, a *cpa* mutant strain that did not produce CPA toxin, still produced NE in an experimental challenge study [11]. Although the expression of *netB* fulfilled the molecular Koch's

Abbreviations: NE, necrotic enteritis; AGPs, antimicrobial growth promoters; qPCR, quantitative real-time PCR.

* Corresponding author.
E-mail address: cw57@msstate.edu (C. Wang).

https://doi.org/10.1016/j.anaerobe.2018.08.010
1075-9964/

postulate, several lines of evidence dispute the importance of *netB* on the pathogenesis. A proportion of *C. perfringens* strains isolated from healthy chickens possess *netB* with some degree of *in vitro* NetB toxin production, but these chickens do not develop NE after challenges with *netB*-positive isolates [12–14]. Low carriage rates of *netB* in *C. perfringens* isolates from NE-affected chicken farms have been noted [15], and isolates without *netB* also produce NE [12,13,15–17]. In addition, use of *netB* positive *C. perfringens* alone, without predisposing factors, failed to consistently reproduce NE in the challenge model [18]. These findings indicate that the pathogenesis of NE is highly complex and other factors should be involved and crucial in the disease development. Meanwhile, a number of studies demonstrated that predisposing factors were required for NE development [19–21], and with a combination of predisposing factors, degradative enzymes, and toxins, *C. perfringens* could conceivably trigger NE, resulting in intestinal tissue destruction, bacterial colonization, and enterotoxicity [10,15,22]. Besides, isolations of *netB*-positive *C. perfringens* from healthy birds further provide the evidence that a simple *C. perfringens* infection without other components is not sufficient to induce NE. Therefore, it is worth examining the possibility that *netB* may originally exist in all *C. perfringens*, not exclusively in NE-producing isolates. Last but not least, Nakano et al. [23] recently found that *netB* and *tpeL* were also present in *C. perfringens* isolated from healthy humans by PCR assay, pointing out the possibility that the presence of those genes may not correlate with the virulence of NE. Based upon these findings, we hypothesized that *C. perfringens* isolates from NE and non-NE chickens may harbor *netB* and other virulence genes regardless of the origin of the isolates. Traditionally, analysis of toxin genes in *C. perfringens* depends on a conventional or multiplex PCR, but quantitative real-time PCR (qPCR) is recognized as the superior technique with higher sensitivity and quantitative ability. Therefore, the aim of this study was to investigate the relevant toxin genes (*cpa*, *cpb*, *etx*, *iap*, *cpe*, *cpb2*, *tpeL*, and *netB*) in NE-producing and non-NE-producing *C. perfringens* by PCR and to quantify the virulence gene, *netB*, by qPCR. This study provides a better understanding of the association of *netB* with clinical NE and also demonstrates a sensitive qPCR assay to detect the presence of toxin gene.

2. Materials and methods

2.1. Isolation and identification of C. perfringens from NE-diseased and non-NE chickens

The jejunum was targeted and collected to isolate *C. perfringens* in this study. NE-diseased chicken was defined by observing clinical symptoms and pathognomonic lesions in the small intestine. On the contrary, non-NE chicken was grossly inspected and determined by the absence of NE lesions in the same site of the gut. Fifteen jejunal tissue samples of NE-diseased chickens were obtained from thirteen broiler farms. For non-NE counterpart, four out of fifteen samples were collected from a processing plant and five jejunums originated from chickens at each different houses within two separated farms. The remaining samples were collected from specific pathogen free (SPF) layers and broilers evenly in untreated control groups of two chicken trails. Afterwards, *C. perfringens* were isolated by streaking scrapings of the jejunal mucus membrane on blood agar plates (Fisher Scientific, Pittsburgh, Pennsylvania, USA) followed by an overnight anaerobic incubation at 37 °C. The anaerobic condition was achieved by using container system sachets (BD Biosciences, San Jose, California, USA) or anaerobic jar (Sigma, St. Louis, Missouri, USA) with anaerobic gas generators (Fisher Scientific). A single colony with double hemolytic zones was subsequently transferred to an egg yolk agar plate (HiMedia, Mumbai, Maharashtra, India) for an additional overnight anaerobic incubation at 37 °C. Following incubation, a colony harboring a positive lecithinase reaction was selected and cultured on a highly selective tryptone sulfite neomycin (TSN) agar plate (Sigma) and incubated anaerobically at 46 °C for an additional 18–20 h. A black colony presumptively identified as *C. perfringens* was then transferred to 10 ml thioglycolate broth (Himedia) and anaerobically incubated at 37 °C overnight for further identification, total DNA extraction, and stock storage. Each strain or isolate was confirmed as *C. perfringens* using RapID ANA II biochemical tests (Fisher Scientific) and PCR assays targeting the *16S rRNA* gene (Table 1).

2.2. Bacterial DNA extraction

Total DNA was isolated from the overnight broth-brown culture inoculated with single colony of *C. perfringens* from plate by using an Ultraclean Microbial DNA Isolation Kit (Mobio, Germantown, Maryland, USA) following the manufacturer's instructions with some modifications. Briefly, the bacterial suspension was mixed with a lysis buffer and 20 μl of 20 mg/ml proteinase K (Fisher Scientific). The mixture was subsequently incubated at 65 °C for 15 min to lyse the bacterial cell wall and to prevent DNase digestion of DNA. In order to avoid DNA shearing, the tube containing the reaction mixture was secured on a flat pad and horizontally vortexed for 10 min. DNA was sequentially eluted with 30 μl 10 mM Tris-HCl (pH 8.0), separated on a 0.8% agarose gel (BD Biosciences) for the quality assurance, and stored at −20 °C until use.

2.3. PCR amplification of C. perfringens toxin genes

Major toxin genes, including *cpa*, *cpb*, *etx*, and *iap*, as well as minor toxin genes, *cpe*, *netB*, *cpb2*, and *tpeL*, were detected by PCR with specific primers (Table 1). ATCC strains 13124, 3626, 12917, 51880, and 27324 were used as reference strains for toxinotyping (A, B, C, D, and E, respectively) and as positive controls for *cpb2* and *cpe*. Strain JP17, kindly provided by Dr. John F. Prescott, was used as the positive control for *netB* and *tpeL*. PCR amplification was performed in a 20-μl volume containing 50 ng template DNA, 10 × *Taq* buffer $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1U *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, Pennsylvania, USA), and 0.2 μM of each primer on a thermocycler (Applied Biosystems GeneAmp PCR System 9700). The protocol was as follows: 1 cycle of 95 °C for 2 min; 35 cycles of 95 °C for 1 min, annealing temperature for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. For *netB*, the assay conditions were modified to: 1 cycle of 95 °C for 3 min; 35 cycles of 95 °C for 30 s, annealing temperature for 30 s, and 72 °C for 30 s; a final extension cycle of 72 °C for 5 min. The amplified products were analyzed on a 1.5% agarose gel and stained with SYBR Safe DNA Gel Stain (Fisher Scientific). PCR amplified products on the gels were extracted, purified, and sequenced to ensure 100% match of reference sequences.

2.4. Quantitative real-time PCR (qPCR)

To estimate relative abundance of *netB* in NE- and non-NE-producing *C. perfringens* isolates, 20 μl reaction mixture was analyzed using a QuantStudio 5 Real-Time PCR System (Fisher Scientific). The mixture contained 10 μl PowerUp SYBR Green Master Mix (Fisher Scientific), 0.5 μM *netB* primer (Table 1), and 100 ng template DNA. Three technical replicates along with corresponding positive and negative controls were included for each plate. The qPCR amplification cycles were as follows: 1 cycle of 95 °C for 2 min; followed by 45 cycles of denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 30 s, and dissociation steps

Table 1
Clostridium perfringens PCR and qPCR targeted genes, primers, and amplicon lengths.

Gene	GenBank accession number	Primers	Sequence (5′–3′)	T_m (°C)	Product (bp)	Reference
cpa	L43545	CPA5L	AGTCTACGCTTGGGATGGAA	55	900	[38]
		CPA5R	TTTCCTGGGTTGTCCATTTC			
cpb	X83275	CPBL	TCCTTTCTTGAGGGAGGATAAA	56	611	[38]
		CPBR	TGAACCTCCTATTTTGTATCCCA			
cpb2	L77965	CPB2L	AGATTTTAAATATGATCCTAACC	53	567	[39]
		CPB2R	CAATACCCTTCACCAAATACTC			
etx	M95206	CPETXL	TGGGAACTTCGATACAAGCA	56	396	[38]
		CPETXR	TTAACTCATCTCCCATAACTGCAC			
iap	X73562	CPIL	AAACGCATTAAAGCTCACACC	57	293	[38]
		CPIR	CTGCATAACCTGGAATGGCT			
cpe	X81849	CPEL	GGGGAACCCTCAGTAGTTTCA	57	506	[38]
		CPER	ACCAGCTGGATTTGAGTTTAATG			
tpeL	EU848493	TPELF	ATATAGAGGCAAGCAGTGGAG	55	466	[37]
		TPELR	GGAATACCACTTGATATACCTG			
netB	GU433338	NETBL	TGATACCGCTTCACATAAAGGTTGG	61	196	*This study*
		NETBR	ATAAGTTTCAGGCCATTTCATTTTTCCG			
16S rRNA	Y12669	16SL	CATCATTCAACCAAAGGAGCAATCC	60	262	*This study*
		16SR	CATTATCTTCCCCAAAGACAGAGC			

at 95 °C for 1 s; 60 °C for 20 s; and 95 °C for 1 s. To differentiate the specific *netB* amplicon from non-specific products, the DNA melting curve was performed to confirm the specific amplicon. Likewise, amplified products were separated on 1.5% agarose gels to confirm that the size was the same as the positive control.

2.5. Efficiency of the qPCR assay

The PCR product of *netB* was cloned into pGEM®-T Easy Vector Systems (Promega, Madison, Wisconsin, USA) to generate plasmid DNA containing specific sequence of *netB*. The amplification efficiency (AE) of the qPCR for *netB* was examined using a 10-fold serial dilution of the plasmid DNA ranged from 1 ng to 100 ag (ag). Standard curve was established based on the threshold cycles (Ct) plotted against the $\log_{10}$ values of the gene copies (Log-copy). The linearity was observed with the equation: $y = -3.4571x + 38.397$ ($R^2 = 0.9999$; AE = 95%), where y is the threshold cycles and x is the amount of the targeted gene with $\log_{10}$ value (Fig. 1).

2.6. Statistical analysis

The average threshold cycle (Ct) from the positive replicate samples was used for calculating the copy number of *netB*, which was expressed as $\log_{10}$ copies/100 ng DNA. After log transformation of the copy number, the difference in copies between NE- and non-NE-producing *C. perfringens* was analyzed with the Wilcoxon Rank-Sum Test using SAS software version 9.4 (SAS Institute, Inc., Cary, North Carolina, USA). Fisher's exact test was used to compare the difference in frequency of *netB, cpb2*, and *tpeL* between NE- and non-NE-producing *C. perfringens* isolates. A level of *0.05* was considered statistically significant.

3. Results

3.1. Isolate identification

Thirty *C. perfringens* isolates were recovered from the jejunum of

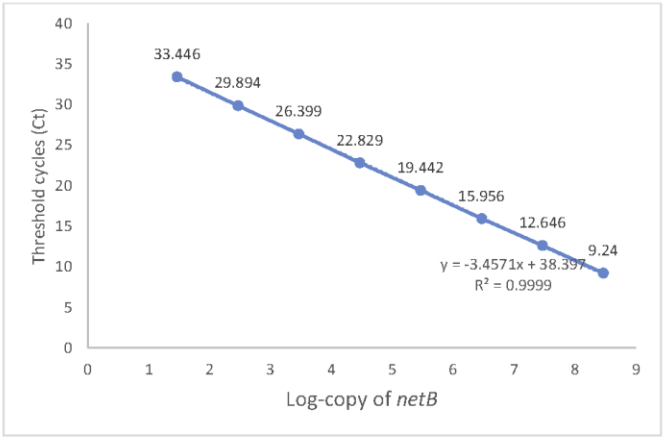

Fig. 1. Standard curve of the *netB* by qPCR. It shows Ct-values of isolate samples plotted versus the log value of quantified *netB* copy numbers. The 10-fold serial dilutions (from 1 ng to 100 ag) were prepared ranging from 2.92×10^8 to 29.2 copies of *netB* gene. The linearity was observed with the equation: $y = -3.4571x + 38.397$ ($R^2 = 0.9999$; AE = 95%).

clinical NE and non-NE chickens in 15 broiler farms, one processing plant, and two experimental control groups (Table 2). All isolates were identified as *C. perfringens* through biochemical tests and *16S rRNA* gene sequence confirmation by PCR.

3.2. Toxinotyping and detection of toxin genes by PCR

All thirty *C. perfringens* isolates were characterized as toxinotype A (Table 2). None of these isolates carried *cpb*, *etx*, *iap*, or *cpe*. Of the thirty isolates, twenty (66.7%) harbored *cpb2* (11 in the NE group and 9 in the non-NE group), two possessed *netB* (6.7%), and two had *tpeL* (6.7%). The prevalence of *cpb2*, *netB* and *tpeL* in NE-producing isolates was higher than that in the non-NE producing isolates. However, no statistical significance was noted. The simultaneous carriage of *netB* and *tpeL* was observed in two isolates.

3.3. qPCR

The carriage rate of *netB* in NE-producing *C. perfringens* was 73% (11/15) compared with 60% (9/15) in the non-NE-producing isolates (Table 3). The NE-producing *C. perfringens* carried a higher copy number of *netB* than the non-NE-producing isolates; however, there was no statistically significant difference ($p > 0.05$). Copies of *netB* in NE-producing isolates varied among different farms. Six NE-producing isolates carried lower *netB* copy number than non-NE producing isolates.

3.4. Comparison of the netB detection by conventional PCR and qPCR

The detection rate of *netB* in total *C. perfringens* isolates was 6.7% (2/30) by PCR compared to 66.7% (20/30) by qPCR. In detail, the *netB* gene was recognized in 13.3% (2/15) and 73.3% (11/15) of NE-producing isolates by PCR and qPCR, respectively. However, *netB* was not found in non-NE producing isolates by PCR, whereas 60% of these isolates (9/15) were positive for *netB* by qPCR (Table 3).

4. Discussion

C. perfringens isolates recovered from NE-producing chickens in this study were all toxinotype A, but were negative for *cpe*, which is consistent with previous studies [5,11]. The pathogenesis of NE has been the subject of considerable interest for over a decade in order to develop effective prevention and mitigation strategies. The NetB toxin, encoded by *netB*, was shown to be an important virulence factor through several experimental challenge studies and epidemiological surveys [12,24,25]. However, a recent study found that *netB* alone, in the absence of other NELoc-1 genes, was unable to restore full virulence in the challenge model performed by Zhou et al. [18]. This finding suggested that other genes present in the same pathogenicity locus, NELoc-1, and their encoded products were required for regulation of *netB* and for full virulence. Furthermore, global gene expression of *netB* harbored by *C. perfringens* was demonstrated to be highly modulated by environmental conditions [26]. These results indicate that *netB* may exist in each *C. perfringens* isolate, and the disease development

Table 2
Distribution of toxin genes and copy number of *netB* in NE- and non-NE-producing *Clostridium perfringens* isolates.

Isolate	Origin	Source	Health status	Type	*cpa*	*cpb*	*etx*	*iap*	*cpe*	*cpb2*	*tpeL*	*netB*		
												PCR	qPCR	Copies/100 ng DNA
JP17	Reference	N/A	N/A	A	+	−	−	−	−	+	+	+	+	$>2.92 \times 10^8$
1N	Broiler	F1	NE	A	+	−	−	−	−	+	+	+	+	$>2.92 \times 10^8$
2N	Broiler	F2	NE	A	+	−	−	−	−	−	−	−	−	−
3N	Broiler	F3	NE	A	+	−	−	−	−	+	−	−	+	7.26×10^3
4N	Broiler	F4	NE	A	+	−	−	−	−	+	−	−	+	4.19×10
5N	Broiler	F5	NE	A	+	−	−	−	−	−	−	−	+	$<2.92 \times 10$
6N	Broiler	F6	NE	A	+	−	−	−	−	−	−	−	+	1.35×10^3
7N	Broiler	F7	NE	A	+	−	−	−	−	+	+	+	+	$>2.92 \times 10^8$
8N	Broiler	F8	NE	A	+	−	−	−	−	+	−	−	+	$<2.92 \times 10$
9N	Broiler	F9	NE	A	+	−	−	−	−	+	−	−	+	1.76×10^3
10N	Broiler	F10	NE	A	+	−	−	−	−	+	−	−	+	$<2.92 \times 10$
11N	Broiler	F11	NE	A	+	−	−	−	−	+	−	−	+	$<2.92 \times 10$
12N	Broiler	F12	NE	A	+	−	−	−	−	+	−	−	+	$<2.92 \times 10$
13N	Broiler	F13; H2	NE	A	+	−	−	−	−	+	−	−	−	−
14N	Broiler	F13; H2	NE	A	+	−	−	−	−	−	−	−	−	−
15N	Broiler	F13; H2	NE	A	+	−	−	−	−	+	−	−	−	−
1C	Broiler	P1	non-NE	A	+	−	−	−	−	+	−	−	+	3.11×10
2C	Broiler	P1	non-NE	A	+	−	−	−	−	+	−	−	+	7.49×10
3C	Broiler	P1	non-NE	A	+	−	−	−	−	+	−	−	−	−
4C	Broiler	P1	non-NE	A	+	−	−	−	−	+	−	−	−	−
5C	Broiler	F14; H1	non-NE	A	+	−	−	−	−	+	−	−	−	−
6C	Broiler	F14; H2	non-NE	A	+	−	−	−	−	+	−	−	+	2.96×10
7C	Broiler	F14; H3	non-NE	A	+	−	−	−	−	+	−	−	+	4.40×10
8C	Broiler	F15; H1	non-NE	A	+	−	−	−	−	+	−	−	+	$<2.92 \times 10$
9C	Broiler	F15; H2	non-NE	A	+	−	−	−	−	+	−	−	+	$<2.92 \times 10$
10C	Layer	EC1	non-NE	A	+	−	−	−	−	−	−	−	−	−
11C	Layer	EC1	non-NE	A	+	−	−	−	−	−	−	−	+	7.23×10
12C	Layer	EC1	non-NE	A	+	−	−	−	−	−	−	−	+	$<2.92 \times 10$
13C	Broiler	EC2	non-NE	A	+	−	−	−	−	−	−	−	−	−
14C	Broiler	EC2	non-NE	A	+	−	−	−	−	−	−	−	+	6.45×10
15C	Broiler	EC2	non-NE	A	+	−	−	−	−	−	−	−	−	−

AbbreviationsF, farm; H: house; P, processing plant; EC, experimental control; NE, necrotic enteritis; non-NE, chickens not affected by NE or healthy.
The copies of netB was calculated by the equation: $y = -3.4571x + 38.397$ ($R^2 = 0.9999$; AE = 95%).
No significant difference of mean log-copies of netB between NE and non-NE group (Wilcoxon Rank-Sum Test; $p > 0.05$).

Table 3
Comparative results of *netB* carriage by PCR and qPCR in NE- and non-NE-producing *Clostridium perfringens* isolates.

		Status	
		NE	non-NE
Conventional PCR	*netB* +	2	0
	netB −	13	15
	Subtotal	15	15
qPCR	*netB* +	11	9
	netB −	4	6
	Subtotal	15	15

AbbreviationsNE, necrotic enteritis; non-NE, chickens not affected by NE or healthy.
No significant difference of prevalence detected by qPCR between NE and non-NE group (Fisher's exact test; $p > 0.05$).

depends on certain regulatory mechanisms to either activate or suppress genes. In other words, *C. perfringens* isolated from NE and non-NE chickens may all possess *netB*, but it may only be expressed under certain conditions, promoting the development of NE. Thus, the carriage and quantity of *netB* in NE- and non-NE-producing *C. perfringens* isolates were re-assessed in this study. The low detection rate of *netB* by PCR (6.7%) was consistent with that of a previous study (4.1%) [15], indicating that NetB was not an important virulence factor, or at least not as important as originally thought. Nevertheless, qPCR data demonstrated that the prevalence of *netB* was high in both NE and non-NE isolates (73% versus 60%, respectively), showing that *netB* is present in the majority of *C. perfringens* regardless of NE production status. No significant difference of prevalence was observed between these two groups regardless of the detection method was used. The *netB* gene could be detected at the amount of 10–20 copies by qPCR, whereas high copy numbers (~10^8) were required for a positive identification by PCR in this study. Abildgaard et al. [13] noted the potential significance of NetB toxin in virulence; however, the presence or absence of *netB* in *C. perfringens* itself is insufficient to predict the pathogenicity. Regarding the quantity of *netB*, NE-producing *C. perfringens* isolates possessed higher copy numbers than non-NE isolates on average. Among eleven NE-producing *C. perfringens* isolates, two had abundant copies of *netB*, while the remaining nine carried relatively low copies of *netB* and yet still established clinical NE, suggesting that the quantity of *netB* in *C. perfringens* is not a reliable indicator of pathogenicity as well.

NE-producing *C. perfringens* strains/isolates typically carry two to five highly conserved, low-copy number plasmids [27]. The *netB*, *cpb2,* and *tpeL* toxin genes are encoded in pathogenicity loci on different large plasmids [27–29]. As *C. perfringens* ordinarily relies on plasmid-encoded toxins to produce NE in chickens [30], the prevalence of *cpb2* and *tpeL*, in addition to *netB*, were selected for this investigation. Several epidemiological studies suggest that *cpb2*-positive *C. perfringens* isolates are highly associated with enteric diseases in domestic animals [31]. In the present study, the high carriage rate of *cpb2* in NE and non-NE isolates was observed by PCR; this result was consistent with other findings [32–35]. We also found that *cpb2* was not differently distributed between isolates from NE-producing and healthy chickens, which disputes that there is an association between *cpb2* and NE, as previously reported by Crespo et al. [32].

Regarding *tpeL*, our PCR data were in accordance with other findings [14,15,36] and revealed low frequencies of *tpeL* carriage in NE-producing isolates. The *tpeL* gene was found only in *netB*-positive isolates. These observations indicate that the putative TpeL toxin may not be essential for NE in chickens due to such a low carriage rate. However, one study demonstrated that several *tpeL*-positive *C. perfringens* in the absence of *netB* causes typical NE in the disease induction model [16], proposing that the TpeL toxin or another toxin plays a role in NE pathogenesis. In another challenge experiment, inoculation of broilers with *tpeL*- and *netB*-positive strains was associated with greater severity of gross lesions compared with strains containing only *netB*, suggesting that *tpeL* potentiates the effect of other virulence attributes of NE strains [37]. Collectively, these data indicate that the role of *tpeL* in pathogenesis remains inconclusive.

In summary, we demonstrated that a qPCR assay was a sensitive and reliable method for characterization and quantification of *netB* in *C. perfringens*. The results provide not only new insights into the prevalence of potential virulence toxin genes in *C. perfringens* populations from NE and non-NE chickens, but also a conclusion that the presence or absence of those genes as well as the quantity of *netB* are insufficient to predict an association with the virulence or pathogenicity. As NE is a multifactorial disease, the understandings of gut microbiota, environmental conditions, and regulation of virulence genes and attributes are recommended and those are rationally required to elucidate the dynamic pathogenesis of *C. perfringens*.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to thank Dr. John F. Prescott (University of Guelph, Ontario, Canada) for providing *netB*- and *tpeL*-positive *C. perfringens* strains, and Dr. Tim Cumming and Dr. Martha Pulido for collecting field samples. Special thanks to Dr. Chuan-yu Hsu (Institute for Genomics, Biocomputing and Biotechnology, Mississippi State University) for assistance in primer design and qPCR. The work was supported by the College of Veterinary Medicine, Mississippi State University.

References

[1] K.K. Cooper, et al., Diagnosing clostridial enteric disease in poultry, J. Vet. Diagn. Invest. 25 (3) (2013) 314–327.
[2] W. Van Der Sluis, Clostridial enteritis is an often underestimated problem, World poult 16 (7) (2000) 42–43.
[3] B. Wade, A. Keyburn, The true cost of necrotic enteritis, World Poultrymeat 31 (2015) 16–17.
[4] D. Liu, et al., Exogenous lysozyme influences Clostridium perfringens colonization and intestinal barrier function in broiler chickens, Avian Pathol. 39 (1) (2010) 17–24.
[5] F. Van Immerseel, et al., Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens, Trends Microbiol. 17 (1) (2009) 32–36.
[6] M. Casewell, et al., The European ban on growth-promoting antibiotics and emerging consequences for human and animal health, J. Antimicrob. Chemother. 52 (2) (2003) 159–161.
[7] L. Timbermont, et al., Necrotic enteritis in broilers: an updated review on the pathogenesis, Avian Pathol. 40 (4) (2011) 341–347.
[8] M.L. Gaucher, et al., Impact of a drug-free program on broiler chicken growth performances, gut health, Clostridium perfringens and Campylobacter jejuni occurrences at the farm level, Poultry Sci. 94 (8) (2015) 1791–1801.
[9] F. Van Immerseel, et al., Clostridium perfringens in poultry: an emerging

threat for animal and public health, Avian Pathol. 33 (6) (2004) 537–549.
[10] A.L. Keyburn, et al., NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens, PLoS Pathog. 4 (2) (2008) e26.
[11] A.L. Keyburn, et al., Alpha-toxin of Clostridium perfringens is not an essential virulence factor in necrotic enteritis in chickens, Infect. Immun. 74 (11) (2006) 6496–6500.
[12] T.G. Martin, J.A. Smyth, Prevalence of netB among some clinical isolates of Clostridium perfringens from animals in the United States, Vet. Microbiol. 136 (1–2) (2009) 202–205.
[13] L. Abildgaard, et al., In vitro production of necrotic enteritis toxin B, NetB, by netB-positive and netB-negative Clostridium perfringens originating from healthy and diseased broiler chickens, Vet. Microbiol. 144 (1–2) (2010) 231–235.
[14] G. Chalmers, et al., Multilocus sequence typing analysis of Clostridium perfringens isolates from necrotic enteritis outbreaks in broiler chicken populations, J. Clin. Microbiol. 46 (12) (2008) 3957–3964.
[15] M.A. Bailey, et al., Low prevalence of netB and tpeL in historical Clostridium perfringens isolates from broiler farms in Alabama, Avian Dis. 59 (1) (2015) 46–51.
[16] L.A. Llanco, et al., Sialidase production and genetic diversity in Clostridium perfringens type A isolated from chicken with necrotic enteritis in Brazil, Curr. Microbiol. 70 (3) (2015) 330–337.
[17] J.A. Smyth, T.G. Martin, Disease producing capability of netB positive isolates of C. perfringens recovered from normal chickens and a cow, and netB positive and negative isolates from chickens with necrotic enteritis, Vet. Microbiol. 146 (1–2) (2010) 76–84.
[18] H. Zhou, et al., Influence of pCP1NetB ancillary genes on the virulence of Clostridium perfringens poultry necrotic enteritis strain CP1, Gut Pathog. 9 (2017) 6.
[19] R.B. Williams, Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity, Avian Pathol. 34 (3) (2005) 159–180.
[20] B. Shojadoost, et al., The successful experimental induction of necrotic enteritis in chickens by Clostridium perfringens: a critical review, Vet. Res. 43 (2012) 74.
[21] R.J. Moore, Necrotic enteritis predisposing factors in broiler chickens, Avian Pathol. 45 (3) (2016) 275–281.
[22] J. Li, et al., Sialidases affect the host cell adherence and epsilon toxin-induced cytotoxicity of Clostridium perfringens type D strain CN3718, PLoS Pathog. 7 (12) (2011) e1002429.
[23] V. Nakano, et al., Multilocus sequence typing analyses of Clostridium perfringens type A strains harboring tpeL and netB genes, Anaerobe 44 (2017) 99–105.
[24] A. Johansson, et al., Genetic diversity and prevalence of netB in Clostridium perfringens isolated from a broiler flock affected by mild necrotic enteritis, Vet. Microbiol. 144 (1–2) (2010) 87–92.
[25] A.L. Keyburn, et al., NetB, a pore-forming toxin from necrotic enteritis strains of Clostridium perfringens, Toxins 2 (7) (2010) 1913–1927.
[26] V.R. Parreira, et al., Comparative transcriptome analysis by RNAseq of necrotic enteritis Clostridium perfringens during in vivo colonization and in vitro conditions, BMC Microbiol. 16 (1) (2016) 186.
[27] D. Lepp, et al., Identification of novel pathogenicity loci in Clostridium perfringens strains that cause avian necrotic enteritis, PLoS One 5 (5) (2010) e10795.
[28] D. Lepp, et al., Identification of accessory genome regions in poultry Clostridium perfringens isolates carrying the netB plasmid, J. Bacteriol. 195 (6) (2013) 1152–1166.
[29] T.L. Bannam, et al., Necrotic enteritis-derived Clostridium perfringens strain with three closely related independently conjugative toxin and antibiotic resistance plasmids, mBio 2 (5) (2011).
[30] J. Li, et al., Toxin plasmids of Clostridium perfringens, Microbiol. Mol. Biol. Rev. 77 (2) (2013) 208–233.
[31] M. Waters, et al., Regulated expression of the beta2-toxin gene (cpb2) in Clostridium perfringens type a isolates from horses with gastrointestinal diseases, J. Clin. Microbiol. 43 (8) (2005) 4002–4009.
[32] R. Crespo, et al., Toxinotypes of Clostridium perfringens isolated from sick and healthy avian species, J. Vet. Diagn. Invest. 19 (3) (2007) 329–333.
[33] A.R. Gholamiandekhordi, et al., Molecular and phenotypical characterization of Clostridium perfringens isolates from poultry flocks with different disease status, Vet. Microbiol. 113 (1–2) (2006) 143–152.
[34] J.Y. Park, et al., Characterization of Clostridium perfringens isolates obtained from 2010 to 2012 from chickens with necrotic enteritis in Korea, Poultry Sci. 94 (6) (2015) 1158–1164.
[35] G.R. Siragusa, et al., Molecular subtyping of poultry-associated type A Clostridium perfringens isolates by repetitive-element PCR, J. Clin. Microbiol. 44 (3) (2006) 1065–1073.
[36] A.L. Keyburn, et al., Association between avian necrotic enteritis and Clostridium perfringens strains expressing NetB toxin, Vet. Res. 41 (2) (2010) 21.
[37] C.F. Coursodon, et al., TpeL-producing strains of Clostridium perfringens type A are highly virulent for broiler chicks, Anaerobe 18 (1) (2012) 117–121.
[38] Y.C. Fan, et al., Incidence and antimicrobial susceptibility to Clostridium perfringens in premarket broilers in Taiwan, Avian Dis. 60 (2) (2016) 444–449.
[39] H.S. Garmory, et al., Occurrence of Clostridium perfringens beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays, Epidemiol. Infect. 124 (1) (2000) 61–67.

附錄二、2018 年度國際家禽論壇口頭發表之研究成果摘要

SCAD

Moderator: Dr. John El - Attrache, CEVA
Room Number: B312
Monday, January 29, 2018

1:00 p.m. M38

Interactions of environmental temperature and dietary protein levels with coccidia vaccination
Jarred Oxford*[GS1], Hector Cervantes[2], Ivan Alvarado[3], Andres Montoya[3], Ricardo Nunes[4], Jomara Bronch[4], Gene Pesti[1] *[1]University of Georgia; [2]Phibro Animal Health; [3]Merck Animal Health; [4]Universidade Estadual do Oeste do Paraná*

1:15 p.m. M39

Evaluation of different coccidia vaccines, with or without a bioshuttle, for control of a pathogenic E. tenella
Laura Tensa*[GS], Grace Albanese, Brian Jordan *University of Georgia*

1:30 p.m. M40

Impact of necrotic enteritis disease models on the growth curve of broilers
Kaylin Chasser*[GS], Kim Wilson, Whitney Briggs, Audrey Duff, Ramesh Selvaraj, Lisa Bielke *The Ohio State University, OARDC*

1:45 p.m. M41

Characterization and Quantification of Toxin Genes in Necrotic Enteritis (NE) Producing and Non-NE Producing Clostridium perfringens Isolated from Chickens
Wenyuan Yang*[GS], Chung-Hsi Chou, Chinling Wang *Mississippi State University*

2:00 p.m. Break

2:15 p.m. M43

The effectiveness of lauric acid and Akkermansia muciniphila against necrotic enteritis in chickens
Chinling Wang*, Hsin-Yi Lu, Wei-Yun Yang, Yue-Jia Lee, Scott Branton *Mississippi State University*

2:30 p.m. M44

Effect of zinc sources and doses on the presence of Necrotic Enteritis in broilers caused by C. perfringens
Greg Mathis*, Brett Lumpkins, Agathe Romeo, Stephane Durosoy *Southern Poultry Research, Inc.*

2:45 p.m. M45

Characteristics of Gallibacterium anatis isolated from poultry samples
Jessica Hockaday*[GS], Alejandro Banda, Jay Kay Thornton, Lifang Yan, Martha Pulido-Landínez *Mississippi State University CVM Poultry Research and Diagnostic Laboratory*

3:00 p.m. Break

3:30 p.m.

USPOULTRY Awards Presentation - Room Number B-313

4:00 p.m. - 6:00 p.m.

Poster Reception - Room Number B-313 Foyer

significantly lower ($p<0.05$) than NC on d22 for M6CP, and remained nearly parallel with NC through d57, though not significantly lower. In Exp 2, d22 BW for NetB1 Low and Supernatant were not significantly lower than NC, but GCP, M6CP, NetB1, and Washed had a significant reduction in BW that required a week or more to meet NC growth. EM played a role in clinical NE, while extremely elevated levels of CP alone were required to produce subclinical NE, where BW was impacted without notable clinical signs. This separation in BW can be used to measure effectiveness of intervention tools against NE.

Key Words: necroticenteritis, growth, Eimeria, Clostridium

M41 Characterization and Quantification of Toxin Genes in Necrotic Enteritis (NE) Producing and Non-NE Producing Clostridium perfringens Isolated from Chickens Wenyuan Yang*[GS], Chung-Hsi Chou, Chinling Wang *Mississippi State University*

Necrotic enteritis (NE) caused by *Clostridium perfringens* is a re-emerging disease in chickens in recent years, contributing to enormous economic losses to the poultry industry. Toxins produced by *C. perfringens* as well as other predisposing factors are responsible for the onset and development of the disease. However, the importance of plasmid encoding toxins NetB, CPB2, and TpeL, to clinical NE cases is controversial. Therefore, we compared the toxinotype and the presence of *netB, cpb2,* and *tpeL* genes, in 15 NE-producing and 15 non-NE producing *C. perfringens* isolates using the conventional PCR and the quantitative PCR (qPCR). Results indicated that all 30 isolates were characterized as toxinotype A, and all were negative for *cpe* gene which is associated with human food poisoning. The *netB* gene was detected in 6.7% of NE-producing isolates by the conventional PCR, whereas 70% by the qPCR. In 15 non-NE producing isolates, no *netB* gene was detected by the conventional PCR but 60% of isolates were positive for *netB* by the qPCR. The presence and copies of *netB* gene were not significantly different between NE and non-NE producing isolates. No difference was observed between NE and non-NE producing isolates in the presence of *cpb2* or *tpeL* gene. The results suggest that neither the presence of *netB, cpb2* and *tpeL* nor the copy number of *netB* genes in *C. perfringens* is correlated with clinical NE. The qPCR should be used to detect the presence of *netB* gene instead of the conventional PCR.

Key Words: NE, Clostridium, toxin, qPCR, netB

M43 The effectiveness of lauric acid and Akkermansia muciniphila against necrotic enteritis in chickens Chinling Wang*, Hsin-Yi Lu, Wei-Yun Yang, Yue-Jia Lee, Scott Branton *Mississippi State University*

Necrotic enteritis (NE) caused by *Clostridium perfringens* (CP) is a multifaceted disease and requires many predisposing factors to facilitate the development of the disease. *C. perfringens* grows favorably in the alkaline and mucin-rich intestinal conditions. *Akkermansia muciniphila* (AM), a mucin-degrading anaerobe and a probiotic supplement, has anti-inflammatory and the improvement of gut integrity effects. Therefore, the objective of this study was to test if lauric acid or *A. muciniphila* can protect chickens against *C. perfringens challenge*. Chickens were divided to nine groups, CP, CP+ *Eimeria*, CP+*Eimeria*+ lauric acid, CP+*Eimeria*+AM, CP+AM, *Eimeria*+AM, AM, *Eimeria* or placebo. Results showed that the NE lesion score in lauric acid or AM treatment group was not significantly different from the CP or the CP+*Eimeria* challenged group. Our qPCR assay also confirmed that chickens with necrotic enteritis lesions had significantly higher numbers of CP in the jejunum, ileum and cecum than the CP challenged birds without NE lesions. In conclusion, neither lauric acid nor *A. muciniphila* supplement prevents chickens against the CP challenge. Interestingly, the composition of gut microbiota seems to influence the development of NE lesions in chickens.

Key Words: Clostridium, enteritis, acid, chickens, probiotics

M44 Effect of zinc sources and doses on the presence of Necrotic Enteritis in broilers caused by C. perfringens Greg Mathis*, Brett Lumpkins, Agathe Romeo, Stephane Durosoy *Southern Poultry Research, Inc.*

Clostridium perfringens-induced Necrotic Enteritis (NE) has become a great concern to the poultry industry, which has resulted in a significant decrease in growth performance, poor feed conversion, and increased mortality. A trial was conducted with 72 cages starting with 8 chicks each, which was divided into 3 groups of 24 cages: one group fed with 80 ppm of Zn from zinc sulfate ($ZnSO_4$) and the others with Zn from potentiated zinc oxide (ZnO) source (Hizox®, Animine), at 80 ppm or at 120 ppm. Then, each group was subdivided into 3 groups of 8 cages: non-challenged birds, challenged birds with non-medicated feed and challenged birds with virginiamycin at 20 ppm. A randomized block design with 8 replications of 8 chicks per cage was used, and $P<0.05$ was used to determine the level of significance. All diets were fed experimental diets *ad libitum* throughout the study. On D14, all the broilers were orally inoculated with a low dose of *E. maxima*. On days 19, 20 and 21, the birds in the challenged groups were orally dosed with *C. perfringens* ~10^8 cfu/mL. Birds and feed were weighed by cage on D0, 14, 21, and 28, and performance parameters were measured. On D21, 3 birds from each cage were sacrificed and examined and scored on the degree of severity of necrotic enteritis lesions, from 0 (normal) to 3 (sloughed and blood small intestine mucosa). The NE model was successful in producing a heavy NE infection. The therapeutic level of the antibiotic Virginiamycin (VIR) improved the performance of all of the challenged birds. Challenged birds fed either level of the potentiated ZnO had improved growth performance compared to ZnSO4 fed birds. The 120 ppm level had the most significant feed conversion ratio and lowest NE mortality of the challenged non-antibiotic groups. These results emphasize the benefits of a potentiated ZnO source in reducing Necrotic Enteritis compared to $ZnSO_4$.

Key Words: Enteritis, zinc, broilers

M45 Characteristics of Gallibacterium anatis isolated from poultry samples Jessica Hockaday*[GS], Alejandro Banda, Jay Kay Thornton, Lifang Yan, Martha Pulido-Landínez *Mississippi State University CVM Poultry Research and Diagnostic Laboratory*

Over the last decade the Poultry Research and Diagnostic laboratory (PRDL) has seen an increase in clinical cases of *Gallibacterium anatis*. *G. anatis* is a naturally occurring commensal bacterial of the upper respiratory system in poultry that has been shown to have pathogenic effects in many avian species. Clinical presentations associated with *G. anatis* include upper respiratory signs, decreased egg production, salpingitis, peritonitis, oophoritis, and airsacculitis and has been isolated in broilers, broiler breeder and commercial layer flocks. Field strains of *G. anatis* collected throughout the Southern US in 2016 and 2017 were evaluated for this study. Samples from layers, broiler breeders, and broiler chickens were cultured, microbial sensitivities performed along with molecular genotyping and phylogenetic analysis. As an emerging pathogen the understanding of appropriate techniques to isolated and further evaluate *G. anatis* is useful to many aspects of poultry medicine and diagnostics.

Key Words: Gallibacterium anatis, Field samples, Chickens