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In vitro and genomic mining studies of anti-*Clostridium perfringens* Compounds Derived from *Bacillus amyloliquefaciens*

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ABSTRACT *Clostridium perfringens* is an important opportunistic microorganism in commercial poultry production that is implicated in necrotic enteritis (NE) outbreaks. This disease poses a severe financial burden on the global poultry industry, causing estimated annual losses of $6 billion globally. The ban on in-feed antibiotic growth promoters has spurred investigations into approaches of alternatives to antibiotics, among which *Bacillus* probiotics have demonstrated varying degrees of effectiveness against NE. However, the precise mechanisms underlying *Bacillus*-mediated beneficial effects on host responses in NE remain to be further elucidated. In this manuscript, we conducted *in vitro* and genomic mining analysis to investigate anti-*C. perfringens* activity observed in the supernatants derived from 2 *Bacillus amyloliquefaciens* strains (FS1092 and BaD747). Both strains demonstrated potent anti-*C. perfringens* activities in *in vitro* studies. An analysis of genomes from 15 *B. amyloliquefaciens*, 11 *B. velezensis,* and 2 *B. subtilis* strains has revealed an intriguing clustering pattern among strains known to possess anti-*C. perfringens* activities. Furthermore, our investigation has identified 7 potential antimicrobial compounds, predicted as secondary metabolites through antiSMASH genomic mining within the published genomes of *B. amyloliquefaciens* species. Based on *in vitro* analysis, BaD747 may have the potential as a probiotic in the control of NE. These findings not only enhance our understanding of *B. amyloliquefaciens*'s action against *C. perfringens* but also provide a scientific rationale for the development of novel antimicrobial therapeutic agents against NE.

Key words: Bacillus amyloliquefaciens, Clostridium perfringens, inhibition, antimicrobial compound

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INTRODUCTION

Necrotic enteritis (NE) is a debilitating disease in broiler chickens, manifesting in both clinical (high mortality) and subclinical (poor performance in growth and higher feed conversion) forms (Lee and Lillehoj 2016, Tsiouris 2016). *Clostridium perfringens* is an important opportunistic pathogen implicated in NE outbreaks in commercial poultry production (Lee and Lillehoj 2021). NE ranks as one of the most economically devastating bacterial enteric ailments, causing annual losses exceeding 6 billion US dollars for the global poultry industry (Wade and Keyburn 2015). NE is a multifactorial disease, necessitating several predisposing and cofactors for an outbreak, including co-infection with coccidiosis, dietary factors (the use of fishmeal and cereal-based diets may lead to increased digesta viscosity and intestinal mucus), immunosuppression, and poor management practices such as high stocking density, ammonia exposure, and heat stress (Prescott et al. 2016). Additionally, the presence of the critical NE B-like toxin (*netB*) gene in *C. perfringens* Type G strains is a key contributing factor (Timbermontet et al. 2009, Prescott et al. 2016). The rise in NE incidence is closely linked to the voluntary reduction or removal of antibiotic growth promoters from feed (Cooper and Songer 2009). Therefore, exploring alternatives to antibiotics is paramount to reducing the growing NE problem.

Extensive efforts have been directed toward developing antibiotic alternatives to safeguard poultry health

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and performance (Gadde et al. 2017a). These alternatives include probiotics, prebiotics, synbiotics, organic acids, enzymes, antimicrobial peptides, hyperimmune egg antibodies, bacteriophages, clay, and metals. Among them, *Bacillus*-based direct-fed microbials have gained prominence in maintaining or restoring the intestinal health of poultry with increasing regulation since the ban on antimicrobial growth promoters. This is mainly owing to their capability to confer gut health benefits and survive the rigorous conditions during chicken feed preparation (Gadde et al. 2017b, Grant et al. 2018, Khalique et al. 2020). These probiotics exert their effects by outcompeting pathogenic bacteria for nutrients, producing natural antimicrobial peptide compounds, and modulating the gut microbiota to promote beneficial microorganisms in the gastrointestinal tract, thus contributing to various immunological benefits (Neveling and Dicks 2021).

Bacillus amyloliquefaciens (Ba), a gram-positive bacterium found in soil and formerly categorized as *B. subtilis* subvariant, is commonly employed as a biocontrol agent for enhancing plant growth and controlling plant diseases (Zhang et al. 2022a). In recent years, it has garnered much attention as a potential probiotic in food animal agriculture, including chickens (Latorre et al. 2015, de Oliveira et al. 2019, Shini et al. 2020, Zhang et al. 2022b). In chickens, the gut microbiome plays a pivotal role in overall health and productivity, in which an imbalance may lead to various health issues, including digestive problems, reduced nutrient absorption, and heightened susceptibility to diseases. *B. amyloliquefaciens* has been demonstrated to effectively ameliorate subclinical NE, thereby enhancing gut health by increasing gut microbiota diversity and reducing the abundance of harmful bacteria (Zhang et al. 2022a). Additionally, it has been shown to improve nutrient absorption, leading to enhanced feed efficiency and overall performance (Latorre et al. 2015, de Oliveira et al. 2019, Shini et al. 2020).

However, the precise mechanisms by which *B. amyloliquefaciens* promotes performance remain to be further defined. This study aims to predict antimicrobial secondary metabolites or their corresponding peptides derived from *B. amyloliquefaciens* strains by comprehensive genomic analysis and test their *in vitro* activities of antimicrobial compounds that can inhibit the growth of pathogenic *C. perfringens*.

MATERIALS AND METHODS

Bacillus Bacteria and Cultures

B. amyloliquefaciens strain FS1092 and D747 (EMFSL lab, ARS, USDA, Beltsville, MD, USA), along with *Bacillus subtilis* 168 (ATCC, Manassas, VA), were cultivated in Tryptic Soy Broth media (TSB, Sigma-Aldrich, St. Louis, MO) at 28°C. *C. perfringens* strains Del1 and LLY_TpeL17 were initially isolated from NE-afflicted chicken farms (Li et al. 2017, Gu et al. 2019). The Del1 and LLY_TpeL17 strain stocks were cultured in Tryptose Sulfite Cycloserine medium (TSC, Perfringens Agar Base, Oxoid, Nepean, Ontario, Canada) with *C. perfringens* selective supplement (D-cycloserine 0.4 mg/mL, Oxoid). Subsequently, the *C. perfringens* strains were anaerobically grown at 37°C in chopped meat glucose (CMG) medium (Anaerobe Systems, Morgan Hill, CA), followed by BYC medium [(3.7% brain heart infusion medium (BD Bacto, Sparks, MD), 0.5% yeast extract (Fisher Scientific, Hampton, NH), 0.05% L-cysteine (Sigma-Aldrich, St. Louis, MO)]. The complete genome sequences for strain FS1092 are available from a previous study (Gonzalez-Escalona et al., 2020), but the genome sequence for strain Ba D747 is unpublished.

Inhibition of C. perfringens *Growth by* B. amyloliquefaciens *Cell-Free Supernatant in Liquid Culture and Well Diffusion Assay*

The cell-free supernatants from *B. amyloliquefaciens* and *B. subtilis* cultures were acquired by centrifugation for 5 min at 6,000 x *g* after culturing 24, 48, and 72 h, followed by filtration through a 0.2-micron pore-size filter (Millipore, St. Louis, MO). The supernatants were stored at 4°C until use.

To determine if the cell-free supernatant from the above bacteria could inhibit the *C. perfringens* growth, the overnight culture from *C. perfringens* Del1 and LLY-TpeL17 strains were diluted in BYC broth, and the cell-free culture supernatant from Ba D747 or TSB broth was added into freshly diluted bacterial culture in 1:10 ratio, and cultivated overnight anaerobically. The optical density at 590 nm (OD590) values was recorded for the bacteria growth, and inhibition capability by Ba D747 was determined by comparison with the TSB control.

To assess the antimicrobial activity of *B. amyloliquefaciens* cell-free supernatant against *C. perfringens* strains and optimal collection times of cell-free supernatant, the agar well diffusion method was employed. TSC broth with 1.5% agar (20 mL) was poured into a sterile round petri dish (90 mm diameter, Biologix Inc, Lenexa, Kansas, MO). After agar solidification, 10 mL of autoclaved TSC agar mixed with 50 μL overnight BYC cultures of *C. perfringens* Del1 and LLY-TpeL17 was poured on the previous agar layer. Wells were created using pipettor tips of 6-mm diameter, and each well was loaded with 100 μL of cell-free supernatant from each *B. amyloliquefaciens* isolates Ba D747, FS1092 or Bs 168, or supernatant collected at 3 different time points. The plates were refrigerated at 4°C for 3 to 4 h to allow the supernatant to be absorbed by the agar. Each test was performed in triplicates. Subsequently, the plates were anaerobically incubated at 37°C overnight. Clear inhibition zones devoid of *C. perfringens* growth around the sample-dropping wells were observed against the background of full bacterial growth on TSC agar plates, and the diameters of these zones were measured. Once the optimal collection time of supernatant was determined,

such supernatant would be used for further inhibition testing from different bacterial sources.

Computational Genomics and Gene Analysis of B. amyloliquefaciens, Bacillus velezensis, *and* B. subtilis *strains*

Detailed genome identification and sequence analysis information for *B. amyloliquefaciens*, related *B. velezensis,* and *B. subtilis* strains is shown in Table 1. Genomic data for the study were retrieved from GenBank. A phylogenetic tree was constructed based on the similarities and differences in DNA, projected RNA, or protein sequences among these organisms. This tree serves as a visual representation of the evolutionary relationships among different species or taxonomic groups (Pearson et al. 2009). To examine the patterns of gene presence and absence across the 14 *B. amyloliquefaciens* strains, we employed the Roary tool, known for its efficiency in constructing comprehensive pan genomes from prokaryotic samples and delineating both core and accessory gene sets (Page et al. 2015, Costa et al. 2020). In this procedure, the genomes of each of the 28 strains listed in Table 1 underwent initial annotation using the Prokka procedure (Seemann 2014). Subsequently, Roary was employed to generate a pan-genome from the gff files produced by Prokka. To establish a core gene alignment, MAFFT with specific options was utilized (Katoh and Standley 2013). Ultimately, the "gene_presence_absence.Rtab" and "accessory_ binary_ genes. fa. newick" output files served as the basis for assessing presence-absence patterns and conducting phylogenetic tree analyses across all strains. Prediction of the secondary/specialized metabolite biosynthetic gene clusters (SM BGCs) was performed using the program "antibiotics and secondary metabolite analysis shell—antiSMASH" in microbial genome mining tasks (Blin et al., 2023).

Table 1. Genomes of *B. amyloliquefaciens* and closely related species strains used for gene mining in this study.

Bacterial species	Strain	Accession#
Bacillus amyloliquefaciens	B15	CP130445.1
	B25	CP065159.1
	BA11	CP071042.1
	BA40	CP018152.1
	C6.7	LN999829.1
	CAU B946	CP079834.1
	D747	Unpublished
	EA19	HE617159.1
	ELA1901024	CP075547.1
	FS1092	JALMGL010000001.1
	GXU-1	JALMGM010000001.1
	H57	LMUC01000001-LMUC01000016
	LM2303	CP038028
	SN16-1	CP021505.1
	SRCM101367	CP014783.1
Bacillus velezensis	19573-3	CP067043.1
	BV5	ASM2453958v1
	BZR 277	CP064845
	BZR 86	CP064846
	DTU001	CP035533.1
	Hx05	CP040672.1
	LF01	CP058216
	M75	CP016395.1
	SRCM101368	CP031694
	WRN014	CP041361.1
	ZL918	CP021338.1
Bacillus subtilis	CP009684.1	168
	AL009126.3	B-1

Statistical Analysis

The OD590 values of bacterial cultures or the diameters of the inhibition zones were subjected to analysis using the GLM procedure of SAS v9.4 for Windows (Cary, NC). Statistically significant differences were defined at $p \leq 0.05$, and all data were presented as mean ± standard deviation for each treatment.

RESULTS

Inhibition of C. perfringens *Growth*

This study aimed to investigate whether these *B. amyloliquefaciens* PS1092 and D747 strains could also suppress the growth of the very pathogenic *C. perfringens* strains, isolated from the NE-afflicted chicken gut. When *B. amyloliquefaciens* D747 supernatant was added to the diluted overnight *C. perfringens* culture at a 1:10 dilution, it exhibited robust inhibition activity against *C. perfringens* LLY_TpeL17 strain, with highly significant differences ($P \leq 0.001$) (Figure 1A).

Next, we determined the optimal time for collecting supernatants from bacterial cultures. Figure 1B illustrates the inhibition zones of Ba D747 cell-free supernatant on the growth of the *C. perfringens* LLY_TpeL17 strain. The diameters of the clear inhibition zones were measured for the supernatants collected from 24, 48, and 72-h cultures, resulting in measurements of 14.5, 18.5, and 19.5 mm, respectively. Notably, the diameter of the inhibition zone for the supernatant collected at the 48-h (18.5 mm) nearly approached that of the 72-h collection (19.5 mm), but was significantly larger than the 24-h collection (14.5 mm). Consequently, the 48-h culture supernatant was selected for our subsequent inhibition studies.

Interestingly, *C. perfringens* colonies initially appeared black on TSC agar with selective supplement (observed at 14-h culture), but their color gradually faded during extended incubation periods (observed at 26-h culture, as shown in Figure 1B).

Figure 1C demonstrates the inhibition zones produced by supernatants from 3 different bacterial cultures on the growth of *C. perfringens*. Ba D747 exhibited superior anti-*C. perfringens* activity compared to FS1092, displaying significant differences ($p \leq 0.01$) as evidenced by the larger inhibition zone diameter against both *C. perfringens* Del1 and LLY_TpeL17 strains. In contrast, the supernatant from Bs168 did not show inhibition ability, as no clear visible inhibition zone was observed (Figures 1C and 1D).

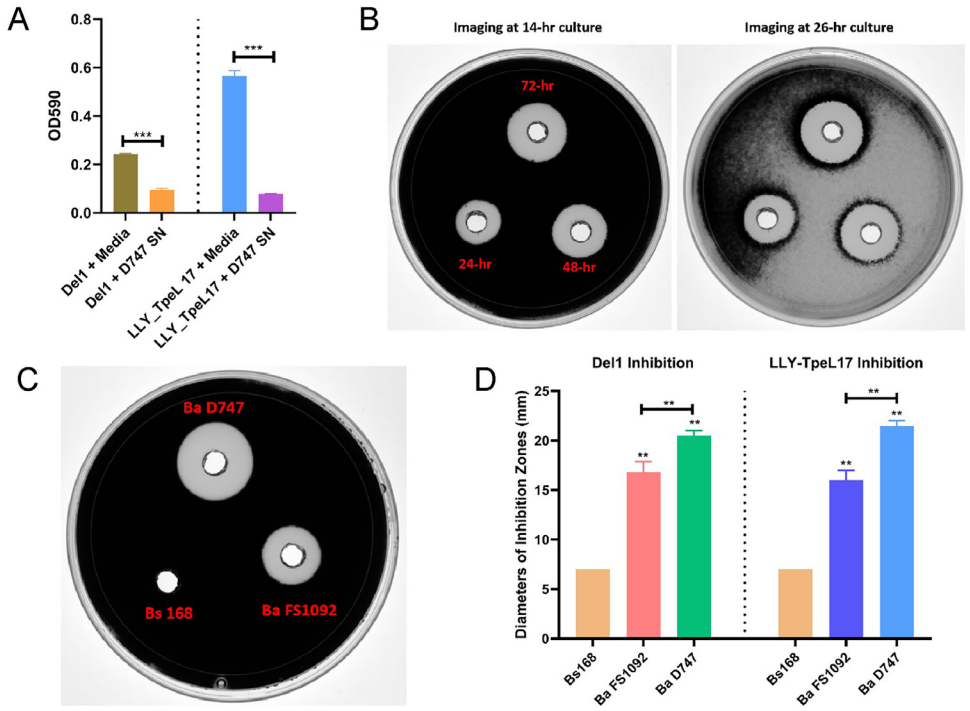

Figure 1. The growth inhibition of *Clostridium perfringens* by cell-free supernatant from *Bacillus amyloliquefaciens* D747 in liquid culture (1A), collected at 3 times of points by *Bacillus amyloliquefaciens* strain D747 (1B) or by culture supernatant from various sources (1C, 1D) in agar well diffusion assay. In 1A, the cell-free supernatants from Ba D747 24-h culture were added into *C. perfringens* cultures of Del1 and LLY_TpeL17 (TpeL17) strains, and OD590 was measured after the overnight culturing anaerobically. In 1b, the *C. perfringens* LLY_TpeL17 strain was seeded in the TSC agar plate, and the cell-free supernatant collected from 3-time points of cultures from *B. amyloliquefaciens* D747 was then loaded on the plate well, and images were taken after 14-h and 26-h culture. In 1C, 3 culture supernatant samples from *B. subtilis* 168 strain, *B. amyloliquefaciens* D747, and FS1092 strains were loaded on the wells of *C. perfringens* LLY_TpeL17-*seed* TSC agar plate. The inhibition zone diameters were measured and analyzed for both *C. perfringens* Del1 and LLY_TpeL17 strains (1D). The horizon line represents the base level of the empty well diameter. All the data were expressed as mean ± standard deviation for each treatment. The symbols of ** and *** represent statistical differences at $P \leq 0.01$, and $P \leq 0.001$, respectively.

Genomic Analysis of B. amyloliquefaciens, Bacillus velezensis *and* B. subtilis *Strains*

Given the robust anti-*C. perfringens* activity displayed by these 2 *B. amyloliquefaciens* strains, it becomes intriguing to delve into the potential gene clusters responsible for the biosynthesis of antimicrobial substances they may generate. The genomic information is publicly available for Ba FS1092 (Genbank Accession#: CP038028), while it is unpublished for Ba D747. Consequently, the genome of Ba FS1092 was mainly utilized as a reference to perform sequence similarity searches against available complete genome sequences of other *B. amyloliquefaciens* strains and closely related species in Genbank. In total, 27 complete genomes were analyzed within this study, comprising 14 *B. amyloliquefaciens*, 11 *B. velezensis,* and 2 *B. subtilis* strains.

The resulting phylogenetic tree for *B. amyloliquefaciens* strains revealed that FS1092 clustered with other Ba strains known for their published anti-*C. perfringens* activities, such as Ba40 and H57 (see Figure 2). Figure 2 also illustrates the Roary matrix for *B. amyloliquefaciens* strains, a tool used for constructing large-scale pan-genomes from prokaryotic samples by identifying both core and accessory genes. Notably, Ba FS1092 may possess some unique genes, as indicated in Table 2 (also refer to Supplementary data). Among these genes, *sdpC* was also found to belong to a cluster of genes in *B. subtilis* responsible for encoding a peptide toxin known as SDP. The SDP toxin appeared to induce autolysis by disrupting the proton motive force during the early stages of sporulation (Lamsa et al. 2012).

Microorganisms possess the capability to synthesize small bioactive compounds as part of their secondary metabolism, which serve critical roles in various bioactivities, including their applications in medicine and agriculture, particularly in antimicrobial contexts. To facilitate the identification of natural product biosynthetic pathways and the prediction of these bioactive metabolites, scientists have developed specialized tools like the antiSMASH software. This software enables the exploration of microbial genomes through genome sequencing and mining, with a particular focus on secondary/specialized metabolite biosynthetic gene clusters (SM BGCs) (Blin et al. 2023). Table 3 provides a comprehensive list of antimicrobial compounds predicted through the genome mining of SM BGCs using the antiSMASH tool. In general, these analyses indicate that most *B. amyloliquefaciences* strains are capable of producing 7 antimicrobial compounds, namely Bacillaene, Bacillibactin, Bacilysin, Deficidin, Fengycin, Macrolactin H (with a high degree of similarity, close to 100%), and Surfactin (with similarity ranging from 78% to 86%). The pilot study of recent genome sequencing of Ba D747 indicated that it may produce an additional antimicrobial compound Thermoactinoamide. On the

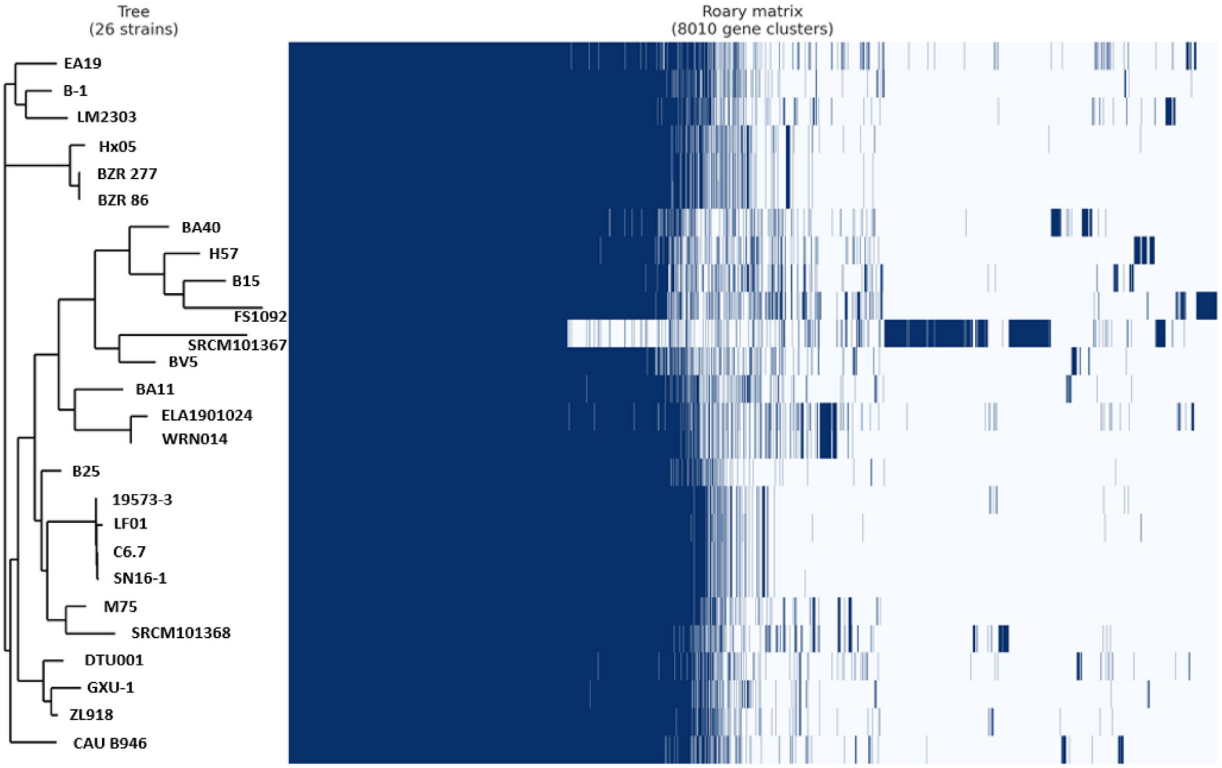

Figure 2. Pangenome analysis for 26 Bacillus genomes (Table 1) using Roary. The left side represents evolutionary relationships among 26 strains based on their core genomes. The right side represents the matrix where conserved core genes and a variable set of accessory genes were either present or absent.

Table 2. Unique genes among 14 *Bacillus amyloliquefaciens* strains.

Genes	Annotation	B15	BA40	FS1092	H57	SRCM101267	B25	B946	BA11	C6.7	EA19	ELA1901024	GXU-1	LM2303	SN16-1
thrZ	Threonine−tRNA ligase 2	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
sdpC	Sporulation delaying protein C	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N
group_1017	hypothetical protein	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N
nasA	Nitrate transporter	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
nasB	Assimilatory nitrate reductase electron transfer subunit	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
nasC	Assimilatory nitrate reductase catalytic subunit	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
group_1758	Tryptophan RNA-binding attenuator protein inhibitory protein	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
group_1847	hypothetical protein	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N
group_1848	HTH-type transcriptional regulatory protein GabR	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
group_2020	hypothetical protein	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
norG_1	HTH-type transcriptional regulator NorG	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
argO	Arginine exporter protein ArgO	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
group_2017	hypothetical protein	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
group_1943	hypothetical protein	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
group_272	hypothetical protein	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N
group_3429	hypothetical protein	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N
group_1163	Thiol-disulfide oxidoreductase YkuV	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N

Y, presence; N, absence.

Table 3. Secondary metabolites predicted by the antiSMASH database.

Bacterial strain	Accession#	Antibiotics produced									
		Bacillaene	*Bacillibactin*	*Bacilysin*	*Difficidin*	*Fengycin*	*Macrolactin H*	*Plantazolicin*	*Surfactin*	*Subtilin*	*Thermoactinoamide*
Ba B15	CP014783.1	100%	100%	100%	100%	100%	100%		86%		
Ba B25	LN999829.1	100%	100%	100%	100%	100%	100%		78%		
Ba BA11	JALMGL010000001.1	100%				80%	100%				
Ba BA40	JALMGM010000001.1	100%	100%	100%	46%	80%	100%		82%		
Ba C6.7	CP130445.1	100%	100%		100%	100%	100%	100%	86%		
Ba CAU B946	HE617159.1	100%	100%	100%	93%	100%	100%	83%	82%		
Ba D747	Unpublished	100%	100%	100%	53%	86%	90%		43%		100%
Ba EA19	CP079834.1	100%	100%	100%	93%	100%	100%	100%	82%		
Ba ELA1901024	CP071042.1	100%	100%	100%	100%	100%	100%		82%		
Ba FS1092	CP038028		100%	100%	100%	100%	100%		82%	100%	
Ba GXU-1	CP065159.1	100%	100%	100%	100%	100%	100%	100%	82%		
Ba H57	LMUC01000001-LMUC01000016	100%	100%	100%	100%	86%	100%		82%		
Ba LM2303	CP018152.1	100%	100%	100%	100%	100%	100%	91%	82%		
Ba SN16-1	CP075547.1	100%	100%	100%	100%	100%	100%	100%	82%		
Ba SRCM101367	CP021505.1	100%	100%	100%		93%			82%		
Bs 168	AL009126.3	100%	100%	100%		100%			82%		

Bs 168 also produces pulcherriminic acid, sporulation killing factor, sublancin 168, and subtilosin A (100% Similarity).
Ba BA40 also produces Amylocyclicin and Mersacidin (100% Similarity).
Ba EA19 also produces Bacillothiazol (100% similarity).
Ba D747 also produces Plipastatin (38% similarity).

other hand, Plantazolicin is predicted to be produced by fewer than half of the Ba strains. In comparison, the commonly studied *B. subtilis* 168 strain, often employed in protease-deficient mutant research, is predicted to produce Bacillaene, Bacillibactin, Bacilysin, Deficidin, Fengycin, and Surfactin.

DISCUSSION

Probiotics offer an effective alternative to antibiotics by nurturing beneficial bacteria in the gastrointestinal tract. They promote nutrient absorption, bolster production traits, and fortify immunity (El-Hack et al. 2020). In this study, we investigated the inhibitory effects of *B. amyloliquefaciens* strains, on pathogenic *C. perfringens* Del1 and LLY-TpeL17 strains, known for carrying the *netB* gene. This gene encodes a pivotal virulence factor, a pore-forming toxin, which contributes to the pathogenesis of necrotic enteritis in broiler chickens (Keyburn et al. 2010). The agar well diffusion study unveiled that culture supernatants from both Ba D747 and FS1092 strains effectively curtailed the growth of *C. perfringens* Del1 and LLY-TpeL17 strains, with Ba D747 demonstrating superior inhibitory activity. It is worth noting that very pathogenic LLY_TpeL17 harbors not only the *netB* gene but also the *tpeL* gene which encodes a large clostridial cytotoxin that is linked to exacerbating the severity of necrotic enteritis in chickens (Coursodon et al. 2012, Prescott et al. 2016).

Since genomic information for Ba D747 was unpublished yet, we resorted to a BLAST alignment analysis that placed D747 and FS1092 in proximity to other Ba strains with documented *C. perfringens* inhibitory activities in the phylogenetic tree. These strains include H57, BA11, and BA40. In genomic mining analysis, Ba FS1092 was predicted as a potential producer of a range of secondary antimicrobial compounds, including Subtilin. Subtilin, initially isolated from *B. subtilis*, is a neutral metalloprotease and an alkaline serine protease with a preference for targeting gram-positive microorganisms (Stein et al. 2005).

Antimicrobial compounds are substances that can kill or inhibit the growth of specific microorganisms, including bacteria, viruses, fungi, and parasites. These compounds demonstrate broad applications in the treatment of infectious diseases and food preservation. In our study, the majority of *B. amyloliquefaciens* strains produced secondary antimicrobial compounds such as Bacillaene, Bacillibactin, Bacilysin, Deficidin, Fengycin, Macrolactin H, and Surfactin. Each of these compounds may potentially suppress the growth of *C. perfringens* at varying degrees. For instance, Bacillaene, originally isolated from *B. subtilis*, inhibits prokaryotic protein synthesis through mechanisms that remain unclear (Rabbee and Baek, 2020). Bacillibactin, a catecholic iron siderophore, plays a pivotal role in facilitating Fe(III) acquisition and is suggested to passively inhibit microbial pathogens (Li et al. 2014; Rabbee and Baek, 2020). Bacilysin acts as an antibiotic that relies on

peptide transporters for entry into target cells, disrupting the biosynthetic pathway of bacterial peptidoglycan or fungal mannoprotein (Rabbee and Baek, 2020). Fengycin, composed of a cyclic octapeptide, is believed to induce cell death in the target organism by compromising cell membrane integrity and altering cell permeability (Rabbee and Baek, 2020). Macrolactin H, a product of polyketide biosynthesis, inhibits bacterial peptide deformylase (Schneider et al. 2007). Deficidin, originally isolated from *Bacillus subtilis*, possesses potent *in vitro* antibacterial activity by affecting the cell wall (Zimmerman et al. 1987). Surfactin, characterized by its amphiphilic structure, functions as a biosurfactant molecule with antimicrobial activity by damaging bacterial cell membranes (Rabbee and Baek, 2020). The additional compound Thermoactinoamide projected to be produced by Ba D747 is a new cyclic hexapeptide, originally extracted from the thermophilic bacterium *Thermoactinomyces vulgaris* strain ISCAR 2354 in Iceland, which can inhibit the growth of Staphylococcus aureus ATCC 6538 (Teta et al. 2017). The lower levels of similarities to some antimicrobial compounds for Ba D747 may result from the partial genome sequencing of this strain with the Illumina sequencing approach.

B. subtilis 168 exhibited no inhibitory effect on *C. perfringens*, in contrast to Ba FS1092 and D747. A comparison of the antimicrobial compounds produced by *B. subtilis* 168 and FS1092 revealed 4 shared (Bacillibactin, Bacilysin, Fengycin, Surfactin) and 2 unique (Deficidin and Macrolactin H) compounds for *B. amyloliquefaciens* FS1092. The presence of Deficidin and Macrolactin H may play pivotal roles in Ba FS1092′s inhibitory activities against gram-positive *C. perfringens* strains, although other factors or variations in compound concentration might also contribute to differences in *C. perfringens* inhibition.

Apart from the production of antimicrobial compounds, *B. amyloliquefaciens* could outcompete *C. perfringens* for nutrients and other resources within the gut environment. Studies involving murine models have demonstrated that the Ba40 strain was able to outperform *C. perfringens* by adhering to gut epithelium and proliferating (Zhao et al. 2016, Jiang et al. 2022). Additionally, *B. amyloliquefaciens* stimulates the host's immune response against *C. perfringens*, reducing the release of pro-inflammatory cytokines (Zhao et al. 2016, Jiang et al. 2022). By producing immunomodulatory compounds such as exopolysaccharides, *B. amyloliquefaciens* activates the host's immune system, enhancing its resistance to bacterial infections (Sung et al. 2022). In essence, the inhibitory effects of *B. amyloliquefaciens* on *C. perfringens* could result from a combination of direct and indirect mechanisms, working synergistically to curtail *C. perfringens* colonization and proliferation in the gut, thereby promoting gut health in chickens.

Interestingly, the inhibitory activity from Ba D747 bacterial culture against pathogenic CP $netB^{+}tpeL^{+}$ LLY-TpeL17 strain was influenced by both culture temperature (28°C and 42°C) and shaking speed (80 rpm and 225 rpm) (data not shown). Supernatants collected from 48-h bacterial cultures at 28°C (225 rpm), and 42°C (80 rpm) demonstrated robust anti-CP activity, whereas those from 42°C (225 rpm) cultures did not. The mechanism remains unclear. Assumingly, the optimal growth temperature range for many *Bacillus* strains is around 30°C to 37°C. At temperatures below this range (such as 28°C), bacterial growth may slow down, allowing more time for the production and accumulation of inhibitory compounds in the culture supernatant. Conversely, at temperatures above the optimal range (such as 42°C) at full aeration (high speed 225 rpm), overgrowth may occur and the synthesis and secretion of inhibitory compounds may be downregulated in response to temperature and aeration changes, potentially reducing the overall production of inhibitory substances.

While these findings hold promise for the use of *B. amyloliquefaciens* as an inhibitory bioagent against *C. perfringens*, further research is imperative to evaluate its safety and efficacy *in vivo*. For example, in one study, administration of lyophilized vegetative *B. amyloliquefaciens* cells with feed did not demonstrate a significant protective effect against necrotic enteritis in an extremely severe experimental broiler NE model, despite the clear *in vitro* inhibitory activity of Ba supernatant against *C. perfringens* strains (Geeraerts et al. 2016). One plausible explanation could be that the established infection model was exceptionally severe, as indicated by a lesion score exceeding 3.0 in the challenge control group. Under such circumstances, the beneficial effects of Ba may have been overshadowed by the severity of the infection. Currently, the lack of comprehensive genomic information on this Ba strain limits the prediction of antibiotic compound activity through genomic mining.

B. amyloliquefaciens also exhibits potential as a vaccine vector that delivers foreign antigens to the immune system and elicits a protective immune response. *B. amyloliquefaciens* possesses several attributes that make it an attractive candidate for vaccine vector development, including spore formation, resilience in challenging environmental conditions, and the ability to produce immunomodulatory compounds. Genetically modified protease-deficient strains of *B. amyloliquefaciens* were developed as a host of efficient and stable expression vectors (Wang et al. 2019). Extensive research is required to construct Ba-specific shuttle vectors and express key antigen targets, either in plasmid-based expression systems or on the spore surface display, targeting pathogenic *C. perfringens in vitro*. Subsequent safety and efficacy evaluations *in vivo* are essential steps in this endeavor.

While direct-fed microbials (DFM) producing antimicrobials may offer benefits in animal health and performance, it is essential to consider their potential implications for antimicrobial resistance, as conventional antibiotics growth promoters, in terms of indirect selective pressures on the microbial populations in the animal's gut, horizontal gene transfer, cross-resistance, and microbial ecological disruption. Some antimicrobial peptides (AMP), for example, Pediocin A produced by

Pediococcus pentaceus FBB61 and Sublacin produced by *B. subtilis* 168, have demonstrated antimicrobial activity against *C. perfringens* type A infection in poultry (Grilli et al. 2009; Wang et al. 2015). However, excessive exposure of pathogens to antimicrobial peptides may lead to the development of AMP-resistant strains (Abreu et al. 2023). Very little information is available on whether antimicrobial substances produced by DFMs generate antimicrobial resistance. Further research is needed to better understand these potential risks and to develop strategies to mitigate them while maximizing the benefits of using DFMs in food animal production.

In summary, our study highlights the potent anti-*C. perfringens* activity of 2 *B. amyloliquefaciens* strains, FS1092 and D747, as demonstrated in *in vitro* studies. Particularly, D747 exhibited superior activity compared to FS1092. Genomic mining analysis reveals that Ba strains with anti-*C. perfringens* activities tend to cluster together. Moreover, Ba strains are predicted to produce 7 major secondary antimicrobial metabolites with broad antimicrobial potential for applications in medicine and agriculture. Based on *in vitro* analysis, BaD747 may have the potential as a probiotic in the control of NE. While further research is required to fully understand the potential benefits of *B. amyloliquefaciens* for animal health, these initial findings highlight these bacterial strains as promising probiotics with a wide range of potential advantages.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

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REFERENCES

Abreu, R., T. Semedo-Lemsaddek, E. Cunha, L. Tavares, and M. Oliveira. 2023. Antimicrobial drug resistance in poultry production: current status and innovative strategies for bacterial control. Microorganisms. 11:953.

Blin, K., S. Shaw, H. E. Augustijn, Z. L. Reitz, F. Biermann, M. Alanjary, A. Fetter, B. R. Terlouw, W. W. Metcalf, E. J. N Helfrich, G. P. van Wezel, M. H. Medema, and T. Weber. 2023. AntiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualization. Nucleic Acids Res. 51(W1):W46–W50.

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

Costa, S. S., L. C. Guimaraes, A. Silva, S. C. Soares, and R. A. Barauna. 2020. First steps in the analysis of prokaryotic pan-genomes. Bioinform Biol Insights 14:1177932220938064.

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

de Oliveira, M. J. K., N. K. Sakomura, J. C. de Paula Dorigam, K. Doranalli, L. Soares, and G. D. S. Viana. 2019. Bacillus amyloliquefaciens CECT 5940 alone or in combination with antibiotic growth promoters improves performance in broilers under enteric pathogen challenge. Poult Sci 98:4391–4400.

El-Hack, M. E. A., M. T. El-Saadony, M. E. Shafi, S. Y. A. Qattan, G. E. Batiha, A. F. Khafaga, A. E. Abdel-Moneim, and M. Alagawany. 2020. Probiotics in poultry feed: a comprehensive review. J. Anim. Physiol. Anim. Nutr. (Berl) 104:1835–1850.

Gadde, U., W. H. Kim, S. T. Oh, and H. S. Lillehoj. 2017. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. Anim Health Res Rev 18:26–45.

Gadde, U., S. Oh, Y. Lee, E. Davis, N. Zimmerman, T. Rehberger, and H. S. Lillehoj. 2017. The effects of direct-fed microbial supplementation, as an alternative to antibiotics, on growth performance, intestinal immune status, and epithelial barrier gene expression in broiler chickens. Probiotics Antimicrobial Proteins 9:397–405.

Geeraerts, S., E. Delezie, R. Ducatelle, F. Haesebrouck, B. Devreese, and F. Van Immerseel. 2016. Vegetative Bacillus amyloliquefaciens cells do not confer protection against necrotic enteritis in broilers despite high antibacterial activity of its supernatant against Clostridium perfringens in vitro. Br. Poult. Sci. 57:324–329.

Grant, A., C. G. Gay, and H. S. Lillehoj. 2018. Bacillus spp. as direct-fed microbial antibiotic alternatives to enhance growth, immunity, and gut health in poultry. Avian Pathol 47:339–351.

Grilli, E., M. Messina, E. Catelli, M. Morlacchini, and A. Piva. 2009. Pediocin a improves growth performance of broilers challenged with Clostridium perfringens. Poult. Sci 88:2152–2158.

Gu, C., H. S. Lillehoj, Z. Sun, Y. Lee, H. Zhao, Z. Xianyu, X. Yan, Y. Wang, S. Lin, and L. Liu. 2019. Characterization of virulent netB+/tpeL+ Clostridium perfringens strains from necrotic enteritis−affected broiler chicken farms. Avian Dis. 63:461–467.

Gu, G., N. Gonzalez-Escalona, J. Zheng, S. Bolten, Y. Luo, A. Mafiz, M. S. Leon, and X. Nou. 2020. Genome sequences of *Brevundimonas naejangsanensis* strain FS1091 and *Bacillus amyloliquefaciens* strain FS1092, isolated from a fresh-cut-produce-processing plant. Microbiol Resour Announc 9(4):e01448–19.

Jiang, Z., W. Su, M. Yang, W. Li, T. Gong, Y. Zhang, C. Wen, X. Wang, Y. Wang, M. Jin, and Z. Lu. 2022. Screening of bacteria inhibiting clostridium perfringens and assessment of their beneficial effects in vitro and in vivo with whole genome sequencing analysis. Microorganisms 10:2056.

Katoh, K., and D. M. Standley. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30:772–780.

Keyburn, A. L., T. L. Bannam, R. J. Moore, and J. I. Rood. 2010. NetB, a pore-forming toxin from necrotic enteritis strains of Clostridium perfringens. Toxins (Basel) 2:1913–1927.

Khalique, A., D. Zeng, M. Shoaib, H. Wang, X. Qing, D. S. Rajput, K. Pan, and X. Ni. 2020. Probiotics mitigating subclinical necrotic enteritis (SNE) as potential alternatives to antibiotics in poultry. AMB Express 10:50.

Lamsa, A., W. T. Liu, P. C. Dorrestein, and K. Pogliano. 2012. The Bacillus subtilis cannibalism toxin SDP collapses the proton motive force and induces autolysis. Mol Microbiol 84:486–500.

Latorre, J. D., X. Hernandez-Velasco, V. A. Kuttappan, R. E. Wolfenden, J. L. Vicente, A. D. Wolfenden, L. R. Bielke, O. F. Prado-Rebolledo, E. Morales, B. M. Hargis, and G. Tellez. 2015. Selection of Bacillus spp. for cellulase and xylanase production as direct-fed microbials to reduce digesta viscosity and Clostridium perfringens proliferation using an in vitro digestive model in different poultry diets. Front. Vet. Sci. 2:25.

Lee, K.-W., and H. S. Lillehoj. 2016. An update on direct-fed microbials in broiler chickens in post-antibiotic era. Anim. Prod. Sci. 57:1575–1581.

Lee, K.-W., and H. S. Lillehoj. 2021. Role of Clostridium perfringens necrotic enteritis B-like toxin in disease pathogenesis. Vaccines 10:61.

Li, B., Q. Li, Z. Xu, N. Zhang, Q. Shen, and R. Zhang. 2014. Responses of beneficial Bacillus amyloliquefaciens SQR9 to different soilborne fungal pathogens through the alteration of antifungal compounds production. Front. Microbiol 5:636.

Li, C., H. S. Lillehoj, U. D. Gadde, D. Ritter, and S. Oh. 2017. Characterization of Clostridium perfringens strains isolated from healthy and necrotic enteritis-afflicted broiler chickens. Avian Dis 61:178–185.

Neveling, D. P., and L. M. T. Dicks. 2021. Probiotics: an antibiotic replacement strategy for healthy broilers and productive rearing. Probiotics Antimicrob Proteins 13:1–11.

Page, A. J., C. A. Cummins, M. Hunt, V. K. Wong, S. Reuter, M. T. Holden, M. Fookes, D. Falush, J. A. Keane, and J. Parkhill. 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 31:3691–3693.

Pearson, T., R. T. Okinaka, J. T. Foster, and P. Keim. 2009. Phylogenetic understanding of clonal populations in an era of whole genome sequencing. Infect Genet Evol 9:1010–1019.

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

Rabbee, M. A., and K. H. Baek. 2020,. Antimicrobial activities of lipopeptides and polyketides of Bacillus velezensis for agricultural applications. Molecules 25:4973.

Schneider, K., X. H. Chen, J. Vater, P. Franke, G. Nicholson, R. Borriss, and R. D. Sussmuth. 2007. Macrolactin is the polyketide biosynthesis product of the pks2 cluster of Bacillus amyloliquefaciens FZB42. J. Nat. Prod. 70:1417–1423.

Seemann, T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069.

Shini, S., D. Zhang, R. C. Aland, X. Li, P. J. Dart, M. J. Callaghan, R. E. Speight, and W. L. Bryden. 2020. Probiotic Bacillus amyloliquefaciens H57 ameliorates subclinical necrotic enteritis in broiler chicks by maintaining intestinal mucosal integrity and improving feed efficiency. Poult. Sci. 99:4278–4293.

Stein, T., S. Heinzmann, S. Dusterhus, S. Borchert, and K. D. Entian. 2005. Expression and functional analysis of the subtilin immunity genes spaIFEG in the subtilin-sensitive host Bacillus subtilis MO1099. J. Bacteriol 187:822–828.

Sung, W. W., Y. Y. Lin, S. D. Huang, and H. L. Cheng. 2022. Exopolysaccharides of Bacillus amyloliquefaciens Amy-1 mitigate inflammation by inhibiting ERK1/2 and NF-kappaB pathways and activating p38/Nrf2 pathway. Int. J. Mol. Sci. 23:10237.

Teta, R., V. T. Marteinsson, A. Longeon, A. M. Klonowski, R. Groben, M. L. Bourguet-Kondracki, V. Costantino, and A. Mangoni. 2017. Thermoactinoamide A, an antibiotic lipophilic cyclopeptide from the icelandic thermophilic bacterium thermoactinomyces vulgaris. J. Nat. Prod. 80:2530–2535.

Timbermont, L., A. Lanckriet, A. R. Gholamiandehkordi, F. Pasmans, A. Martel, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2009. Origin of Clostridium perfringens isolates determines the ability to induce necrotic enteritis in broilers. Comp. Immunol. Microbiol. Infect. Dis. 32:503–512.

Tsiouris, V. 2016. Poultry management: a useful tool for the control of necrotic enteritis in poultry. Avian Pathol 45:323–325.

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

Wang, H., X. Zhang, J. Qiu, K. Wang, K. Meng, H. Luo, X. Su, R. Ma, H. Huang, and B. Yao. 2019. Development of Bacillus amyloliquefaciens as a high-level recombinant protein expression system. J. Ind. Microbiol Biotechnol 46:113–123.

Wang, S., X. Zeng, Q. Wang, J. Zhu, Q. Peng, C. Hou, P. Thacker, and S. Qiao. 2015. The Antimicrobial peptide sublancin ameliorates necrotic enteritis induced by Clostridium perfringens in broilers. Am. Soc. Anim. Sci. 93:4750–4760.

Zhang, H., Y. Zhou, H. Xu, C. Liang, and Z. Zhai. 2022. Bacillus amyloliquefaciens BLCC1-0238 alone or in combination with mannan-oligosaccharides alleviates subclinical necrotic enteritis in broilers. Probiotics Antimicrob. Proteins 14:158–168.

Zhang, Y., Y. Li, S. Liang, W. Zheng, X. Chen, J. Liu, and A. Wang. 2022. Study on the preparation and effect of tomato seedling disease biocontrol compound seed-coating agent. Life (Basel) 12.

Zhao, P. Y., H. L. Li, M. Mohammadi, and I. H. Kim. 2016. Effect of dietary lactulose supplementation on growth performance, nutrient digestibility, meat quality, relative organ weight, and excreta microflora in broilers. Poult. Sci. 95:84–89.

Zimmerman, S. B., C. D. Schwartz, R. L. Monaghan, B. A. Pelak, B. Weissberger, E. C. Gilfillan, S. Mochales, S. Hernandez, S. A. Currie, E. Tejera and, et al. 1987. Difficidin and oxydifficidin: novel broad spectrum antibacterial antibiotics produced by Bacillus subtilis. I. Production, taxonomy and antibacterial activity. J. Antibiot (Tokyo) 40:1677–1681.