Non-antibiotic proteins Colicins and Salmocins as novel antibacterial agents for control of foodborne pathogens *Escherichia coli* and *Salmonella enterica*

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List of abbreviations

Abbreviation	Meaning
aa	amino acid
ATCC	The American Type Culture Collection
AU	arbitrary units
bp	base pairs
BSA	bovine serum albumin
C-terminal	carboxy-terminal
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CE	crude extract
ColM	colicin M (example for abbreviation of all colicins)
CFU	colony forming units
CP	coat protein
CPMV	cowpea mosaic virus
Da	dalton, atomic mass unit
dH2O	deionized water
DEC	diarrheagenic *E. coli*
DMP	dry milk powder
DNA	desoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
dpi	days post inoculation
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures GmbH)
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FDA	The U.S. Food and Drug Administration
Fig.	figure
FW	fresh weight
gDNA	genomic DNA
g	gravitational constant
GFP	green fluorescent protein
GOI	gene of interest
GRAS	Generally Recognized as Safe
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HUS	hemolytic uremic syndrome
(i)	insert
kDa	kilo dalton
LB	left border (of T-DNA)
LB-Medium	Luria-Bertani medium
LF	left flank
LPS	lipopolysaccharide
MES	2-(N-morpholino) ethanesulfonic acid

MIC	minimal inhibitory concentration
MoClo	modular cloning
MP	movement protein
mRNA	messenger RNA
MS	mass spectrometry
MW	molecular weight
NCBI	The National Center for Biotechnology Information The U.S. National Library of Medicine
N-terminal	amino-terminal
nt	nucleotide
NTR	non-translated region
NTS	non-typhoidal *Salmonella*
OD_{600}	optical density at 600 nm
ON	overnight
ORF	open reading frame
PCR	polymerase chain reaction
PVX	potato virus X
PWD	post-weaning diarrhea
RB	right border (of T-DNA)
RBS	ribosome-binding-site
RF	right flank
RNA	ribonucleic acid
RP	recombinant protein
rpm	rounds per minute
rRNA	ribosomal RNA
RT	room temperature
s	second
SalE1b	salmocin E1b
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SD	standard deviation
STEC	Shiga toxin-producing *E. coli*
Taq	*Thermus aquaticus*
T-DNA	transfer DNA
Ti- plasmid	tumor inducing plasmid
TMV	tobacco mosaic virus
tRNA	transfer RNA
TSP	total soluble protein
TVCV	turnip vein clearing virus
U	unit
UPEC	uropathogenic *E. coli*
USDA	The United States Department of Agriculture
UTI	urinary tract infection
(v)	vector
vol.	volume
v/v	volume/volume

VTEC	vero toxin-producing *E. coli*
w/v	weight per volume
WHO	World Health Organization
wt	wild type

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1. Introduction

1.1 The antibiotic resistance crisis

In 1928 Alexander Fleming discovered penicillin produced by the fungus *Penicillium notatum*, which was the beginning of the modern era of antibiotics (Sengupta, Chattopadhyay, & Grossart, 2013).

Antibiotics are chemical substances produced by microorganisms that can kill other microorganisms (bactericidal) or inhibit their growth (bacteriostatic). These compounds have successfully been used to prevent and treat a wide variety of acute or chronic bacterial infections and are fundamental for modern medicine (CDC, 2013; Gould & Bal, 2013; Wright, 2014).

Antibiotics target essential bacterial processes: inhibiting synthesis- and metabolic pathways or disrupting cell wall and membrane structures (O'Connell et al., 2013). The activity spectrum can vary; while glycopeptide activity is limited to gram-positive organisms, ß-lactams are broad-spectrum antibacterials, targeting bacterial processes across many species (O'Connell et al., 2013).

Since their discovery, many new antibiotics, which are mainly natural products from microbial sources, have been therapeutically used. Of these, numerous have been chemically modified (semisynthesis) to broaden the antibiotic arsenal (Mitcheltree et al., 2021; Wright, 2016). Only 3 clinically used classes are solely derived from de novo chemical synthesis (O'Connell et al., 2013).

Resistance to an antibacterial can be defined as the continued growth of the microorganism despite the presence of cytotoxic concentrations of the antibacterial by resisting its growth inhibitory or killing activity (Verraes et al., 2013; Wright, 2007).

The evolutionary battle of antibiotic compounds and bacterial resistance by natural selection has been taking place for millions of years. Therefore, it is not surprising that clinically significant resistance has developed to every antibacterial ever used (natural and non-natural compounds), sometimes within years of their introduction (Aslam et al., 2018; Munita & Arias, 2016; O'Connell et al., 2013). This resistance development has been greatly accelerated by the careless and ubiquitous use of antibiotics in human and animal healthcare (Larsson & Flach, 2022; Sengupta et al., 2013).

Additionally, there has been a recent lack of new drug development, mainly due to the reduced financial incentives for pharma companies to invest in new products but also due to challenging regulatory requirements for approval (Aslam et al., 2018; Bartlett, Gilbert, & Spellberg, 2013;

Tacconelli et al., 2018). However, as the gap between the clinical need for new antibiotics and new drug discoveries becomes larger, there is an urgent need for new antimicrobials (Tacconelli et al., 2018).

Today, bacterial infections have once again become a threat, with only a few antibiotics of last resort remaining against multiresistant pathogens. In 2013, the CDC stated that humankind is now in a "post-antibiotic era" (CDC, 2013; Lushniak, 2014; Sengupta et al., 2013). In 2019, it was estimated bacterial antimicrobial resistance caused 1.27 million deaths, making it a leading global health issue, especially in the poorest countries (Murray et al., 2022). This number could reach 10 million by 2050 (Coates, Hu, Holt, & Yeh, 2020; Murray et al., 2022).

Many once susceptible multidrug-resistant (MDR) organisms have acquired drug resistance by a variety of mechanisms. These involve decreased antimicrobial permeability, the deactivation or removal of the drug, and the bypassing or alteration of the drug's target (Mor-Mur & Yuste, 2009; Munita & Arias, 2016; Wright, 2016). The formation of biofilms can also greatly decrease drug susceptibility (Aslam et al., 2018; Wright, 2016).

Mutations that lead to antibiotic resistance can arise by two main mechanisms: spontaneous random mutations that are beneficial for the survival of the microorganism or horizontal gene transfer (HGT), which spreads acquired resistance among different species of bacteria using, e.g., multi-resistance integrons, mobile plasmids or transposons (Aslam et al., 2018; Larsson & Flach, 2022; Munita & Arias, 2016; O'Connell et al., 2013; Read & Woods, 2014). Due to natural selection, resistant bacteria have a reproduction advantage in an environment where antimicrobials are present - outcompeting their antimicrobial-sensitive competitors (Melnyk, Wong, & Kassen, 2015).

1.2 Food-borne infections

1.2.1 The impact of food-borne infections

Foodborne diseases, also referred to as food poisoning, often result from the intake of food contaminated with pathogenic bacteria, viruses, or parasites.

Typical symptoms usually include diarrhea and vomiting, but depending on the pathogen, short-term complications can occur, which may involve bloody diarrhea and sepsis or long-term complications such as kidney disease or recurring intestinal inflammation (Scallan 2011 (Hoffmann, Maculloch, & Batz, 2015; Scallan et al., 2011).

In the year 2010, foodborne diseases caused an estimated 600 million illnesses worldwide, leading to 420,000 deaths. Foodborne diseases are becoming a growing burden for public health and global economies, causing a substantial amount of disability and mortality (WHO, 2017).

The USDA estimates that there are 48 million foodborne illnesses annually in the US, meaning that 1 in 6 people will suffer from a foodborne disease every year (Hoffmann et al., 2015).
For food poisoning from fresh meat, the most concerning pathogens are *Salmonella*, *Campylobacter,* and enterohaemorrhagic *E. coli* (including serotype O157:H7), which are often linked to large foodborne outbreaks (Sofos, 2008; Suo et al., 2010).
Concerning its death toll and economic impact, non-typhoidal *Salmonella* are the most significant foodborne pathogens. It is the main cause of hospitalization (35%) and deaths (28%) and is therefore also the pathogen causing the highest economic burden: ($3.7 billion (24%) of the total $15.5 billion) (Hoffmann et al., 2015). Most of the food poisonings occurred from contaminated poultry or vegetables and fruits, but red meats, eggs, and fish are also often contaminated (Diez-Gonzalez, 2007).
Because of its ability to infect humans with relatively low bacterial cell numbers and to cause complications that lead to kidney failure and death, *E. coli* O157:H7 is considered one of the most dangerous pathogens (Puligundla & Lim, 2022; Rohatgi & Gupta, 2021; Suo et al., 2010).
As the international food trade increases and becomes more global, this causes pathogens and resistant pathogens to be distributed around the globe so that small individual outbreaks can lead to multicounty outbreaks (WHO, 2017).

1.2.2 Foodborne infections caused by Shiga toxin-producing *E. coli* (STEC)

Escherichia coli is a thoroughly studied member of the Enterobacteriaceae family with rod-shaped, non-spore-forming cells that can have flagella for motility (Ishii & Sadowsky, 2008; Mathusa, Chen, Enache, & Hontz, 2010). The majority of strains of this highly diverse group of gram-negative bacteria do not pose a threat to humans. However, Shiga toxin-producing E. coli (STEC) strains regularly cause outbreaks with symptoms of mild to bloody diarrhea infections (Coombes et al., 2008; Ishii & Sadowsky, 2008; Mathusa et al., 2010; Newell & La Ragione, 2018). The infection can also lead to hemorrhagic colitis or hemolytic uremic syndrome (HUS), a life-threatening condition that often occurs in young children and can cause kidney failure. These symptoms are caused by Shiga-toxins 1 and 2 that inhibit protein synthesis (Bryan, Youngster, & McAdam, 2015; Colello, Etcheverria, Di Conza, Gutkind, & Padola, 2015; Coombes et al., 2008; Paton & Paton, 2002).

Enterohemorrhagic *E. coli* (EHEC) are an important subset of the STEC serotypes that are highly pathogenic and account for many severe clinical illnesses such as HUS (Coombes et al., 2008; Newell & La Ragione, 2018). Worldwide they are one of the top causes of bacterial enteric infections. According to the CDC in the US, they account for an annual number of 100,000 illnesses, 3,000 hospitalizations, and 90 deaths (Schulz et al., 2015). The EHEC serotype O157:H7 is the most prevalent and virulent EHEC for human infections. (Delannoy, Beutin, & Fach, 2013; Ishii & Sadowsky, 2008; Puligundla & Lim, 2022). In the US, it alone causes 63,000 foodborne illnesses, 2,100 hospitalizations, and 20 deaths per year (Hoffmann et al., 2015). Globally, O157:H7 accounts for an estimated 2.8 million foodborne illnesses per year (Ameer, Wasey, & Salen, 2022).

However, 6 non-O157:H7 EHEC serotypes are of growing concern: The serotypes O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28 have been defined by the FDA and USDA Food Safety and Inspection Service (FSIS) as the "Big 6" and account for the vast majority of non-O157:H7 STEC infections in the US (Brooks et al., 2005; Delannoy et al., 2013; Schulz et al., 2015). The so-called "Big 7" panel includes all "Big 6" and O157:H7 serotypes together (Schulz et al., 2015). Another STEC serotype, O104:H4, caused a widespread outbreak in Germany in 2011 through contaminated sprouted fenugreek seeds from Egypt. This emerging strain caused a surprisingly high incidence of HUS in adult women (68%) and killed 53 patients (Beutin & Martin, 2012). This outbreak increased the overall awareness of STEC and put pressure on the EU´s public health services for heightened STEC surveillance in animals, humans, and food (Newell & La Ragione, 2018).

1.2.3 **Foodborne infections caused by *Salmonella enterica***

Salmonella is a non-spore-forming gram-negative bacterium from the *Enterobacteriaceae* family. The cells are motile, rod-shaped, and facultative anaerobic (Jajere, 2019).

The genus *Salmonella* consists of 2 species which are *S. bongori* and *Salmonella enterica*. *S. enterica* is divided into 6 subspecies (*arizonae, diarizonae, enterica, houtenae, indica, salamae*) with more than 2500 serovars (Agbaje, Begum, Oyekunle, Ojo, & Adenubi, 2011; De Cesare, 2018). According to the Kauffman-White classification, *Salmonella* serovars are differentiated based on their somatic O (lipopolysaccharide), flagellar H, and capsular Vi antigenic representation (Agbaje et al., 2011; Brenner, Villar, Angulo, Tauxe, & Swaminathan, 2000; de Jong, Parry, van der Poll, & Wiersinga, 2012).

More than 2500 serotypes belong to *S. enterica* subsp. *enterica* (De Cesare, 2018). On the one hand, they consist of typhoidal *Salmonella* serotypes that are only adapted to humans and higher

primates and include serovars such as *Salmonella* Typhi, Paratyphi A, Paratyphi B, and Paratyphi C (Feasey, Dougan, Kingsley, Heyderman, & Gordon, 2012).

On the other hand, they consist of non-typhoidal *Salmonella* (NTS) serotypes that are either host-specific like *Salmonella* Dublin to cattle or *Salmonella* Gallinarium to poultry that rarely infect humans or non-host-specific serotypes infecting a broad vertebrate host range including humans (e.g., *Salmonella* Typhimurium and *Salmonella* Enteritidis) (Agbaje et al., 2011; de Jong et al., 2012). Serotypes are commonly named after a condition they supposedly provoke (e.g., Typhimurium: typhoid fever in mice) or after the site of discovery (e.g., *Salmonella* Muenchen) (Feasey et al., 2012).

Non-host-specific NTS are very significant foodborne bacterial pathogens that contaminate many different food products (Brown et al., 2017). *Salmonella* is widely present in food animal species and can pass through the entire food chain from animal feed until it reaches the end consumer. Salmonellosis is usually contracted by the consumption of contaminated food of animal origin (mainly eggs, meat, poultry, and milk), but also other foods, such as green vegetables contaminated by manure, have been linked to outbreaks (De Cesare, 2018; McDermott, Zhao, & Tate, 2018).

The pathogen is ubiquitous and robust, being able to multiply at temperatures from 7 to 45°C and can tolerate pH ranges between 4.0 and 9.5, which allows survival in harsh environments for extended periods (De Busser, De Zutter, Dewulf, Houf, & Maes, 2013; De Cesare, 2018).

Salmonella infection (salmonellosis) symptoms usually include diarrhea, fever, and abdominal cramps that may last for several days. While most infections are mild, severe systemic infections requiring hospitalization occur in ~5% of all cases and are more frequent in infants, elderly and immune-impaired patients (Agbaje et al., 2011; de Jong et al., 2012; Feasey et al., 2012; M. A. Gordon, 2008; Pham & McSorley, 2015).

Salmonella cells can survive passage through the stomach, colonizing the small and large intestines. After invading the intestinal epithelium cells, they remain intracellularly inside a modified phagosome (Salmonella-containing vacuole) where they multiply and produce toxins (e.g., the typhoid toxin, a genotoxin that directly damages DNA (DNAse activity)) (de Jong et al., 2012; Martinovic, Andjelkovic, Gajdosik, Resetar, & Josic, 2016; Steele-Mortimer, 2008). The released toxins also induce the release of cytokinins leading to an inflammatory reaction that can cause, e.g., mass cell death leading to the typical symptoms of an Enteritidis (Crum-Cianflone, 2008; de Jong et al., 2012; Pham & McSorley, 2015)

The *Salmonella* serotypes Typhimurium and Enteritidis are the most prevalent cause for salmonellosis in the US and the EU. Enteritidis alone accounts for more than half of all cases in the EU (De Cesare, 2018).

On a global scale, NTS causes an estimated 93.8 million illnesses per year (80.3 million are foodborne), leading to an estimated 155,000 deaths (Majowicz et al., 2010). While in many countries, it is the most common foodborne pathogen, in the EU, it holds second place, causing 94,000 confirmed cases in 2016 (De Cesare, 2018; McDermott et al., 2018).

Foodborne diseases through *Salmonella* and other agents have been increasing. The industrialized mass production and the globalization of food markets amplify the global distribution of pathogens and multidrug-resistant pathogens. (Besser, 2018; Majowicz et al., 2010).

The pathogenicity islands primarily determine the virulence factors of *Salmonella* (e.g., SPI1, SPI2). The plasticity of virulence plasmids and their operons as well as their easy interchangeability between serovars, make *Salmonella* potent at adapting to new environmental factors (Fluit, 2005). This interchange can include antibiotic resistances that are mostly encoded on plasmids or the multidrug-resistant region of *Salmonella* Genomic Island (SGI1). This is greatly accelerated by the global mobility of contaminated food products (Agbaje et al., 2011; Fluit, 2005). The source of antibiotic resistance in isolated strains comes mainly from antibiotic use in the food industry. The number of strains resistant to certain antibiotics usually correlates with the duration of their use in animal husbandry. Therefore resistances against tetracycline, sulfamethoxazole, and streptomycin are the most prominent (McDermott et al., 2018). Multidrug antibiotic resistance was first observed in the 1980s and since then has increased substantially. In present times, resistances to almost all antimicrobial classes have been documented (M. A. Gordon, 2008; Hahn-Lobmann et al., 2019). In 2014 10% of the strains isolated and sampled for resistance from humans in the US showed multidrug resistance (resistant to 3 or more antimicrobial classes). In the EU, this number was higher, with 26% of the isolates being MDR (McDermott et al., 2018). For human medicine, patients with salmonellosis are only treated with antibiotics if the disease becomes severe or invasive or if the patients are young children or elderly, then antibiotics are crucial to save lives (McDermott et al., 2018).

1.2.4 Decontamination practices for food products

Apart from good hygiene and management practices throughout the food chain to avoid foodborne pathogens, postharvest decontamination strategies for food products are necessary to assure the safety of the end consumer (WHO, 2017).

Antibiotics are not suitable for food treatment to control foodborne pathogens as they enhance the selection for resistant bacteria on the foods, which are then transferred to humans (Bai, Kim, Ryu, & Lee, 2016; Verraes et al., 2013). Therefore, the food industry uses several post-harvest decontamination methods for contact surfaces and the food itself: these include physical (e.g., heat treatment, ionizing radiation), chemical (e.g., organic acids, chlorine), and biological (e.g., bacteriocins, bacteriophages, bacteriophage endolysins, polyphenols) methods or a combination (Dincer & Baysal, 2004; Puligundla & Lim, 2022; Sohaib, Anjum, Arshad, & Rahman, 2016).

Most biological food decontamination methods have the advantage of not modifying the taste and quality of the food products through physiochemical interactions.

The peptide bacteriocin nisin has been approved by the EU, the WHO, and the FDA. It also received Generally Recognized as Safe (GRAS) status as a food substance for food preservation and has been used globally for many years (1.3.1) (Schneider et al., 2018; Sohaib et al., 2016).

Other biological interventions on food include the use of bacteriophages directly (viruses capable of killing bacteria) or bacteriophage endolysins (antibacterial proteins). All three substances have been approved as food antimicrobials in the USA, as they are ubiquitous in a normal human diet and the gut microbiota (Puligundla & Lim, 2022; Schulz et al., 2015). As food antimicrobials, some bacteriocins (e.g., colicins: antimicrobial proteins from *E. coli*) (Stephan et al., 2017) bacteriophage mixtures (e.g., SalmoFresh^{TM} for controlling *Salmonella* or Listex^{TM} P100 against *Listeria monocytogenes*) (M. Sharma, 2013), as well as endolysins to control *Clostridium perfringens* (Kazanaviciute, Misiunas, Gleba, Giritch, & Razanskiene, 2018) have also achieved GRAS status by the US Food and Drug Administration (FDA).

Several companies offer commercially available bacteriophage mixtures for the decontamination of food products from, e.g., *Salmonella*, STEC O157:H7, and *L. monocytogenes* (Polaska & Sokolowska, 2019; M. Sharma, 2013). Intralytics, Inc. (US) developed the first regulatory accepted mixture named ListShield^{TM} for food treatment against *Listeria monocytogenes* (Perera, Abuladze, Li, Woolston, & Sulakvelidze, 2015). The bacteriophage mixture SalmoFresh^{TM} against pathogenic *Salmonella* serovars has also shown promising activity on foods such as chicken breast fillets (Sukumaran, Nannapaneni, Kiess, & Sharma, 2016).

1.3 Bacteriocins and colicin-like bacteriocins

1.3.1 Introduction to bacteriocins

Apart from broad-spectrum antibiotics and lysozymes, microbes also produce an extensive and diverse array of bacteriocidal peptides or proteins named bacteriocins as part of their defense system. Bacteriocins are mostly peptides that are ribosomally synthesized and produced by almost every bacterial species examined to date (D. M. Gordon & O'Brien, 2006; Riley & Wertz, 2002). The classification of bacteriocins into different classes has been subject to change, but as of recent, they are separated into 3 distinct classes (Table 1).

Table 1: Classification of bacteriocins.
(Alvarez-Sieiro, Montalban-Lopez, Mu, & Kuipers, 2016; Heilbronner, Krismer, Brotz-Oesterhelt, & Peschel, 2021; Kumariya et al., 2019)

class		size	characteristics	example molecules
class I		~19-50 aa	post-translationally modified peptides with uncommon amino acids (e.g. lanthionine), only in gram-positive bacteria, heat stable	nisin, gassericin A, microcin B17
Ia	lantibiotics			
Ib	laborynthopeptins			
Ic	sanctibiotics			
class II		<10 kDa	unmodified short cationic peptides, by far largest group among gram-positive bacteriocins, heat stable	pediocin PA-1, enterocin A, bactofencin A, microcin L
IIa	pediocin-like bacteriocins			
IIb	two peptides unmodified bacteriocins			
IIc	circular bacteriocins			
IId	unmodified, linear non-pediocin-like bacteriocins			
class III		>10 kDa	unmodified large proteins usually composed of different domains, heat-labile	enterolysin A, colicins

The production of bacteriocins provides an ecological advantage over bacterial competitors to the producing strain, shaping the microbial diversity and the stable coexistence in microbiomes of ecological niches such as the human colon or the vagina (Kleanthous, 2010). The killing spectrum of bacteriocins can vary significantly from very narrow, only targeting closely related strains to very broad, targeting many different species. Nevertheless, most bacteriocins have a narrow spectrum, which sets them apart from commonly used antibiotics that are usually broadly active (Kleanthous, 2010; Riley & Wertz, 2002).

The most widely studied and characterized groups of bacteriocins are lantibiotics, produced by lactic acid bacteria (e.g., nisin, lacticin, and pediocin), offering a broad activity range against gram-positive bacteria (Diez-Gonzalez, 2007; Mota-Meira, LaPointe, Lacroix, & Lavoie,

2000). The name usually indicates the bacteriocin origin: for example, pesticins are produced by *Yersinia pestis* (Kleanthous, 2010).

The most successful bacteriocin in terms of industrial use is the polycyclic peptide nisin, produced by *Lactococcus lactis*. It has received approval as a food additive by many national food safety agencies, e.g., receiving GRAS status ("Generally Recognized As Safe") by the FDA, and has been successfully applied as a food substance for the preservation of many food products, including dairy products, vegetables and meats (S. C. Yang, Lin, Sung, & Fang, 2014) (Cotter, Hill, & Ross, 2005).

Bacteriocins possess a promising potential for the reduction of pathogenic and harmful bacteria on food, as antibiotics and their residues are prohibited for food preservation, and chemical preservatives are not very sought after (Cotter et al., 2005). The narrow activity spectrum of most bacteriocins causes significantly less collateral resistance in non-targeted bacteria (Cotter, Ross, & Hill, 2013; Hols, Ledesma-Garcia, Gabant, & Mignolet, 2019).

When bacteriocins are used in mixtures and in combination with other methods of food preservation, they have an even higher activity potency and an even lower resistance-inducing effect (Kumariya et al., 2019). Treating patients with a mixture of antibiotics (combination therapy) with antibiotics is known to broaden the activity spectrum of individual antibiotics and lower the formation of antimicrobial resistance (Hughes & Andersson, 2017; Tangden, 2014). Mixing bacteriocins should have the same effect. For candidates for food applications, the specific activity against the targeted pathogenic strains, the stability and activity when applied on the food substrate, and the overall expected development of resistance should be assessed (Cotter et al., 2005; Diez-Gonzalez, 2007).

Ideally, bacteriocins for food treatment have a limited technical effect that minimally alters the characteristics of the food. This way, they can be declared as food processing aids, which allows not to label them in the final product (e.g., GRAS in the US) (Hahn-Lobmann et al., 2019; Soltani et al., 2021).

1.3.2 **Colicins – bacteriocins from *E. coli***

1.3.2.1 Introduction to colicins

Colicins were discovered by the Belgian microbiologist André Gratia in 1925 (Gratia, 1925). and are the most extensively studied bacteriocins from gram-negative bacteria (Riley & Wertz, 2002). They belong to the class III bacteriocins (heat-labile proteins, >10 kDa) (Table 1). Colicins are high molecular mass proteins with a range of 30-80 kDa and an average of 500 to

600 amino acids, the largest being colicin D (ColD) (74.7 kDa, 697 residues) and the smallest being ColM (29.5 kDa, 271 residues) (Volkmar Braun & Patzer, 2013; Cascales et al., 2007; Kleanthous, 2010).

Group A colicins are encoded by/from small multicopy plasmids (~6-10 KB) that contain only the colicin operon and then released into the surrounding medium by cell lysis. Group B colicins, however, are encoded by/from large monocopy plasmids (~40 KB) that harbor several genes and are not released into the medium but are actively transported outside of the cell (Cascales et al., 2007; D. M. Gordon & O'Brien, 2006).

Colicinogenic strains of *E. coli* produce colicins as an SOS response to, e.g., nutrient depletion, DNA damage, and high temperatures (Hols et al., 2019; Lancaster, Wintermeyer, & Rodnina, 2007). This gives them an ecological advantage over non-colicinogenic *E. coli* strains and related *Enterobacteriaceae*, for which they are lethal (Cascales et al., 2007; Kleanthous, 2010; S. C. Yang et al., 2014).

It is estimated that over 30% of *E. coli* isolates produce colicins. Among the *E. coli* in the animal gut, the percentage is higher (Volkmar Braun & Patzer, 2013; V. Braun, Patzer, & Hantke, 2002; Cascales et al., 2007; D. M. Gordon & O'Brien, 2006; Lancaster et al., 2007; J. Smarda & Smajs, 1998).

1.3.2.2 Structure and biology of colicins

The antimicrobial activity colicins exert on a sensitive cell is defined by 3 steps. Each step is carried out by one of the colicin protein´s 3 domains, which each account for ~1/3 of the protein. In the first step, the central receptor domain (R) binds to an outer membrane receptor. In the second step, the N-terminal translocation domain (T) mediates the translocation from the outer membrane receptor to the target inside the cell, where in the 3rd step, the C-terminal catalytic/activity domain can fulfill its cytotoxic function (Figure 1) (Cascales et al., 2007; Hahn-Lobmann et al., 2019; O. Sharma, Zakharov, Zhalnina, Yamashita, & Cramer, 2013).

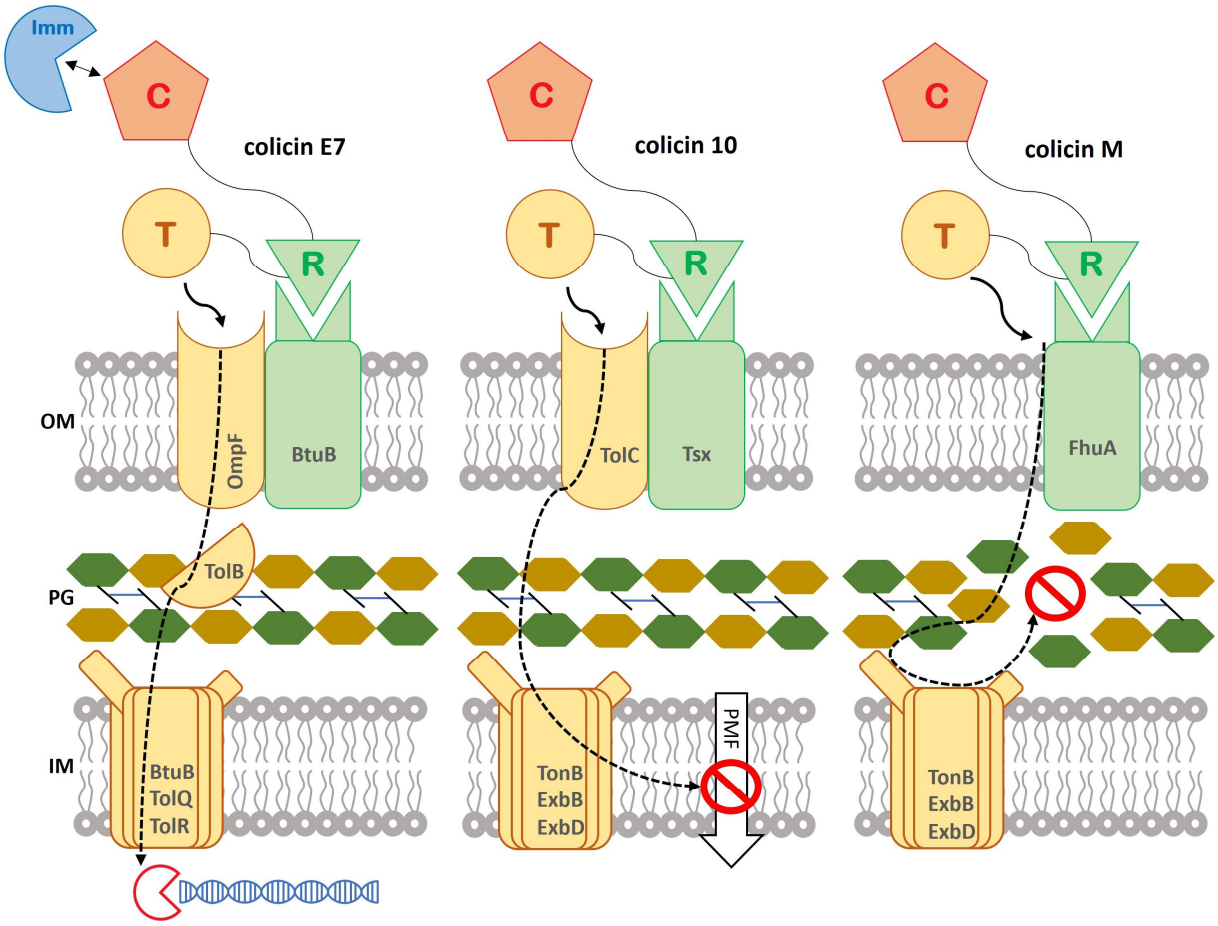


Figure 1: Mechanisms of receptor binding, translocation, and activity illustrated for a colicin with nuclease activity (colicin E7), pore-forming activity (colicin 10), and with murein synthesis inhibiting activity (colicin M). The receptor-binding domain (R) of colicin binds to the outer membrane (OM) receptor of the target cell. The translocation of the colicin molecule into the cell is mediated by the interaction of the translocation domain (T) with a cell translocation machinery (Tol or Ton systems). Colicins with nuclease activity (e.g. colicin E7) are translocated across the outer membrane, cell wall, and inner membrane (IM) to the cytoplasm, where they hydrolase DNA or RNA of the target cell; nucleic acid degradation is catalyzed by the cytotoxic domain (C) of colicin. Pore-forming colicins (e.g. colicin 10) are translocated across the outer membrane and cell wall to the inner membrane. The cytotoxic domain is inserted into the inner membrane and forms a pore that destroys the proton motive force (PMF), thus impairing the membrane integrity. The murein synthesis inhibiting colicin M is translocated across the outer membrane; it exerts its cytotoxic activity in the periplasm by enzymatic degradation of lipid I and lipid II peptidoglycan intermediates. The cleavage occurs between the lipid moiety and the pyrophosphoryl groups; this results in the arrest of peptidoglycan polymerization (Adapted from Hahn et al. 2019 (Hahn-Lobmann et al., 2019)).

Colicins attach themselves to receptors in the outer membrane of the bacteria with high affinity to enter the bacterial target cell (Kleanthous, 2010). The receptors used by colicins are mostly monomeric porins that allow the diffusion of hydrophilic molecules like vitamin B12 (BtuB receptor) or iron chelate complexes (e.g., Tsx and Cir receptors) (Kleanthous, 2010; Lancaster et al., 2007). The narrow target range of colicins is due to their narrow specificity for a particular receptor (Volkmar Braun & Patzer, 2013; S. C. Yang et al., 2014). The quantity of a particular receptor type in the outer membrane can depend on nutrient availability and physiological conditions (Sibinelli-Sousa, de Araujo-Silva, Hespanhol, & Bayer-Santos, 2022).

Colicins can be divided into 2 groups depending on the inner membrane proteins they use as translocation machinery. These groups were defined due to the observed cross-resistances. Group A colicins (A, E1-E9, K, L, N, S4, U, and Y) use the Tol system consisting of TolA, TolB, TolQ, TolR, and Pal whereas group B colicins (B, D, H, Ia, Ib, M, 5, and 10) use the Ton system consisting of TonB, ExbB, and ExbD (V. Braun et al., 2002; Buchanan et al., 2007; Cascales et al., 2007; Lancaster et al., 2007). Many of these translocation proteins are also used for cell entry by bacteriophages (Cascales et al., 2007; Kim, Tarr, & Penfold, 2014).

The activity of colicins is thought to follow first-order kinetics meaning that a single colicin can kill an entire sensitive cell (Cascales et al., 2007; Kleanthous, 2010; Pressler, Braun, Wittmann-Liebold, & Benz, 1986).

The cytotoxic domain of colicins needs to reach either the periplasm for inhibition of peptidoglycan biosynthesis activity, the inner membrane for a pore-formation activity, or the cytoplasm for nuclease activity (Volkmar Braun & Patzer, 2013). The antimicrobial activity includes the formation of pores which causes depolarization of the cytoplasmatic membrane (colicins A, B, E1, Ia, Ib, K, L, N, U, 5, and 10) or an enzymatic degradation activity. There are 3 types of enzymatic degradation activities of colicins: 1) non-specific DNase activity, resulting in random degradation of DNA (colicins E2, E7, E8, and E9), 2) highly specific RNase activity (16S RNase and tRNases) which ultimately arrests protein synthesis (colicins E3, E4, E5, E6, and D) and 3) diphosphoric diester hydrolase activity, resulting in degradation of peptidoglycan precursors which inhibits murein synthesis (colicins M) (Cherier, Patin, Blanot, Touze, & Barreteau, 2021; El Ghachi et al., 2006; Lancaster et al., 2007).

1.3.2.3 Immunity proteins

A bacterial cell can protect itself from its own or exogenous colicin by producing an immunity protein (10-18 kDa). The immunity protein is encoded by the same plasmid as the colicin and is highly specific to the individual colicin (Cascales et al., 2007). It tightly binds to the enzymatic activity domain (DNase, RNase, tRNase), thereby deactivating it, or it embeds itself in the inner membrane, inhibiting pore-formation (Volkmar Braun & Patzer, 2013; Cascales et al., 2007; Kleanthous, 2010). Endogenously produced enzymatic colicins are thereby inactivated until they reach the outer membrane receptor of their target (Cascales et al., 2007; O. Sharma et al., 2013).

1.3.2.4 Preliminary colicin studies

Scientists at Nomad Bioscience GmbH have transiently expressed and tested 23 colicins as well as their respective immunity proteins in *Nicotiana benthamiana* and other plants. Expression levels are as high as up to 30% of total soluble protein (TSP) or 3 g of protein per kg of fresh green biomass (Schulz et al., 2015).

Research aimed to evaluate the feasibility of plant-produced colicins for commercial use. Colicins were examined in the form of total soluble protein (TSP) in plant material extracts (2.6.2) or as purified protein (2.7.4).

Antimicrobial activity experiments with TSP extracts revealed that colicins M, E2, E6, E7, Ia, K, and 5 had the broadest and highest activity on *E. coli* "Big 7" STEC strains (major foodborne *E. coli* strains) (2.1.1.2) and strain DH10B (pICH37713). ColM and ColIa are the most broadly active colicins against STEC strains, with ColM also having good activity on O157:H7. The strain with the highest susceptibility to the colicins tested was O104:H4 (Schulz et al., 2015).

While ColU only had high activity on one STEC strain, it showed the highest overall activity against 69 *E. coli* clinical isolates with multiple antibiotic resistances tested by Nomad Bioscience in collaboration with the St. George´s University, Institute for Infection & Immunity (London, UK) (Nomad Bioscience internal data).

At Nomad Bioscience GmbH, the use of certain colicin mixtures or blends had synergistic effects for the antimicrobial activity on STEC strains (Schulz et al., 2015). A mixture of TSP extracts containing ColM or ColE7 showed high activity against all STEC strains. Depending on the strain, the mixture reduced the colony-forming units (CFU) in broth culture by 2 to over 6 logs at doses below 10 mg of colicins per liter. Additionally, a mixture of the ColM (3 mg/kg) and ColE7 (1 mg/kg) applied to contaminated pork meat reduced *E. coli* O157:H7 by 2.3 logs at 1 h and 2.7 logs at 1 d. Longer storage periods, up to 72 h, led to a further decrease and no regrowth, hinting at an ongoing technical effect (Schulz et al., 2015).

The potency of this colicin mixture shows that mixtures of 2 or more colicins with complementary or synergistic activities could substantially lower the colicin doses needed for treatment. Instead of ColE7, colicins ColE6, ColIa, ColK, or Col5 could also be used in combination with ColM.

Mixtures of bacteriophage endolysins or bacteriocins have shown a lower mutation rate because more than one beneficial spontaneous mutation in a short time period is necessary to render the cell resistant, guaranteeing its survival and reproduction (Schmelcher, Donovan, & Loessner, 2012). The use of colicin mixtures has also been shown to significantly lower the occurrence of colicin resistance (Schamberger & Diez-Gonzalez, 2005). Mixtures of bacteriocins or

bacteriophage endolysins have also shown to be more potent, having increased activity which, among other methods, was shown by a reduction of the minimal inhibitory concentration (MIC) (Bouttefroy & Milliere, 2000; Hanlin, Kalchayanand, Ray, & Ray, 1993; Mulet-Powell, Lacoste-Armynot, Vinas, & Simeon de Buochberg, 1998; Parente, Giglio, Ricciardi, & Clementi, 1998; Schmelcher et al., 2012).

Colicins are proteins which make them susceptible to the human digestive tract. Researchers at Nomad Bioscience GmbH tested to what extent purified colicins (ColM, E7, K and U) are digested and inactivated in simulated gastric and intestinal fluids. The colicins were entirely degraded to 2-3 kDa size peptides which should not have any residual activity on the gut microflora. These peptides are also predicted to be too small to cause allergic reactions (Schulz et al., 2015).

Colicins in TSP extracts were also analyzed by mass spectrometry-based sequencing revealing that plant-expressed colicins are correctly expressed and identical to their native microbial-produced counterparts (Schulz et al., 2015). Native colicins are found in the human gastrointestinal tract and in the environment, with a long history of human exposure (Cascales et al., 2007; D. M. Gordon & O'Brien, 2006). Consequently, Nomad Bioscience GmbH had successfully approved its plant-made colicins using the GRAS (Generally Recognized As Safe) regulatory pathway of FDA, USA, which declares them safe for food use as processing aids (GRAS Notices GRN593 (2015), GRN676 (2016) and GRN775 (2018)). In addition, plant-made colicins have also been approved by USDA/FSIS (USA, Directive 71201.1 (2017). A detailed overview of colicins used in this study and their characteristics is shown in Table 2.

Table 2: Overview of all colicins used in this study and their characteristics.
Accession numbers from Uniprot/Swiss-Prot or NCBI (marked with asterisks)

bacteriocin	source organism	colicin group	receptor	translocation	activity	MW (kDa)	pI	pos. cat1 intron	Accession No.
ColE1	*E. coli*	group A	BtuB	TolC, TolAQ	pore	57,279			P02978
ColE2	*E. coli*	group A	BtuB	OmpF, TolABQR	DNase	61,561	9,02	V508	P04419
ColE3	*E. coli*	group A	BtuB	OmpF, TolABQR	16S rRNase	57,96	9,04	G518	P00646
ColE5	*E. coli*	group A	BtuB	OmpF, TolABQR	tRNase	58,254	5,99	G468	P18000
ColE6	*E. coli*	group A	BtuB	OmpF, TolABQR	16S rRNase	58,011	8,79	G517	P13477
ColE7	*E. coli*	group A	BtuB	OmpF, TolABQR	DNase	61,349	9,69	K522	Q47112
ColE8	*E. coli*	group A	BtuB	OmpF, TolABQR	DNase	60,872	9,16	G513	P09882

ColE9	*E. coli*	group A	BtuB	OmpF, TolABQR	DNase	61,587	8,78	V546	P09883
ColD	*E. coli*	group B	FepA	TonB, ExbBD	tRNase	74,683	4,96	G647	P17998
DF13	*E.coli (E. cloacae)*	group A	IutA	TolAQR, OmpF	16S rRNase	59,278	9,35	G528	P00645
ColA	*C. freundii*	group A	BtuB	OmpF, TolABQR, OmpA, LPS	pore	62,992	7,78		Q47108
ColN	*E. coli*	group A	OmpF	TolAQR	pore	41,696	9,62		P08083
ColS4	*E. coli*	group A?	OmpW	OmpF, TolABQR, (TonB, ExbBD)	pore	54,085	5,43		Q9XB47
ColK	*E. coli*	group A	Tsx	OmpF, OmpA, TolABQR	pore	59,611	5,53		Q47502
Col5	*E. coli*	group B	Tsx	TolC, TonB, ExbBD	pore	53,137	5,4		Q47500
Col10	*E. coli*	group B	Tsx	TolC, TonB, ExbBD	pore	53,342	5,33	(V313)	Q47125
ColU	*Shigella boydii*	group A	OmpA	OmpF, TolABQR	pore	66,289	8,49		CAA72509.1 *
ColR	*E. coli*	group A	OmpA	OmpF, TolABQR	pore	67,471	8,85		T2D1N2
Col28b	*S. marcescens*	group A	OmpA	OmpF, TolABQR	pore	47,505	9,36		Q06308
ColY	*E. coli*	group A	OmpA	?	pore	67,161	9,3	(V451)	Q9KJ98
ColB	*E. coli*	group B	FepA	TonB, ExbBD	pore	54,742	4,82		P05819
ColIa	*E. coli*	group B	Cir	TonB, ExbBD, Cir	pore	69,429	9,51		P06716
ColIb	*E. coli*	group B	Cir	TonB, ExbBD, Cir	pore	69,923	9,02		P04479
ColM	*E. coli*	group B	FhuA	TonB, ExbBD	inhibition of murein synthesis	29,453	8,9	G81	P05820

1.3.2.5 Prior work done with colicins on curing post-weaning diarrhea

Another important issue is the extensive use of antibiotics for animal health. In the US, 80% of the antibiotics sold are used in animal husbandry; only 20% are used for treatment in humans (Diez-Gonzalez, 2007). They are used to treat acute infections and prophylactically to prevent disease development, which improves overall animal health and promotes growth (Ben Lagha, Haas, Gottschalk, & Grenier, 2017). One of these economically important infections is post-weaning diarrhea (PWD), an enteric disease that significantly impacts the growth performance of piglets (Laxminarayan et al., 2013). It weakens the piglets through diarrhea, dehydration,

and a loss of appetite, which can ultimately cause considerable retardation in growth (Chen, Gao, Jiao, & Liu, 2004; S. A. Cutler, Lonergan, Cornick, Johnson, & Stahl, 2007; Melin, Mattsson, Katouli, & Wallgren, 2004; Rhouma, Fairbrother, Beaudry, & Letellier, 2017). PWD can also be lethal, accounting for about 50% of all deaths that result from diarrhea in piglets.

PWD is commonly caused by enterotoxigenic *E. coli* (ETEC) strains of the O149 serogroup with F4 or F18 adhesins (Rhouma et al., 2017). The disease often goes along with virus infections such as rotavirus, which can contribute to the severity of the disease (Melin et al., 2004).

The illness typically occurs after the piglets are weaned at 4 weeks of age (post-weaning phase) when the piglets are exposed to sudden changes in their environment and biological stress, strongly impacting the immune system and leaving them vulnerable to disease. (Campbell, Crenshaw, & Polo, 2013; S. A. Cutler et al., 2007; Fairbrother, Nadeau, & Gyles, 2005; Rhouma et al., 2017).

The prophylactic and growth-promoting antibiotic treatment practices in animal husbandry, such as PWD treatment, have increased the formation of resistance to many commonly used antibiotics, making the disease untreatable (S. A. Cutler et al., 2007; Fairbrother et al., 2005; Larsson & Flach, 2022). Additionally, the antibiotics and resistant bacteria ultimately find their way to the end consumer and into the environment, where they can cause severe harm (Ben Lagha et al., 2017; Wepking et al., 2019). Therefore, the use of antibiotics for prophylactic and growth-promoting purposes has been banned by regulations in many countries (EU ban in 2006 (Aslam et al., 2018; Heo et al., 2013), leaving only veterinary prescriptions for acute diseases (Ben Lagha et al., 2017; Koluman & Dikici, 2013). A new EU directive (regulation (EU) 2019/4 and (EU) 2019/6) goes one step further and restricts the use of antibiotics to an absolute minimum, banning all routine use.

Alternative treatments such as vaccinations and special diets containing, e.g., zinc, copper, or probiotics have been explored but remain inconsistent in alleviating the infections (Fairbrother et al., 2005; Heo et al., 2013). Alternative antimicrobials for PWD treatment are urgently needed (Sara Anne Cutler, 2007; Rhouma et al., 2017).

In search of new antibiotic alternatives to cure PWD, Cutler et al. 2007 and Stahl et al. 2014 focused on colicins. Stahl et al. 2004 inhibited the growth of PWD strains with ColE1 and ColN *in vitro,* and Cutler et al. 2007 demonstrated an effect of ColE1 on PWD in piglets at a dosage of 8.25 mg ColE1/0.5 kg feed (S. A. Cutler et al., 2007; Stahl, Callaway, Lincoln, Lonergan, & Genovese, 2004).

The purified ColE1 protein as a feed additive lowered the incidences and the severity of the illness and promoted the piglet´s growth performance. However, only a high dose (16.5 mg/kg feed) could significantly cure PWD, eliminating all clinical signs of PWD, while the lower dose of 11 mg/kg only slightly delayed infection. (S. A. Cutler et al., 2007). As colicins are susceptible to digestive proteases (Schulz et al., 2015), it is unclear how much intact colicin arrived at the area of infection to cause this effect (dose-effect relationship). Delivering the colicin inside a gastro-resistant medical capsule to assure gastric protection and later intestinal release should enable more intact colicin protein to reach the intestine.

Miller et al. 2015 analyzed cheap and effective ways of delivering acid-labile macromolecules to the small intestine (Miller et al., 2015). They conclude that double Delayed-Release capsules (DRcaps® (Capsugel, Morristown, NJ, USA)), meaning a size 0 DRcap® in a size 00 DRcap®, coated with 4 layers of an 8,1% w/v Eudragit L100-55 (Evonik Industries, Essen, Germany) polymer coating solution, offer the best *in vivo* and *in vitro* patterns for duodenal release. DRcaps® (Capsugel, Morristown, NJ, USA) have a low moisture content and are marketed to be more resistant to gastric fluid than gelatin capsules, with a stomach disintegration time of 52 min instead of 5 min for the latter. They are promoted to be ideally suitable for probiotics and enzymes and could be used to transport colicins to the intestine of the piglets.

1.3.3 **Salmocins – colicin-like bacteriocins from *Salmonella***

1.3.3.1 Colicin activity against *Salmonella*

With the increase in antibiotic resistance of pathogenic *Salmonella* strains and the substantial lack of new drug development, alternatives to current antibiotic treatments are desperately needed (Majowicz et al., 2010).

Scientists at Nomad Bioscience wanted to identify bacteriocins that can serve as natural non-antibiotic antibacterials for broad-spectrum *Salmonella* pathogen control. The sensitivity of certain *Salmonella* isolates to colicins has been reported in the literature previously (Fredericq, 1957; Graham & Stocker, 1977; Guterman, Wright, & Boyd, 1975; Wray & Clarke, 1974).

In total, all 23 *E. coli* colicins (1.3.2.5) available for plant expression at Nomad Bioscience were tested against 35 *Salmonella* strains (2.1.1.3). Only a few of the 23 colicins showed antimicrobial activity, presumably because of the species-specificity known for bacteriocins (Graham & Stocker, 1977; Guterman et al., 1975). These colicins were ColIa, ColIb, ColM, Col5, and Col10, all group B colicins and pore-forming except for ColM. The only colicin from Group A that showed activity was ColS4.

Their overall activity on *Salmonella* was, however, low. While some strains were not sensitive, for most strains, activity was below 100 AU/µg colicin and, in isolated cases, above 1000 AU/µg colicin.

The observed lack of group A colicins activity corresponded to literature data showing that the *Salmonella* serovar Typhimurium is insensitive to group A colicins E1, E2, and E3 (Guterman et al., 1975). However, some *Salmonella* strains have been found to be sensitive to group B colicin M (Graham & Stocker, 1977).

The colicins were also tested against 20 clinical *Salmonella* isolates by a Nomad Bioscience scientist in collaboration with the Institute for Infection & Immunity (St. George´s University, London, UK). Many isolates showed no sensitivity to any colicins tested, except for ColIa and ColM (Nomad internal data).

From all these studies, it became evident that the tested colicins would not be able to control a broad range of disease-relevant *Salmonella* serovars.

1.3.3.2 Selection of salmocin candidates for feasibility test

Therefore an exploration into the bacteriocins produced by *Salmonella* was conducted. The existence of colicin-like bacteriocins in *Salmonella* has been known for more than 60 years (Fredericq, 1952; Joerger, 2020). Several studies have evaluated the presence and type of colicins for the potential taxonomic differentiation of *Salmonella* serovars (Atkinson, 1973; Barker, 1980; Campos & Hofer, 1988; Patankar & Joshi, 1985; Vicente & de Almeida, 1984). The discovered colicins were also characterized according to their type of antimicrobial activity and the inhibition zones and compared to known *E. coli* colicins with similar attributes (Atkinson, 1973). Furthermore, colicin-encoding plasmids such as IncI1 were examined (Ayala, Krane, & Hartl, 1994; Barker, 1980; Fricke et al., 2009; Johnson et al., 2010; Kaldhone et al., 2019; Kaldhone et al., 2018; Vicente & de Almeida, 1984). In one study, the Salmonella bacteriocins were isolated (Nedialkova et al., 2014). In most studies, the *Salmonella* bacteriocins´ activity was tested on *Salmonella,* whereas some also tested their activity on *E. coli* (Atkinson, 1973; Kaldhone et al., 2019; Nedialkova et al., 2014; Spriewald, Glaser, Beutler, Koeppel, & Stecher, 2015; Wray & Clarke, 1974). There have also been suggestions for naming them “Salmonellins” (Atkinson, 1967) or “Salcols” (Atkinson, 1973; Patankar & Joshi, 1985). While some salmocins like ColIb have received some more focused attention (Nedialkova et al., 2014; Spriewald et al., 2015), to our knowledge, no one has worked on these salmocins in greater detail, including salmocin protein isolation, characterization of their activity spectrum and their expression in different hosts.

Due to the high species specificity of bacteriocins, colicin-like bacteriocins from *Salmonella* should be more uniquely adapted to the specific receptor and translocation protein arrangements of the *Salmonella* cell wall (Cascales et al., 2007).

When we searched the National Center for Biotechnology Information (NCBI) database *for* homologs to *E. coli* colicins in *Salmonella enterica*, it became evident that group B colicins seem to be widely distributed in *Salmonella* with a 99-100% identity in amino acid sequence. This evolutionary exposure may have increased resistance against group B colicin. Additionally, it may have caused the widespread distribution of group B colicin immunity proteins.

Consequently, it was assumed that the *Salmonella* bacteriocin counterparts to *E. coli* group A colicins would be the most promising bacteriocin candidates. It was assumed that because they are less prevalent in *Salmonella*, the existing resistance should be less ubiquitous. Therefore, the colicin-like bacteriocins in *Salmonella* should be homologous in protein sequence to group A colicins.

The NCBI database search for homologs of group A colicins from *E. coli* in *Salmonella enterica* was done using the blastp suite (BLASTP 2.6.1+ program (Altschul et al., 1997). To ensure sufficient similarity, the amino acid sequence homology cutoff for the activity domains was set to ≥70% identity. For the translocation domain identity, the cut-off was set to ≤80% (except for SalE3 with 95% identity) to ensure a sufficient difference. An example of these queries is shown in Schneider et al. 2018 (Schneider et al., 2018) (7.2).

6 promising *Salmonella* colicins that offer different modes of activity (3.4.1 Table 24) were chosen for further investigation.

1.3.4 Evolution and modularity of colicin-like bacteriocins – a hope for future diversification?

In gram-negative bacteria, the variety of bacteriocins seems to have been facilitated by its configuration and conserved domain structure (Riley & Wertz, 2002). As colicins domains often share regions of high sequence similarity, DNA fragments that encode for functional domains could have easily been exchanged (V. Braun et al., 2002; Riley & Wertz, 2002). This theory of exchange is also supported by findings of high sequence homology in the domains´ flanking DNA regions, making them prone to recombination events (Volkmar Braun & Patzer, 2013).

Individual domains have been shown to be functionally independent of the other domains: isolated receptor domains can bind to receptors, and isolated activity domains can form

membrane pores or target their substrates (Cascales et al., 2007). Some colicins have also exhibited activity when receptor (ColIa) or translocation (ColE1 and ColA) domains were deleted or truncated, most likely due to redundancies with the function of the other domains (Cascales et al., 2007; Jakes, 2012; Jakes & Finkelstein, 2010). Translocation domains have been shown to be interchangeable between enzymatic colicins displaying that the translocation mechanism must be relatively generic. However, the affinity of the translocation domain to the different translocation machinery proteins can vary slightly (Cascales et al., 2007).

These characteristics should make domain rearrangement between colicin-like bacteriocins feasible. The reengineering of bacteriocins through the rearrangement of their domains has successfully been done for antimicrobial peptides, creating new antimicrobials with altered activity characteristics (Brogden & Brogden, 2011; Schmelcher, Tchang, & Loessner, 2011). Active chimeric antimicrobials have also been made by combining, e.g., antimicrobial peptides or domains of antimicrobial proteins with bacteriophage endolysins (Gerstmans, Criel, & Briers, 2018; Lukacik et al., 2012).

Before this research was started, a scientist at the subsidiary company of Nomad Bioscience GmbH, Nomads UAB (Vilnius, Lithuania), created a novel and functional interspecies bacteriocin by combining the receptor and translocation domain of a pyocin (antimicrobial proteins from *Pseudomonas aeruginosa*) with the activity domain of ColU. This interspecies bacteriocin was successfully expressed in plants (unpublished data). The company recently constructed a chimeric molecule of 2 porin-type *Pseudomonas* bacteriocins that shows promising antimicrobial activity in *Pseudomonas* infection models (Paskevicius et al., 2022).

Pyocin (S1 or S2) colicin (ColE3 or ColE2) chimeras were also successfully constructed in a 1996 study, creating a new kind of pyocin, with RNAse activity. Additionally, a chimeric protein with antimicrobial activity against *Pseudomonas aeruginosa* and *E. coli* was constructed (Kageyama, Kobayashi, Sano, & Masaki, 1996).

1.4 Determining the potential of a bacteriocin

Many pathogenic bacteria are increasingly displaying resistance against the available antibiotic arsenal. Therefore, the product lifetime value of new antimicrobials for the treatment depends on the target pathogen's ability to develop resistance against them.

New antimicrobials need to be highly effective and, if possible, target the bacterial cell in a way that makes resistance to them harder to come by (Tyers & Wright, 2019).

Resistance, being usually the result of an alteration of the targeted vital cell components, can come at a fitness cost to the organism (Guo, Abdelraouf, Ledesma, Nikolaou, & Tam, 2012; Linkevicius, Anderssen, Sandegren, & Andersson, 2016; Melnyk et al., 2015; Riley, 1998).
Additionally, the antimicrobial activity of new antimicrobials should be tailored to microbial processes, efficiently arresting growth at low doses and, therefore, only having minor toxicity for the patient. They should also be sufficiently selective so they do not harm the benign microbiome (Laxminarayan et al., 2013; Singh, Young, & Silver, 2017).
Resistance acquirement should be minimally vulnerable to lateral gene transfer. It should engage multiple targets (e.g., penicillin to penicillin-binding proteins), as only engaging one target would have much more resistance liabilities (Tyers & Wright, 2019).
The calculation of the minimal inhibitory concentration (MIC) with a consecutive determination of the mutation rate offers essential information on the resistance potential of bacteria against antimicrobials.
The MIC is defined as the lowest concentration of an antibacterial agent required to inhibit the visible growth of a microorganism after 16-20 h incubation (Andrews, 2001). The determination of the MIC for antimicrobial substances is an integral part of modern microbiology practice for testing the effectiveness of new antibiotics or for monitoring the development of resistance in bacteria (Andrews, 2001; Martinez, 2014; Wiegand, Hilpert, & Hancock, 2008). Additionally, possible synergistic effects can be discovered by determining the MIC for individual antimicrobials and mixtures thereof. While the MIC is an indicator of antimicrobial efficacy, it is also crucial for the assessment of the mutation rate via the Luria-Delbrück fluctuation assay (Pope, O'Sullivan, McHugh, & Gillespie, 2008).
The mutation rate is the rate at which a bacterial strain develops a spontaneous resistance-conferring mutation for a particular antimicrobial (Foster, 2006; Rosche & Foster, 2000).
“A mutation rate is an estimation of the probability of a mutation occurring per cell division and corresponds to the probability of a mutation occurring in the lifetime of a bacterial cell” (Pope et al., 2008). It differs from the mutation frequency, “the proportion of mutant bacteria present in a culture” (Pope et al., 2008).
The mutation rate can be determined by performing fluctuation assays initially described by Luria and Delbrück (Luria & Delbruck, 1943). “A fluctuation assay consists of determining the distribution of mutant numbers in parallel cultures; the mutation rate is obtained from analyzing that distribution” (Rosche & Foster, 2000). Several parallel cultures in a non-selective medium are inoculated with a small number of cells (e.g., 2000 CFU). During the subsequent growth, the cells divide many times, accumulating random mutations until the cultures reach saturation.

The cells are then plated onto selective media with a certain antimicrobial concentration that only allows resistant cells to survive. From the number of mutants, the mutation rate of the test strain against the antimicrobial can be calculated (Hall, Ma, Liang, & Singh, 2009; Radchenko, McGinty, Aksenova, Neil, & Mirkin, 2018; Rosche & Foster, 2000).

Measurements of in vitro rates of mutation against new antimicrobials are often conducted as they give a perspective of the risk of antimicrobial resistance formation, which can have implications for the potential product lifetime (L. L. Ling et al., 2015; Ragheb et al., 2019; Windels et al., 2019). Promising candidates should have a mutation frequency below 10^{-8} at four times the minimal inhibitory concentration (MIC) (Tyers & Wright, 2019).

1.5 The MagnICON® plant transient expression platform

In nature, *Agrobacterium tumefaciens* causes crown gall disease in plants by integrating a single-stranded transfer DNA (T-DNA) from its tumor-inducing plasmid (Ti-plasmid) into the host genome. In 1983 it became evident that the Ti-plasmid could be genetically modified to contain a selectable marker and a multiple cloning site for the insertion of any gene of interest (GOI) and that the T-DNA and the vir-regions on the plasmid could be separated into 2 separate replicons (Hoekema, Hirsch, Hooykaas, & Schilperoort, 1983; Peyret & Lomonossoff, 2015). This tandem was called the binary vector system, and genetically engineered *Agrobacterium* became widely used as a molecular tool for stable transformation and transient gene expression in plants (Krenek et al., 2015).

The production of recombinant proteins in plants is still not as widely used as the earlier invented and quite common microbial and animal-cell-based expression systems that have well-established regulatory frameworks in place (Giritch, Klimyuk, & Gleba, 2017).

Nevertheless, the advantages of plant-expressed pharmaceuticals in plants as a robust, highly scalable, low-cost, and safe system are becoming more and more evident (Leuzinger et al., 2013; Pogue et al., 2010). The Magnifection technology optimizes the cost-effectivity and safety of plant expression (Giritch et al., 2017). This technology was developed by ICON Genetics in Halle, Germany, and is trademarked as magnICON® (Giritch et al., 2017). Magnifection couples the strengths of 3 biological systems: *Agrobacterium* efficiently delivers the recombinant T-DNA to the plant cells, where the replication machinery of a plant virus promotes its rapid amplification, leading to high levels of protein expression in a plant production host that offers good posttranslational capabilities and low production costs (Y.

Gleba, Klimyuk, & Marillonnet, 2005; Marillonnet, Thoeringer, Kandzia, Klimyuk, & Gleba, 2005).

Rather than on stable transformation, the approach relies on transient amplification of the viral vectors (Y. Gleba, Klimyuk, & Marillonnet, 2007). This saves costs and time as the production is easily and quickly manipulable, and the maximum protein yield is reached between 4-10 days after inoculation of *Agrobacterium*, offering high scalability and rapid manufacturing cycles (Giritch et al., 2017; Peyret & Lomonossoff, 2015). It is also a safer technology as it depends on episomal DNA transcription instead of DNA integration, and there are no inheritable changes to the production plant. This means that no foreign genes are transmitted by pollen or insects (Hahn, Giritch, Bartels, Bortesi, & Gleba, 2015; Komarova et al., 2010; Pogue et al., 2010). Additionally, with a plant production host, there is no contamination possibility with human or animal pathogens during up- and downstream processing (Y. Gleba et al., 2005; Komarova et al., 2010; Pogue et al., 2010).

MagnICON® uses deconstructed minimal-virus systems. These viruses retain the required viral elements for the GOI's fast and efficient expression and lack unwanted viral elements such as a high species specificity or the ability to form infectious viral particles. The absence of inefficient viral functions such as its infectivity, cell-to-cell movement, shutoff of the plant cellular synthesis, and silencing suppression can be provided by non-viral elements (molecular machines) (Y. Gleba et al., 2007; Y. Y. Gleba, Tuse, & Giritch, 2014). Additionally, the GOIs are codon-optimized for plant expression, and plant introns are added to longer viral genes to facilitate processing (Marillonnet et al., 2005). The most commonly used RNA-based plant viruses for this technology are tobacco mosaic virus (TMV), potato virus X (PVX), alfalfa mosaic virus (AMV), and cowpea mosaic virus (CPMV) (Y. Gleba et al., 2007; Pogue et al., 2010).

The *Agrobacterium* provides systemic delivery of a DNA copy of the viral replicon to the host plant if applied using vacuum infiltration or spraying, whereas the RNA viral vector derived from the T-DNA of *Agrobacterium* is capable of localized cell-to-cell or systemic movement (Y. Gleba et al., 2007). For TMV-based vectors, the viral replicons are designed for local cell-to-cell spread through the movement protein (MP). The CP responsible for systemic movement has been removed to allow for higher expression and the integration of larger genes (Y. Gleba et al., 2007; Peyret & Lomonossoff, 2015). For PVX-based vectors, the viral replicons are usually capable of systemic movement enabled by coat protein (CP), which is essential for both types of viral movement, local cell-to-cell and systemic, in this virus species (Y. Y. Gleba et al., 2014). An example for these expression vectors are the vectors pICH29912 and pICH0421

used in this thesis (Figure 2). TMV and PVX are non-competing plant viruses, and their backbones are often used for the co-expression of proteins in the same cell. This is useful when expressing an antibody's heavy and light chain or a bacteriocin and its respective immunity protein (Giritch et al., 2017; Peyret & Lomonossoff, 2015).

Even if TMV and PVX are non-competing viruses, protein expression from co-infiltrated viral vectors can be lower than when using only one vector (Y. Y. Gleba et al., 2014).

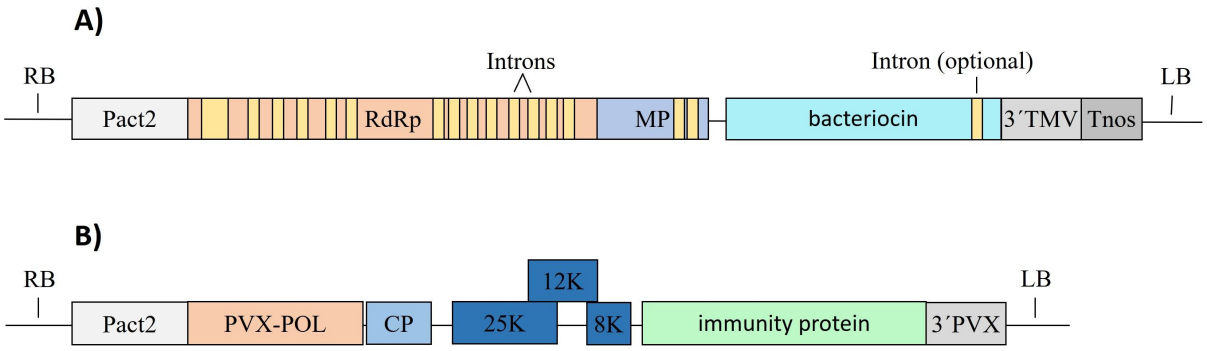


Figure 2: Schematic representation of the plasmid constructs pICH29912 (TMV +MP) and pNMD0674 (PVX + CP) with T-DNA regions.
The plasmids are used for transient *Agrobacterium*-mediated co-expression of bacteriocins and their immunity proteins in plants. A) A bacteriocin-expressing TMV-based vector (pICH29912) capable of cell-to-cell movement. B) A bacteriocin-immunity protein-expressing PVX-based vector (pNMD0674) capable of systemic movement. Figure legend: LB and RB: binary left and right borders, respectively; Pact2, *Arabidopsis thaliana* actin 2 promoter; Tnos, nos terminator; RdRp, RNA-dependent RNA polymerase of TVCV (Turnip Vein-Clearing Virus); MP, movement protein, has 2 introns; Gene of interest has 1 optional intron; 3´TMV, 3´untranslated region of TMV; P35S, CaMV 35S promoter; PVX-Pol (PVX RdRp); RdRp, RNA-dependent RNA polymerase; CP (PVX coat protein); 25K, 12K, 8K, PVX triple gene block; 3´PVX, 3´CP coding sequence and 3´untranslated region of PVX (Schneider et al., 2018)

Using the MagnICON® system, the expression of transient protein can reach up to 5mg/ g of fresh green biomass (FW) or up to 80% of soluble protein, approaching the biological limits of plant-based production (Bendandi et al., 2010; Giritch et al., 2017; Y. Y. Gleba et al., 2014; Hahn et al., 2015; Schulz et al., 2015).

Plant inoculation of *A. tumefaciens* or agroinfiltration is the process of bringing diluted suspensions of *agrobacteria* carrying T-DNAs that encode viral replicons in direct contact with the plant cells by injecting them into the intercellular cavities of the leaf tissue (Y. Y. Gleba et al., 2014; Pogue et al., 2010). There are 3 main *Agrobacterium* inoculation methods. Infiltration of *Agrobacterium* or agroinfiltration is done in two ways: either by needleless syringe or by a mild vacuum (~0.8-1.0 bar). A plant can also be inoculated with *Agrobacterium* by spraying, whereby the plants are sprayed with a suspension of *Agrobacterium* cells supplemented with a surfactant (Y. Gleba et al., 2007; Y. Y. Gleba et al., 2014; Komarova et al., 2010; Leuzinger et al., 2013).

Agroinfiltration by vacuum uses negative pressure to flood the intercellular plant leaf space with a suspension of *Agrobacterium* cells. The unpruned plant is dipped into the infiltration solution containing agrobacteria while the vacuum is applied. The vacuum withdraws the air from the plant tissue, and during vacuum release, the infiltration solution is pressed into the plant tissue. While infiltration efficiency is slightly less than with syringe infiltration, vacuum infiltration is far less labor-intensive for large-scale production (Hahn et al., 2015).
When using plant inoculation by spraying, the surfactant facilitates the entry of agrobacteria into intercellular spaces of plant leaves. Agroinfiltration by needleless syringe is labor-intensive and more suitable for small-scale screening approaches, but plant inoculation by vacuum or spraying can be highly scalable and is used in industrial applications (Y. Y. Gleba et al., 2014; Klimyuk, Pogue, Herz, Butler, & Haydon, 2014).
The TMV-based protein expression in plants usually reaches its maximum attainable yield (referred to as the optimal harvesting time point) between 4-8 days post inoculation (dpi) (Schneider et al., 2018; Schulz et al., 2015). After the optimal harvesting time point, recombinant protein yield can start to reduce due to cytotoxic effects. The optimal harvesting time point is determined for each newly expressed recombinant protein.

The native Australian species *Nicotiana benthamiana* has been extensively used for research since the 1940s (Bally 2018). It has become a key production platform for molecular farming due to its high susceptibility to virus-delivered gene expression and gene silencing (insertion in the RNA-dependent RNA polymerase gene NbRdRP1m; (S. J. Yang, Carter, Cole, Cheng, & Nelson, 2004), ease of cultivation, and high recombinant protein expression rates (up to 5mg/g FW). (Goodin, Zaitlin, Naidu, & Lommel, 2008; Pogue et al., 2010). The FDA recognizes the expression host as safe for animal and human consumption if the final product is sufficiently purified (GRN 775 (2018); GRN802 (2019); GRN 824 (2019), GRN 910 (2020); GRN 920 (2020)). For all its advantages, multiple product candidates in clinical evaluation (e.g., vaccines, antibodies, therapeutics) are already being expressed in *N. benthamiana* by companies such as Kentucky BioProcessing, Inc. (KBP), Fraunhofer IMB, iBio CMO, Icon Genetics GmbH, and Nomad Bioscience GmbH (McNulty et al., 2020; Stephan et al., 2017).

1.6 Objective of research

The overall aim of this thesis was to address the ever-increasing need for developing new antimicrobials due to the accelerated formation of antimicrobial resistance combined with a lack of new drug development.

Nomad Bioscience has successfully produced and analyzed a number of plant-produced recombinant colicins. This research aims to further deepen the understanding of the antimicrobial activity of colicin-like bacteriocins and their potential for industrial and medical use.

The practical value of new antimicrobials for the treatment of pathogenic bacteria depends on the target pathogen's ability to develop resistance against them. Therefore, the calculation of the minimum inhibitory concentrations with a consecutive determination of the resistance mutation rates are essential data required to assess the potential of any said antimicrobial protein as a medicine or a food antimicrobial. This should be done for individual colicins and mixtures thereof.

Given a similar molecular architecture of colicin-like bacteriocins, namely their modular design that comprises receptor-binding, translocation, and cytotoxicity domains, the simple other way of producing novel bacteriocins should be engineering by swapping domains coming from different natural bacteriocins. Obtained chimera should be tested for and compared with the parental molecules concerning antimicrobial activity potency and breadth.

While colicins have shown great *in vitro* capabilities, they have only seldomly been tested *in vivo*. Therefore, the feasibility of colicin mixtures should be tested in an animal study on a Post-Weaning Diarrhea- causing ETEC strain.

On a global scale, NTS causes an estimated 93.8 million illnesses per year (80.3 million are foodborne), leading to an estimated 155,000 deaths. Additionally, antibiotics have been mostly banned in animal husbandry in the EU and in other countries and are not suitable for food treatment, leaving the food industry with limited decontamination options. Much attention has been given to colicins, natural non-antibiotic proteins of the bacteriocin class, to control the related pathogen *Escherichia coli*.

However, the main goal of this thesis will be to test colicin-like bacteriocin candidates from *Salmonella*, named salmocins. Their expression as soluble recombinant proteins should be determined along with their activity spectra on *Salmonella*. Furthermore, the potential of salmocins for *Salmonella* food decontamination in the food industry should be analyzed.

If salmocins identical to their native bacterial counterparts could be expressed in plants at high yields and offer good antimicrobial activity on a wide variety of *Salmonella* strains, this would further highlight the feasibility and benefits of plant-based bacteriocin production.

2. Materials and methods

2.1 Bacterial strains and growth conditions

2.1.1.1 Pathogenic bacteria

2.1.1.2 Pathogenic *Escherichia coli* strains

Table 3: Pathogenic *E. coli* serotypes.
Shiga toxin-producing *E. coli* (STEC); Enterohemorrhagic *E. coli* (EHEC); Enterotoxigenic *E. coli* (ETEC); American Type Culture Collection (ATCC); Centers for Disease Control and Prevention (CDC).

organism	culture collection reference #	serotype	characteristics	source of supply	used for experiments
E. coli (STEC)	CDC 03-3014	O26:H11	positive for virulence genes *stx1* and/or *stx2* and *eae*	Big 7 STEC QC Set (#5219, Microbiologics Inc., St. Cloud, Minnesota USA)	3.2.3; 3.4.3.2
	CDC 00-3039	O45:H2			3.2.3; 3.4.3.2
	CDC 06-3008	O103:H11			3.2.3; 3.4.3.2
	CDC 2010C-3114	O111:H8			3.2.3; 3.4.3.2
	CDC 02-3211	O121:H19			3.2.3; 3.4.3.2
	CDC 99-3311	O145:NM			3.2.3; 3.4.3.2
	ATCC® 35150™	O157:H7			3.2.3; 3.4.3.2
E. coli (STEC)	ATCC® BAA-2326™	O104:H4	positive for virulence gene *stx2*	QC strain (#01104, Microbiologics Inc.)	3.4.3.2
E. coli (ETEC)	CVI-1000	O149:K91	(F4 (K88ac), LT+, STb+)	SGS Nederland, DGS Shiphol, Douglassingel 25, 1119 MC Shiphol-Rjik	3.3.2; 3.3.6

All strains (except for CVI-1000) were used exclusively for antimicrobial screens of bacteriocins. They were streaked individually from liquid nitrogen permanent culture on LB agar medium and grown ON at 37°C. Then they were individually inoculated into LB broth and grown ON at 37°C and 150 rpm. The strains were handled in a biosafety level 2 (BSL-2) laboratory (Biosolutions Halle GmbH, Halle, Germany).

E. coli serotype O149:K91:F4ac (LT+, STb+) was isolated from a farm pig with PWD and designated CVI-1000 (Animal and Science Group, Lelystad, Netherlands) (Nabuurs, Hoogendoorn, & van Zijderveld-van Bemmel, 1996). It was used for the animal study.

2.1.1.3 Pathogenic *Salmonella* strains

Table 4: *Salmonella enterica ssp. enterica* strains.

No.	culture collection: culture collection reference No.	Serovar/ Serotype	Serotype designation (Subspecies [space] O antigens [colon] Phase 1 H antigen [colon] Phase 2 H antigen)	source of supply	Rank according to CDC 2012 Report[+]
1	ATCC® 13076™*	**Enteritidis**	I 1,9,12:g,m:-	(#0345P, Microbiologics Inc.)	1
2	ATCC® 49223™*		I 9,12:g,m	(#01103P, Microbiologics Inc.)	
3	ATCC® 14028™*	**Typhimurium**	I 4,5,12:i:1,2	(#5068P, Microbiologics Inc.)	2
4	ATCC® 13311™*		I 4,5,12:i:1,2	(#0421P, Microbiologics Inc.)	
5	ATCC® 6962™*	**Newport**	I 6,8:e,h:1,2	(#01095P, Microbiologics Inc.)	3
6	ATCC® 10721™*	**Javiana**	I 1,9,12:l,z28:1,5	LGC standards	4
7	ATCC®BAA-1593™		I 9,12:-:1,5	LGC standards	
8	ATCC® 8387™*	**Montevideo**	I 6,7:g,m,s:-	LGC standards	6
9	ATCC®BAA-1675™	**Infantis**		LGC standards	7
10	ATCC® 8388™*	**Muenchen**	I 6,8:d:1,2	LGC standards	8
11	ATCC® 8326™*	**Heidelberg**	I 4,5,12:r:1,2	(#01151P, Microbiologics Inc.)	9
12	ATCC® 9115™*	**Bareilly**	I 6,7:y:1,5	LGC standards	10
13	ATCC® 8391™*	**Thompson**	I 6,7:k:1,5	LGC standards	12
14	ATCC® 9712™*	**Saintpaul**	I 1,4,5,12:e,h:1,2	LGC standards	13
15	ATCC® 9239™*	**Oranienburg**	I 6,7:m,t:-	LGC standards	14
16	ATCC®BAA-2739™	**Mississippi**	I 13,23:b:1,5	LGC standards	15
17	ATCC® 9270™*	**Anatum**	I 3,10:e,h:1,6	(#01095P, Microbiologics Inc.)	17
18	ATCC® 51957™*	**Agona**	I 4,12:f,g,s:-	(#01154P, Microbiologics Inc.)	19
19	ATCC® 8392™*	**Berta**	I 9,12:f,g,t:-	LGC standards	20
20	ATCC® 15480™*	**Dublin**	I 1,9,12:g,p:-	LGC standards	
21	ATCC® 6960™*	**Derby**	I 1,4,12:f,g:-	LGC standards	
22	ATCC® 10723™*	**Cerro**	I 18:z4,z23:-	LGC standards	
23	DSM 10062	**Senftenberg**	I 1,3,19:g,s,t:-	Leibniz Institute DSMZ	
24	ATCC® 9263™*	**Kentucky**	I (8),20:i:z6	LGC standards	
25	ATCC® 51958™*	**Mbandaka**	I 6,7:z10:e,n,z15	LGC standards	
26	ATCC® 10708™*	**Cholerasius**	I 6,7:C:1,5	(#01095P, Microbiologics Inc.)	
27	ATCC® 12002™*	**Tallahassee**	I 6,8:z4,z32:-	(#01095P, Microbiologics Inc.)	
28	ATCC® 9150™*	**Paratyphi A**	I 1,2,12:a:-	(#01095P, Microbiologics Inc.)	
29	NCTC 6017	**Abony**	I 4,12,27:b:e,n,x	(#0890P, Microbiologics Inc.)	
30	ATCC® 13036™*	**Pullorum**	I 9,12:-:-	(#0604P, Microbiologics Inc.)	
31	ATCC® 15611™*	**Vellore**	I 1,4,12,27:z10:z35	(#0342P, Microbiologics Inc.)	
32	ATCC® 9842™*	**Bispebjerg**	I 4,12:a:enx	(#01056P, Microbiologics Inc.)	
33	NCTC 4840	**Poona**	I 13,22:z:1	(#0851P, Microbiologics Inc.)	
34	DSM 4883	**Gallinarum**	I 9:-:-	Leibniz Institute DSMZ	
35	DSM 13674	**Gallinarum**	I 9,12:-:-	Leibniz Institute DSMZ	
36	ATCC® 700136™*	**Braenderup**	I 6,7:e,h:e,n,z15	LGC standards	11
37	17-00918	-	I 4,[5], 12:i:-	Robert Koch Institute	5

[+]National *Salmonella* Surveillance Annual Report 2012, Table 1a (CDC, 2014).

Serotypes are ranked 1-20 according to the number of laboratory-confirmed human *Salmonella* infections reported to the CDC. All *Salmonella* strains were streaked individually from liquid nitrogen permanent culture on LB agar medium and grown ON at 37°C. Then they were individually inoculated to LB broth and grown ON at 37°C and 150 rpm. All strains were handled in a biosafety level 2 (BSL-2) laboratory (Biosolutions Halle GmbH, Halle, Germany). Strains 1-36 were used in experiments 3.4.3.1. Strains 1, 3, 5, 6, 8, 9, 10, 11, 14 and 37 were used in experiment 3.2.3. Strains 1 and 3 were used in experiment 3.4.4. Nalidixic acid-resistant derivatives of *S. enterica* ssp. *enterica* serovars Enteritidis (strain ATCC®13076™*), Typhimurium (strain ATCC®14028™*), Newport (strain ATCC® 6962™*), Javiana (strain ATCC® 10721™*), Heidelberg (strain ATCC® 8326™*), Infantis (strain ATCC®BAA-1675™) and Muenchen (strain ATCC® 8388™*)) were used in experiments 3.4.5.1 and 3.4.5.2.

Strain suppliers were Microbiologics, Inc. (St. Cloud, USA), LGC Standards (Teddington, UK), Leibnitz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and Robert Koch Institute, national reference centre for salmonellosis and other enteric pathogens (Wernigerode, Germany).

2.1.2 **Non-pathogenic bacteria**

2.1.2.1 *Escherichia coli*

***E. coli* ATCC® 25922™** (Microbiologics, Inc. (St. Cloud, USA)

Used for the determination of the minimal inhibitory concentration of colicins. Grown overnight (ON) at 37°C in LB broth with shaking at 150 rpm or on LB agar

ElectroMAX™ DH10B™ cells from Invitrogen™ (Fisher Scientific GmbH, Schwerte, Germany). Genotype: F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139Δ(*ara*, *leu*)7697 *gal*U *gal*K λ⁻*rps*L *nup*G.

Used to determine the minimal inhibitory concentration and mutation rate of colicins to a *E. coli* DH10B strain. Grown ON at 37°C in Müller-Hinton Bouillon (MHB) (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) with shaking at 150 rpm or on Müller-Hinton agar (MHA) (prepared from the above mentioned MHB supplemented with 0.75% (w/v) agar bacteriology grade (AppliChem GmbH, Darmstadt, Germany).

***E. coli* DH10B**

Used for cloning of plasmids. Grown ON at 37°C in LB broth with shaking at 150 rpm or on LB agar. Depending on the plasmid-mediated resistance, the growth medium was supplemented with kanamycin (AppliChem GmbH, Darmstadt, Germany), carbenicillin (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), and streptomycin (AppliChem GmbH, Darmstadt, Germany) at 50 µg/ml.

***E. coli* DH10B (pICH 73311)**

Used as an *E. coli* DH10B strain for antimicrobial activity determination in soft agar overlay assay (2.8.1).

This DH10B strain carries a plasmid for the expression of genes providing red-orange color to the cells, facilitating visual inspection of bacterial growth inhibition in soft agar overlay assay. It is grown ON at 37°C in LB broth (shaking at 150 rpm) or on LB agar supplemented with kanamycin and streptomycin at 50 µg/ml.

ElectroMAX™ DH10B™ T1 Phage-Resistant Competent Cells from Invitrogen™ (Fisher Scientific GmbH, Schwerte, Germany).

Genotype: F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139Δ(*ara*, *leu*)7697 *gal*U *gal*K λ⁻*rps*L *nup*G *ton*A.

Used as a positive control for ColM resistance (*fhuA* mutation) in DH10B. Grown ON at 37°C in LB broth (Shaking at 150 rpm) or on LB agar.

2.1.2.2 *Agrobacterium tumefaciens*

***Agrobacterium tumefaciencs* ICF320** (Bendandi et al., 2010) carrying binary vectors was cultivated at 28°C in LBS medium (modified LB medium containing 1% soya peptone (Duchefa, Haarlem, Netherlands) or LBS agar supplemented with 50 µg/ml kanamycin (plasmid-mediated resistance) and 50 µg/ml Rifampicin (Duchefa, Haarlem, Netherlands) for ~24 h at 180 rpm.

2.1.2.3 Other bacteria

Micrococcus luteus supplied by BioSolutions Halle GmbH (Halle (Saale), Germany). Original source: Institute of Biology/Microbiology of the Martin-Luther-Universität Halle-Wittenberg. Cells were grown ON at 37°C, 150 rpm in LB broth or on LB-agar.

2.2 Nucleic acid isolation

2.2.1 Isolation of genomic DNA from *E. coli*

ElectroMAX™ DH10B™ cells (Invitrogen™) were inoculated into LB broth from -80°C glycerol culture and grown ON at 37°C and 180 rpm. For genomic DNA isolation, the PureLink® Genomic DNA Kit (Fischer Scientific GmbH, Schwerte Germany) was used according to the manufacturer's instructions. The DNA concentration was measured with the Biotek™ Synergy™ HTX Multi-Mode Microplate Reader (Model: HTX S1LA; BioTek Instruments, Inc., Winooski, VT, USA).

2.2.2 Isolation of plasmid DNA from *E. coli*

E. coli DH10B carrying plasmids was inoculated into LB broth from -80°C glycerol culture or from LB-agar plate culture and grown ON. For plasmid DNA isolation, the GeneJET Plasmid Miniprep Kit (Fisher Scientific GmbH, Schwerte, Germany) was used according to the manufacturer's instructions. Plasmid DNA was eluted in sterile water and stored at -20°C.

2.2.3 Isolation of mRNA from plant material

Plant leaf material, snap-frozen in liquid nitrogen upon harvest and stored at -80°C in 2 ml Eppendorf® tubes, was ground using the Quiagen TissueLyser II (Quiagen GmbH, Hilden, Germany) at a frequency of 30 Hz for 1 min and then placed on ice. The mRNA isolation was done with the NucleoSpin® RNA Plant kit (Machery-Nagel GmbH & Co. KG, Düren, Germany), according to the manufacturer's protocol.

The mRNA was eluted with 60 µl RNase-free water and stored at 4°C. The mRNA quality and quantity were measured with the Biotek™ Synergy™ HTX Multi-Mode Microplate Reader and analyzed by gel electrophoresis.

2.3 Nucleic acid manipulation

2.3.1 DNA-amplification by PCR

2.3.1.1 PCR for cloning

The 50 µl PCR reactions for cloning were conducted with different polymerases and their accompanying kit components: The KOD Hot Start DNA Polymerase (71086-3 Sigma-Aldrich, Taufkirchen, Germany) was used for GC-rich sequences. The Phusion™ High-Fidelity DNA Polymerase (2 U/µl) (Fisher Scientific GmbH, Schwerte, Germany) was used for all other sequences. Both polymerases offer proofreading capability and were used according to the

manufacturer's instructions. 200 µM dNTP (Fisher Scientific GmbH, Schwerte, Germany), 0.1 µM forward oligonucleotide, and 0.1 µM reverse oligonucleotide were added to the reactions.

2.3.1.2 Colony PCR

Colony PCRs were used to screen *E. coli* DH10B individual colonies for successful cloning events. After a plasmid transformation and ON incubation on LB-agar, bacterial colonies were picked with a pipette tip, transferred to a new LB-agar plate, and then the pipette tip was shortly dipped into the tube with the PCR reaction mixture.

The 25 µl PCR reactions contained: 1x PCR loading dye (20x loading dye: 30% sucrose, 6 mM tartrazine yellow, 3.5 mM cresole red (Hodges, Boddy, Thomas, & Smith, 1997) 200 µM dNTP (Thermo Fisher Scientific, Schwerte Germany), 0.1 µM forward oligonucleotide, 0.1 µM reverse oligonucleotide, 0.025 U Taq polymerase, and dH_2O. The PCR program used is shown in Table 5.

Table 5: Program for colony PCR

step	temperature	time
1. cell lysis/denaturing	95 °C	5 min
2. denaturing	95 °C	20 sec
3. annealing	55-65 °C*	30 sec
4. elongation	72 °C	60 sec/kb
cycle steps 2-4 for 30 cycles		
5. final elongation	72 °C	5 min

*~5°C below melting temperature of primers

2.3.1.3 Analytical PCR on plasmids

This PCR was conducted to confirm the proper directional insertion of a cloning reaction and/or proper sequence of such insert. For the 50 µl PCR reactions, the same components, concentrations, and PCR program were used as for the colony PCR. Additionally, 20-40 fmol plasmid DNA was added.

2.3.1.4 PCR on genomic DNA for sequencing

For the amplification, the gDNA concentration was adjusted to 10 ng/µl in sterile water.

The 50 µl reactions contained: 1x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, 1% (v/v) Triton X-100, 0.1% (w/v) gelatine), 200 µM dNTP (Thermo Fisher Scientific,

Schwerte, Germany), 0.1 µM forward oligonucleotide, 0.1 µM reverse oligonucleotide, 0.025 U Taq polymerase, 50 ng gDNA and dH_2O. The PCR program used is shown in Table 6.

Table 6: Program for PCR on gDNA

step	temperature	time
1. cell lysis/Denaturing	95 °C	5 min
2. denaturing	95 °C	20 sec
3. annealing	55-65 °C*	30 sec
4. elongation	72 °C	~1 kb/min at 72°C
cycle steps 2-4 for 30 cycles		
5. final elongation	72 °C	5 min

2.3.2 Reverse transcription of mRNA to cDNA

The reverse transcription of mRNA to cDNA was conducted with the RevertAid H Minus First Strand cDNA Synthesis Kit (Fisher Scientific GmbH, Schwerte, Germany), according to the manufacturer's instructions. The reactions in 0,2 ml Eppendorf® PCR tubes were incubated in the PCR machine (Biometra TRIO PCR machine (Analytik Jena AG, Jena Germany)) for 60 min at 42°C, then for 5 min at 70°C. The cDNA was stored at -20°C until further use.

2.3.3 Restriction digestion of plasmid DNA

Isolated DNA was digested by restriction enzymes (New England Biolabs GmbH, Frankfurt am Main, Germany) according to the manufacturer´s instructions. Restriction digestions for analytical and cloning purposes contained 1x buffer (New England Biolabs GmbH, Frankfurt am Main, Germany), 5-10 units of enzyme/µg DNA depending on endonuclease efficiency in the buffer, ~200 ng DNA for analytical digestion or ~500 ng DNA for cloning digestion, and deionized water (dH_20) added to a total volume of 20 µl.

The reaction was incubated for 1-3 h at the optimal temperature, depending on the DNA concentration and enzyme efficiency in the buffer. The cleaved DNA was then separated by agarose gel electrophoresis for analysis or further downstream manipulation.

2.3.4 DNA Ligation

DNA ligation for cloning was performed in a 20 µl reaction volume containing 1 U T4 DNA ligase (Thermo Fisher Scientific GmbH, Darmstadt, Germany), 1x T4 DNA ligase buffer, ~50

ng of the vector and ~150 ng of the insert in dH_20. Incubation was done for 1-3 h at room temperature or ON at 4°C. The vectors were transformed into competent *E. coli* DH10B cells.

2.3.5 Golden-Gate/Modular Cloning

Golden-Gate/Modular Cloning (MoClo) (Weber, Engler, Gruetzner, Werner, & Marillonnet, 2011) was used to assemble constructs for protein expression in plants, which are described in (Schneider et al., 2018) and 1.5. The expression constructs are designed for easy insertion of the gene of interest with the MoClo approach.

A standard MoClo cloning reaction had a volume of 20 µl, which included 40 fmol of each vector, 15 U *Bsa*I (New England Biolabs GmbH, Frankfurt am Main, Germany), 15 U T4 DNA ligase (Thermo Fisher Scientific GmbH, Darmstadt, Germany), 1x T4 DNA Ligase buffer and dH_2O to 20 µl.

The cloning reaction was conducted in a PCR thermocycler: Step 1: 37°C for 2 min, Step 2: 20°C for 4 min, Step 3: 50°C for 5 min, and Step 4: 80°C for 5 min. Steps 1 and 2 were cycled 30-35 times. The reaction was then transformed into competent *E. coli*.

2.3.6 DNA purification

PCR products or restriction fragments were separated by gel electrophoresis and excised from the agarose gel with a scalpel, weighed, and purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania). DNA was eluted in sterile water and stored at -20°C.

2.3.7 Transformation of competent *E. coli* cells

A 50 µl aliquot of chemically competent *E. coli* cells was thawed on ice for 2 min and mixed with the DNA, e.g., 20 µl of a cloning reaction or 1 µl of plasmid. The mixture was further incubated on ice for 10-20 min. Subsequently, it was heat-shocked at 42°C for 45 s in a water bath, followed by immediate cooling on ice for 1 min. After adding 500 µl of LB medium, the mixture was incubated at 37°C for 1 h. Finally, the bacterial suspension was plated on selective LB agar plates and incubated ON at 37°C. Individual bacterial clones from the plates were selected using, e.g., blue-white screening, and their plasmids were isolated (2.2.2) to analyze them by one or a combination of different screening methods, including colony PCR, restriction digestion, and sequencing (2.4.2). Bacterial clones with a correct plasmid were grown in liquid LB-medium ON at 37°C, and 180 rpm, and a -80°C glycerol stock was prepared.

2.3.8 Transformation of *Agobacterium tumefaciens*

A 50 µl aliquot of electro-competent cells *of A. tumefaciens* strain ICF320 was thawed on ice for 2 min and mixed with 1 µl of plasmid DNA. The mixture was then incubated on ice for 10-15 min before transferring it to a pre-cooled cuvette (electroporation cuvettes EP 201, Cell Projects Ltd, Kent, UK). Next, the cuvette with the cells was pulsed in the electroporator (MicroPulsar^{TM} Electroporater, Bio-Rad, Hercules, USA), set to the *Agrobacterium tumefaciens* specific program (2.2 kV for ~5 ms). Afterward, the cuvette was removed immediately and cooled on ice for 1-2 min before adding 500 µl LBS medium. The cell suspension was then transferred to an Eppendorf tube and incubated for 2 h at 28°C before plating them on selective LB plates. Plates were incubated at 28°C for 48 h. The ICF320 cells were collected from the plate, inoculated into 5 ml selective liquid LBS medium and incubated at 28°C for 48 h before a -80°C glycerol stock was prepared.

2.4 Nucleic acid analysis

2.4.1 Agarose gel electrophoresis

To perform agarose gel electrophoresis, the gel matrix agarose was added into TAE buffer (40 mM Tris (hydroxymethyl) aminomethane, 20 mM acetate, and 50 mM Ethylenediamine Tetraacetic Acid) in concentrations of 0.8-2% (w/v)). 1% ethidium bromide solution (Carl Roth + Co. KG, Karlsruhe, Germany) or MIDORI Green Advance DNA Stain (Nippon Genetics Europe GmbH, Düren, Germany) (~0.2-0.5 µg/ml) were added to the gel for DNA visualization. The DNA samples were supplemented with 6x loading buffer (Fisher Scientific GmbH, Schwerte, Germany) and loaded onto the gel with a molecular-weight size marker (e.g., GeneRuler 1 kb Plus DNA Ladder, Fisher Scientific GmbH, Schwerte, Germany). After electrophoresis, the gel was analyzed under UV light and recorded by an imaging system.

2.4.2 DNA sequencing and software-assisted analysis of nucleotide sequences

Sanger sequencing was performed by Seqlab (Seqlab Sequence Laboratories Göttingen GmbH, Göttingen, Germany).

Sequence data files were compared with reference sequences created and compiled in Vector NTI Advance® suite (version 11.5.2; Thermo Fisher Scientific) using the alignment tool Sequencher (ver. 4.10.1; Gene Codes Corporation)

2.5 Transient *Agrobacterium*-mediated expression of proteins in plants

2.5.1 Plant growth under greenhouse conditions

All *Nicotiana benthamiana* and *Spinacia oleracea* 'Frühes Riesenblatt' plants were grown in the greenhouse at day temperatures of 19-23 °C and night temperatures of 17-20 °C, with a humidity of 35-70% and light for 12h. If not stated otherwise, five-week-old spinach and 6-week-old *N. benthamiana* plants were used for plant inoculation with *A. tumefaciens*.

2.5.2 Agroinfiltration with needleless syringe

Six-week-old *N. benthamiana* plants were pruned 1-2 days before infiltration, leaving only 4-5 large leaves. The *Agrobacterium* cultures for bacteriocin expression were inoculated from -80°C glycerol stocks and grown ON at 28°C and 180 rpm in 5 ml LBS medium supplemented with 50 µg/ml kanamycin and 50 µg/ml rifampicin). For infiltration, the culture was diluted to OD_{600}=1.5 (Ultrospec 3100 Pro Spectrophotometer (GE Healthcare Life Sciences, Chalfont St. Giles, UK) in the infiltration medium (10 mM MES (pH 5.5), 10 mM $MgSO_4$) and further diluted 1:100 with infiltration medium. For the co-agroinfiltration of plants with a bacteriocin and its respective immunity protein-expressing constructs, both cultures, adjusted to OD_{600} = 1.5 were combined 1:1 and diluted 1:50 with the infiltration medium. The *Agrobacterium* culture was infiltrated into the abaxial side of 3-4 leaves with a 1-ml needleless syringe using the alternating spot-infiltration method (Figure 3). The plants were then incubated under greenhouse conditions until harvest.

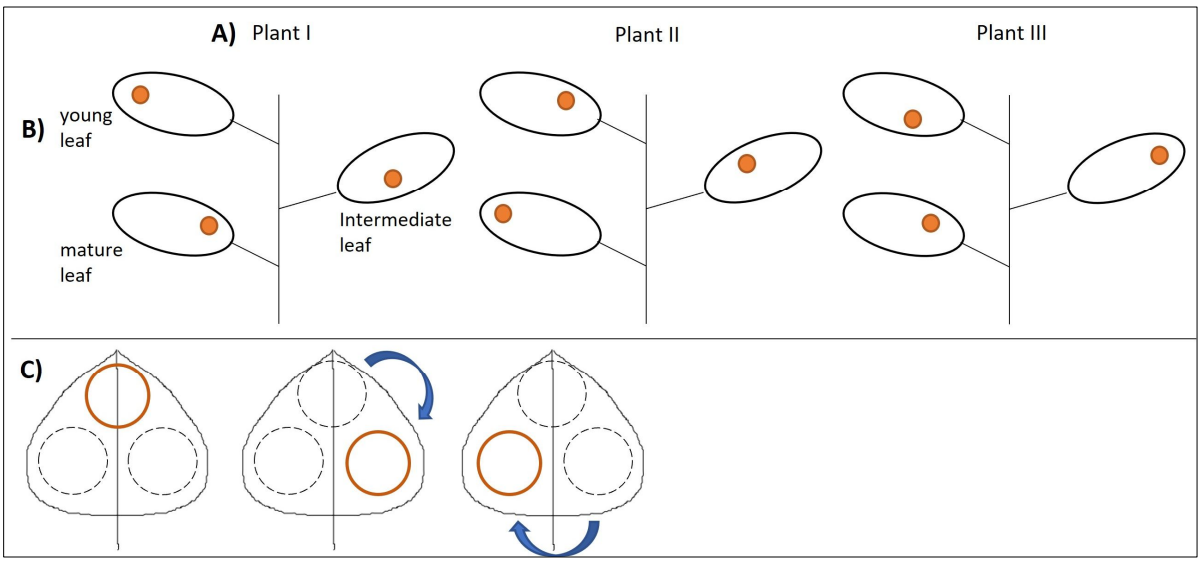


Figure 3: Alternating spot-infiltration setup to determine the optimal harvesting time point of a plant-expressed bacteriocin.
The inoculation spots by needleless syringe (orange) were (A) distributed over 3 plants (I, II, and III) (B) 3 different leaf ages (young, intermediate, and mature leaf), and (C) rotated clockwise over different areas of the leaf blade. On each day of harvest, a sample of the bacteriocin expressing leaf material was harvested: 1 leaf disc from each of the infiltration spots (a pool of 9 leaf discs), offering a good overall expression representation in the plants.

2.5.3 Agroinfiltration of plants with vacuum

For the infiltration mixture, a 5 ml LBS medium culture was inoculated with *Agrobacterium* (ICF320) from -80°C glycerol stock and incubated at 28°C and 130 rpm ON. This ON culture was then added to a 100 ml LBS medium culture and incubated ON at 28°C at 130 rpm. The 100 ml ON culture was diluted to OD_{600} = 1.4 with infiltration medium (10 mM MES (pH 5.5), 10 mM $MgCl_2$) before being further diluted 1:100 in the infiltration medium. The infiltration mix was filled into a 10 l bucket and placed inside a vacuum bell jar.

Six-week-old *N. benthamiana* plants were pruned 1-2 days before infiltration leaving only medium to large-sized leaves. Shortly before infiltration, the plants were watered to open up the stomata. The plant pots were covered with tin foil to prevent soil from falling out when upside down.

Individual plants were submerged upside down into the infiltration solution, the pot held by 2 sticks lying on the rim of the bucket. The bell jar for vacuum generation was sealed, and a vacuum of ~0.8-1.0 bar was created with a vacuum pump and maintained for ~30 s before releasing the vacuum. Infiltrated leaves showed a dark green color where the solution had reached the intercellular space. The plant was supported by a stick and returned to the greenhouse for plant incubation.

2.5.4 Plant inoculation with *A. tumefaciens* by spraying

Preparation of the *Agrobacterium* infiltration mixture (up to the dilution step) and the pruning of plants was described in 2.5.2. The ON *Agrobacterium* culture was diluted to OD_{600} = 1.4 in infiltration medium (10 mM MES (pH 5.5), 10 mM $MgCl_2$), then further diluted to 1:100 in 250 ml infiltration medium and filled into a 250 ml plastic sprayer flask (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The mix was supplemented with 0.1% (v/v) Silwet L-77 (Kurt Obermeier GmbH & Co. KG, Bad Berleburg, Germany), a surfactant to enhance the transfection by spraying. The spraying was carried out in a special cabinet with exhaust ventilation that traps and removes all aerosols produced by spraying. Each of the leaves was sprayed 3 times on the adaxial and abaxial leaf surface. The plants were then incubated under greenhouse conditions until harvest.

2.5.5 Harvest and storage of bacteriocin-containing plant material

2.5.5.1 Small-scale harvest

This procedure was used for harvesting bacteriocins infiltrated with the alternating spot-infiltration method (2.5.2). Plant material was harvested using cork borers of 1cm diameter. If not stated otherwise, 9 leaf discs per sample originating from 3 leaves of three plants were harvested, pooled as one sample, and transferred into a 2 ml Safe-Lock Eppendorf tube (2.5.2, Figure 3). Additionally, 2 stainless steel balls (3.175 mm; Kugel Winnie, Bamberg, Germany) were added to facilitate later pulverization. After determining the fresh weight (FW), the samples were snap-frozen in liquid nitrogen and stored at -80°C.

2.5.5.2 Larger-scale harvest

This procedure was used for the production of larger amounts of bacteriocin. The infiltrated leaf material was harvested by collecting the leaf blades and removing the major veins and non-infiltrated leaf areas. The leaf material was ground in liquid nitrogen with a precooled mortar and pestle to a fine powder. The frozen powder was transferred to precooled 50 ml Falcon tubes and stored at -80°C. Any thawing until storage at -80°C was avoided.

2.5.5.3 Production of dry leaf powder for leaf-derived colicin formulation

Agroinfiltration of *N. benthamiana* leaves with vacuum (2.5.3). All leaves were harvested, and major leaf veins and uninfiltrated leaf areas were removed. Next, the leaves were cut into ~2 x

2 cm pieces, pooled, and weighed. For later reference (2.10.1), a sample of the leaf material was harvested as described in 2.5.5.2.

The cut-up leaves were dried in Miracloth "bags" 60x40 cm, made by sealing rectangle pieces of Miracloth tissue at the edges using metal staples. Bags were placed into a drying oven with circulating air at 37°C for ~ 48h or until the plant material was dried entirely with a "crunchy-brittle" texture. The dried leaf material was then blended to a fine powder in a waring blender (Memory Blender (RMB), Rotor, Switzerland) at a maximum velocity twice for 15s or until completely fine. Due to the colicins´ heat sensitivity, the blender was given time to cool off between blending cycles. The dried leaf material was stored at room temperature in glass jars, sealed with clamps and Parafilm® M sealing film (Thermo Fisher Scientific, Darmstadt, Germany) to prevent moisture intake.

2.5.6 Determination of the optimal harvesting time point for plant expressed bacteriocins

When analyzing the expression of recombinant bacteriocins in plants, the optimal harvesting time point with the maximal attainable protein yield was determined. The leaf phenotype during protein expression was documented by a camera, focusing on the leaf tissue condition and signs of tissue necrosis.

Furthermore, the bacteriocin-containing plant material was harvested on several dpi (days post inoculation) (typically 4-8 dpi). The plant protein was then crudely extracted (2.6.1) for analysis by SDS-PAGE with Coomassie staining (2.7.2) to determine the time point of maximal attainable protein yield.

2.6 Preparation of bacteriocin-containing plant extracts

2.6.1 Crude extracts of plant material

Samples of plant material, harvested in small-scale and stored at -80°C were placed into the adapter sets (pre-chilled at -80°C) of the TissueLyser II (Quiagen GmbH, Hilden, Germany) and pulverized at a frequency of 30 Hz for 1 min.

After pulverization, 5 vol. of 2x Laemmli buffer (4% SDS (w/v), 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue, 0.125 M Tris-HCl, pH 6.8 (Laemmli, 1970)) were added.

Samples were then vortexed and incubated at 95°C for 10 min, vortexed again and centrifuged for 10 min at 13,000 rpm. The supernatant was transferred to a new Eppendorf tube.
Crude extract samples were either directly analyzed via SDS-PAGE or stored at -20°C. For SDS gel analysis, typically, 7.5 µl crude extract were loaded, which corresponds to 1.5 mg FW of plant leaf biomass.

2.6.2 **Total soluble protein (TSP) extracts of plant material**

Extraction of the TSP from plants was done with neutral or acidic extraction buffers. If small-scale harvested samples were used, the pulverization of the plant material was done with the TissueLyser II as described in 2.5.5.1. Large-scale harvested samples had already been pulverized before storage (2.5.5.2). The Eppendorf tubes or Falcon tubes were transferred to ice, and 5 vol. of pre-chilled (4°C) extraction buffer were added. The neutral HEPES-containing extraction buffer consisted of 50 mM HEPES pH 7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 300 mM NaCl (Schulz et al., 2015). The acidic extraction buffers were e.g., phosphate buffers at pH 4.0, or pH 5.5. Samples were vortexed and incubated on ice for 10 min with further vortexing every 5 min. The samples were then centrifuged for 10 min at 13,000 rpm and 4°C (small-scale samples) or for 10 min at 4500 rpm and 4°C (large-scale samples). After this, the supernatant was transferred to a new tube, and samples were analyzed.

2.7 Protein analysis

2.7.1 **Determination of protein concentration by Bradford assay**

The Bradford protein assay was used for the quantification of the total soluble protein (TSP) concentration in plant extracts (Bradford, 1976). The assay was performed using a microplate reader (Biotek Synergy HTX Multi-Mode Reader). BSA (Protein standard (bovine serum albumin), 1mg/ml BSA in 0.15 M NaCl, 0.05% sodium azide; Sigma-Aldrich Chemie GmbH, Munich, Germany) was diluted with dH_2O and used as a protein standard for the calibration curve. TSP extracts were also diluted with dH_2O. The assay was performed in a 96-well plate (P96 Maxisorb, Nunc Immunoplate, Thermo Scientific). Standard and samples were measured in duplicate. Per well, 10 µl of the protein standard series and the TSP samples were added. In the next step, 200 µl of Coomassie Blue G250 (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories, Munich, Germany) were added to each well. The plate was

incubated for 5 min at RT, placed into the reader, and mixed by orbital shaking inside the reader. The absorbance of the solution was measured at 595nm.

For concentration measurements of purified protein, copper-based protein quantification using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) was performed according to the manufacturer's instructions.

2.7.2 Protein analysis by SDS-PAGE and Coomassie-Staining

Proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie-stained using PageBlue Protein Staining Solution (Thermo Fisher Scientific). The gels were prepared as described in Table 7.

Table 7: Reagents and composition of the SDS resolving and stacking polyacrylamide gels

	resolving gel (ml, total 10 ml)			stacking gel (ml, total 10 ml)
gel percentage	**10%**	**12%**	**15%**	**4%**
dH_2O	4.00	3.35	2.45	6.10
1,5 M Tris HCL pH8.8*	2.50	2.50	2.50	---
0,5 M Tris HCL pH6.8*	---	---	---	2.50
10% SDS*	0.10	0.10	0.10	0.10
30% acrylamide/bis-acrylamide*	3.33	4.00	5.00	1.30
10% ammonium persulfate*	0.05	0.05	0.05	0.05
TEMED**	0.01	0.01	0.01	0.01

* Roth, Karlsruhe, Germany; ** Sigma-Aldrich, Munich, Germany

SDS-PAGE equipment originated from Bio-Rad (Feldkirchen, Germany). PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo Fisher Scientific, Darmstadt, Germany) was used as a marker.

TSP plant extracts or other protein-containing solutions were supplemented 1:2 with 2x Laemmli buffer (Laemmli, 1970), 4% SDS (w/v), 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue, 0.125 M Tris-HCl, pH 6.8) and denatured at 95°C for 10 min before loading them onto the polyacrylamide gel.

Electrophoresis was performed in 1x Running Buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) at 400 mA/130 V. After electrophoresis, the gels were placed in a tray on an orbital shaker for Coomassie staining and shaken for 10 min minutes in 25% (v/v) isopropanol, 10% (v/v) acetic acid for protein fixation. Subsequently, they were shaken in distilled water for 2 min and then for 4-18 h in Page Blue™ Protein Staining Solution (Fisher Scientific GmbH,

Schwerte, Germany). To destain the gels, they were shaken in dH_2O for 3-4 h with 2 paper tissues for Coomassie absorption. The gels were documented by scanning with a flatbed scanner.

2.7.3 Semi-quantitative protein estimation by SDS-PAGE and Coomassie staining

The estimation of recombinant protein as a percentage of TSP was done by comparison of the recombinant protein bands to a BSA protein standard (Protein standard (bovine serum albumin), ampule, 1mg/ml BSA in 0.15 M NaCl, 0.05% sodium azide; Sigma-Aldrich Chemie GmbH, Munich, Germany) on Coomassie-stained polyacrylamide-gels. TSP samples and the BSA standard dilution series were mixed 1:2 with 2x Laemmli buffer and incubated for 10 min at 95°C before subjecting them to electrophoresis. The recombinant protein bands were then visually compared to the BSA bands, assuming that protein bands with similar strength/intensity had a very similar protein quantity. An example of this semi-quantitative protein estimation can be seen in (3.4.2.2 Figure 26).

2.7.4 Protein purification

The affinity chromatography-purified bacteriocins were obtained from Nomad Bioscience GmbH researcher Anett Stephan. For the methods of purification used, please refer to (Stephan et al., 2017) for colicins and (Schneider et al., 2018) for salmocins. Recombinant protein quantification for purity determination of purified protein was performed by CGE (Capillary Gel Electrophoresis).

2.8 Antimicrobial activity testing of bacteriocins

2.8.1 Semi-quantitative determination of bacteriocin activity by soft agar overlay assay

A soft agar overlay assay is a semi-quantitative determination method for antimicrobial activity by growth inhibition described in (Schulz et al. 2015).

15-20 ml LB agar medium (1.5% (w/v) agar) were supplemented with antibiotics if required and poured into quadratic Petri dishes (~120x120x17mm). The bacterial test cultures were streaked on LB agar from liquid nitrogen permanent culture and grown ON at 37°C and 180 rpm. Consequently, a 5 ml liquid LB medium culture was inoculated from the streaked plate, and the culture was grown ON at 37°C and 180 rpm.

On the day of the experiment, the liquid culture was pre-diluted to OD_{600} = 1.0. LB soft agar (0.8% (w/v) agar) was melted, and 25 ml aliquots were cooled down to 55°C. The aliquots were then supplemented with antibiotics if required and seeded with 250 µl of the pre-diluted test culture (~1×10^7 CFU/ml bacterial cells or 0.14 ml/cm^2). The supplemented soft agar was poured into the Petri dish overlaying the pre-poured LB agar.

The bacteriocin solutions were applied to the agar in serial dilutions. The bacteriocin-containing solutions were either plant extracts (extracted with 5 vol. HEPES buffer (50 mM HEPES pH 7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 300 mM NaCl) or purified protein solutions. The bacteriocin solutions were serially diluted (usually 1:2) in HEPES buffer or 1% (w/v) milk powder (Carl Roth, Karlsruhe, Germany) solution and spotted onto the soft agar in 5 µl droplets. As a control, uninfiltrated plant material was analyzed within each experiment. The plates were incubated at 37°C ON for ~20h.

Soft agar overlay assay plates were visually inspected for bacteria growth inhibition. For each bacteriocin, the dilution factor of the highest dilution creating a visible growth inhibition in the bacterial lawn was recorded. The reciprocal of the dilution factor corresponds to the arbitrary activity units (AU) per mg fresh weight of plant material. The colicin-specific activity was defined as AU/µg recombinant protein, which can be calculated after assessing the bacteriocin content of the solution (2.7.1, 2.7.3).

2.8.2 **Quantitative activity determination of selected salmocins in broth culture**

Purified bacteriocins salmocin E1a (SalE1a), SalE1b, SalE7, ColM, and ColIb were provided by Anett Stephan (Nomad Bioscience GmbH) (2.7.4). For each individual batch of purified protein, the recombinant protein content was determined as described in 2.7.1. and 2.7.3.

The bacteriocins were dissolved in H_2O to a concentration of 0.1 mg/ml = 0.01% (w/w), and further dilutions were prepared with sterile 1 x phosphate-buffered saline (1x PBS), pH 7.4. The antibiotic kanamycin (kanamycin sulfate (AppliChem GmbH, Darmstadt, Germany) was also dissolved in this buffer to a concentration of 0.075 mg/ml.

The 2 *Salmonella enterica* strains ATCC®13076™ of serovar Enteritidis, and ATCC®14028™ of serovar Typhimurium (2.1.1.3) were streaked from liquid nitrogen permanent culture on LB-plates and grown ON. A liquid LB culture was then inoculated from the plate and grown ON at 37°C and 150 rpm. On the day of the experiment, the LB culture was diluted to OD_{600}=0.05 in 25 ml LB medium. The cultures were grown to OD_{600}~0.3 and diluted to OD_{600}=0.1 with pre-chilled (4°C) LB medium to halt further growth temporarily. The bacterial cultures were mixed

1:1 and diluted to a final density of OD_{600}=0.0001 (~$1.5x10^4$ CFU/ml) in pre-chilled (4°C) LB medium. Fourteen ml of bacterial solution were aliquoted into 100 ml Erlenmeyer flasks. These were then incubated at 4°C until antimicrobial treatment. For each treatment type, 3 technical replicates were prepared (N=3). The bacterial solutions were supplemented with either 1 ml bacteriocin solution, 1 ml negative control solution (1x PBS pH 7.4), or 1 ml kanamycin solution (0.075 mg/ml).

The following treatments were tested individually: SalE1a (0.1 and 0.01 µg/ml), SalE1b (1 and 0.1 µg/ml) and SalE7 (1 and 0.1 µg/ml). Additionally, a salmocin-colicin mixture combining SalE1b (0.03µg/ml), ColM (0.01 µg/ml) and ColIb (0.01 µg/ml) was tested. Kanamycin was also tested at a concentration of 5µg/ml. The treated cultures were incubated at 37°C or 10°C and agitated in an orbital shaker at 150 rpm for 24 h.

At T=0 h (before the addition of bacteriocin, carrier or antibiotic solutions) and upon T=2 h, T=4 h or T=24 h of antimicrobial treatment, viable bacteria were determined using dilution plating on LB agar. The plates were incubated at 37°C for 16-20 h, and colony-forming units (CFU) were enumerated.

2.8.3 **Analysis of bacteriocin activity on various meat matrices**

2.8.3.1 Antibacterial efficacy of salmocin mixtures on skinless poultry meat

The experimental procedures for testing salmocin activity against *Salmonella enterica* on chicken meat were described in (Schneider et al., 2018).

For better differentiation between the introduced *Salmonella* strains and the naturally occurring resident *Salmonella* strains, nalidixic-resistant mutants of strains of *S. enterica* ssp. *enterica* serovars Enteritidis (strain ATCC®13076™*), Typhimurium (strain ATCC®14028™*), Newport (strain ATCC® 6962™*), Javiana (strain ATCC® 10721™*), Heidelberg (strain ATCC® 8326™*), Infantis (strain ATCC®BAA-1675™) and Muenchen (strain ATCC® 8388™*) were used. All media employed for bacterial growth were supplemented with 25 µg/mL nalidixic acid. On XLD-agar (according to ISO 6579 (Xylose-Lysin-Desoxycholat Agar, #TN 1196, Sifin)), all the strains mentioned above appear as white colonies with a dark/black center allowing differentiation between introduced and non-introduced *Salmonella* that appear as plain white colonies.

All work with these strains was performed in biocontainment laboratories (BioSolutions Halle GmbH), compliant with their respective national and regional biosafety requirements.

The 7 *Salmonella* strains were streaked individually from liquid nitrogen permanent culture on LB agar medium and grown ON at 37°C. Next, they were individually inoculated to LB broth and grown ON at 37°C and 150 rpm. They were then diluted to OD_{600}=0.05 with LB broth and grown to exponential phase (OD_{600}~0.3) at 37°C, 150 rpm.

For *Salmonella* contamination of the poultry, the bacterial cultures were diluted with LB medium to OD_{600} = 0.1 and mixed in equal parts 1:1:1:1:1:1:1. The culture was then further diluted with LB medium to OD_{600} = 0.001 (~2×10^5 CFU/ml).

The chicken breast fillet (200-300 g fillets) for the experiment was purchased from an Aldi Nord supermarket (Halle (Saale), Germany) and stored at 4°C until use. The meat packaging was disinfected with 70% ethanol before opening. The chicken breast fillets were cut into pieces of about 20 g weight, pooled, and contaminated with 1 ml of the 7 strain *Salmonella* mixture (~2×10^5 CFU/ml) per 100 g of meat, corresponding to ~3 logs CFU/g. The meat was then incubated at room temperature for 30 minutes with hand massaging to ensure the bacteria attached to the meat surfaces. Initial *Salmonella* contamination was enumerated by dilution plating on XLD-agar.

For salmocin treatment, small atomizer flasks (Zerstäuberflaschen 2-4 bar (Roth GmbH + Co. KG, Nürnberg, Germany) were sterilized and rinsed with 70% ethanol. The chicken breast trimmings were treated by spraying with TSP extracts (10 ml/kg) prepared with a HEPES-containing buffer (50 mM HEPES pH 7.0, 10 mM K acetate, 5 mM Mg acetate, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 300 mM NaCl) from *Nicotiana benthamiana* plants expressing salmocins or *Nicotiana benthamiana* WT plant material as a negative control ("carrier"). Treatments were made up of extracts containing an individual salmocin (3 mg/kg SalE1a) or mixtures of salmocin-containing extracts in a high dose (3 mg/kg SalE1a, 1 mg/kg SalE1b, 1 mg/kg SalE2, 1 mg/kg SalE7) and a low dose (0.3 mg/kg SalE1a, 0.1 mg/kg SalE1b, 0.1 mg/kg SalE2, and 0.1 mg/kg SalE7). The treated meat trimmings were then further incubated at room temperature for 30 min and mixed by hand every 15 min.

Subsequently, aliquots of meat trimmings corresponding to ~40 g were packed into BagFilter ®400 P sterile bags (Interscience, St Nom la Bretèche, France) with 4 biological replicates per treatment (N=4). In total, meat samples were incubated at room temperature for 1.5 h during contamination and salmocin treatment before being weighed, sealed and stored at 10 °C. The bags were closed with closing clips (BagClip®400, Interscience, Saint Nom la Brètache, France) and stored for 1 h, 24 h, and 72 h at 10 °C. This temperature represents realistic industrial meat processing conditions that still allow for bacterial growth at suboptimal conditions.

As a control for potential background microbes, non-contaminated and non-treated samples (N=4) were prepared, stored, and analyzed for bacterial counts together with the contaminated and treated samples.

To assess the meat samples' initial bacterial contamination levels (N=3), meat samples that had been contaminated but not treated were sampled at 0 h.

For analysis of bacterial populations, poultry aliquots were homogenized with 4 vol. peptone water (Roth, Karlsruhe, Germany) using Bag Mixer® 400CC homogenizer (Interscience, St Nom la Bretèche, France) (settings: gap 0, time 30 s, speed 4). After homogenization, the microbial suspension from the filtered part of the bag was collected. The colony-forming units (CFU) of *S. enterica* were enumerated after plating undiluted or 1:10 dilutions of the bacterial suspension with peptone water on XLD-medium (Sifin Diagnostics, Berlin, Germany) and incubating them at 37°C ON.

2.8.3.2 Antibacterial efficacy of salmocin mixtures on beef, tuna, and whole egg matrices

The experimental procedures for testing salmocin activity on *Salmonella enterica* ssp. *enterica* on beef meat, tuna meat, and whole egg were described in (Hahn-Lobmann et al., 2019).

Nalidixic resistant mutants of *S. enterica* ssp. *enterica* serovars Enteritidis (strain ATCC®13076™*) and Typhimurium (strain ATCC®14028™*) were used. All media employed for bacterial growth were supplemented with 25 µg/ml nalidixic acid, and all work with pathogenic *S. enterica* was performed in biocontainment laboratories (BioSolutions Halle GmbH).

The 2 *Salmonella* strains were handled as described for the 7 strains in 2.8.3.1 up until the mixing of the bacterial cultures. The bacterial cultures were diluted with LB medium to OD_{600} = 0.1 and mixed 1:1. The culture was then further diluted with LB medium to OD_{600}=0.005 = (~$5x10^6$ CFU/ml) in the 1st experiment and to OD_{600} = 0.001 (~2×10^5 CFU/ml) in the 2nd and 3rd experiment.

The food matrices (raw beef (round roast), frozen tuna fillet steaks, and eggs) were bought from a local Kaufland supermarket (Halle (Saale), Germany). The tuna was thawed ON at 4°C. The packaging, it was disinfected with 70% ethanol before opening. The egg shells were also disinfected with ethanol. The beef and tuna were cut into ~20-gram pieces. The egg white and yolk were homogenized together (whole egg) for 2 min at 1000 rpm in a waring blender (Memory Blender (RMB), Rotor, Switzerland).

The meat matrices and the whole egg were contaminated with 10 ml/kg of the bacterial solution containing the 2 *Salmonella* strains at OD_{600} = 0.005 (~$5x10^6$ CFU/ml) in the 1st experiment and at OD_{600} = 0.001 (~$2x10^5$ CFU/ml) in the 2nd and 3rd experiment. The exact contamination load was enumerated by dilution plating of the contamination solution. Contaminated food samples were incubated at RT for 30 minutes with occasional hand-mixing to enable bacterial attachment. The whole egg sample was mixed using a magnetic agitator at 620 rpm for 1 min, incubated at RT for another 30 min, and occasionally mixed.

Purified SalE1b powder (2.7.4) was dissolved in a buffer consisting of 20 mM citric acid pH 7.5, 20 mM Na_2HPO_4, and 100 mM NaCl. The buffer without salmocin was used as a negative control ("carrier").

SalE1b was tested at an application rate of 5 mg/kg in experiment 1 and at an application rate of 0.5 mg/kg meat in experiments 2 and 3. The contaminated meat samples and the egg samples were treated with SalE1b or the control applied by pipette at 20 ml/kg, followed by hand mixing or magnetic stirrer mixing for the egg and further incubation for 30 min at RT, with occasional mixing. The total incubation time at RT during these 2 procedures (contamination and treatment) was ~2 h.

Subsequently, aliquots of meat trimmings corresponding to ~40 g were packed into BagFilter ®400 P sterile bags (Interscience, St Nom la Bretèche, France) in replicates (N=4). For whole egg, 40 ml of whole egg homogenate were placed into sterile sample bags (BagFilter®400 P) (N=4). The bags were weighed and closed with closing clips (BagClip®400) and stored for 1 h and 48 h at 10 °C. This temperature represents realistic industrial meat processing conditions that still allow for bacterial growth at suboptimal conditions.

To assess the meat's initial bacterial contamination levels, meat samples (N=3), which had been contaminated but not treated, were sampled at 0 h. As a control for potential background microbes, non-contaminated and also non-treated samples (N=4) were prepared for each food matrix and stored and enumerated together with the contaminated and treated samples.

Aliquots were sampled at 1 h and 48 h post-treatment to determine bacterial viability. Samples were mixed with 4 vol. peptone water and homogenized in a bag mixer (Bag Mixer ®400CC ® homogenizer). After homogenization, the microbial suspension from the filtered part of the bag was collected. The recovered bacterial solutions were serially diluted with peptone water and plated on XLD Agar (Sifin Diagnostics) for bacterial enumeration. The plates were incubated at 37°C for 16-20 h and CFU enumerated.

2.9 Analysis of colicin resistance development

2.9.1 Determination of the MIC by agar dilution method

The protocol employed follows the standardized guidelines of the CLSI (Clinical & Laboratory Standards Institute) and the EUCAST (European Committee on Antimicrobial Susceptibility Testing) and is closely adapted from (Wiegand et al., 2008).

ElectroMAX™ DH10B™ *Escherichia coli* cells (Fisher Scientific GmbH, Schwerte, Germany) was streaked for single colony from -80°C glycerol culture on Mueller Hinton Agar (MHA) (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) plates (Mueller Hinton Broth (MHB) supplemented with 0.75% (w/v) agar bacteriology grade (AppliChem GmbH, Darmstadt, Germany)).

5-8 colonies of equal size were pooled and inoculated to 4 ml MHB culture. The culture was incubated at 37°C and 180 rpm for 2-3 h until it reached an OD_{625} = 0.2-0.3, which corresponds to ~$1x10^8$ CFU/ml. The OD_{625} was adjusted to 0.2, and further diluted to $1x10^7$ CFU/ml with MHB and was spotted onto the agar dilution series (see below).

The purified colicins (2.7.4) were resuspended in 1xPBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH7.4) and supplemented with 0.1mg/ml BSA (Bovine serum albumin (Protein standard (BSA), ampule, 1mg/ml BSA in 0.15 M NaCl, 0.05% sodium azide; Sigma-Aldrich Chemie GmbH, Munich, Germany)). Stock solutions of 1, 0.1, and 0.01 µg colicin/µl were prepared by dilution with 1xPBS pH7.4 supplemented with 0.1mg/ml BSA (Albumin Fraction V (pH 7.0)) (AppliChem GmbH, Darmstadt, Germany) and a further dilutions series were prepared thereof. Typically a twofold-dilution series with 10 dilutions up and down from 1µg/µl was used (Wiegand et al., 2008).

If mixtures of colicins were analyzed, the colicins were diluted to equal protein concentrations and mixed 1:1, the final protein concentration being the sum of the evenly mixed colicins.

25 ml aliquots of melted MHA medium (0.75% agar) pre-warmed to 51°C were supplemented with 0.1 mg/ml BSA and with appropriate amounts of colicin stock solutions. Plates were stored at 4°C until use.

A total of 3 spots of 1 µl (spot size 5-8 mm) of each bacterial test culture was applied to each MHA plate of the antimicrobial dilution series. After incubation at 37°C for 16-20 h, the MIC was assessed. The MIC was defined as the lowest concentration of the antimicrobial substance that inhibits the visible growth of the test microbe; the growth a single colony or a faint film from the inoculum was disregarded (Wiegand et al., 2008). The cell number of the test culture

was determined by plating 10^{-3} and 10^{-4} on MHA plates and CFU count upon incubation ON at 37°C.

2.9.2 **Determination of the mutation rate by fluctuation assay**

ElectroMAX™ DH10B™ *Escherichia coli* cells (Fisher Scientific GmbH, Schwerte, Germany) was streaked from -80°C glycerol culture for single colony on MHA plates and incubated at 37°C ON.

5-8 colonies of equal size were pooled and inoculated to 4 ml MHB culture. The culture was incubated at 37°C and 180 rpm for 2-3 h until it reached an OD_{625} = 0.2-0.3, which corresponded to ~$1x10^8$ CFU/ml. The OD_{625} was adjusted to 0.2, and the culture was stepwise diluted with MHB: first to $1x10^6$ CFU/ml (OD_{625} = 0.002) and further diluted again 1:1000 to a cell density of 1-$2x10^3$ CFU/ml. To confirm the cell density of this starter culture, 100µl of the starter culture was plated on MHA for bacterial enumeration. From this starter culture, 2 ml each were aliquoted into 30 culture tubes, and these parallel cultures were incubated at 37°C, 180 rpm for ~18h.

100 µl of each of the parallel cultures were plated on MHA supplemented with individual colicin or colicin mixtures at a concentration of 10-fold MIC (prepared as described in 2.9.1). Depending on the expected mutation rate, the cultures were diluted 1:10 before plating. The plates were incubated at 37°C for 20 h, and the numbers of resistant mutants (r) were counted. Of 30 mutants selected for resistance to individual colicin or colicin mixtures, each originating from a different parallel culture, -80°C glycerol cultures were prepared (preferably 15 large, 10 medium, and 5 small colonies) for further analysis and characterization (2.9.3).

To count the total number of viable cells exposed to mutational events (N_t), the cell density of 3 of the parallel cultures were enumerated by dilution plating on MHA and incubation at 37°C ON.

To calculate the mutation rate (M) from the counted number of mutants per plate (r) and the number of total viable cells (N_t), the webtool FluCalc (http://flucalc.ase.tufts.edu/) (Radchenko et al., 2018) employing the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) calculation method was used.

2.9.3 **Verification of colicin resistance phenotype**

The *Escherichia coli* mutants obtained from the fluctuation assays were considered resistant because they had grown on 10x MIC of the colicin or colicin mixtures. 30 resistant clones with

different colony morphologies were stored as glycerol culture from each of the fluctuation assays. These mutants were retested to verify and determine the degree of their resistance.
Bacterial clones were tested against colicin-containing plant extracts by soft agar overlay assay (2.8.1). Colicins IA, M, U, and K were expressed in *N. benthamiana* using agroinfiltration with syringe (2.5.2) of the colicins Ia, M, U, and K. The plant material was harvested in small-scale (2.5.5.1), and the TSP was extracted with 5 vol. HEPES-containing buffer (2.6.2). The extracts were serially diluted 1:2 with 1% milk powder starting at undiluted and applied to the soft agar. The ElectroMAX^{TM} DH10B^{TM} WT sensitivity to colicins was analyzed in parallel and served as a reference. The sensitivity of the WT strain to the colicin was set to 100%. The sensitivity of the mutant below 100% implied some degree of resistance.

2.10 Encapsulation of colicins for oral delivery in animal studies

2.10.1 Yield and stability of colicin activity in dried plant material in comparison to fresh plant material

In order to measure the stability of colicin activity in colicin-containing dried leaf material, the activities of colicins extracted from dried (2.5.5.3) and frozen leaf powder (-80°C) (prepared as described in 2.5.5.2) of the same batch of harvested plant material were compared. Colicin-expression constructs were agroinfiltrated into *N. benthamiana* by syringe (2.5.2). The leaves were harvested, cut into pieces, and dried at 37°C. As the drying procedure reduced the plant material's weight by ~90%, 1 g of the frozen leaf powder was compared to ~100 mg dry leaf powder (2.5.5.3). Therefore, extraction of the plant material was done with 5 vol. HEPES-containing buffer (pre-chilled (4°C)) for the frozen leaf powder and ~50 vol. of the buffer for the dried leaf powder.
Both TSP extracts were then analyzed by Bradford assay (2.7.1), semi-quantitative protein estimation via SDS-PAGE (2.7.3) and soft agar overlay assay (2.8.1) for estimation of the recombinant protein content in order to compare specific antimicrobial activities.

2.10.2 **Production of capsules for oral colicin delivery**

2.10.2.1 Filling of the capsules

First dissolution test series:

For the first dissolution test series, the inner size 0 capsule of the dosage form configuration (DRcaps® Size 0, Natural TR.V700, Type: Coni-snap (Capsugel, Lonza, Morristown, USA) was filled with a test substance (mixture of milk powder and 5% (w/w) crystal violet) and a metal steel bar using the ProFunnel (X-0 Funnel, Harm. Code 901890, Torpac Inc., Fairfield, USA). The ProFunnel was used to fill individual capsules and assess the weight of the filler substance. The size 0 capsule was inserted into an outer size 00 capsule (DRcaps®). This capsule was then coated with an enteric coating (2.10.2.2).

For the pilot study, the capsules were made as described above, but they were filled only with milk powder.

Second dissolution test series:

For the second dissolution test series, the dosage form configuration consisted only of the size 0 DRcap without the outer size 00 capsule. It was filled with the same test substance as for the first test series but with 5 stainless steel balls (3.175 mm; Kugel Winnie, Bamberg, Germany) instead of a metal steal bar. This capsule was then coated with the enteric coating (2.10.2.2).

Animal study:

For the animal study, the dosage form configuration size 0 DRcaps® were filled with 5 metal steel balls (3.175 mm, ~100 mg/ball; Kugel Winnie, Bamberg, Germany) and one of the 2 colicin-containing formulations (leaf-derived colicin formulation or purified colicin formulation) or with milk powder as a negative control. The caps were filled using the Profiller 1100 (size 0 Torpac Inc., Fairfield, USA) according to the manufacturer's instructions.

To achieve the appropriate dose per capsule, milk powder was used as a filler (non-active ingredient/excipient) for the colicin-containing formulations.

A leaf-derived colicin formulation capsule contained 63 mg ColIb-, 124 mg ColM-, 103 ColU-, and 30 mg ColK-containing dry leaf powder mixed with 40 mg milk powder (~11%(w/w)) making a total of 360 mg of ingredients per capsule. The uncoated capsules together with the 5 metal balls weighed ~1030 mg.

A purified colicin formulation capsule contained 18 mg ColIb-, 12 mg ColM-, 16 mg ColU-, and 7 mg ColK purified colicin, mixed with 367 mg of milk powder (~87% (w/w)) (Carl Roth, Karlsruhe, Germany) making a total of ~420 mg of ingredients per capsule. The uncoated capsules together with the 5 metal balls weighed ~1090 mg.

The weight uniformity of the colicin formulation inside the capsules was checked for 100 capsules. Only 10% of capsules exceeded a 5% weight deviation. None of the capsules tested exceeded 10% weight deviation.

2.10.2.2 Enteric coating of capsules for enteric colicin delivery

This coating recipe differed from the used literature sources on enteric coatings of capsules: Dodds et. al 2008 could also solubilize a 12% polymer concentration of Eudragit S100 (Evonik, Essen, Germany) but used only ethanol as a diluent and Miller et al. 2015 used acetone, isopropanol, and water as diluents (Miller et al., 2015; Ruth Dodds, 2008). The 2 recipes were modified to offer the highest acid resistance achievable for tabletop dip coating.
The final recipe for the coating solution using the enteric coating agent Eudragit® L100 (Evonik, Essen, Germany) is shown in Table 8. Only acetone (40% of diluents) and isopropanol (60% of diluents) were used as a diluent with no addition of water, making drying at ambient air quicker. Additionally, the plasticizer triethyl citrate (10% of dry polymer) was used. After the final coating, the capsules were sprinkled with talcum powder as an anti-tacking agent.

Table 8: Ingredients for 500 ml enteric dip coating solution with a 12% polymer concentration

ingredient	function	quantity (g)	quantitiy (ml)
Eudragit® L 100	polymer	60.0	-
triethyl citrate	plasticizer	6.0	-
acetone	diluent	-	173.6
isopropanol	diluent	-	260.4

To prepare the dip-coating solution, the diluents were combined and stirred at 200 rpm. Next, the polymer was slowly and gradually added to avoid clump formation, and the solution was stirred until the polymer was completely dissolved (1-3 h). During mixing the beaker was covered with Parafilm® M sealing film (Thermo Fisher Scientific, Darmstadt, Germany) to prevent the evaporation of the diluents. After the polymer had dissolved, the plasticizer triethyl citrate (Applicem GmbH, Darmstadt, Germany) was added. The coating solution was stored in a closed bottle at RT for further use. The solution was stirred for 5 min at 100 rpm before use.

2.10.2.3 Dip coating of the capsules with an enteric coating agent

For dip coating of capsules with Eudragit® L100, the ProCoater base kit, ProCoater holders, and the ProCoater drying rack from Torpac (Torpac Inc., Fairfield, NJ, USA) were used. This device is made for the enteric coating of small batches of capsules for research purposes.

The capsules were coated according to the manufacturer's instructions and the recommended literature (Ruth Dodds, 2008).

2.10.3 **Dosage form configuration dissolution testing**

For dissolution tests of pharmaceutical dosage form configurations, the guidelines from method B of the USP general chapter on dissolution <711> for delayed-release dosage forms, U.S. Pharmacopeia (The United States Pharmacopeial Convention USP, 2011), and methods described in (Miller et al., 2015) were adapted to the equipment available at Nomad Bioscience GmbH.

In a 2 liter beaker, 1200 ml of acidic solution (0.1 N HCl, pH 1 (1st test series) or pH 4 (2nd test series)) was stirred by a magnetic agitator at 150 rpm at 37°C. The test dosage form was hung into the solution inside a stainless steel tea tong - one capsule per tea tong and 2 tea tongs per beaker. Once the capsules were exposed to the acidic medium (e.g., pH 1.0 - pH 4.0, 0.1 N HCl) for 2 h, they were immediately shifted to a phosphate buffer (0.05 mol/l, pH 7.0, 37°C) and stirred by a magnetic agitator at 150 rpm for an additional 60 min. The capsule´s condition was documented after the acidic incubation and twice during pH 7.0 incubation.

2.10.4 **Animal study and analysis**

The work with and on the piglets was done by veterinary technicians at the Central Veterinary Institute (CVI) (Lelystad, Netherlands) of the Wageningen University (Netherlands). The piglet study was planned to run for 16 days, from the piglets' arrival until necroscopy at the (CVI).

Table 9: An overview of the piglet study timeline conducted at the Central Veterinary Institute in Lelystad, Netherlands.
Not all study days and activities are shown.

study day	**-3**	**-2**	**-1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
activity description													
oral gavage of test products	x	x	x	x	x	x	x	x	x	x	x	x	
clinical health observation	x	x	x	x	x	x	x	x	x	x	x	x	x
challenge rotavirus (RV277)		x											
rectal fecal samples			x	x	x	x	x	x	x	x	x	x	x
challenge ETEC (CVI-1000)				x	x								
necroscopy													x

On day -6 the piglets (breed: “Topig 20”) arrived freshly weaned from the supplying farm at 4 weeks of age with a weight of 10-12 kg. The 5 treatment groups consisted of 10 piglets each.

Each treatment group was kept in separate rooms, separated into groups of 5 animals (Figure 4).

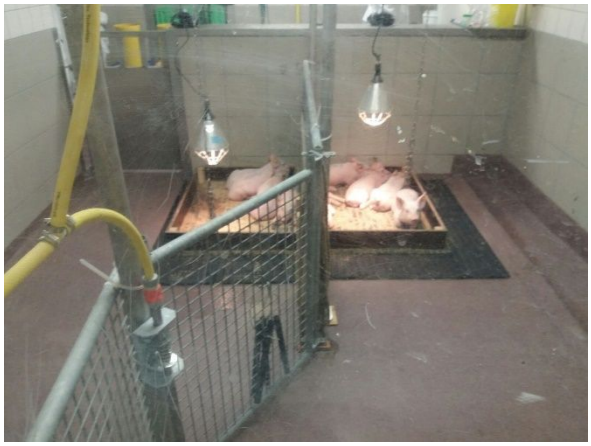


Figure 4: A treatment group of piglets in a separate room of the CVI divided into 2 groups of 5 animals by a fence.

Two groups received the leaf-derived colicin formulation protein, and 2 groups received the purified colicin formulation. One group received the negative control in the form of milk powder, which was also used as an excipient for treatment groups 1-4.

A plan and timeline of the study is shown in Table 9.

The oral test product (colicin capsules) was administered from day -3 until day +8 (Table 9).

To avoid damage to the capsules and the enteric coating, capsules were gavaged to the piglets. This meant retraining the piglet in an upright position, forcefully opening the mouth, and inserting a mouth gag. In the next step, the capsule was placed at the posterior boundary of the oral cavity with a dosing gun, and the piglet was stroked along the neck to stimulate swallowing (Figure 5).

Figure 5: The oral gavaging procedure of the capsules performed by technicians at the CVI.
Every capsule was gavaged individually and was a stressful event for the technicians and the piglets.

A dosing gun is a tube-like instrument for oral gavage of test substances. The steel dosing gun was used on days -3 until 0. The silicone dosing gun was used for the remainder of the feedings. As of this date, the vitality parameters such as appetite/feed intake, temperature, overall behavior, feces consistency, bodyweight etc. were examined regularly.

On day -2 a rotavirus challenge (2 ml suspension containing 10^6 particles/ml of enteric rotavirus, strain RV277 (Debouck & Pensaert, 1979) was administered to the piglets to increase their susceptibility to an *E. coli* infection. The piglets were infected with *E. coli* (ETEC strain CVI-1000) (2.1.1.2), isolated by the CVI. The 5 ml bacterial challenge with CVI-1000 was performed on day 0 and again on day 1 and had a bacterial density of 10^9 CFU/ml.

Faecal samples were obtained by rectal swab or manual rectal stimulation. The first fecal samples were analyzed on the day before the first *E. coli* challenge (-1) to exclude a prior ETEC-caused diarrheal infection. They were checked for consistency and water content to determine the level of diarrhea and were analyzed for the shedding of the test strain CVI-1000 by dilution plating for bacterial enumeration (Figure 6)(Figure 7).

Figure 6: Different daily fecal samples of piglets from the animal study in the microbiology lab of the CVI. In the back right are dilution tubes for bacterial enumeration by dilution plating.

The study was conducted by permission of the Dutch Ministry of Agriculture (Ref: DGVgzVVP/V951872) and by permission of the animal experimental commission.

Source of the animals was: VOF van Beek, Lelystad; the farm of the supplier was a high health status pig farm with regular serological monitoring of infection status and was free of major porcine pathogens and no anamnesis of post-weaning diarrhea.

Piglets were supplied directly after weaning which is important for susceptibility of post-weaning diarrhea, typically present in the first week after weaning; Therefore, the piglets had no acclimatization period.

The vitality parameters such as appetite/feed intake, temperature, overall behavior, bodyweight, etc., were examined regularly throughout the study.

At the end of the study, animals were euthanized and pathologically examined during necroscopy.

Stool samples were serially diluted in saline water (BioTRADING Benelux B.V., Mijdrecht, Netherlands) and plated on selective HIS agar (5% sheep blood, 50 mg/ml streptomycin, 25 mg/ml tetracycline and 50 mg/ml vancomycin (BioTRADING Benelux B.V., Mijdrecht, Netherlands)). Plates with 1-100 hemolytic colonies of F4 positive *E. coli* were counted. Ten F4-positive *E. coli* colonies were tested per day by a specific *E. coli* CVI-1000 antibody developed at the Central Wageningen Institute.

CVI-1000 is hemolytic (ß-hemolysis on blood agar) and was distinguishable from other *E. coli* (Figure 7).

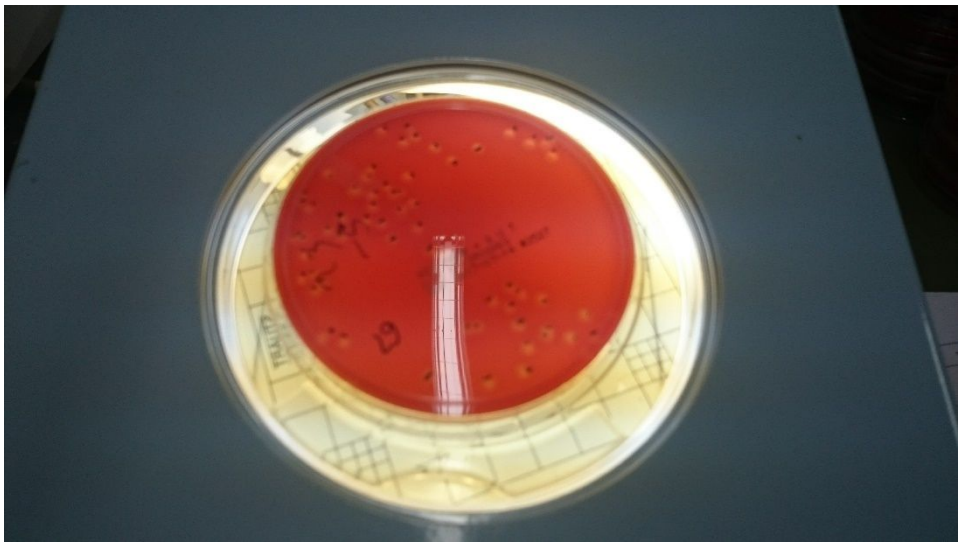


Figure 7: Plated dilution of a fecal sample on selective HIS agar.
Because CVI-1000 is hemolytic, a clear halo formed around the colonies.

3. Results

3.1 Evaluation of the potential of colicin resistance development in *E. coli* ElectroMAX™ DH10B™

3.1.1 Selection of colicins and the DH10B test strain

Four colicins (Table 10) known to have a high activity on *E. coli* and possibly work synergistically due to various modes of entry and activity were chosen to be tested individually and in mixtures on *E.coli* ElectroMAX™ DH10B™.

Table 10: Colicins chosen for the MIC assessment and mutation rate analysis.
They offer 4 complementary ways of entry by targeting 4 different receptors, using both translocation systems, and offering different modes of activity.

colicin	**group**	**receptor**	**translocation**	**activity**
ColM	group B	FhuA	TonB, ExbBD	inhib. of murein synth.
ColK	group A	Tsx	OmpF, OmpA, TolABQR,	pore-forming
ColIa	group B	Cir	TonB, ExbBD	pore-forming
ColU	group A	OmpA	OmpF, TolABQR, LPS	pore-forming

Before the determination of the mutation rate of ElectroMAX™ DH10B™ to colicins by Luria-Delbrück fluctuation assays (2.9.2; 1.4), the minimal inhibitory concentration (MIC) of the colicins for ElectroMAX™ DH10B™ was determined (2.9.1; 1.4).

E. coli ElectroMAX™ DH10B™ was chosen as the test strain as it is genetically characterized and can be handled under low biosafety conditions (2.1.2.1) (Durfee et al., 2008).

Cultures of Agrobacterium carrying constructs for ColM, ColK, ColK, and ColU plant expression (2.1.2.2)(7.1.1.1) were infiltrated into *N. benthamiana* by syringe (2.5.2) and the colicin-containing plant material was harvested in larger scale (2.5.5.2). TSP extracts from the plant material were then used for affinity chromatography-purification of colicins, provided by Dr. Anett Stephan (2.7.4).

For each batch of purified protein, the exact protein content was determined using Bradford assay (2.7.1), and the purity was estimated from Coomassie-stained SDS PAGE (2.7.2)

3.1.2 MIC determination of individual colicins and colicin blends for *E. coli* ElectroMAX™ DH10B™

The determination of the MIC (minimal inhibitory concentration) of antimicrobial substances is standard laboratory practice to test the efficacy of new antibiotics or monitor the development of bacterial resistance. The MIC is defined as the lowest concentration of an antibacterial agent required to inhibit the visible growth of a microorganism after 16-20 h incubation (2.9.1) (Andrews, 2001; Wiegand et al., 2008).

For MIC assessment, the agar dilution method was used. The bacteria were applied to agar plates containing increasing concentrations of the antibacterial agent.

For the agar dilution method, the antibacterial agent must be diluted into the melted liquid agar (>50°C) before pouring the plates. As colicins are generally non-heat stable proteins, their heat sensitivity for agar incorporation was tested in an activity experiment.

Purified colicin aliquots of ColM and ColE7 were incubated in a water bath at a range of temperatures from 48°C to 58°C for 2 min (control 4°C). Immediately afterward, they were cooled on ice, and their activity was tested in a soft agar overlay assay (2.8.1). ColM activity was stable until 52°C and then started to decrease. ColE7 activity began to decrease at ≤56°C. Therefore, the agar dilution method was performed at 50°C for all experiments, which is the lowest possible temperature for handling liquified agar before it solidifies.

To test the established MIC determination method, the MIC of kanamycin for *E. coli* ATCC® 25922™, which is a common reference strain for MIC determination, was assessed. Kanamycin has a MIC distribution (distribution of attained MIC values from various institutions and isolates) of 1-4 ng/µl (Fass & Barnishan, 1979) or 0.25-8 ng/µl (European Committee on Antimicrobial Susceptibility Testing (EUCAST) database, version 5.26) on *E. coli* ATCC® 25922™ (2.1.2.1). The MIC obtained was 8 ng/µl, which is valid as it is within one two-fold dilution of the MIC distribution from Fass & Barnishan 1979 and within the MIC distribution of the EUCAST database (Andrews, 2001; Turnidge & Paterson, 2007; Wiegand et al., 2008).

After initial MIC determinations for ColM, an attempt was made to stabilize colicins, potentially increasing antimicrobial activity reflected by a lower MIC. This was done by including the stabilizing agents BSA and Triton X-100 in the colicin solutions and the agar (Mitsui & Mizuno, 1969; Schaller, Dreher, & Braun, 1981). BSA could also act as a protease "decoy," protecting colicin integrity. The agar diffusion was further altered by reducing the agar concentration to 0.75% (w/w), thereby reducing the viscosity (Cabo, Murado, Gonzalez, & Pastoriza, 1999).

These adaptations were tested for all 4 colicins in different combinations. In conclusion, the addition of 1µg/µl (0.1%) BSA to all purified colicin protein dilutions and the agar dilution series for MIC determination, in combination with the reduction of the agar content from 1.5% (w/w) to 0.75%, provided the lowest MIC for colicins. These adaptions had no adverse effects on the growth of ElectroMAX™ DH10B™.

The MIC values for all individual colicins and mixtures were determined as described in 2.9.1 and are presented in Table 11. Overall, the MICs are very similar, and the mixing of colicins did not reduce the MIC.

Table 11: MIC values determined for individual colicins and the colicin blends as described in 2.9.1. The agar dilution method was applied using Mueller Hinton Broth supplemented with 0.75% agar and 0.1% BSA. The MIC of rifampicin was determined as control.

	antimicrobial	antimicrobial concentration (ng/µl)	MIC (ng/µl)
individual colicins	M	M (0.00024)	0.00024
	K	K (0.00098)	0.00098
	Ia	Ia (0.00049)	0.00049
	U	U (0.00024)	0.00024
colicin blends	M + K	M (0.00012) + K (0.00012)	0.00024
	M + K + Ia	M (0.00016) + K (0.00016) + Ia (0.00016)	0.00049
	M + K + Ia + U	M (0.00012) + K (0.00012) + Ia (0.00012) + U (0.00012)	0.00049
antibiotic	rifampicin	rifampicin (25.00000)	25.00000

3.1.3 Determination of mutation rates for individual colicins and colicin blends by fluctuation assay

As for the MIC, the methodology for performing fluctuation assays to determine the mutation rate was adapted from literature (Foster, 2006; Pope et al., 2008; Rosche & Foster, 2000) and tested by reproducing published data. Durfee et al. 2008 calculated the mutation rate for DH10B against rifampicin that occurs due to spontaneous non-lethal point mutations in the *rpoB* gene every 2,34 x 10^9 cell divisions of ElectroMAX™ DH10B™ (Durfee et al., 2008).

In reproducing the published data, the methodology chosen differed from the procedures used by Durfee et al. 2008: Mueller-Hinton agar was used instead of LB agar because as it had also been used for the MIC determination. The parallel culture volume was enlarged to 2 ml, and the cultures were grown to the stationary phase (~18h) instead of early stationary phase as in Durfee et al. 2008. Additionally, as for the MIC determination, all dilutions and media

containing the colicins were supplemented with 0.1% BSA, and the agar content was reduced from 1.5% (w/v) to 0.75% (2.9.2).

While Durfee et al. 2008 obtained a mutation rate of 2,43 x 10^9 for DH10B exposed to rifampicin, the mutation rate of 4,15 x 10^9 determined in this study was in the same range.

Mutation rates were determined for the same individual colicins and colicin blends as in 3.1.2 using the webtool FluCalc (http://flucalc.ase.tufts.edu/). FluCalc is based on the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) calculation method (Radchenko et al., 2018). It is considered to be the best calculation method available because it is valid for the entire range of mutation rates (Foster, 2006; Hall et al., 2009; Radchenko et al., 2018; Rosche & Foster, 2000). The mutation rates can be seen in Table 12.

Table 12: The estimated number of mutations and the mutation rates obtained by Luria-Delbrück analysis for individual colicins and colicin blends for ElectroMAX™ DH10B™ with 95% confidence intervals (CI), calculated with the webtool FluCalc (http://flucalc.ase.tufts.edu/) (Radchenko et al., 2018).
FluCalc uses the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) method. All of the obtained mutants were counted as legitimate mutants. A 10x MIC of the individual colicins and colicin blends was used (Table 11). Concentrations of invidual colicins: ColM (0.0024 ng/µl); ColK (0.0098 ng/µl); ColIa (0.0049 ng/µl); ColU (0.0024 ng/µl). Concentrations of colicin mixtures: ColM (0.0012 ng/µl) + ColK (0.0012 ng/µl); ColM (0.0016 ng/µl) + ColK (0.0016 ng/µl) + ColIa (0.0016 ng/µl); ColM (0.0012 ng/µl) + ColK (0.0012 ng/µl) + ColIa (0.0012 ng/µl) + ColU (0.0012 ng/µl).

resistance for		number of mutations (m)	**mutation rate** (M, mutations/ generation)	95% CI range upper bound	95% CI range lower bound
rifampicin (n=1)		15.50	1.87x 10^{-9}	2.52	1.29
individual colicins (n=1)	colicin M	1571.93	1.71x 10^{-7}	1.95	1.48
	colicin K	540.53	6.29x 10^{-8}	7.51	5.14
	colicin Ia	2782.52	3.57x 10^{-7}	3.98	3.17
	colicin U	99.09	1.65x 10^{-8}	2.02	1.31
2-colicin blend (n=1)	colicins M + K	462.19	5.78x 10^{-8}	5.14	6.45
3-colicin blend (n=1)	colicins M + K + Ia	15.53	3.88x 10^{-9}	5.23	2.69
4-colicin blend (n=3)	colicins M + K + Ia + U	0.04	6.81x 10^{-10}	15.88	1.04

These experiments demonstrated that mixtures of colicins with different modes of entry and activity on the cell could substantially reduce the mutation rate. ColU showed the lowest mutation rate for an individual colicin, almost 28-fold lower than for ColIa. As expected, the mutation rate was very low when 3 and 4 colicins were combined. The 4-colicin blend fluctuation assay was performed 3 times (n=3) to get a total of 30 resistant clones for later analysis as mutant numbers were low.

3.1.4 Verification of colicin resistance and analysis of colicin sensitivity

Thirty clones from each of the fluctuation assays were stored and further analyzed to verify that they were indeed all resistant to the colicin/s they were selected against (2.9.3).

First, the 30 clones selected on the 4 colicins blend were tested to determine if they were indeed resistant to all 4 colicins. Developing a quadruple resistance by spontaneous mutations in such a short period of time seemed unlikely.

The clones were grown and spotted on MHA containing 10 x MIC of the 4-colicin blend supplemented with 0,1% BSA and with 0.75% agar content as in the MIC determination experiments. This was the same colicin concentration they were selected on, but none of the supposedly resistant mutants grew on these plates except for mutant clone 16, which also appeared to have a different colony morphology and color. This clone was tested on selective media and analyzed genetically to verify that this clone was indeed a multiple-colicin-resistant ElectroMAX™ DH10B™.

A nested PCR was applied to determine the bacterial genotype of clone 16 and the original ElectroMAX™ DH10B™ strain. The first PCR used the primers fD1 and rP2 (7.1.2) to amplify the bacterial 16S-rDNA.

The PCR product was sent for sequencing with the 2 sequencing primers EUB800 and Eco1392 (7.1.2).

BLAST analysis of the amplified 16S rDNA sequences against the NCBI database (blastn suite (Altschul et al., 1997)) revealed that the ElectroMAX™ DH10B™ WT sequence had a 100% identity match to *E. coli* K12 16S rDNA, but clone 16 showed a 100% identity to *Micrococcus luteus* 16S rDNA.

In a second PCR, the PCR product obtained in the first PCR was used as a template for amplification with 2 *E. coli* K12 specific primers K12L and K12R (7.1.2). This PCR would only yield a PCR product if the DNA originated from an *E. coli* K12 isolate. ElectroMAX™ DH10B™ WT showed a PCR product identifying it as *E. coli* K12, but there was no amplification product for the multiresistant clone 16 (Figure 8).

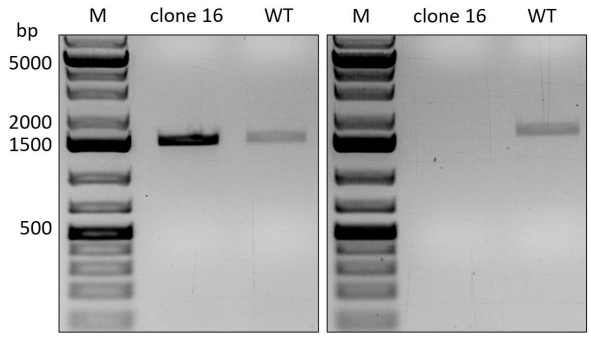


Figure 8: Analysis of genomic DNA of the 4-colicin blend insensitive ElectroMAX™ DH10B™ mutant clone 16 by PCR.
Determination of the clone 16 genotype by PCR. Left: PCR product of the 16S-rDNA amplification for clone 16 and ElectroMAX™ DH10B™ WT. Right: Amplification on the 16S-r-DNA PCR product from the first PCR (left) with *E. coli* K12 specific primers. PCR reactions were analyzed by agarose gel electrophoresis.

Additionally to the genotype, the phenotype of clone 16 was analyzed on different selective agar media where it was streaked out in parallel with *E. coli* ElectroMAX™ DH10B™ WT strain and a *Micrococcus luteus* strain (2.1.2.3). Here, it showed the same growth pattern and color as *Micrococcus luteus*.

The genetic and phenotypic analysis of clone 16 confirmed that this clone is not a progeny of *E. coli* ElectroMAX™ DH10B™ but a *Micrococcus luteus* contamination.

All the 4-colicin resistant mutants were sensitive to the 10 x MIC of the 4 colicin blend, against which they had been selected in the fluctuation assays. Therefore, all mutant clones obtained and stored from the fluctuation assays were analyzed for their degree of insensitivity against ColM, ColIa, ColU, and ColK. This analysis was done by soft agar overlay assay with the mutant strains embedded in the soft agar (2.9.3).

As a control, the ElectroMAX™ DH10B™ WT was used as the benchmark for 100% sensitivity to a colicin. If a mutant displayed a reduced sensitivity, it was calculated as a percentage of that sensitivity. The sensitivity pattern of a mutant to each colicin could provide information on the type of mutation responsible for the resistance.

The results of colicin mutant characterization for colicin sensitivity are shown in Table 13 for colicin-insensitive mutants selected for colicin resistance with individual colicins and in Table 14 for colicin-insensitive mutants selected for colicin resistance with colicin blends in fluctuation assays.

Table 13: Quantification of the degree of colicin sensitivity mutants (ElectroMAX™ DH10B™) attained in the fluctuation assays with selection for an individual colicin (3.1.3).
Colicin sensitivity to the 4 colicins was assessed by soft agar overlay assay (2.9.3) and is shown as a percentage of *E. coli* ElectroMAX™ DH10B™ WT sensitivity (=100% sensitivity). The colors emphasize lower levels of sensitivity: white cells show sensitivity levels between 100-51%, grey cells 50-11%, yellow cells 10-1%, and orange cells visualize percentages <1%.

ColM mutants				
No.	colicins tested			
	ColM	ColK	ColIa	ColU
1	0.000			
2	0.002			1.563
3	0.000			
4	0.195			50
5	0.000			
6	0.000			
7	0.000			
8	0.195			
9	0.000			
10	0.098			
11	0.391			50
12	0.000			
13	0.000			
14	0.195			
15	0.000			50
16	0.000			
17	0.024			
18	0.003			
19	0.001			
20	0.002			
21	0.391			
22	0.391			
23	0.001			
24	0.391			
25	0.391			
26	0.000		0.003	13
27				
28				6.250
29	0.391			
30	0.391			
WT				

ColK mutants				
No.	colicins tested			
	ColM	ColK	ColIa	ColU
1		0.003		
2		0.006		
3		0.001		
4		0.195		
5		0.002		
6		6.250		
7		50		
8		0.001		
9		0.024		
10		0.024	50	
11		0.391		
12		0.012		
13		0.001		
14		0.003	0.391	0.003
15		0.003	0.098	0.000
16		0.049		0.391
17		0.024		
18		0.024	50	0.098
19		0.006		50
20		0.003		0.098
21				
22		0.006		0.002
23		0.002		0.003
24		0.003		0.002
25		0.012		
26				
27		0.098	6.250	13
28		0.006	6.250	0.006
29		0.000		0.000
30		0.000	1.563	0.002
WT				

ColIa mutants				
No.	colicins tested			
	ColM	ColK	ColIa	ColU
1			0.098	
2			0.098	
3			0.098	
4			0.049	
5			0.049	
6			0.098	
7			0.098	
8			0.098	
9			0.098	
10			0.098	
11			0.098	
12			0.098	
13			0.098	
14			0.098	
15			0.024	
16			0.098	
17			0.098	
18			0.098	
19			0.098	
20			0.098	
21			0.098	
22			0.098	
23			0.098	
24			0.098	
25			0.098	
26			0.098	
27	0.000		0.098	
28	6.250	0.391	0.098	
29	0.000		1.563	
30	0.000		0.098	
WT				

ColU mutants				
No.	colicins tested			
	ColM	ColK	ColIa	ColU
1				1.563
2				0.781
3				0.781
4				3.125
5				3.125
6				3.125
7				1.563
8				3.125
9			3.125	1.563
10				0.781
11		0.024		0.195
12		0.002		0.002
13		0.012		0.049
14		0.006		0.781
15		0.006		0.006
16		0.391		6.250
17		0.006		0.098
18		0.012		0.049
19		0.006		0.049
20		0.002		0.001
21		0.003		0.001
22				6.250
23		0.024		0.781
24		0.003		0.003
25		0.002		0.098
26		0.391		0.781
27		0.006		0.098
28		0.002		0.003
29				3.125
30		0.012		0.098
WT				

The vast majority of the mutants, with a few exceptions, showed a high insensitivity to the colicin they were selected with.

Table 14: Quantification of the degree of colicin sensitivity mutants (ElectroMAX™ DH10B™) attained in the fluctuation assays with selection for multiple colicins (3.1.3).
Colicin sensitivity to the 4 colicins was assessed by soft agar overlay assay (2.9.3) and is shown as a percentage of *E. coli* ElectroMAX™ DH10B™ WT sensitivity (=100% sensitivity). The colors emphasize lower levels of sensitivity: white cells show sensitivity levels between 100-51%, grey cells 50-11%, yellow cells 10-1%, and orange cells visualize percentages <1%.

ColM + ColK mutants				
No.	colicins tested			
	ColM	ColK	ColIa	ColU
1	0.000			
2	0.006			
3	0.024			
4	0.024			
5	0.024			
6	0.000			
7	0.000			
8				
9	0.000			
10	0.024			
11	0.024			
12	1.563			
13	1.563			
14	0.391			
15	0.000			
16	0.391			
17	0.391			
18	0.000			
19	0.098			
20	0.000			
21	0.098			
22	0.391			
23	0.098			
24	0.000			
25	0.391	50		
26	0.000		0.391	
27	0.000		0.391	
28	0.000		0.391	
29	0.000		0.391	
30	0.000	50	0.049	
WT				

ColM + ColK + ColIa mutants				
No.	colicins tested			
	ColM	ColK	ColIa	ColU
1	0.000	0.000	0.000	0.000
2	0.781			
3				
4				
5				
6	0.195			
7	1.563			
8				
9				
10				
11	6.250			
12				
13	0.098			
14				
15	0.000			
16				
17	0.000			
18	6.250			
19	50			
20				
21				
22				
23	50			
24	0.000	0.000	0.000	0.000
25	25			
26	0.000			
27	0.000			
28	0.000			
29	50			
30	0.000			13
WT				

ColM + ColK + ColIa + ColU mutants				
No.	colicins tested			
	ColM	ColK	ColIa	ColU
1				
2				
3				
4				
5				
6			50	
7				
8			25	
9				50
10				
11			50	
12	50		50	50
13				25
14			50	50
15				50
16	0.000	0.000	0.000	0.000
17				50
18				50
19			25	50
20				
21				
22				
23				
24				50
25				
26				
27				
28				
29				50
30				50
WT				

The majority of the colicin mutants selected with 2- and 3-colicin blends showed insensitivity to ColM, but none of the 2-colicin resistant mutants showed insensitivity to ColK; instead, some had a strong insensitivity to ColIa. Of the colicin mutants selected with 3-colicin blends, none showed insensitivity to ColK and ColIa. The colicin mutants selected with the 4-colicin-blend only displayed minor insensitivities, mostly towards ColU. Clone 16 of the 4-colicin-resistant mutants had been tested and identified as a contamination. As clone 1 and 24 of the 3-colicin-resistant mutants were completely resistant against all 4 colicins, they were also genotypically analyzed with the same method used for clone 16 (see above) of the 4-colicin-resistant mutants. Phenotypically they displayed a different growth phenotype than ElectroMAX™ DH10B™ WT. Genotypically, they turned out to be gram-positive bacterial contaminations as well. When blasted against the NCBI database, clone 1 shared 99.58% identity with *Staphylococcus haemolyticus,* and clone 24 shared 100% identity with *Bacillus frigoritolerans*.

Mutations conferring ColM insensitivity or resistance to ColM seemed to occur more frequently than for the other 3 colicins tested, which is also evident from the determined mutation rate (Table 12). None of the mutants obtained from the colicin blend fluctuation assays were verified to be resistant to all of the colicins they were selected with.

The mutation rate was recalculated, taking into account the number of actual mutants after the verification of colicin sensitivity (see above). For each fluctuation assay, the results obtained for the 30 re-analyzed mutants was extrapolated to the total number of mutants identified. For the 2-colicin blend, for example, only 2 genuinely resistant clones were found to have an insensitivity to both colicins, so only every 15th mutant was counted as a real mutant (Table 15). For the 3- and 4-colicin blend, no resistant clone was found. Hence, the minimal possible number of mutations (1 mutation (m) for all the cells at risk (N_t) was entered into the mutation rate calculation. Therefore, the adapted mutation rates were below this number. For the 4 individual colicins, a relatively similar mutation rate was observed. Combining the colicins into mixtures lowered the mutation rate substantially. For the 3-4 colicin blends, no mutants could be verified among the 30 obtained mutants. Therefore, the mutation rate was below the lowest possible mutation rate detectable with this web tool and its calculation method.

Table 15: Recalculation of the estimated number of mutations and the mutation rates obtained by Luria-Delbrück analysis for the individual colicins and the colicin blends for ElectroMAX^{TM} DH10B^{TM} with 95% confidence intervals (CI) considering only confirmed colicin resistant mutants (3.1.4).
Numbers were calculated with the webtool FluCalc (http://flucalc.ase.tufts.edu/) (Radchenko et al., 2018). FluCalc uses the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) method. A 10x MIC of the individual colicins and colicin blends was used (Table 11). Concentrations of invidual colicins: ColM (0.0024 ng/µl), ColK (0.0098 ng/µl), ColIa (0.0049 ng/µl), ColU (0.0024 ng/µl). Concentrations of colicin mixtures: ColM (0.0012 ng/µl) + ColK (0.0012 ng/µl), ColM (0.0016 ng/µl) + ColK (0.0016 ng/µl) + ColIa (0.0016 ng/µl), ColM (0.0012 ng/µl) + ColK (0.0012 ng/µl) + ColIa (0.0012 ng/µl) + ColU (0.0012 ng/µl).

resistance for		number of mutations (m)	**mutation rate** (M, mutations/ generation)	95% CI range upper bound	95% CI range lower bound
rifampicin (n=1)		15.50	1.87×10^{-9}	2.52	1.29
individual colicins (n=1)	colicin M	1571.93	1.71×10^{-7}	1.95	1.48
	colicin K	540.53	6.29×10^{-8}	7.51	5.14
	colicin Ia	2782.52	3.57×10^{-7}	3.98	3.17
	colicin U	99.09	1.65×10^{-8}	2.02	1.31
2-colicin blend (n=1)	colicins M + K	55.93	6.99×10^{-9}	8.59	5.52
3-colicin blend (n=1)	colicins M + K + Ia	0.21	$<52.85 \times 10^{-12}$	124.30	7.79
4-colicin blend (n=3)	colicins M + K + Ia + U	0.21	$<41.11 \times 10^{-12}$	96.68	6.06

3.1.5 **Analysis of colicin resistance mechanisms**

ColM resistant mutants obtained in fluctuation assay were analyzed for the type of mutation conferring the resistance. The most probable mechanism to render bacterial cells resistant to ColM is the mutation of the *fhuA* receptor gene for ColM.

Therefore, for all 28 mutant clones, the ColM receptor gene *fhuA* was amplified from gDNA with primers specific to the *fhuA* ORF or its flanking regions (7.1.2). The amplified *fhuA* ORF (2534 bp) (2.3.1.4) was then sequenced with the primers used for amplification (2.4.2). If no mutation was found in *fhuA*, the genes essential for the TonB translocation pathway, *tonB*, *exbB,* and *exbD*, were amplified and sequenced (2.3.1.4) (2.4.2), and if possible, genetic mutations were analyzed.

Agarose gel electrophoresis analysis revealed that PCR product of expected size (2534 bp) was amplified with *fhuA* flanking oligonucleotides for 19 mutant clones. Sequence analysis showed a non-altered sequence (Table 16). Nevertheless, one-third of the mutants (9 out of 28) showed a larger gene amplification product due to the insertion of DNA. For mutant clones 3, 12, 16, and 26, the PCR resulted in a larger gene amplification product due to DNA insertion. The inserts´ identity was identified as the active transposable element IS1 with a length of 768 bp (Matsutani, 1994) (NCBI database). Five of the mutants had a DNA insertion with no significant homology to any known DNA insertion sequence, with clones 7 and 13 having the same insertion sequence. For 2 of the mutants, the insertion sequence could not be determined even though additional sequencing primers were used. Clones 27 and 28 were 100% ColM sensitive when retested and were not analyzed further. In the end, all identified mutations were caused by insertions, and no single point mutations were found. Roughly half of the identified mutations were receptor gene-related, while the other half was translocation gene-related. There seems to be a correlation between the degree of sensitivity and the resistance type, as receptor mutations lead to a higher degree of resistance than a translocation machinery mutation (Table 16).

Table 16: Analysis of 28 ElectroMAX™ DH10B™ ColM resistant mutants obtained in the ColM fluctuation assay for potential mutations in the ColM receptor gene or the translocation genes.
Mutants were tested on their ColM sensitivity in 3.1.4, shown here under (ColM sensitivity (% of ElectroMAX™ DH10B™ WT sensitivity). ElectroMAX™ DH10B™ WT has a 100% sensitivity. The sequenced genes without mutation are highlighted in green. The sequenced genes with a mutation in the receptor gene are highlighted in red, while those with a mutation in the translocation machinery are highlighted in orange. If the gene was not sequenced or the sequencing results were unambiguous, it is highlighted in gray. If the number of inserts and the insert lengths were determinable, this information is provided.

		receptor *fhuA*		translocation				
DH10B™ mutant clone Nr.	ColM sensitivity (% of ElectroMAX™ DH10B™ sensitivity)	length PCR	No. and length of Inserts	*tolB*	*exbB*	*exbD*	No. and length of Inserts	cross resistance to
1	0.0001	3500	1 (713bp)					
2	0.0015	2534						ColU
3	0.0002	3000	1 (777bp)					
4	0.1953	2534					1	
5	0.0002	3000						
6	0.0004	3500	1 (1132bp)					
7	0.0001	3500	2 (340bp; 291bp)					
8	0.1953	2534					1	
9	0.0004	2534						
10	0.0977	2534						
11	0.3906	2534					1	
12	0.0004	3000	1 (777bp)					
13	0.0004	3500	1 (1338bp)					
14	0.1953	2534					1	
15	0.0002	2534						
16	0.0004	3000	1 (777bp)					
17	0.0244	2534						
18	0.0031	2534						ColU
19	0.0008	2534						
20	0.0015	2534						
21	0.3906	2534						
22	0.3906	2534						
23	0.0008	3500	1 (718bp)					
24	0.3906	2534						
25	0.3906	2534						
26	0.0004	2534					1 (779bp)	Colla + ColU
29	0.3906	2534						
30	0.3906	2534						
WT	100.0000	2534						

In general, 10/28 mutants had a mutation in the receptor gene, while 11/28 mutants had a mutation in the translocation machinery genes, mostly in *exbB*. For 7 of the mutants, no mutation could be found in the receptor or translocation genes responsible for ColM cell entry. Mutants displaying a receptor mutation were not analyzed for mutations in the genes responsible for the translocation machinery.

3.1.6 Analysis of ColM-resistant clones with *fhuA* mutations

Complementation analysis was performed to exclude second-site mutations and verify that *fhuA* gene mutation was, in fact, the cause for ColM resistance. For complementation, *fhuA* was introduced to the bacteria on the plasmid pNMD41440 under control of the OBX12 promoter. The plasmid pNMD41440 and its corresponding empty vector pNMD0319 (7.1.1.4) as negative control were transformed into ElectroMAX™ DH10B™ cells, ElectroMAX™ DH10B T1 phage-resistant cells (2.1.2.1) (mutation in *fhuA*) and into ColM resistant mutants with a verified *fhuA* mutation obtained from fluctuation assay. The clones 12, 13, and 23 with a known insertion in the *fhuA* ORF were analyzed for *fhuA* complementation (Table 16). Clone 2 was chosen for *fhuA* complementation control as it had an intact *fhuA* ORF and should not be affected by the *fhuA* complementation. All strains were analyzed for ColM sensitivity by soft agar overlay assay (2.8.1).

As expected, ColM resistant clones with known *fhuA* mutation (12, 13, 23) and ElectroMAX T1 phage-resistant cells could be reverted to ColM sensitivity by *fhuA* vector (pNMD41440) complementation (Figure 9). The sensitivity of ElectroMAX™ DH10B™ WT (pNMD41440) to ColM was even increased by several logs compared to the WT empty vector control (pNMD0319). Higher *fhuA* expression levels in the cells conferred by plasmid-based expression could facilitate ColM cell entry (Olschlager, Turba, & Braun, 1991). However, the ColM sensitivity of clone 2 was not changed by plasmid-based *fhuA* expression. This could indicate that Clone 2 had a second-site mutation responsible for its higher ColM tolerance.

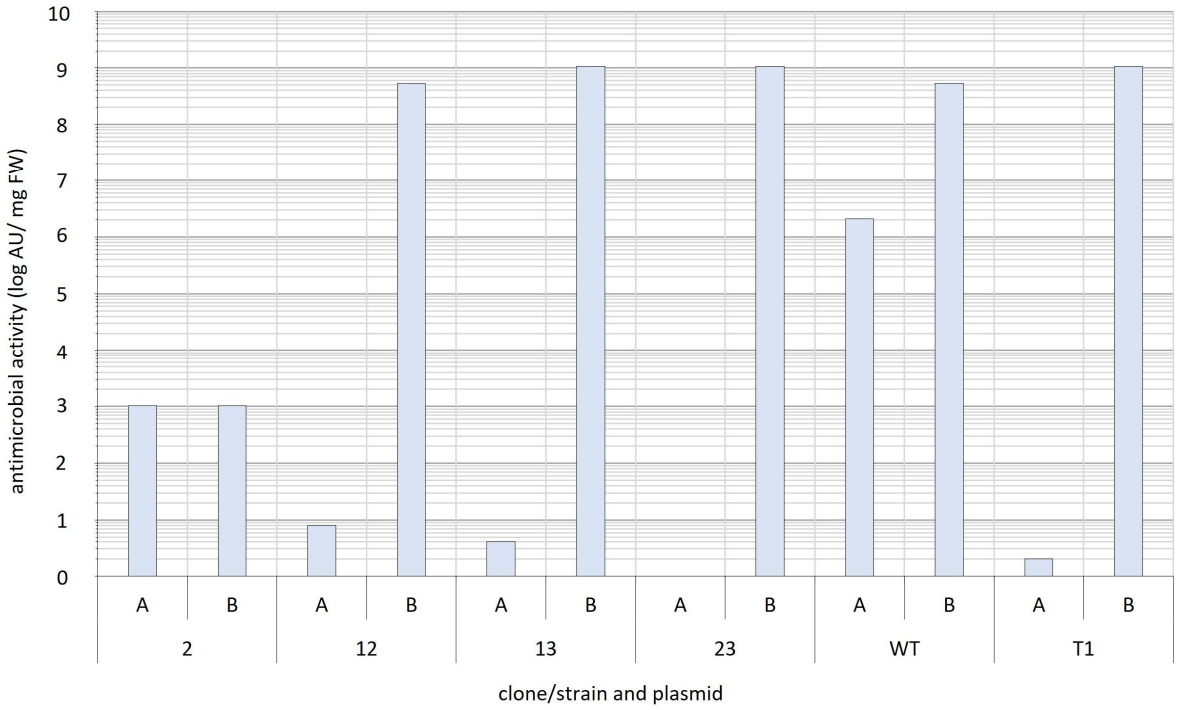


Figure 9: Antimicrobial activity assay (2.8.1) of complemented *fhuA* mutants with purified ColM protein (2.7.4). Complementation of ColM resistant mutants that have a verified *fhuA* mutation (clone 12, 13, and 23 have a *fhuA* insertion, T1 has a mutation) or no verified mutation (clone 2 and WT) with a FhuA expression construct pNMD41440 (B) and the corresponding empty vector pNMD0319 (A). WT: ElectroMAX™ DH10B™ cells; T1: ElectroMAX™ DH10B™ T1 Phage-Resistant Competent Cells.

3.2 Diversifying the arsenal of bacteriocins by creating colicin-like bacteriocin chimeras with novel and potentially improved characteristics

For colicin-like bacteriocins, the modularly structured domains can be seen as interchangeable building blocks (similar to Lego®). Domains from different colicin-like bacteriocins can be fused to create new chimeric proteins termed "legocins" by Nomad Bioscience.

The potential legocins could broaden the antimicrobial arsenal by offering alternative antimicrobial molecules in case of resistance formation against the parental molecule.

It would also be of great value to discover legocins with improved characteristics compared to the parental molecules. These characteristics could include a wider activity range, higher expression levels in plants, or favorable protein purification characteristics.

3.2.1 Intraspecies legocins with ColM and ColIb activity domains

For the annotation of the bacteriocin domains, their protein sequences (GenBank database) were blasted against the GenBank protein database using the BLASTP 2.6.1+ tool (protein-protein BLAST®; NCBI) (Altschul et al., 1997). The retrieved sequences were also compared using the program, ClustalW2, ClustalOmega or EMBOSS Needle from EMBL-EBI.

The selection of the colicin and salmocin domains for the generation of legocins was hampered because many of the colicins were missing domain annotations in common databanks.

Therefore, several of the annotations used for domain swapping were taken from literature (7.3) or when literature data was insufficient, predicted via sequence alignment to homologous colicins. The domain annotations of the bacteriocins used for the legocin cloning are described in 7.3.

Two bacteriocin modules were combined to generate one legocin. The TR-module consisted of the translocation and receptor domain of one bacteriocin, while the A-module consisted of the activity domain of a different bacteriocin (Figure 10). Recombined, they offer different modes of entry for an individual activity domain.

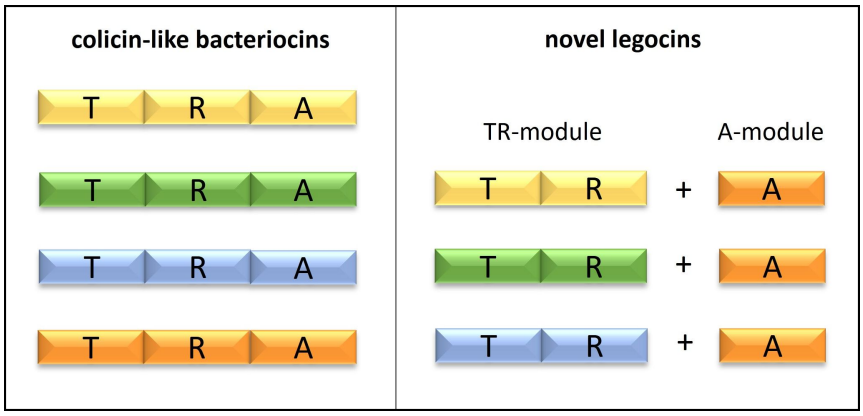


Figure 10: An exemplified domain swapping of 4 natural colicin-like bacteriocins with their 3 domains (T= translocation, R = receptor, A= activity) to 3 new legocins.
The novel legocins consisted of 2 modules: The TR-module consisted of the T+R domains of the first 3 bacteriocins combined with the A-module that consisted of the A domain of the fourth bacteriocin.

Translocation and receptor domains for the TR-modules originated from colicins that use a variety of different receptors and have good activity on the "Big 7" STEC strains. The A-modules of the legocins were taken from bacteriocins with high and broad activity on the "Big 7" STEC strains (1.3.2.4) (Schulz et al., 2015). Nuclease activity domains were avoided, as they have to be co-expressed with their respective immunity protein, potentially lowering the yield. Additionally, their mode of action seems to be more narrowly specific.

Legocin modules were amplified from plasmids containing the colicin sequences synthesized and codon-optimized for *N. benthamiana* (7.1.1.1). The oligonucleotides used for module amplification (7.1.2) added *BsaI* restriction sites for Golden-Gate cloning (2.3.5). The amplified DNA was blunt-end cloned (2.3.4) into the intermediate vector pICH41021 (7.1.1.4), and using Modular Cloning (2.3.5), the legocin modules were cloned into the final binary expression vector pICH29912 (TMV + MP) (1.5, Figure 2). Twelve novel legocins with a ColM or ColIb activity domain were made (7.1.1.3) (Table 17).

Table 17: Colicin domain swapping to create 12 novel legocins (7.1.1.3).
The receptors, the translocation pathway proteins, and the mode of activity for the legocin are shown. The protein size prediction was performed using the Compute pI/Mw tool from ExPASy SIB bioinformatics (Artimo et al., 2012).

legocin name	TR-modules				A-modules		predicted size (kDa)
	translocation and receptor domain				cytotoxic activity domain		
	bacteriocin	colicin group	receptor	translocation	**bacteriocin**	activity	
TR_{ColA}-A_{ColIb}	**ColA**	A	BtuB	OmpF, TolABQR, OmpA, LPS	**ColIb**	pore	60,8
TR_{ColA}-A_{ColM}					**ColM**	inhibition of murein synth.	57,2
TR_{ColE1}-A_{ColIb}	**ColE1**	A	BtuB	TolCAQ	**ColIb**	pore	56,4
TR_{ColE1}-A_{ColM}					**ColM**	inhibition of murein synth.	52,8
TR_{ColE3}-A_{ColIb}	**ColE3**	A	BtuB	OmpF, TolABQR	**ColIb**	pore	66,2
TR_{ColE3}-A_{ColM}					**ColM**	inhibition of murein synth.	62,3
TR_{ColIA}-A_{ColIb}	**ColIa**	B	Cir	TonB, ExbBD	**ColM**	inhibition of murein synth.	66,2
TR_{ColK}-A_{ColIb}	**ColK**	A	Tsx	OmpF, OmpA, TolABQR	**Col Ib**	pore	59,3
TR_{ColK}-A_{ColM}					**ColM**	inhibition of murein synth.	55,7
TR_{ColM}-A_{ColIb}	**ColM**	B	FhuA	TonB, ExbBD	**ColIb**	pore	33,1
TR_{ColN}-A_{ColIb}	**ColN**	A	LPS, OmpF	OmpF, TolAQR	**ColIb**	pore	39,1
TR_{ColN}-A_{ColM}					**ColM**	inhibition of murein synth.	35,4

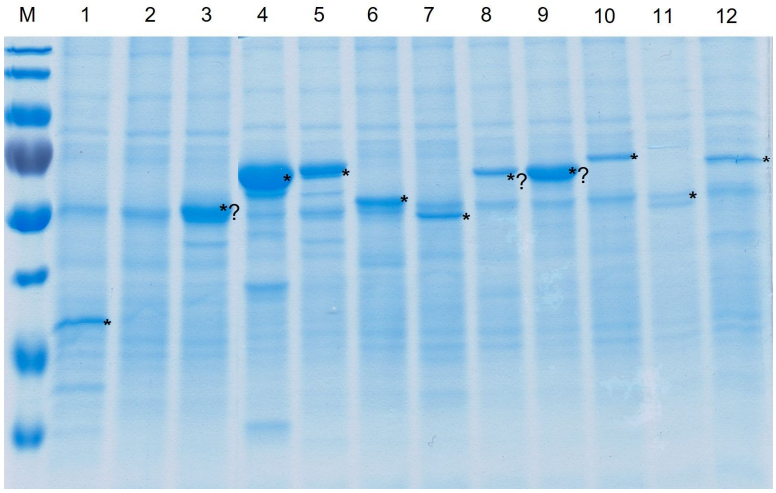
For legocin expression analysis, the 12 legocin expression vectors were transformed into the *Agrobacterium* strain ICF320 (2.3.8), which was then inoculated into *N. benthamiana* by syringe infiltration (2.5.2). Next, the optimal harvesting time point was determined (2.5.6), and the legocin-containing plant samples from this time point were extracted with HEPES-containing extraction buffer (2.6.2) with subsequent protein analysis by SDS-PAGE with Coomassie staining (2.7.2) and the determination of antimicrobial activity by soft agar overlay assay (2.8.1).

While 11 of the 12 legocins with ColIb and ColM activity domains were expressed well and soluble in HEPES buffer, all legocins had a significantly lower antimicrobial activity than the corresponding TR-module parental molecules (Table 18).

Table 18: The legocins with a ColIb and ColM activity domain and their antimicrobial activity on DH10B (pICH73311).
The legocins consisted of TR-modules from 6 different colicins and A-modules from 2 colicins. Legocin expression constructs were agroinfiltrated into *N. benthamiana* by syringe infiltration. The protein from the harvested plant material was extracted by HEPES containing buffer, and their antimicrobial activity was determined by soft agar overlay assay (2.8.1) and compared to the donor colicins.

plasmid	legocin		activity	optimal day of harvest post infiltration
pNMD	TR	A	AU/mg FW	
45928	ColN	ColIb	256	5
45942	ColM	ColIb	256	5
46011	ColA	ColIb	4096	5
45961	ColK	ColIb	128	6
46035	ColE3	ColIb	8	6
45901	ColE1	ColIb	16	5
45911	ColE1	ColM	0	5
45931	ColN	ColM	0	5
45971	ColK	ColM	0	6
45991	ColIa	ColM	0	5
46021	ColA	ColM	0	6
46041	ColE3	ColM	0	5
3680	ColN		262144	5
10221	ColM		4194304	6
25831	ColA		131072	6
15252	ColK		67108864	7
15521	ColE3		524288	5
3650	ColE1		262144	4
19141	ColIa		2097152	6
25861	ColIb		4194304	5

The legocins with a ColM activity domain showed no activity, while the legocins with a ColIb activity domain showed very low activity. The highest activity was seen for the legocin A_{colA}-TR_{ColIb}. Nevertheless, the legocins were mostly well expressed and soluble in the extraction buffer (Figure 11). This lack of activity may have been caused by incorrect domain annotation. Therefore, further chimeras were created in the pursuit of diversifying the antimicrobial arsenal and making novel highly and broadly active legocins.



	legocin		
No.	TR	A	MW (kDa)
1	N	Ib	39,065
2	M	Ib	33,112
3	A	Ib	60,837
4	K	Ib	59,340
5	E3	Ib	66,186
6	E1	Ib	56,430
7	E1	M	52,800
8	N	M	35,435
9	K	M	55,711
10	Ia	M	66,177
11	A	M	57,208
12	E3	M	62,556

Figure 11: Expression analysis of the legocins with a ColIb and ColM activity domain by resolving the extracted plant protein by an SDS-PAGE.
Agroinfiltration for legocin expression was done by needleless syringe (2.5.2). The legocin-containing plant material was harvested at the optimal harvesting time point in small-scale (2.5.5.1) and extracted with HEPES-containing buffer (2.6.2). TSP corresponding to 1.5 mg FW was resolved on a 12% SDS-PAGE with Coomassie staining (2.7.2). Asterisks indicate recombinant protein. Asterisks with a question mark indicate a discrepancy between the expected molecular weight and the protein band.

3.2.2 Intraspecies legocins with a ColU activity domain

ColU has often been used throughout this work showing good antimicrobial activity. A scientist of the subsidiary company Nomads UAB (Vilnius, Lithuania) had created a chimera of a pyocin and ColU with antimicrobial activity (Šarūnas Paškevičius, unpublished data) (1.3.4). The ColU activity domain was not annotated in the literature, so it was annotated using alignments with other colicins. This ColU activity domain annotation was used (7.3).

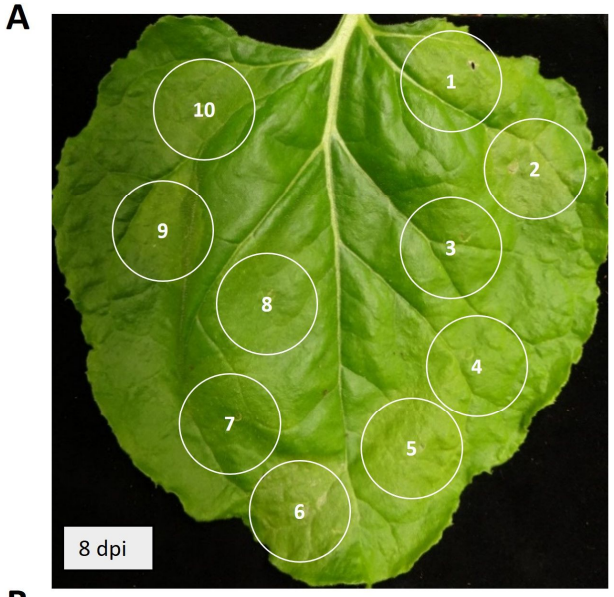
A DNA sequence encoding the ColU activity domain was combined with the corresponding DNA sequences coding for the TR-modules of ColIb and ColM that had been amplified with appropriate 4 bp overhangs for *BsaI* Golden-Gate cloning (3.2.1). By cloning these TR-module encoding sequences to the ColU activity domain encoding sequence, a cloning artifact of 3 or 6 additional bp (1 or 2 aa) between the modules was created. For the TR-modules encoding sequences amplified for ColIb activity domain encoding sequence attachment, an additional 3 bp encoding for lysine (K) (basic, positive charge, long aliphatic side chain) was added between the modules (designation: $A_{K\text{-}ColU}$). For the TR-modules encoding sequences amplified for ColM activity domain encoding sequence attachment, an additional 6 bp encoding for glycine (G) (neutral charge, very short side chain) and serine (S) (polar/uncharged, short side chain) were added between the modules (designation: $A_{GS\text{-}ColU}$) (7.3). To test if these cloning artifacts had any adverse consequences for the legocins, the TR-modules encoding sequences for cloning to the ColIb activity domain encoding sequence and the TR-modules encoding sequences for

cloning to ColM activity domain encoding sequence were all cloned to the ColU activity domain encoding sequence and compared (Figure 12).

When shuffling domains of proteins according to literature and homology alignments, it is uncertain if they will function in the absence of the other protein parts that may be required for cross-domain interactions. The proximity to novel protein domains may also hinder correct protein folding. Therefore, it was tested if a linker sequence between the TR- and A-modules would benefit the activity of the TR_{ColK}-A_{ColU} legocin. This legocin consisted of the ColK translocation and receptor domain with the addition of the first 30 (N-terminal) amino acids (designation: $TR_{ColK+30\ aa}$) of the ColK activity domain. This TR-module was then combined with the activity domain of ColU that additionally contained the last 30 (C-terminal) amino acids of the ColU receptor domain in front of the activity domain (designation: $A_{ColU–30\ aa}$). This created a 60 aa linker between both legocin modules (7.3).

The legocins were cloned (7.1.1.3) and analyzed as described in 3.2.1. Figure 12A shows plant phenotype caused by legocin expression at 8 dpi. At this time point, all legocins with an A-module from ColU had the highest attainable protein yield and healthy leaf tissue with only slight decolorization. Therefore, 8 dpi was defined as the optimal time point for harvesting the legocins with an A-module from ColU.

Figure 12B shows the expression levels of the legocins and their solubility when extracted with HEPES-containing buffer (2.6.2) at 8 dpi.



		legocin		
No.	pNMD	TR	A	MW (kDa)
1	48101	ColE1	K-ColU	58.925
2	48111	ColN		41.560
3	48121	ColK		61.835
4	48131	ColA		63.332
5	48141	ColE1	GS-ColU	58.941
6	48151	ColN		41.576
7	48161	ColK		61.851
8	48172	ColIa		72.317
9	48182	ColA		63.348
10	48251	ColK+30	ColU-30	68.129

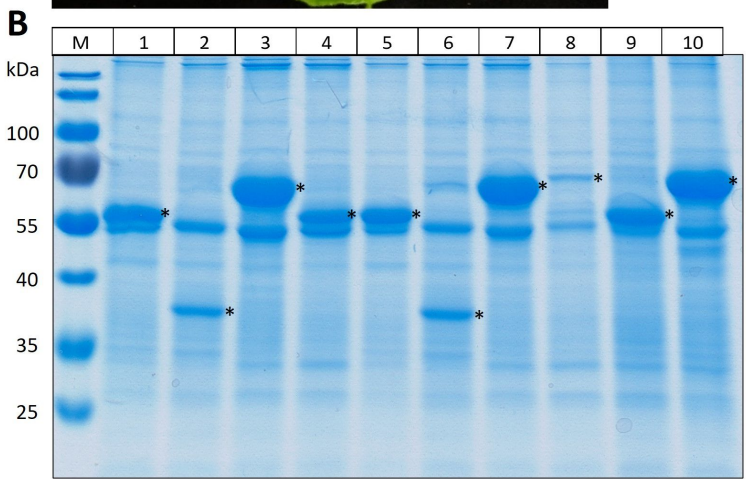


Figure 12: Expression analysis of the legocins with a ColU activity domain by leaf phenotype (A) and by resolving the extracted plant protein using an SDS-PAGE (B).
TR-modules with different 4 bp overhangs for ColIb activity domain cloning (K-ColU) and for ColM activity domain cloning (GS-ColU) were used and combined with A_{ColU}. Agroinfiltration for legocin expression was done by needleless syringe (2.5.2). The legocin-containing plant material was harvested at 8 dpi in small-scale (2.5.5.1) and extracted with HEPES-containing buffer (2.6.2). TSP corresponding to 1.5 mg FW was resolved on a 12% SDS-PAGE with Coomassie staining (2.7.2). Asterisks indicate recombinant protein.

All legocins were very well expressed, especially the legocins with a ColK TR-module, which coincides with the expression of ColK (Nomad Bioscience internal data). In the SDS-PAGE, not all legocins had the protein size predicted. However, ColK is known to run higher than ColA even though ColK is ~3.4 kDa smaller.

Table 19 shows the antimicrobial activity of the ColU activity domain legocins compared with the respective parental molecules. Contrary to the ColIb and ColM activity domain legocins, these legocins had a much higher activity that was either equal or even higher than the corresponding parental molecules' activity.

Table 19: Comparison of the antimicrobial activity of legocins with a ColU activity domain and the parental molecules on DH10B (pICH73311), assessed by soft agar overlay assay (2.8.1).
The Legocins consisted of TR-modules from 6 different colicins and the A-modules of ColU. Agrobacterial cultures carrying legocin expression constructs were inoculated into *N. benthamiana* by syringe infiltration. The protein from the harvested plant material (8 dpi) was extracted by HEPES containing buffer, and their antimicrobial activity was determined by soft agar overlay assay and compared to the wt colicins

No.	vector	modules		activity
	pNMD	TR	A	AU/mg FW
1	48100	ColE1	K-ColU	33554432
5	48140	ColE1	GS-ColU	33554432
2	48110	ColN	K-ColU	16384
6	48150	ColN	GS-ColU	1048576
3	48120	ColK	K-ColU	33554432
7	48160	ColK	GS-ColU	33554432
4	48130	ColA	K-ColU	131072
9	48180	ColA	GS-ColU	131072
8	48170	ColIa	GS-ColU	65536
10	48250	ColK+30aa	ColU-30aa	67108864
	3650	ColE1		262144
	3680	ColN		262144
	15252	ColK		67108864
	25831	ColA		131072
	19141	ColIa		2097152
	15271	ColU		67108864

The antimicrobial activities are presented in AU/mg FW of plant biomass, not in specific activity (AU/µg recombinant protein). Therefore, the activities can be significantly affected by colicin expression levels and can only serve as a rough estimate for the antimicrobial activities. The expression levels of the legocins can be compared well when taking Figure 12 into account. The high antimicrobial activity for TR_{ColK}-A_{ColU} and TR_{ColE1}-A_{ColU} legocins is most likely due to their higher expression levels.

In general, the newly created chimeric proteins all have a similar activity to their parental molecules and could be used to widen the colicin-like antimicrobial arsenal.

The legocins with the TR-module from ColE1 (pNMD48100, PNMD48140) had a 128x higher antimicrobial activity than ColE1. In some cases, the different overhangs (K-ColU and GS-ColU) between TR- and A-modules of the legocins made a difference in antimicrobial activity. The legocin with the TR-module from ColN for cloning with the GS-ColU activity domain (pNMD48150) had a 64x higher antimicrobial activity than the legocin with the TR-module

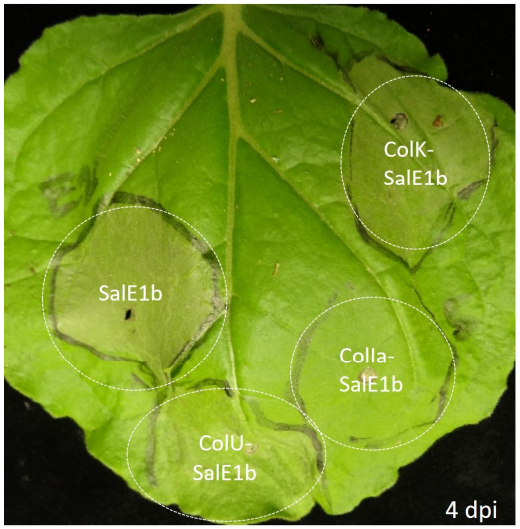
from ColN for cloning with the K-ColU activity domain (pNMD48110). Legocins with the TR-module of ColK and colA had the same antimicrobial activity as ColK and ColA. The ColIa-ColU legocin is less active than ColIa. The legocin with the extra 60 aa linker between the TR- and A-module (pNMD48250) did not have significantly better antimicrobial activity than the equivalent legocins without the linker (pNMD48120, pNMD48160). Nevertheless, this randomly chosen linker also did not reduce the antimicrobial activity compared to the legocin with the non-linker and the parental molecules.

3.2.3 **Interspecies legocins with a SalE1b activity domain**

Additionally to intraspecies legocins, consisting only of colicin domains, five interspecies legocin chimeras with the TR-modules derived from colicins and the A-module derived from a salmocin were made. During the scientific work for this thesis, Salmocin E1b (SalE1b) (3.4.1 Table 24) was found to be a highly potent bacteriocin with a broad-ranged and high antimicrobial activity (3.4.3). For the TR-module, similar to 3.2.1 and 3.2.2, colicins that use different receptors and have a high antimicrobial activity on the “Big 7” STEC strains were chosen.

The legocins were cloned (7.1.1.3) and analyzed as described in 3.2.1. Two of the planned legocins (TR_{ColM}-A_{SalE1b}) and (TR_{ColE3}-A_{SalE1b}) could not be cloned successfully.

The expression phenotype in *N. benthamiana* was documented and is shown in (Figure 13). At 4 dpi, the infiltrated leaf areas showed changes in color which turned into tissue necrosis 5 dpi (not shown).

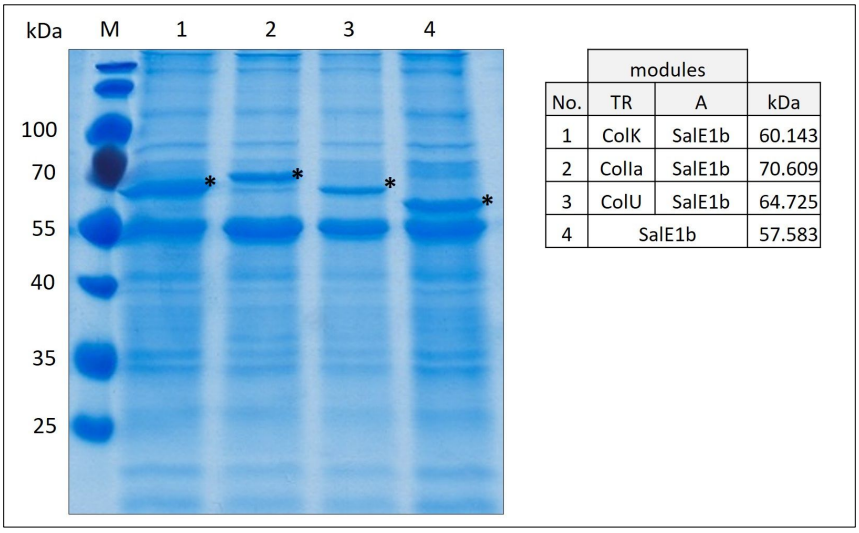


	modules		predicted
pNMD	TR	A	size (kDa)
45981	ColK	SalE1b	60.143
46005	Colla	SalE1b	70.609
48281	ColU	SalE1b	64.725
28204	SalE1b		57.583

Figure 13: Plant expression phenotype of the 3 legocins with a SalE1b A-module at 4 dpi.

The highest attainable yield of the 3 legocins with a SalE1b activity domain was achieved at 4 dpi (

Figure 14) (2.5.6). The leaf tissue condition is also acceptable at 4dpi. Therefore, 4 dpi was defined as the optimal harvesting time point, which coincides with the optimal harvesting time point of the parental molecule SalE1b.



	modules		
No.	TR	A	kDa
1	ColK	SalE1b	60.143
2	Colla	SalE1b	70.609
3	ColU	SalE1b	64.725
4	SalE1b		57.583

Figure 14: Expression levels for the legocins with a SalE1b A-module at 4dpi, analyzed by SDS-PAGE with Coomassie staining.
The legocin-containing plant material was harvested at 4 dpi in small-scale (2.5.5.1) and extracted with HEPES-containing buffer (2.6.2). TSP corresponding to 1.5 mg FW was resolved on a 12% SDS-PAGE with Coomassie staining (2.7.2). Asterisks indicate recombinant protein.

The legocins showed expression levels similar to SalE1b. The TR_{ColK}-A_{SalE1b} legocin had a higher expression level than TR_{ColIa}-A_{SalE1b} and TR_{ColU}-A_{SalE1b}.

Furthermore, the antimicrobial activity of the legocins against *E. coli* DH10B (pICH73311) was assessed by soft agar overlay assay (2.8.1). The legocins showed higher activity than SalE1b and slightly lower activity than their parental colicin molecules (Figure 15).

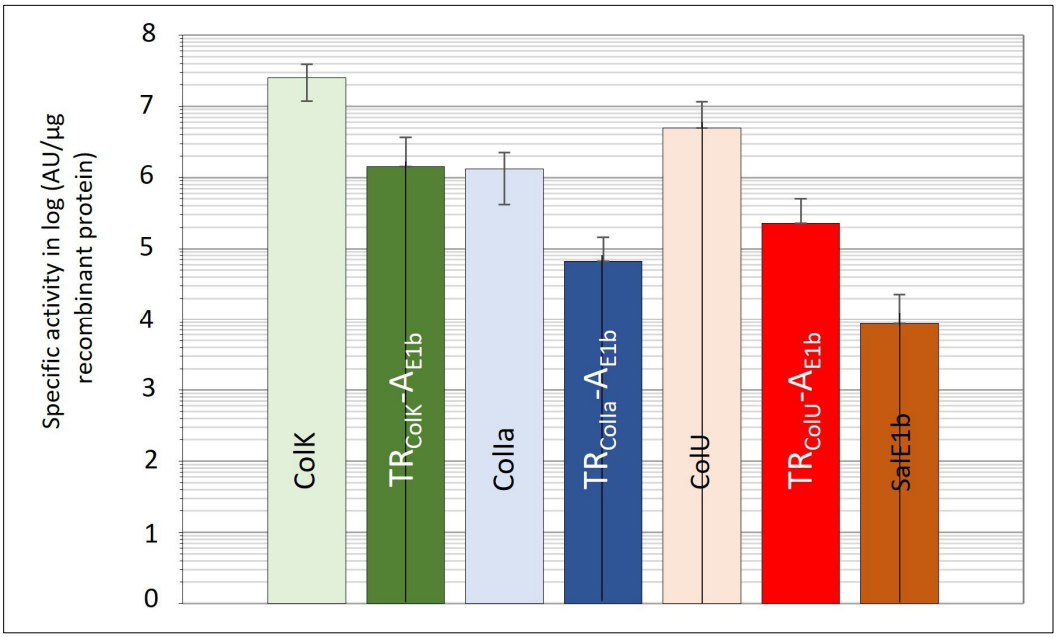


Figure 15: The specific antimicrobial activity of legocins (Leg) with a SalE1b activity domain and the corresponding parental molecules on DH10B (pICH73311).
Activity assessed by soft agar overlay assay (2.8.1) using TSP extracts (2.6.2) of legocin-containing plant material. (n=3, error bars correspond to SD).

The results from Figure 15 underline that the legocins had a lower activity against DH10B (pICH73311) than the corresponding parental molecules.

As the new legocins are chimeras of colicins and salmocins from 2 different bacterial species, their antimicrobial activity was tested against the 10 most frequently reported *Salmonella* serotypes, ranked according to the number of laboratory-confirmed human *Salmonella* infections reported to the CDC in 2012 (CDC, 2014) (2.1.1.3) (Figure 16) and the Big 7 STEC strains (2.1.1.2) (Figure 17) by soft agar overlay assay (2.8.1).

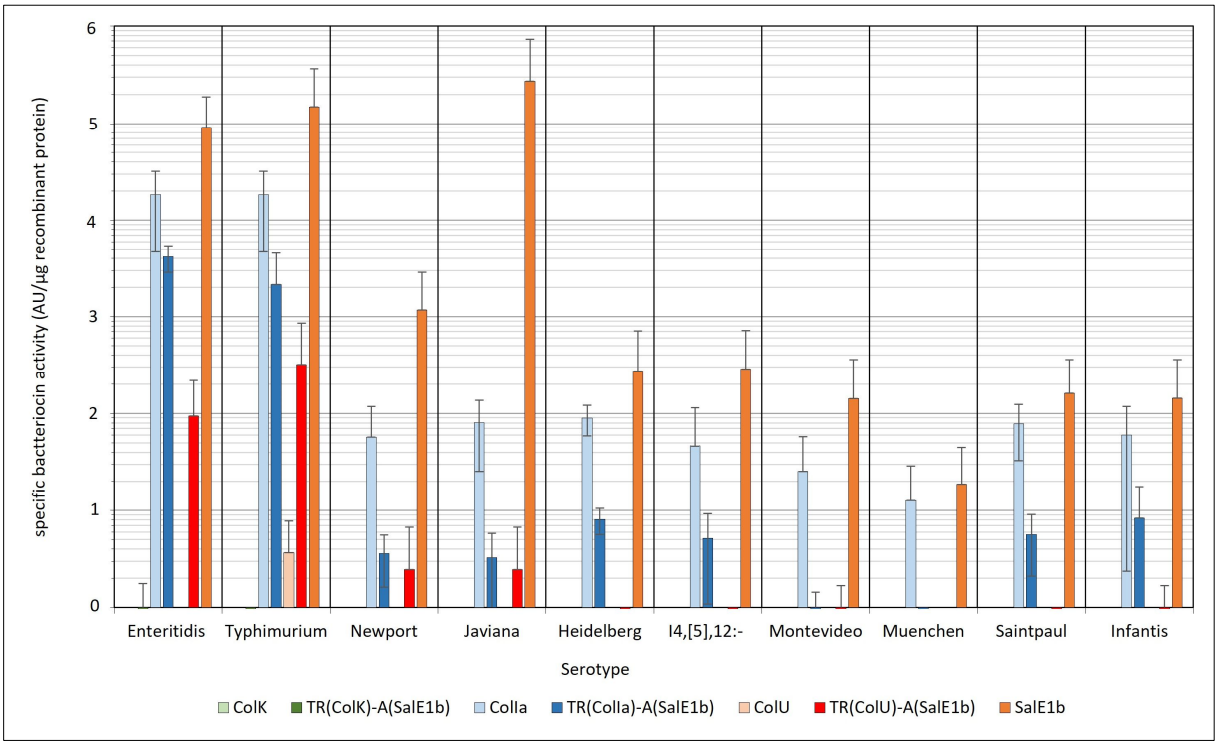


Figure 16: The specific antimicrobial activity of legocins with a SalE1b A-module on 10 *Salmonella* strains compared to the corresponding parental molecules.
The antimicrobial activity was assessed by soft agar overlay assay (2.8.1) using TSP extracts (2.6.2) of legocin-containing plant material. (n=3, error bars correspond to SD). S. enterica ssp. enterica serovars Enteritidis (strain ATCC®13076™*), Typhimurium (strain ATCC®14028™*), Newport (strain ATCC® 6962™*), Javiana (strain ATCC® 10721™*), Heidelberg (strain ATCC® 8326™*), I 4,[5], 12:i:- (strain ATCC®17-00918), Montevideo (ATCC® 8387™*), Muenchen (strain ATCC® 8388™*), Saintpaul (ATCC® 9712™*) and Infantis (strain ATCC®BAA-1675™) were used (2.1.1.3).

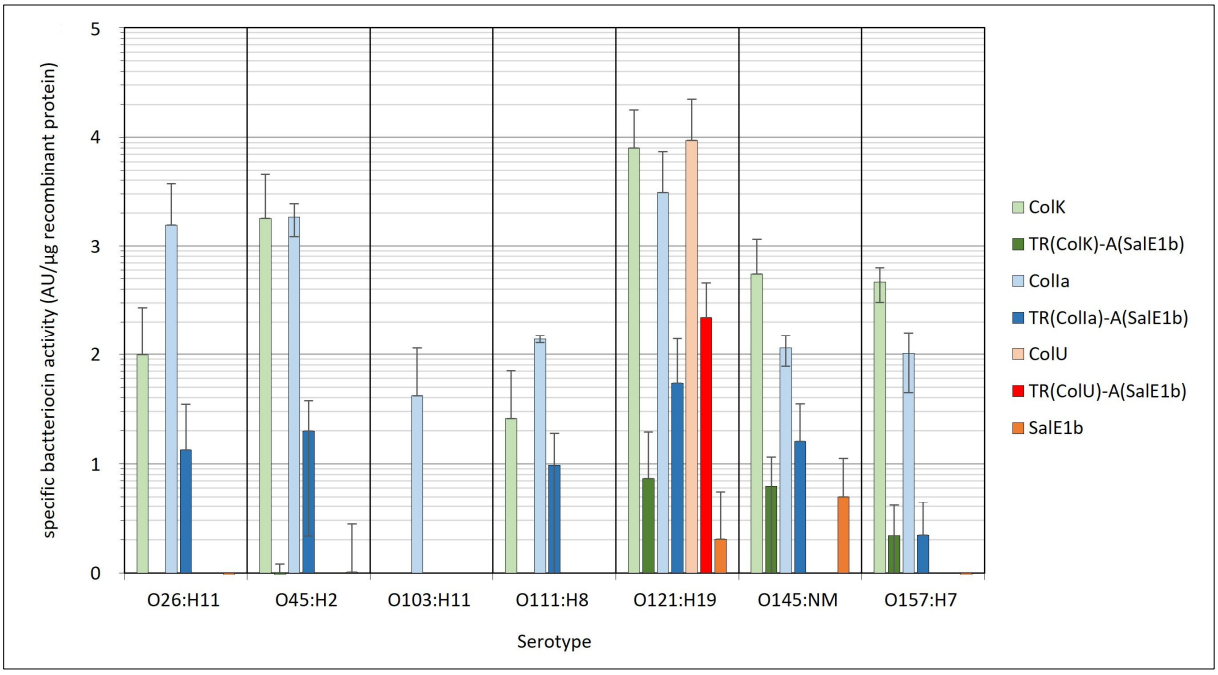


Figure 17: The specific antimicrobial activity of legocins with a SalE1b A-module on the "Big 7" STEC strains compared to the corresponding parental molecules.
The antimicrobial activity was assessed by soft agar overlay assay (2.8.1) using TSP extracts (2.6.2) of legocin-containing plant material (n=3, error bars correspond to SD). STEC strains O26:H11 (CDC 03-3014), O45:H2 (CDC 00-3039), O103:H11 (CDC 06-3008), O111:H8 (CDC 2010C-3114), O121:H19 (CDC 02-3211), O145:NM (CDC 99-3311), O157:H7 (CDC 35150) were used (2.1.1.2).

Overall, SalE1b had higher antimicrobial activity on all *Salmonella* strains than the legocins with a SalE1b A-module (Figure 16). However, the legocins have a broader activity range than SalE1b and include some of the STEC strains, especially the legocin TR_{ColIa}-A_{SalE1b} (Figure 17). Nevertheless, compared to ColIa, TR_{ColIa}-A_{SalE1b} showed a lack of activity on the strains O26:H11 and O45:H2.

Overall, parental colicins had much higher activity on the STEC strains than the legocins with the corresponding TR-module (Figure 17).

The species specificity of the legocins was mostly but not solely determined by their TR-module. Most notably, ColU-SalE1b legocin had broader activity (Enteritidis, Newport, Javiana) and higher activity on *Salmonella* strains than ColU. The TR_{ColIa}-A_{SalE1b} legocin, on the other hand, was not as broadly and highly active on the *Salmonella* strains as ColIa (Figure 16).

The newly created legocins did not offer higher and broader activity ranges on *E. coli* than the corresponding parental colicins ColK, ColIa, and ColU. However, their activity range included some *Salmonella* strains. Ultimately, promising new colicin-like molecules were created that could potentially broaden the antimicrobial arsenal against STEC and *Salmonella* strains.

3.3 Analysis of colicin activity *in vivo*: animal study for the determination of the colicin effect on enterotoxigenic *E. coli*

3.3.1 Overview of the animal study

This study aimed to investigate if a mixture of orally administered plant-produced colicins can prevent and cure post-weaning diarrhea (PWD), an infectious disease in piglets (1.3.2.5). The pig is a model organism for human diseases. Successful treatment could substantiate colicin´s safety and efficacy for future animal and human health applications (Meurens, Summerfield, Nauwynck, Saif, & Gerdts, 2012). As colicin efficacy against *E. coli* has been proven *in vitro* and on biological surfaces, they could potentially serve as an antibiotic alternative for pig farmers, broadening their antibacterial arsenal.

The piglets would be infected with a common post-weaning diarrhea-causing Enterotoxigenic *E. coli* (ETEC) strain CVI-1000 (2.1.1.2) and then treated with a mixture of colicin proteins (ColM, ColU, ColK, ColIb) in 2 different types of drug formulations. One formulation contained leaf-derived colicins and the other contained affinity-purified colicins.

The pilot and the final study were conducted in collaboration with the Central Veterinary Institute (CVI) (Lelystad, Netherlands) of the Wageningen University (Wageningen, Netherlands). All work with the piglets throughout the pilot- and the final animal study was done by the institute's veterinary technicians at their facilities in Lelystad.

The preparations for this experiment were conducted at Nomad Bioscience GmbH within the framework of this thesis. The antimicrobial activities of all available plant-expressed colicins were tested against the test strain CVI-1000 to choose the most effective colicin mixture for treatment. The colicin proteins for the drug formulations were produced and tested for the stability of their antimicrobial activities after prolonged storage. Furthermore, a feasible dosage form (mixture of active ingredients and inactive ingredients (excipient) in a particular dose within a specific configuration (e.g., capsule)) for colicin delivery and release to the piglets´ small intestine was tested and produced. On-site support during the final study, including the bacterial enumeration of the piglets' feces and the final evaluation of the data, was also done within the framework of this thesis.

Prior to the experiment, a test dosage form was tested in a pilot study to verify the correct drug delivery.

The timeline for the final study was developed in collaboration with the CVI (2.10.4, Table 9). Briefly, the piglets were challenged with rotavirus and the ETEC strain CVI-1000 (2.1.1.2). The oral test product was administered 1 day prior to the challenges until the end of the study

(12 days). The piglet´s bacterial shedding of ETEC and their overall health were examined throughout the study. At the end of the study, the animals were euthanized and pathologically examined in a necroscopy.

3.3.2 **Selection of the colicins and the expression host**

A mixture of colicins (colicin "cocktail") with possible synergistic effects was required in order to target CVI-1000 effectively, possibly reducing the dose and delaying or even hindering the formation of bacterial resistance.

Nomad Bioscience GmbH has successfully transiently expressed 23 colicins in *N. benthamiana* (Schulz et al., 2015). In order to identify the most effective colicin mixture to target CVI-1000, all of these 23 colicins (in some cases together with their respective immunity proteins) (7.1.1.1) (Schulz et al., 2015) were expressed in *N. benthamiana*. ColE1, tested in Cutler et al. 2007 study, had low expression levels and caused strong necrosis of plant tissue in *N. benthamiana*. Therefore, it was not tested.

The *Agrobacteria* carrying the colicin expression constructs were infiltrated into the plants by syringe (2.5.2). Colicin-containing plant material was harvested in small-scale (2.5.5.1), and the TSP was extracted with HEPES-containing buffer (2.6.2). The specific antimicrobial activity of the extracts against CVI-1000 was assessed by soft agar overlay assay (2.8.1) and semi-quantitative protein estimation (2.7.1, 2.7.3).

For 13 of the 23 colicins, antimicrobial activity on CVI-1000 was observed (Figure 18).

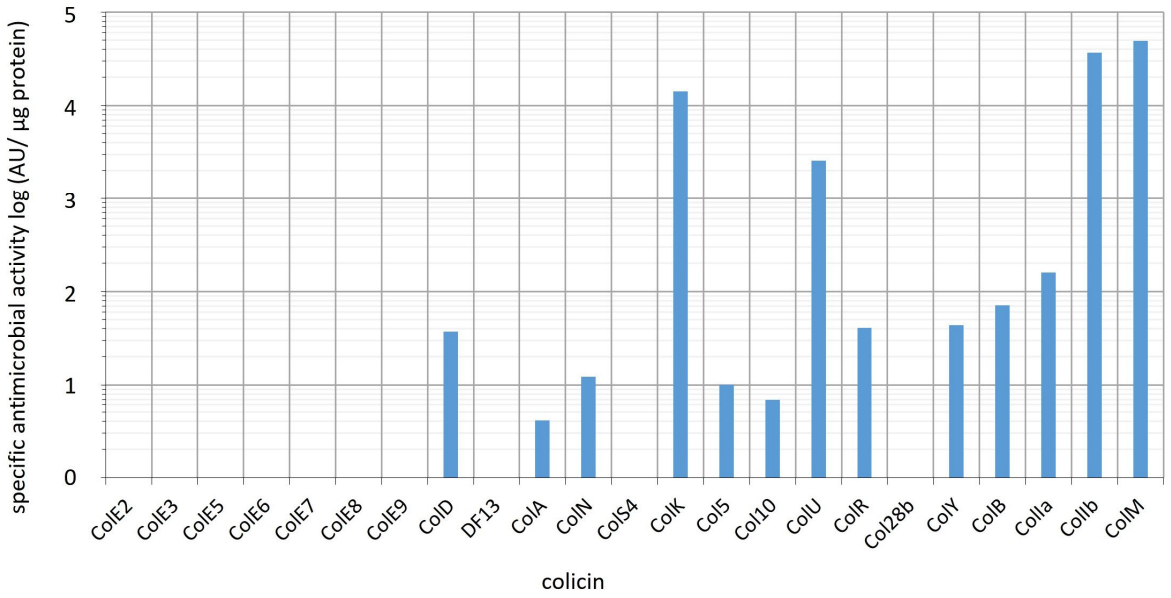


Figure 18: Specific antimicrobial activity of 23 colicins on ETEC strain CVI-1000.
N. benthamiana TSP extracts containing colicins (2.6.2) were analyzed by soft agar overlay assay (2.8.1) (n=1). Colicins ColE2, ColE6, ColE7, ColE8, ColE9, ColD and DF13 were co-expressed with the corresponding immunity proteins (ImmE2, ImmE6, ImmE7, ImmE8, ImmE9, ImmD and ImmDF13 respectively).

In conclusion, 9 out of 23 colicins had a specific antimicrobial activity between 4 and 160 AU/µg protein. Four of the colicins had a much higher activity (ColIb - 36,526 AU/µg protein, ColM - 49,045 AU/µg protein, ColU - 2565 AU/µg protein, and ColK - 14,079 AU/µg protein). A repetition of the experiment confirmed the initial activity data. Conveniently, the 4 colicins with the highest activity were also expected to work synergistically, as they use various receptors, both translocation pathways, and offer 2 modes of activity (Table 20). Therefore, they were chosen for the animal study on piglets.

Table 20: The 4 colicins selected for the animal study.
Overview of the receptors, the translocation machinery, and the modes of action they use.

colicin	**group**	**receptor**	**translocation**	**type of activity**
ColIb	group B	Cir	TonB, ExbBD	pore-forming
ColM	group B	FhuA	TonB, ExbBD	inhibition of murein synthesis
ColU	group A	OmpA	OmpF, TolABQR, LPS	pore-forming
ColK	group A	Tsx	OmpF, OmpA, TolABQR,	pore-forming

N. benthamiana was chosen as the production host for colicin expression. Protein production in edible plants offers advantages for animal or human studies, as *N. benthamiana* contains toxic alkaloids such as nicotine and anabasine (Sisson & Severson, 1990). However, the yields achieved in the tested edible plants (*Spinacia oleracea* 'Frühes Riesenblatt') were not sufficient for the amounts of colicin needed for this experiment.

3.3.3 Determination of the colicin formulations and the dosage form

In the animal study, the efficacy of a colicin mixture being administered in 2 different drug formulations was tested. One formulation was based on affinity-purified colicin ("purified colicin formulation") (2.7.4), with most non-recombinant host-cell proteins and plant metabolites such as alkaloids and polyphenols removed, resulting in a colicin preparation of ~90% purity (Stephan et al., 2017) to allow a very accurate correlation of cause and effect for the drug in the piglet model. However, the purification process is relatively costly and time-consuming.

The second formulation was more cost-effective: dried leaf tissue containing recombinant colicin was processed into dried leaf powder. The resulting powder was used in the "leaf-derived colicin formulation" and does not require any expensive treatment and storage conditions, making it more feasible for industrial purposes, provided that the colicin activity can be conserved. Drying of the protein-containing leaf material has proven to be a promising method for conserving functional recombinant proteins such as antibodies and cellulases (Joh, McDonald, & VanderGheynst, 2006). Furthermore, the conservation of antimicrobial active ColM in dried leaf material had already been tested successfully at ICON Genetics GmbH (Halle, Germany). (Nomad Bioscience GmbH internal data).

3.3.3.1 Colicin stability in dried plant leaf material

For the expression of the recombinant colicins, *N. benthamiana* plants were agroinfiltrated by vacuum with *A. tumefaciens* (2.5.3), carrying the colicin-expression constructs (7.1.1.1). The colicin-containing leaf material was harvested in batches, 1 for each colicin, and processed to a dried leaf powder (2.5.5.3). For each dried leaf powder batch stored at room temperature (RT), the antimicrobial activity and the yield of recombinant protein in the TSP extracts were compared to the TSP extracts of harvested and frozen leaf material (-80°C) of the same infiltration batch (2.10.1) (Table 21).

Table 21: Activities (against *E. coli* DH10B (pICH73311)) and yields of colicin-containing TSP extracts from colicin-containing dry leaf powder (dry) and colicin-containing frozen leaf powder (frozen) of the same infiltrated plant batches (2.10.1) of the first 4 colicin test batches (ColIb batch #1, ColM batch #1, ColU batch #1, ColK batch #1) (n=1).
The plant material was analyzed 4 d after completion of the drying process (4 d of storage) (2.5.5.3).

colicin	storage of plant material	concentration in TSP extract (µg/µl)	% colicin of TSP extract	yield bacteriocin (mg/g FW)	antimicrobial activity (AU/µg bacteriocin)
Ib	dry (RT)	0.45	43	0.97	3386873
	frozen (-80°C)	2.05	14	1.44	4566969
M	dry (RT)	0.62	44	1.36	300293
	frozen (-80°C)	2.14	23	2.46	332873
U	dry (RT)	0.63	33	1.04	1576142
	frozen (-80°C)	1.69	14	1.18	2769907
K	dry (RT)	0.97	33	1.60	4094720
	frozen (-80°C)	2.77	13	1.80	7279756

The specific antimicrobial activity of colicins derived from dried leaf powder was very similar to those derived from frozen leaf powder (Table 21). Only a minor decrease in activity was observed for the dried leaf powder.

The yield of ColIb and ColM from dry leaf powder was reduced by ~30-40% compared to the frozen leaf powder. However, for ColU and K, the yield was almost identical, ~5% lower than for the frozen leaf powder. This shows that the yield is only partially reduced by the drying procedure, depending on the colicin. The yield of TSP from the dry leaf powder is reduced, while the percentage of colicin of the TSP is elevated compared to the frozen leaf powder, which is also visible in the SDS-PAGE analysis (Figure 19). Also, no significant protein degradation was found.

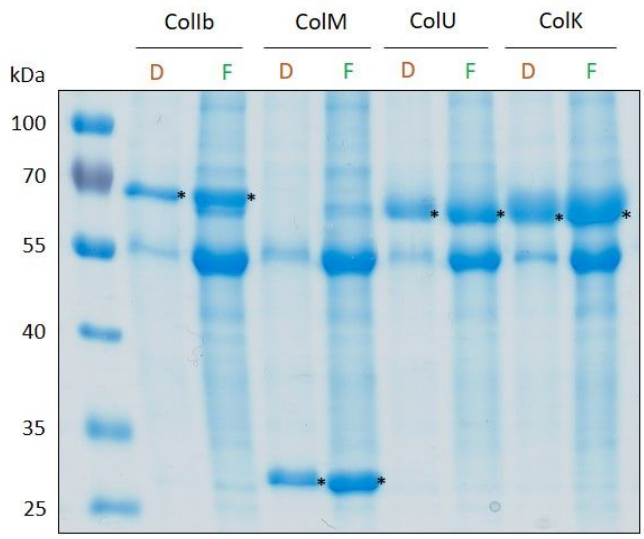


Figure 19: Comparison of colicin content in dried leaf powder (D) and frozen leaf powder (F) of the same colicin-containing *N. benthamiana* test batch (ColIb batch #1, ColM batch #1, ColU batch #1, ColK batch #1) by SDS-PAGE (12% gel) with Coomassie staining.
TSP extracts of material used in Table 21 corresponding to 1.5 mg FW loaded, and the asterisks mark the colicins: ColIb – 69.9kDa, ColM – 29.5kDa, ColU - 66.3kDa, ColK – 59.6kDa.

Furthermore, the colicin-containing dried leaf powder was tested for antimicrobial activity after prolonged periods of storage at RT (Figure 20).

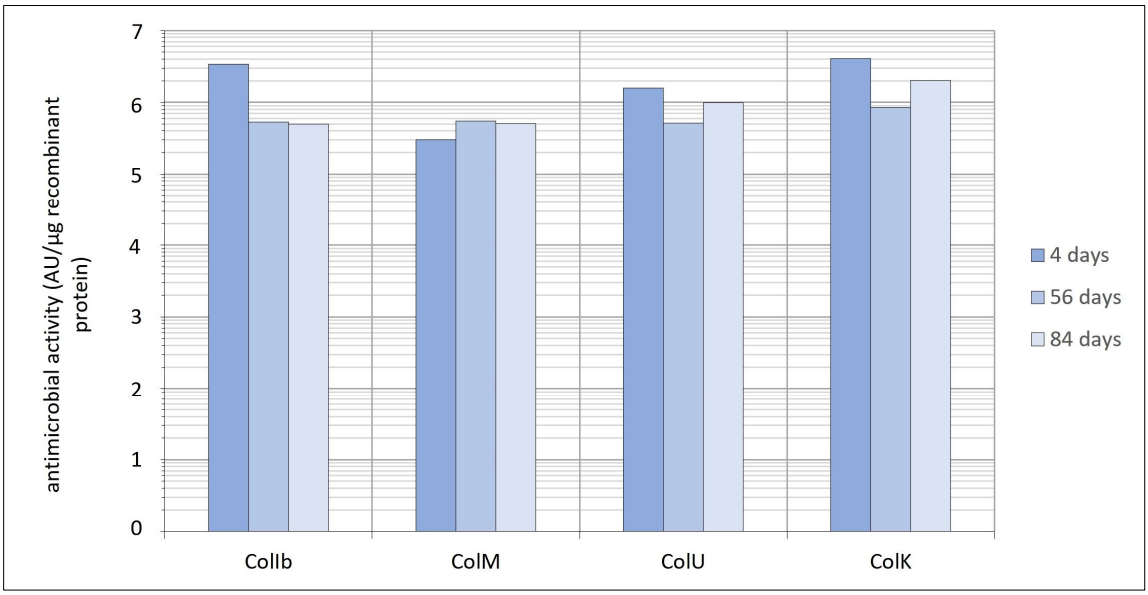


Figure 20: Specific antimicrobial activity of colicins extracted from dry leaf powder test batches (ColIb batch #1, ColM batch #1, ColU batch #1, ColK batch #1) upon different periods of storage against *E. coli* DH10B (pICH73311).
Extraction of TSP from the dry leaf powder was done as described in 2.10.1 and for Table 21.

The test batches of dry leaf powder containing ColIb, CoM, ColU, and ColIb were tested on their specific antimicrobial activity 4, 56, and 84 days after drying. Apart from some activity fluctuation, probably due to experimental variance, the antimicrobial activity levels stayed

constant throughout storage. In summary, there was no significant loss of yield and activity when colicin-containing dry leaf powder was stored for prolonged periods.

3.3.3.2 Large-scale production of colicins

For each drug formulation, 2 doses of colicin proteins, one lower (10 mg/kg feed (5 mg/day/piglet: 1.5 mg ColM, 1.5 mg ColU, 1 mg ColK and 1 mg ColIb)) and one higher (20 mg/kg feed (10 mg/day/piglet: 3 mg ColM, 3 mg ColU, 2 mg ColK and 2 mg ColIb)) than the successful dose of 16.5 mg ColE1/kg feed in Cutler et al.´s study (S. A. Cutler et al., 2007) (1.3.2.5) was planned.

In the next step, several larger batches of colicin-containing dry leaf powder, each with 1 of the 4 colicins, were produced. For this, the plants were inoculated with *A. tumefaciens* harboring colicin-expression constructs by infiltration using vacuum (2.5.3), and the leaf material was harvested and processed to dry leaf powder (2.5.5.3).

Every produced batch of dried leaf powder was tested for antimicrobial activity of the contained recombinant colicin proteins against *E. coli* CVI-1000 at different time points (2.10.1) to assure the preservation of the antimicrobial activity during drying and storage. The test batches (numbered #1) of all 4 colicins were tested 3 times (Figure 20), the second batches (numbered #2) were tested twice, and the third and fourth batches (numbered #3 and #4 respectively)were tested at least once (data not shown). How often the batches were tested depended on the time gap between the production date and the animal trial. Overall the stability of antimicrobial activity in the larger production batches was similar to the antimicrobial activity of the test batches and remained stable over several weeks of storage until the final study. The dry weight of the plant material was usually between 8 and 10% of the initial fresh weight (Table 22).

Table 22: Overview of the recombinant colicin-containing dried leaf powder batches for the leaf-derived colicin formulation in terms of the fresh weight, dry weight, colicin yield (2.10.1), and the total amount of colicin per batch.
The colicin yield was calculated by semi-quantitative protein estimation (2.7.1; 2.7.3) of extracted plant material (2.6.2).

		plant material			
bolicin	batch #	fresh weight (g)	dry weight (g)	yield (mg colicin/g DW)	total amount colicin/batch (mg)
ColIb	1	75	6	8.52	50.27
	2	708	62	9.43	582.77
ColM	1	67	6	7.76	44.23
	2	517	49	5.60	272.16
	3	535	48	3.73	177.18
	4	270	29	7.91	226.23
ColU	1	72	6	9.18	53.70
	2	615	55	6.10	336.11
	3	287	28	6.91	192,10
	4	251	24	6.32	150.04
ColK	1	74	7	19.97	133.60
	2	422	34	21.11	715.63

For the purified colicin formulation, affinity-purified colicin was used. The purified colicin batches were produced and analyzed for protein concentration (2.7.1), protein integrity (2.7.2) and activity (2.8.1) by Anett Stephan (Researcher at Nomad Bioscience GmbH) to assure consistent quality.

As mentioned earlier, 2 doses for each colicin-containing formulation type (purified colicin formulation and the leaf-derived colicin formulation) were used for the treatment of the piglets (3.3.1).

The entire animal study required the production of 585 mg ColM, 585 mg ColU, 390 mg ColK, and 390 mg ColIb for each of the 2 colicin formulation types.

3.3.3.3 The configuration of the dosage form

Researchers at Nomad showed that colicins are susceptible to digestive enzymes of the human digestive tract in combination with gastric acids and duodenal bile salts (1.3.2.4) (Schulz et al., 2015). Therefore, when administered orally to cure an intestinal disease, colicins need be protected by a dosage form configuration that enables them to bypass the acidic environment of the stomach (pH 1-3 and pepsin enzymes) and the duodenum (pH 5-6 and bile salts and, e.g., trypsin) without degradation (D. F. Evans et al., 1988; Whitcomb & Lowe, 2007).

Miller et al. 2015 concluded that double delayed-release capsules (DRcaps® (Capsugel, Morristown, NJ, USA)), meaning a smaller size 0 inside a bigger size 00 capsule, coated with 4 layers of an 8,1% w/v Eudragit L100-55 (delayed-release coating for drug delivery to the duodenum (Evonik Industries, Essen, Germany)), offer the best gastric protection and *in vitro* and *in vivo* duodenal release patterns of a drug for pigs (Miller et al., 2015).

Therefore, the dosage form configuration of choice was this capsule configuration coated with a gastric-resistant Eudragit® coating that offered the appropriate release pattern. F4 and F18 Fimbrial type positive strains, like CVI-1000, are usually prevalent throughout the whole length of the jejunum and the ileum of the small intestine, which in piglets spans a length of ~10 m (Fairbrother et al., 2005; McCance, 1974). The protection must then dissolve and release the colicins at the correct intestinal location. This enteric release should occur at a pH of >6.0 (D. F. Evans et al., 1988; McEwan, Schousboe, & Skadhauge, 1990).

For these reasons, the capsule coating Eudragit® L 100 (Evonik Industries, Essen, Germany) was chosen, which offers protection up to pH 6.0 and is made for jejunum release (pH 6-7) of drugs. The coating should also ensure protection from duodenal bile fluids dissolving after the duodenum over the first sections of the jejunum. The passage time of food through the small intestine is usually 3 (±1) hours in humans (Camilleri et al., 1989; Degen & Phillips, 1996) , which is very similar in pigs. Therefore, the capsule must dissolve quickly at pH6 and higher.

The coating solution with Eudragit® L100 was adapted to allow tabletop coating by referring to an article by (Ruth Dodds, 2008), the Eudragit technical staff, and Miller et al. 2015 with the aim to achieve a highly acid-resistant coating solution with a tabletop dip-coating device (2.10.2.2). For the encapsulation of the colicins, a tabletop device was used (2.10.2.1).

In their study, Miller *et al.* used double DRcaps® (size 0 capsule inside a size 00 capsule) for drug delivery (Miller et al., 2015). This combination was also adapted for the piglet study as these capsule sizes could carry the appropriate amount of colicin formulation while being swallowable by 4-week-old piglets.

3.3.3.4 The first dissolution test series for the optimized dosage form configuration.

For the pharmaceutical industry, the dissolution of dosage form configurations, such as capsules and tablets, is tested *in vitro* in standardized dissolution apparatuses (USP Apparatus 1, 2, 3, 4) described in the U.S. Pharmacopeia (The United States Pharmacopeial Convention USP, 2011).

Their primary function is to keep a test product submerged while creating a water flow and mimicking peristaltic movement.

While apparatus 1 has a basket that spins with the test product inside, apparatus 2 has a paddle that spins with the test product lying on the vessel's bottom. As these apparatuses were not available, a simple setup combining mechanisms from apparatus 1 and 2 was built. A basket kept the capsules submerged (similar to apparatus 1), and a magnetic agitator kept the fluid inside the vessel moving (similar to apparatus 2), allowing an estimation of the capsules´ dissolution characteristics (2.10.3).

In the first dissolution test of the dosage form configuration, the size 0 DRcaps® capsules were filled with a test substance, put into a size 00 DRcaps® capsules (2.10.2.1), and coated with 1, 2, 3 or 4 layers of 12% Eudragit® L100 enteric coating (2.10.2.2) (2.10.2.3).

This dissolution test series showed that double DRcaps® (size 0 capsule inside side 00 capsule) require a minimum of 2 coating layers of Eudragit® L100 for reliable gastric protection and timely intestinal dissolution. After a 2 h incubation at pH 1, the capsules showed no signs of disintegration, but after 40 min at a pH of 7 the capsules showed a considerable change in shape and were very soft and fragile during handling. Presumably, they would be prone to rupture at this stage due to the peristaltic movement. Leakage of the capsules occurred at 80 min at pH 7. However, 2 coatings should not be exceeded as the 3x or 4x coated capsules at pH 7 took over 100 min for leakage to occur. Capsules with 1 coating layer did not offer proper gastric protection showing an ingress of the pH 1 test solution within the 2 h incubation.

The passage of gastric-acidity resistant capsules, the integrity of the capsules after transport through the stomach, and the capsules' disintegration in the small intestine in piglets was tested in a pilot study (3.3.4). For this, the test dosage form, double-coated double DRCaps® capsules (2.10.2.2) (2.10.2.3) filled with milk powder (2.10.2.1), were sent to the CVI (Lelystad, Netherlands).

3.3.4 **Results of testing the planned dosage form *in vivo* (pilot study)**

The pilot study was conducted by the CVI employees (4 weeks before the final study) to examine if the chosen dosage form configuration was suitable for delivering the colicins to a piglet's small intestine. Additionally, the stress level of capsule administration to the piglets by gavage was assessed.

In the pilot study, three 4-6 week old piglets were used for the capsule-gavage-testing of 6 test dosage forms (2x coated (Eudragit L100) (2.10.2.3) double DRcaps® capsules (size 0 inside

size 00) filled with milk powder sent from Nomad Bioscience GmbH These capsules were filled as described in 2.10.2.1 for the first dissolution test series but without adding 5% (w/w) crystal violet. The capsules were color-marked to make them traceable inside the piglet's body for the pathological examination. The capsules were administered to the piglets 18 h and 4 h before euthanization and necroscopy by the gavaging procedure, allowing for gastric passage to the intestine, which was estimated to take 3-4 h (Miller et al., 2015). For the gavage procedure, pigs were kept and restrained in an upright position, the mouth was opened by a mouth gag, and the capsules were placed as far back as possible in the mouth. By stroking along the neck, swallowing was stimulated.

The pilot study revealed that no more than 2 capsules per piglet treatment should be administered, as the gavage of each capsule is stressful for the piglets and laborious for the staff. This affected the dosages of colicin formulations used in the final study (3.3.5).

The pilot study also led to new insights for optimizing the dosage form configuration: when the necroscopy was performed 18 h after the first gavaging, all capsules were still found floating at the surface of the gastric juices inside the stomach. The capsules had not proceeded to the intestine with the rest of the food. The veterinary staff concluded that the capsules' buoyancy had kept them from reaching the stomach exit (pylorus) when the stomach emptied partially after digestion. Due to piglets' constant food uptake, their stomach only empties partially so that undigested and floating substances are held back in the stomach.

This meant that the capsules' buoyancy had to be reduced by diminishing airspace and buoyant capsule material. For this, the outer 00 size capsule was removed from the dosage form configuration, which meant that the active ingredient filled size 0 DRcap® would not be inserted into a size 00 capsule. The buoyancy of a double-coated and filled size 0 capsule was still to high; therefore, 5 stainless steel balls (2.10.2.1) were added to make the capsule sink in water.

The veterinary doctors also concluded that the piglets' stomach pH was at a pH of 3-4 and, therefore, higher than the pH used in the first dissolution test series (3.3.3.4) and used by Miller et al. 2015 (Miller et al., 2015). According to them, this is primarily due to the piglet's frequent eating pattern.

The dissolution properties of this optimized dosage form configuration with different numbers of enteric coating layers were redetermined in a second dissolution test series. The optimized dosage form configuration consisted of a size 0 DRcap® capsules. For the tests, they were filled with a mixture of milk powder and 5% (w/w) crystal violet with an addition of 5 stainless steel balls (2.10.2.1) and coated 1-4 times with Eudragit® L100 (2.10.2.2; 2.10.2.3). This should

determine the number of coating layers necessary for proper enteric release while also analyzing if a gastric pH of 4.0 instead of pH 1.0 would affect the dissolution properties (2.10.3) (Figure 20).

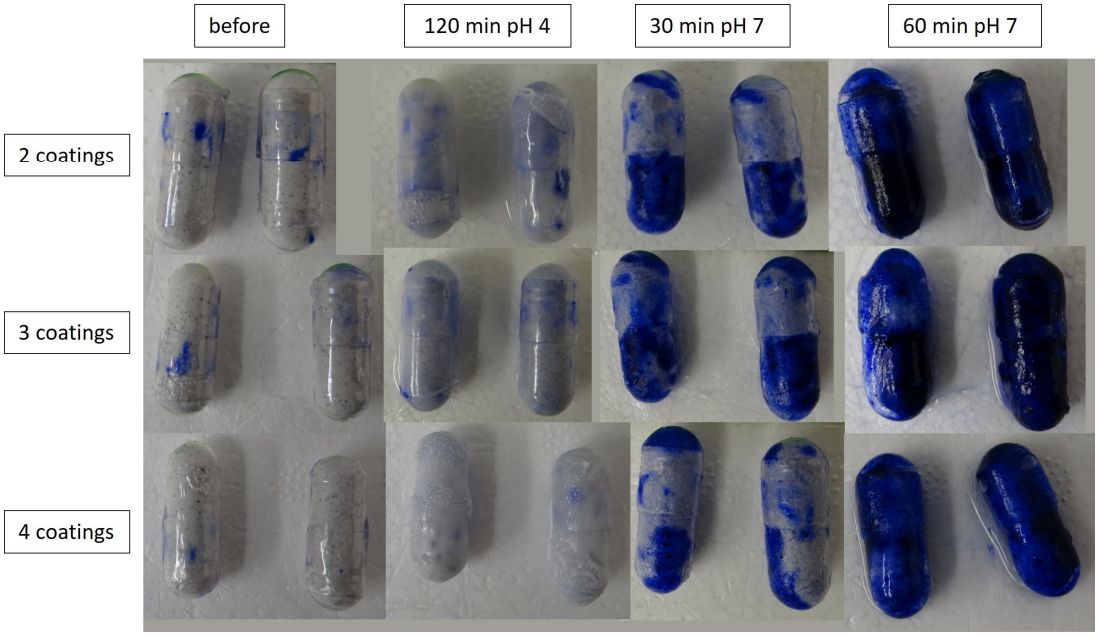


Figure 21: The second dissolution test series (2.10.3) for the optimized dosage form configuration. Size 0 DRcaps® capsules were filled with a mixture of milk powder, 5% (w/w) crystal violet, and 5 stainless steel balls (2.10.2.1) and coated with 2, 3, and 4 layers of enteric coating (Eudragit L100) (2.10.2.3). Capsules are shown prior to treatment, then upon a 120 min incubation at pH 4.0 (150 rpm, 37°C) and subsequent incubation at pH 7.0 (150 rpm, 37°C) for 30 and 60 min.

This study concluded that 2 coating layers were sufficient for gastric protection at pH 4.0 as the capsules showed no signs of disintegration after 2 h. Intestinal dissolution at pH 7.0 was timely, as the capsules showed changes in shape, and leakage occurred before 60 min of incubation. The capsules with 3 or 4 coating layers had similar dissolution characteristics as the 2-layer coated capsules.

3.3.5 **Assembly of the final capsules**

The pilot study concluded that the piglets of each treatment group could only receive a maximum of 2 capsules (dosage forms) per day. This mainly affected the higher dose (10 mg/0.5 kg feed) of the leaf-derived colicin formulation that required the capsule volume of 4 capsules. Therefore, both treatment doses of the leaf-derived colicin formulation were halved. The planned dosage of the purified colicin formulation remained unchanged, as the higher dose only required the volume of 2 capsules.

Additionally, to achieve the correct doses of colicin per capsule, milk powder was used as an inactive ingredient (excipient) to extend the volume of the formulations (2.10.2.1). This was done before the pilot study's final results, which meant that the addition of 5 metal balls to the capsules to minimize buoyancy reduced the planned colicin dose per capsule. For the reasons stated above, the final colicin doses used in the experiment (Table 23) differed (~-22% for the purified colicin formulation and ~-64% for the leaf-derived colicin formulation) from the planned amounts presented in 3.3.3.2.

The final study was done as a double-blind study. Therefore, all treatment groups received 2 capsules as a daily treatment. This meant that the lower dose would consist of one capsule with the colicin-containing formulation, and the other would be a capsule with dry milk powder (DMP), identical to the capsules administered to the control group or placebo treatment (Table 23).

Table 23: Final colicin doses for the 5 different treatment groups in the animal study.
The total number of capsules required for each treatment group, consisting of 10 piglets receiving 12 feedings, is shown. All treatments consisted of 2 capsules to blind the technicians to the treatment. Therefore, daily lower dose treatments were complemented with a dry milk powder (DMP) containing capsule.

			colicin protein in (mg/day/animal)					
sample group	treatment type	colicin dose	total	ColM	ColU	ColK	ColIb	No. of capsules
1	leaf derived colicin formulation	low	1.8	0.6	0.6	0.4	0.4	120 + (120 DMP)
2		high	3.6	1.0	1.0	0.7	0.7	240
3	purified colicin formulation	low	3.9	1.2	1.2	0.8	0.8	120 + (120 DMP)
4		high	7.8	2.3	2.3	1.6	1.6	240
5	control with DMP	nil	0.0	0.0	0.0	0.0	0.0	240

Before encapsulation, the antimicrobial activity of the final colicin-containing formulations was assessed by soft agar overlay assay (2.8.1) to assure that both mixtures still had the expected activity.

DRCaps® capsules size 0 were filled as described in 2.10.2.1.

3.3.6 **On-site evaluation of the piglet animal study**

The detailed study plan was developed in collaboration with the CVI and the methodology within the framework of this thesis is explained in 2.10.4.

Due to the pathological effects caused by capsule administration using a metal dosing gun, the study had to be terminated early and can only serve as an indication for the effectiveness of a colicin treatment of CVI-1000 infection in piglets. There is data on fecal samples from day +1 of the study directly after the 1st oral challenge day with ETEC until day +5 when the study was discontinued (Figure 22).

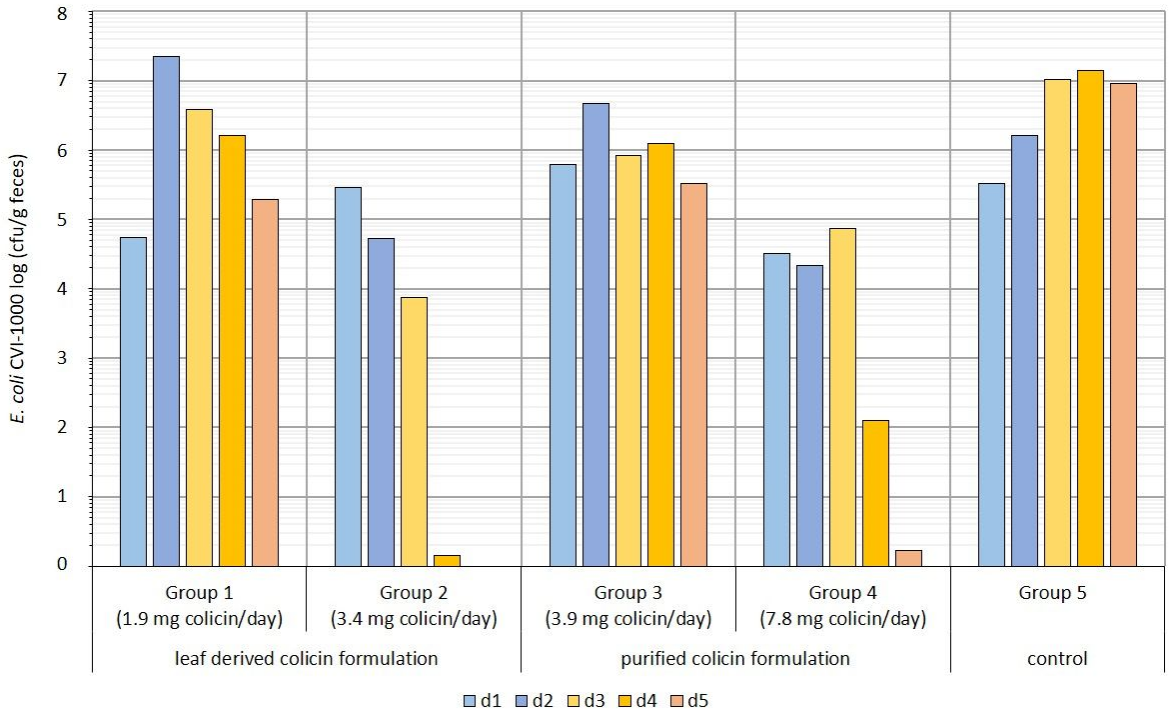


Figure 22: Antimicrobial effect of mixed colicin formulations (ColIb (20%), ColM (30%), ColU (30%) and ColK (20%)) on ETEC strain CVI-1000 in piglets.
The mean CFU count of CVI-1000 in the piglets' fecal samples from day +1 to day +5 of the study. Fecal shedding of CVI-1000 was enumerated by dilution plating. Groups 1 and 2 were treated with the leaf-derived colicin formulation, and groups 3 and 4 were treated with the purified colicin formulation. Group 5 received a negative control treatment. The CFU/g feces is the group's mean of the viable piglets on that day. If piglets had no feces or were dead, they were not included in the mean.

The capsule administration technique had caused strong confounding factors that greatly minimized this study's validity in determining a clear dose-response relationship for the colicin treatment of a PWD infection in piglets. However, a certain dose effect was observed (Figure 22). The high doses of both colicin formulations showed a clearly visible reduction in the bacterial load compared to the low doses and the negative control. Low doses of both formulations also showed an effect compared to the negative control. In the end, it is unclear what dose ultimately reached the intestine because not all capsule remains, in the form of the metal balls, were found in the intestine or the fecal samples. Nevertheless, the dose that arrived had a distinct antimicrobial effect on the amount of CVI-1000 shedding.

The receptor status for susceptibility to CVI-1000 was not tested during the necroscopy of the piglets due to the confounding factors of the study. However, it is known that a high percentage of flemish pig breeds are susceptible to F4 and/or F18-positive bacteria (Nguyen et al., 2017). Due to time-related and financial reasons, the animal study could not be repeated using an optimized approach of dosage form administration.

3.4 Screening salmocin candidates for yield and antimicrobial activity

3.4.1 Generation of binary constructs for TMV-based recombinant protein expression in plants.

Scientists at Nomad Bioscience identified candidates of colicin-like bacteriocins for broad-spectrum *Salmonella* control (1.3.3.2). The screening of salmocins for suitability as antimicrobial protein product candidates to target *Salmonella* was described in Schneider et al. 2018 and revealed novel bacteriocin candidates (Table 24) (Schneider et al., 2018) (7.2). For the 3 salmocins with a potential nuclease activity (SalE3, SalE2, and SalE7), the corresponding salmocin immunity proteins were synthesized (SImmE3, SImmE2, and SImmE7) to suppress cytotoxicity for the expression host by co-expression.

Table 24: The donor constructs encoding salmocins or salmocin immunity proteins were generated in preliminary work and provided for cloning.
Salmocin sequences were obtained from gene synthesis and were codon-optimized for *N. benthamiana* (7.1.1.2).

No.	salmocin	activity	MW (kDa)	GenBank assession No.
1	SalE2	DNase	61.96	KTM78572.1
2	SalE3	RNase	61.71	GAS18013.1
3	SalE7	DNase	62.26	KSU39545.1
4	SalE1a	pore-forming	52.81	OIN35410.1
5	SalE1b	pore-forming	57.58	OIN32443.1
6	Spst	degradation of murein	33.23	ESF65298.1

immunity protein	specificity	GenBank assession No.
SimmE2	SalE2 (DNase)	KTM78571.1
SimmE3	SalE3 (RNase)	GAS18012.1
SimmE7	SalE7 (DNase)	KSU39546.1

The salmocin-coding sequences in the 6 donor vectors pNMD0337-0342 (7.1.1.2) were cloned into the TMV-based assembled expression vector pICH29912 (1.5, Figure 2) (7.1.1.2) for cytosolic plant expression using Modular Cloning (2.3.5).

The immunity protein-coding sequences in the 3 donor vectors pNMD0343-0345 (7.1.1.2) were cloned into the potato virus X (PVX)-based assembled expression vector pNMD0674 (1.5, Figure 2) using Modular Cloning (2.3.5). These vectors were used for cytosolic co-expression with their respective salmocin TMV-based expression vectors.

The plasmids were transformed into the *Agrobacterium* strain ICF320 for plant expression (2.3.8).

3.4.2 Determination of the optimal harvesting time point and estimation of the recombinant protein yield for transiently expressed salmocins using *N. benthamiana* as a host

3.4.2.1 Determining the optimal harvesting time point

Agrobacterium cultures harboring constructs for the expression of nuclease salmocin (SalE3, SalE2, and SalE7) were infiltrated by syringe (2.5.2) either alone or as a mix with cultures carrying the corresponding immunity protein constructs (SImmE3, SImmE2, and SImmE7) to determine which option provides lower cytotoxicity and higher yield. The salmocin-containing plant material was harvested (2.5.5.1) on days 4, 5, 6, 7, and 8 post infiltration, and the phenotype was documented to assess the optimal harvesting time point (2.5.6) (Figure 23).

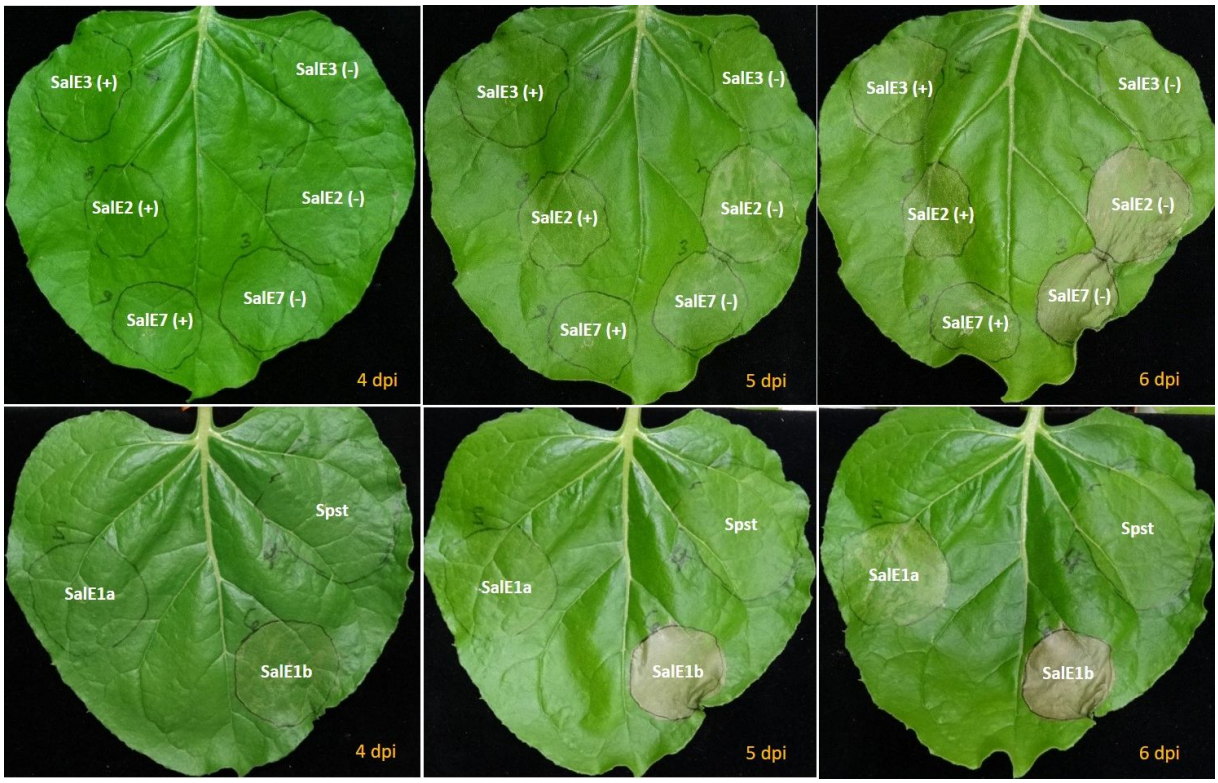


Figure 23: Plant expression phenotype examination of salmocin in *N. benthamiana* at 4, 5, and 6 dpi. SalE3, SalE2, and SalE7 were expressed with (+) and without (-) their respective immunity proteins. *Agrobacterium* cultures harboring constructs for the expression of salmocin or salmocin immunity protein were inoculated into the plants using syringe infiltration. (2.5.2) One representative leaf of intermediate age per plant is shown.

The expression of all salmocins caused strong leaf necrosis on days 7 and 8 post infiltration (not shown). SalE1b expression already showed high cytotoxicity at 5 dpi.

SalE2 and SalE7 were less cytotoxic when co-expressed with their immunity proteins SimmE2 and SimmE7, respectively. For SalE3, however, there was no visible difference between individual expression and co-expression with SimmE3. In addition to the leaf phenotype, the optimal harvesting time point was assessed by SDS-PAGE with Coomassie staining (2.5.6) (Figure 24).

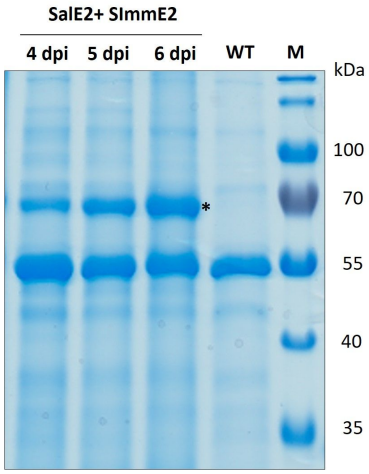


Figure 24: Example of assessing the highest attainable yield for a recombinantly expressed bacteriocin to determine the optimal harvesting time point over a time course of 3 days.
Agrobacterial cultures harboring either SalE2 or SImmE2 expression constructs were co-infiltrated into *N. benthamiana* leaves using syringe (2.5.2). The infiltrated leaf material was harvested (2.5.5.1) at 4, 5, and 6 dpi, and crude extracts (2.6.1) of the plant material were analyzed by SDS-PAGE with Coomassie staining (1.5 mg FW loaded) 2.7.2. The harvesting time point with the most intense specific protein band (SalE2: 61.9 kDa) corresponds to the highest protein yield (asterisk).

Based on the phenotypic evaluation of salmocin-expressing leaves (Figure 23) and the assessment of the highest attainable yield of recombinant protein (Figure 24), the optimal harvesting time point was determined (summarized in Table 25).

Table 25: The optimal harvesting time point for transiently expressed salmocins in *N. benthamiana*.
Salmocin expression constructs were agroinfiltrated by syringe.

pNMD construct	salmocin/ immunity protein	activity	MW (kDa)	optimal harvesting time point(dpi)
28160 + 28220	SalE2 + SImmE2	DNase	61.96	6
28150	SalE3	RNase	61.71	5
28170 + 28230	SalE7 + SimmE7	DNase	62.26	5
28190	SalE1a	pore-forming	52.81	5
28200	SalE1b	pore-forming	57.58	4
28180	Spst	degradation of murein	33.23	6

While SalE3 showed similar plant cytotoxicity individually and in co-expression with SImmE3, the yield was higher without SImmE3. In contrast, SalE2 and SalE7 were significantly less cytotoxic to the plant in co-expression with SImmE2 and SImmE7, respectively, and provided a higher yield.

3.4.2.2 Salmocin yield estimation and testing different methods of agroinfiltration

Agrobacterium cultures carrying constructs coding for the salmocins were inoculated into *N. benthamiana* by syringe infiltration (2.5.2), and the plant material was harvested (2.5.5.1) for TSP extraction with HEPES-containing buffer (2.6.2) to assess salmocins´ solubility in this buffer by SDS-PAGE with Coomassie staining (2.7.2) at the optimal harvesting time point (Figure 25).

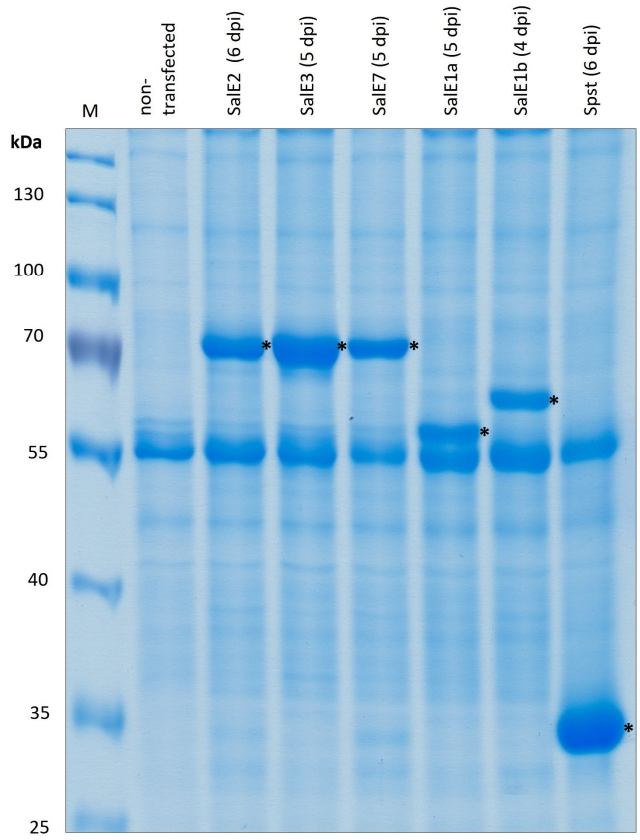


Figure 25: Salmocin expression in *N. benthamiana*.
Agroinfiltration of constructs for salmocin expression was done using syringe (2.5.2). Plant material was harvested in small-scale (2.5.5.1) at the optimal harvesting time point (dpi) and extracted with HEPES-containing buffer (2.6.2). Plant extracts corresponding to 3 mg FW plant material were resolved in 10% polyacrylamide gel and stained with Coomassie (2.7.2) The Asterisks mark recombinant proteins: SalE2 - 61.96 kDa, SalE3 - 61.71 kDa, SalE7 - 62.26 kDa, SalE1a - 52.81 kDa, SalE1b - 57.58 kDa, Spst - 33.23 kDa.

All salmocins were well expressed and soluble in HEPES-containing buffer. The highest recombinant protein level was observed for Spst and SalE3 (Figure 25). Total protein concentration in the TSP extract was determined by Bradford assay (2.7.1), and the recombinant salmocin yield was estimated semi-quantitatively using visual comparison with BSA on Coomassie-stained polyacrylamide gel (2.7.3) (Figure 26).

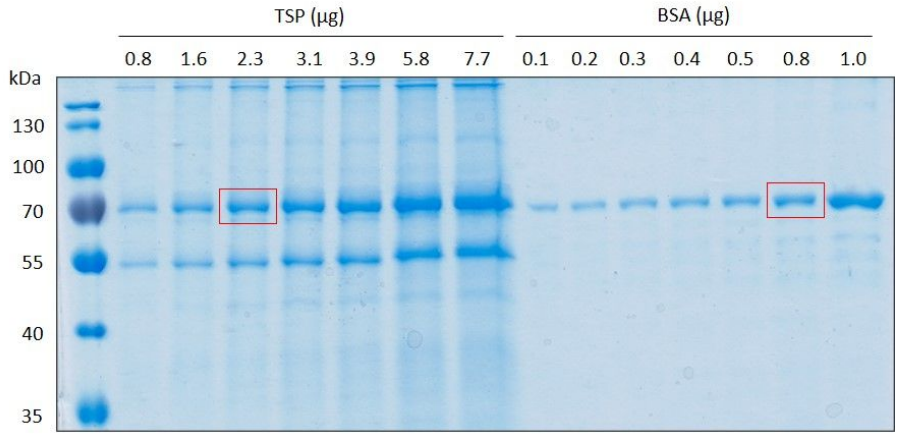


Figure 26: Example of a semi-quantitative estimation of recombinant SalE2 content in TSP extract from *N. benthamiana* leaf material co-expressing SalE2 and SImmE2, harvested in small-scale (2.5.5.1). Proteins were resolved by 12% SDS-PAGE with Coomassie staining (2.7.3).
The proportion of recombinant protein in extracted TSP (left) was estimated by visual comparison with a BSA standard (right). Here, the proportion of SalE2 was estimated to be 35% of TSP

Table 26 summarizes the yield of recombinant salmocins expressed in *N. benthamiana* at the optimal harvesting time point.

Table 26: Yield of recombinant salmocins expressed in *N. benthamiana* upon syringe infiltration of *Agrobacterium* cultures carrying TMV-based expression constructs.
Salmocin-containing plant material was harvested (2.5.5.1) at the optimal harvesting time point (dpi). Proteins were extracted with HEPES-containing buffer (2.6.2), and the protein concentration was estimated semi-quantitatively using SDS-PAGE with Coomassie staining (2.7.1) (2.7.3). The average and standard deviation (SD) of 3 independent experiments are shown.

salmocin	harvest (dpi)	yield (mg/g FW)		yield (% of TSP)	
		average	SD	average	SD
SalE2	6	1.7	0.3	25.0	0.0
SalE3	5	1.6	0.2	37.0	10.4
SalE7	5	1.4	0.3	18.0	6.9
SalE1a	5	1.3	0.2	20.3	3.1
SalE1b	4	1.2	0.1	25.7	3.1
SpSt	6	1.8	0.3	47.0	23.6

For industrial production, however, needleless syringe infiltration is too labor-intensive. Other *Agrobacterium* inoculation methods like vacuum infiltration and spraying are much easier to

automate and, therefore, more scalable and cost-efficient (1.5). For this reason, the salmocin yields with these inoculation methods were explored.

Agrobacterium cultures carrying constructs for expression of the 6 salmocins and the corresponding immunity proteins (SImmE2, SImmE7) (7.1.1.2) were inoculated into *N. benthamiana* by vacuum infiltration (2.5.3). The infiltrated leaf material was then harvested (2.5.5.1), and the protein was extracted using either Laemmli buffer (crude extracts) (2.6.1) or a citric extraction buffer (2.6.2) and resolved by SDS-PAGE (2.7.2) (Figure 27, Figure 28). Additionally, the optimal harvesting time point was determined (2.5.6).

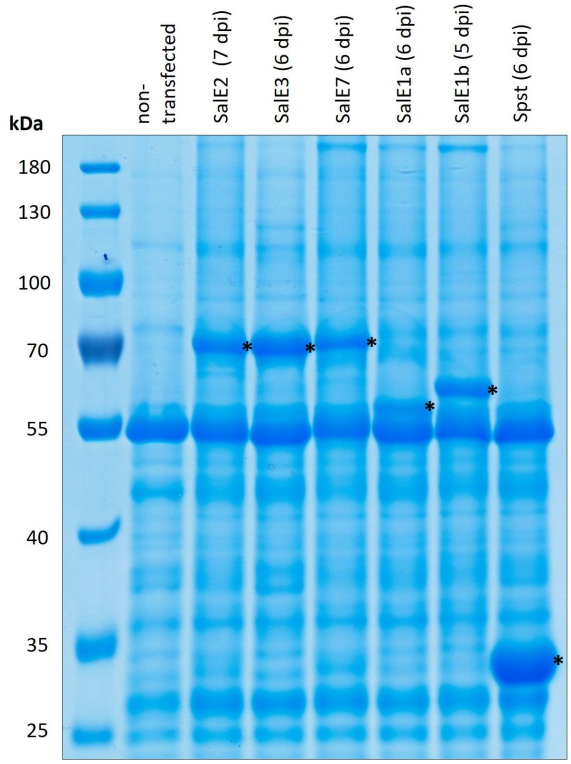


Figure 27: Crude extraction of transiently expressed salmocins using Laemmli buffer.
Recombinant proteins accumulated in *N. benthamiana* leaves upon vacuum infiltration (2.5.3) with *Agrobacterium* cultures carrying TMV and PVX vectors for the expression of salmocins and corresponding immunity proteins. Crude extracts (2.6.1) were prepared from plant material harvested in small-scale (2.5.5.1) at the optimal harvesting time point (shown in dpi). Plant extracts corresponding to 3 mg FW were resolved by 10% SDS-PAGE with Coomassie staining (2.7.2). The Asterisks mark recombinant proteins: SalE2 - 61.96 kDa, SalE3 - 61.71 kDa, SalE7 - 62.26 kDa, SalE1- 52.81 kDa, SalE1b - 57.58 kDa, Spst - 33.23 kDa.

The same plant material was also used for a protein extraction test with acidic conditions (2.6.2) (Figure 28).

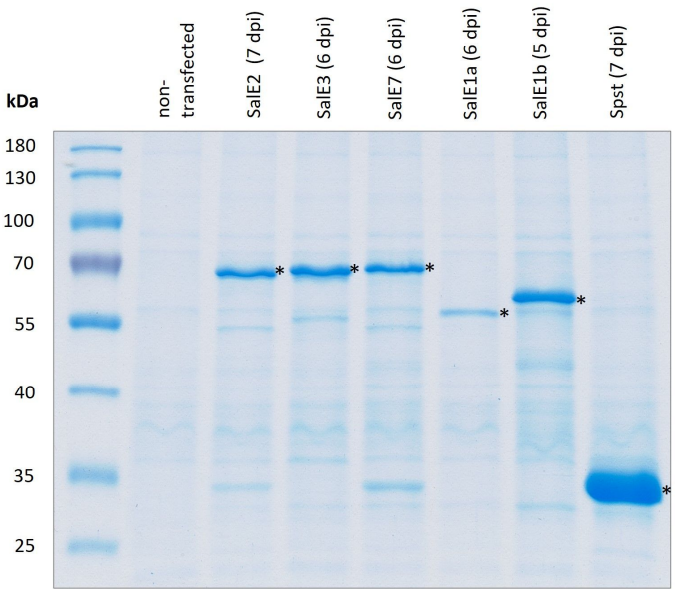


Figure 28: Salmocin extraction test with citric acid buffer pH 4.0 (20 mM citric acid pH 4.0, 20 mM NaH_2PO_4, 30 mM NaCl) (2.6.2).
Recombinant proteins accumulated in *N. benthamiana* leaves upon vacuum infiltration (2.5.3) with *Agrobacterium* cultures carrying TMV and PVX vectors for the expression of salmocins and corresponding immunity proteins. Plant material was harvested at the optimal harvesting time point (shown in dpi) (2.5.5.1). Plant extracts corresponding to 1.5 mg FW were resolved by a 12% SDS-PAGE with Coomassie staining (2.7.2). The Asterisks mark recombinant proteins: SalE2 - 61.96 kDa, SalE3 - 61.71 kDa, SalE7 - 62.26 kDa, SalE1- 52.81 kDa, SalE1b - 57.58 kDa, Spst - 33.23 kDa.

The salmocins were well expressed when using vacuum agroinfiltration. The acidic buffer provided a more selective extraction of the salmocins, which could facilitate downstream protein purification processes (Figure 28).

The inoculation of *A. tumefaciens* cultures carrying the salmocin expression constructs into *N. benthamiana* by spraying was tested with 2 different concentrations of *Agrobacterium*: cultures at OD_{600} = 1.5 were diluted 1:100 or 1:1000 in the infiltration medium (2.5.4) (Figure 29). The resulting salmocin-containing plant material was handled as described above for the vacuum-infiltrated plant material but extracted with a HEPES-containing buffer (2.6.2).

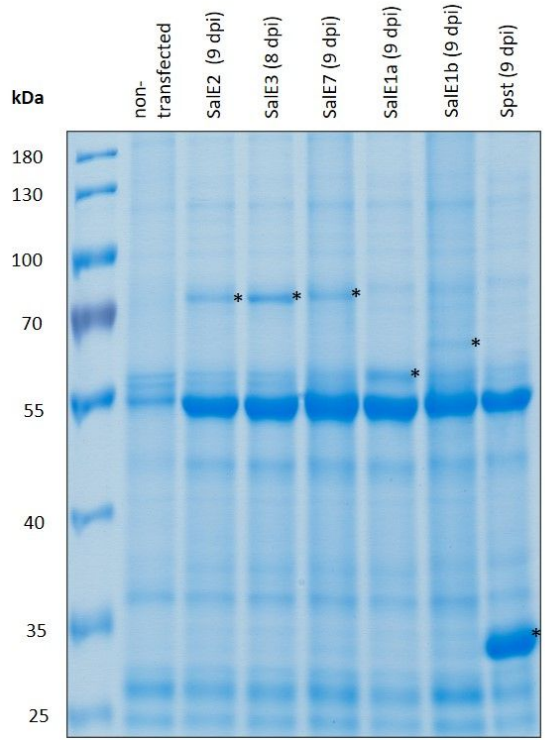


Figure 29: Transient expression of salmocins in *N. benthamiana* upon spray inoculation with *Agrobacterium* (OD_{600} = 1.4, diluted 1:100 in infiltration medium) carrying TMV and PVX vectors for salmocins and the corresponding immunity proteins (2.5.4).
Plant material harvested at the optimal harvesting time point (shown in dpi) (2.5.5.1) was extracted with HEPES-containing buffer (2.6.2). Plant extracts corresponding to 3 mg FW plant material were resolved by a 10% SDS-PAGE with Coomassie-staining (2.7.2). The Asterisks mark recombinant proteins: SalE2 - 61.96 kDa, SalE3 - 61.71 kDa, SalE7 - 62.26 kDa, SalE1- 52.81 kDa, SalE1b - 57.58 kDa, Spst - 33.23 kDa.

The lower *Agrobacterium* dilution (OD_{600} = 1.4, diluted 1:100 in infiltration medium) provided higher expression levels than the higher dilution (OD_{600} = 1.4 diluted 1:1000) for all salmocins. Overall the salmocin expression levels achieved with inoculation by spraying appear to be lower than for vacuum and syringe agroinfiltration. Furthermore, recombinant protein accumulation took longer than for the other 2 inoculation methods. Nevertheless, the cost-efficiency of inoculation by spraying may render it feasible in large industrial settings.
Table 27 summarizes the optimal harvesting time points providing the highest accumulation of recombinant salmocins in *N. benthamiana* leaves for 3 methods of *Agrobacterium* delivery.

Table 27: The optimal harvesting time points providing the highest recombinant salmocin yield. *N. benthamiana* leaves were inoculated with *Agrobacterium* cultures using 3 different methods: syringe infiltration, vacuum infiltration, and spraying.

		optimal harvesting time point by inoculation type (dpi)		
pNMD construct	salmocin/ immunity protein	syringe	vacuum	spraying
28160 + 28220	SalE2 + SImmE2	6	7	9
28150	SalE3	5	6	8
28170 + 28230	SalE7 + SImmE7	5	6	9
28190	SalE1a	5	6	9
28200	SalE1b	4	5	9
28180	Spst	6	6	9

3.4.2.3 Salmocin expression in spinach, an edible plant

After using *N. benthamiana* as the production host, the expression of salmocins was also tested in an edible plant. Compared to *N. benthamiana*, an edible plant host should contain little to no alkaloids (1.5) so that the salmocin-containing plant material or the plant extracts could be used in clinical trials or animal health studies (e.g., as presented in 3.3) without further purification steps, reducing production costs.

Therefore, the salmocins were expressed in spinach (*Spinacia oleracea* 'Frühes Riesenblatt'), which has often been shown to be the edible plant with the highest yield for colicins expressed by TMV-based vectors delivered by *Agrobacterium* strain ICF320 (Nomad Bioscience GmbH internal data). The optimal harvesting time point was assessed (2.5.6). *Agrobacterium* cultures carrying salmocin expression constructs were inoculated into the plants by syringe infiltration as described above for *N. benthamiana* (2.5.2) (Figure 30).

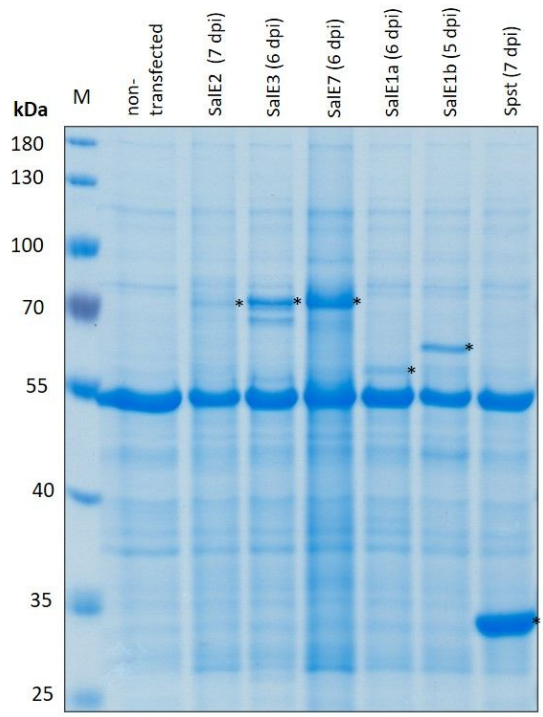


Figure 30: Transient expression of salmocins in *Spinacia oleracea*. Agroinfiltration of constructs for salmocin expression was done using syringe (2.5.2).
Plant material was harvested at the optimal harvesting time point (shown in dpi) and extracted with 150 mM NaCl (2.6.2). Plant extracts corresponding to 3 mg FW plant material were resolved in 12% polyacrylamide gel and stained with Coomassie (2.7.2). The Asterisks mark recombinant proteins: SalE2 - 61.96 kDa, SalE3 - 61.71 kDa, SalE7 - 62.26 kDa, SalE1- 52.81 kDa, SalE1b - 57.58 kDa, Spst - 33.23 kDa.

3.4.2.4 Sequence verification for salmocin gene transcripts in *N. benthamiana*

The coding sequences of SalE3, SalE2, and SalE7 for the expression in plants were synthesized with the insertion of a CAT1 intron to prevent the cytotoxicity in *E. coli* DH10B cells used for cloning. The intron must then be spliced out in the plant host.

To verify correct intron splicing *in planta*, the total plant RNA was extracted (2.2.3) and reverse transcribed (2.3.2). The salmocin sequences were amplified (2.3.1.3) (7.1.1.2), and the PCR products were analyzed using agarose gel electrophoresis (2.4.1) and DNA sequencing. The correct splicing of the salmocin transcripts was confirmed with no mutations or changes introduced and no remnants of the CAT1 intron remaining.

3.4.2.5 Analysis of salmocin integrity via mass spectrometry

Salmocin-containing plant material was extracted with 5x volumes of 20 mM Na citrate, 20 mM NaH_2PO_4, 30 mM NaCl, pH 5.5 (2.6.2), and the extract was resolved by SDS-PAGE

(2.7.2). The gel was Coomassie-stained, and the recombinant protein bands containing 5 µg of protein were excised from the gel and sent to the Fraunhofer Institute for Cell Therapy and Immunology (IZI; Halle, Germany) for matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) analysis described in (Schneider et al., 2018).
The MS analysis of the primary structure of the salmocins revealed that they were correctly expressed in plants. Their identities could be confirmed by searching their MS/MS datasets against the NCBI non-redundant database. Additionally, some post-translational modifications were observed. In particular, the N-terminal methionine of SalE2, SalE7, SalE1a, and SalE1b was cleaved, whereas SalE7 and SalE1a had undergone N-terminal acetylation. Further details are described in Schneider et al. 2018.

3.4.3 Functionality test: determination of salmocin activity spectrum on *Salmonella enterica*

3.4.3.1 Analysis of antimicrobial activity of salmocins against pathogenic *Salmonella*

The salmocins were tested for their antimicrobial activity spectrum against 36 *Salmonella* strains. The collection encompasses 17 (for 2012) and 16 (for 2013) of the 20 *Salmonella* serotypes, with the highest number of reported human infections in the US (CDC, 2014, 2016) (2.1.1.3, Table 4). With a few exceptions, they have each caused more than 100 human *Salmonella* infections each and well over 31,000 in total in the US in 2012.
The degree and range of antimicrobial activity was assessed by soft agar overlay assay (2.8.1). Soft agar plates were seeded with *Salmonella* strains (2.1.1.2, Table 2, No. 1-35) and with *E. coli* DH10B (pICH73311) as a control. In short, the 6 salmocins were expressed in *N. benthamiana* upon inoculation of *Agrobacterium* carrying the expression constructs (7.1.1.2) by syringe infiltration (2.5.2). The TSP from the plant material was extracted with HEPES-containing buffer (2.6.2) and then serially diluted 1:2 in the same buffer. The dilutions were then applied to the pre-seeded soft agar plates using a multichannel pipette. (2.8.1). Additionally, the protein yield of the salmocins was assessed by combining Bradford assay (2.7.1) and semi-quantitative protein estimation by SDS-PAGE. The values obtained were used to calculate the specific antimicrobial activity (AU/ µg protein) (2.7.3).
The experiments revealed a surprisingly broad activity range (
Figure 31).

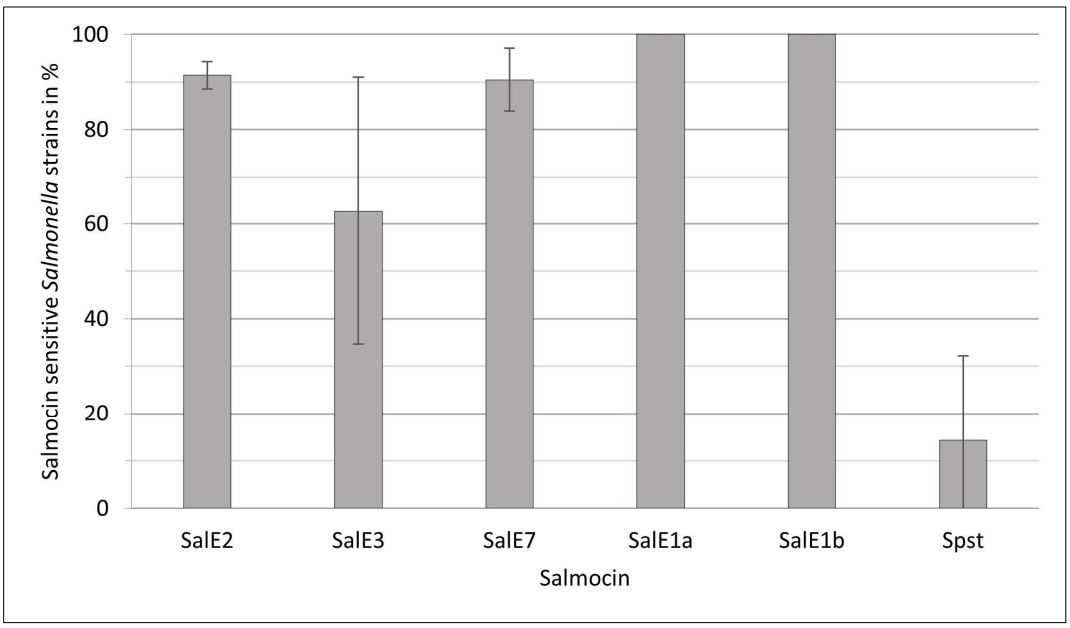


Figure 31: The percentage of the tested *Salmonella* strains (2.1.1.3, Table 4, No. 1-35) sensitive to the individual salmocins.
Assessment of antimicrobial activity by soft agar overlay assay (2.8.1) using salmocin-containing TSP plant extracts (2.6.2). Average and standard deviation of 3 independent experiments.

While SalE1a and SalE1b were active against all of the strains, SalE2 and SalE7 were active against the vast majority of them. SalE3 was active against a little more than half of the strains, while Spst showed a narrow activity range.

The average specific activity of the salmocins was very high. SalE1a and SalE1b showed the highest values, followed by SalE2 and SalE7, which had a 2 log lower activity (Figure 32).

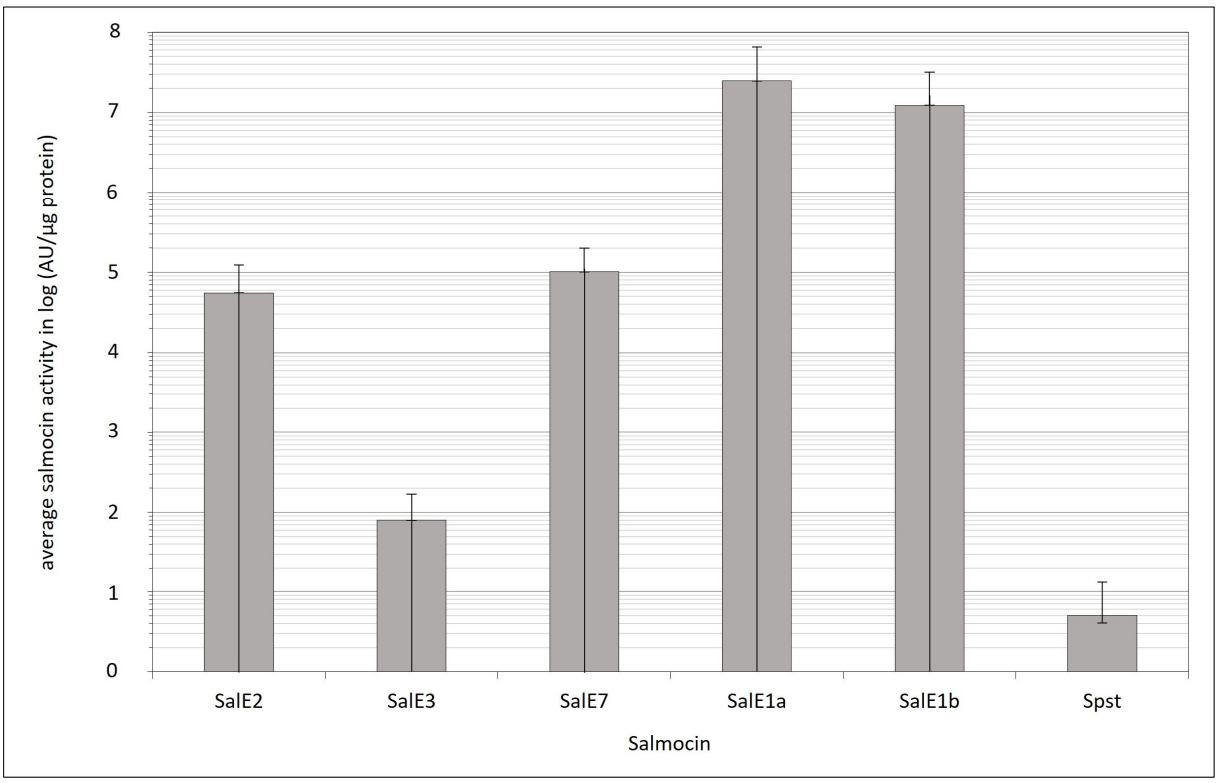


Figure 32: The average specific killing activity of the salmocins against *Salmonella* strains.
Salmonella strains (2.1.1.3, Table 4, No. 1-35).
Assessment of antimicrobial activity by soft agar overlay assay (2.8.1) using salmocin-containing TSP plant extracts (2.6.2). Average and standard deviation of 3 experiments.

When analyzing the range and level of salmocin activity (
Figure 31 and Figure 32), 4 distinct activity groups can be formed according to their activity range and level of activity (Schneider et al., 2018):

Group1 consists of SalE1a and SalE1b, which showed the highest average activity against all strains, exceeding 10^7 AU/µg bacteriocin. They were also active against all 35 tested strains, which make SalE1a and SalE1b g promising product candidates.

Group 2 consists of SalE2 and SalE7, which had an average activity lower than 10^5 AU/µg protein. While they were not active against all strains, the activity range was still extensive.

Group 3 consists only of salmocin E3, which, at 10^2 AU/µg protein, showed a substantially lower average activity than group 1 and 2 salmocins. It also offered a narrower range of activity.

Group 4 includes Spst alone, which had a very low average activity below 10^2 AU/µg protein against all strains. Furthermore, it was only active against a few strains.

Furthermore, the the average specific activity of each of the salmocins on all tested *Salmonella* strains was assessed (
Figure 33 -
Figure 38).

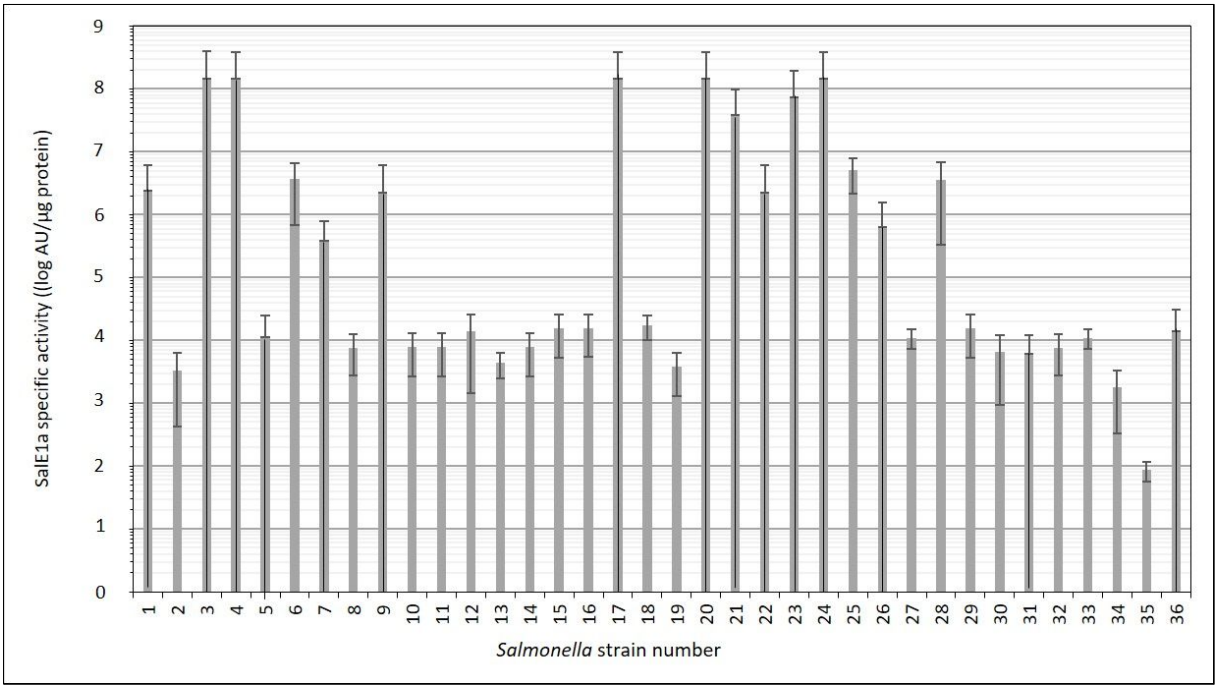


Figure 33: Specific activity of SalE1a on each *Salmonella* strain.
Assessment of antimicrobial activity by soft agar overlay assay (2.8.1) using salmocin-containing TSP plant extracts (2.6.2). Average and standard deviation of N=3 (for strains No. 1-35) and N=2 (for strain No. 36) independent experiments.

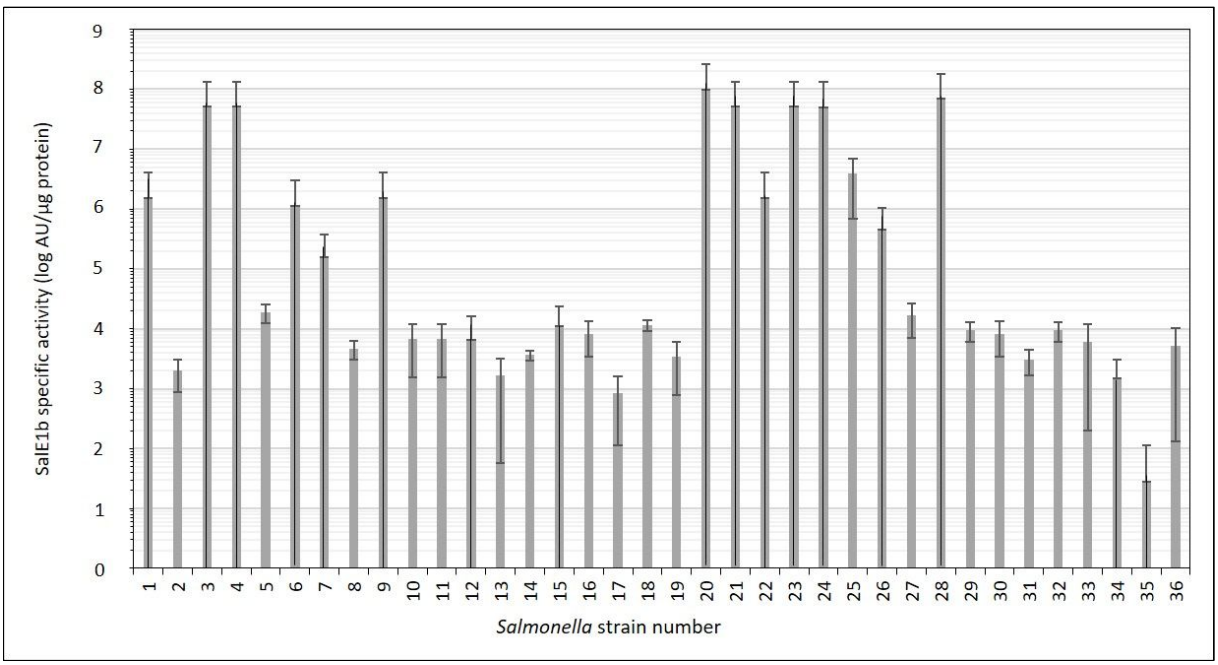


Figure 34: Specific activity of SalE1b on each *Salmonella* strain.
Salmonella strains (2.1.1.3, Table 4, No. 1-36).
Assessment of antimicrobial activity by soft agar overlay assay (2.8.1) using salmocin-containing TSP plant extracts (2.6.2). Average and standard deviation of N=3 (for strains No. 1-35) and N=2 (for strain No. 36) independent experiments.

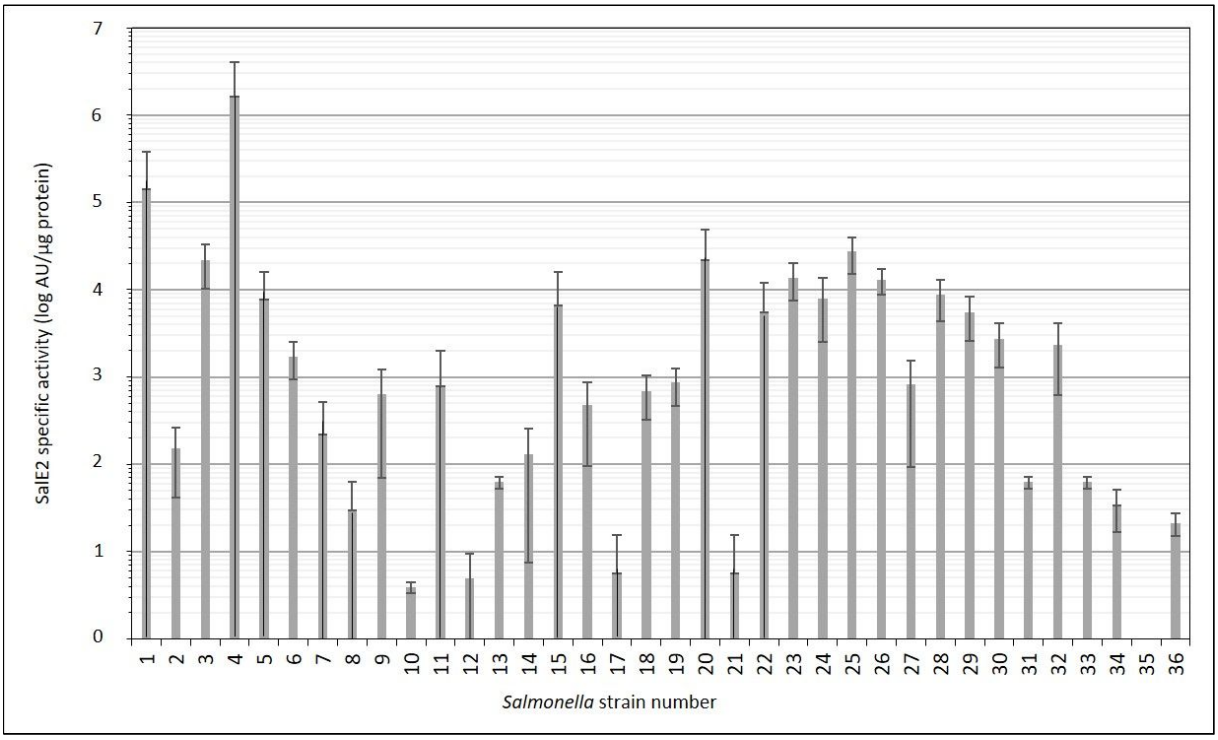


Figure 35: Specific activity of SalE2 on each *Salmonella* strain.
Salmonella strains (2.1.1.3, Table 4, No. 1-36).
Assessment of antimicrobial activity by soft agar overlay assay (2.8.1) using salmocin-containing TSP plant extracts (2.6.2). Average and standard deviation of N=3 (for strains No. 1-35) and N=2 (for strain No. 36) independent experiments.

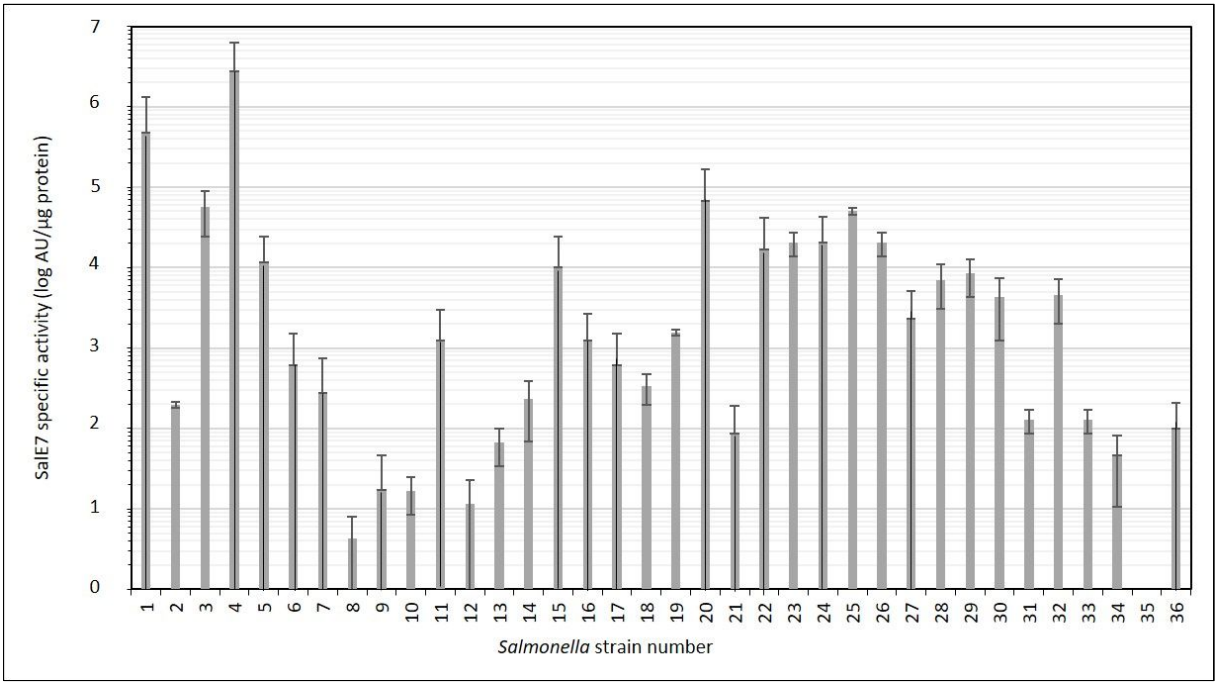


Figure 36: Specific activity of SalE7 on each *Salmonella* strain
Salmonella strains (2.1.1.3, Table 4, No. 1-36).
Assessment of antimicrobial activity by soft agar overlay assay (2.8.1) using salmocin-containing TSP plant extracts (2.6.2). Average and standard deviation of N=3 (for strains No. 1-35) and N=2 (for strain No. 36) independent experiments.

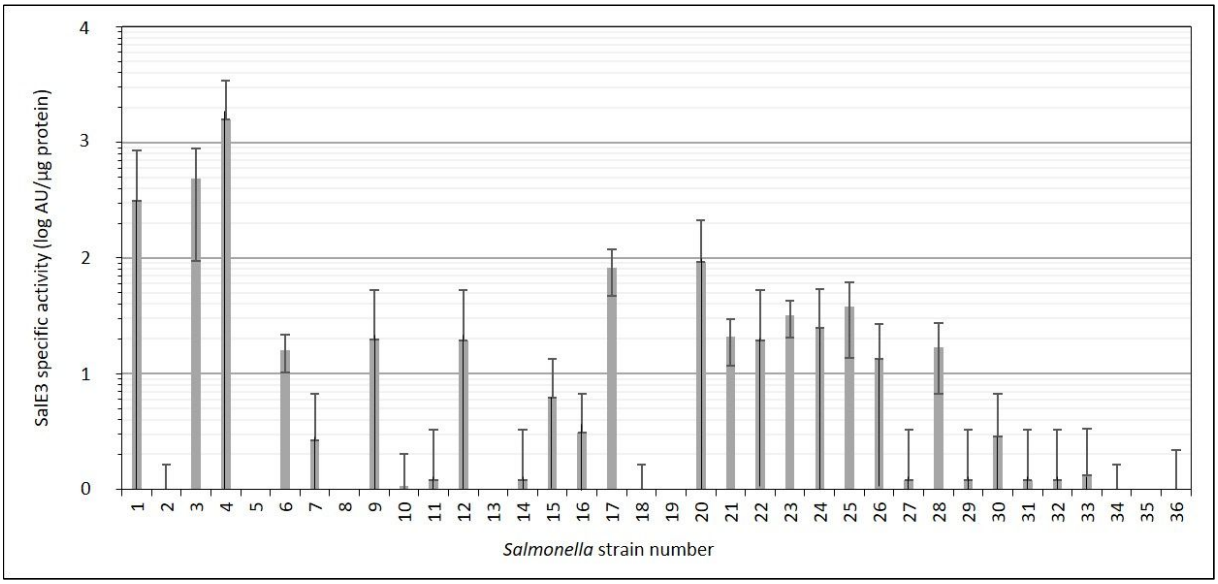


Figure 37: Specific activity of SalE3 on each *Salmonella* strain.
Salmonella strains (2.1.1.3, Table 4, No. 1-36).
Assessment of antimicrobial activity by soft agar overlay assay (2.8.1) using salmocin-containing TSP plant extracts (2.6.2). Average and standard deviation of N=3 (for strains No. 1-35) and N=2 (for strain No. 36) independent experiments.

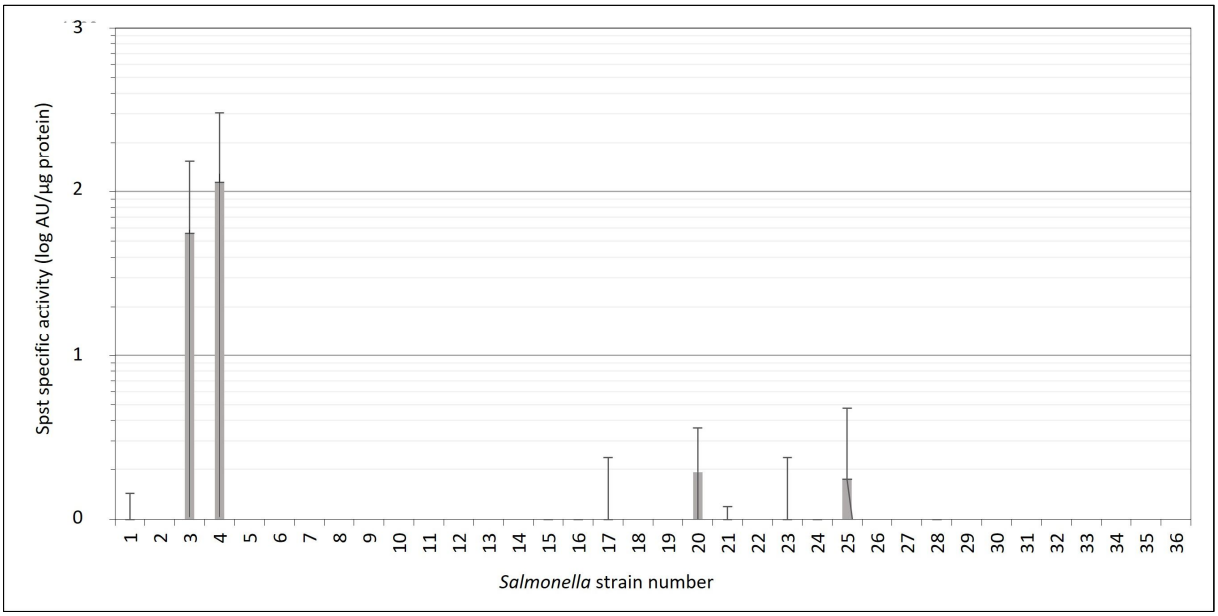


Figure 38: Specific activity of Spst on each individual *Salmonella* strain.
Salmonella strains (2.1.1.3, Table 4, No. 1-36).
Assessment of antimicrobial activity by soft agar overlay assay (2.8.1) using salmocin-containing TSP plant extracts (2.6.2). Average and standard deviation of N=3 (for strains No. 1-35) and N=2 (for strain No. 36) independent experiments.

The level of activity and the activity range of salmocins were unexpectedly high and showed good potential for future product development.

3.4.3.2 Analysis of the antimicrobial activity of salmocins against pathogenic *E. coli* strains

The antimicrobial activity of the salmocins was also tested on the "Big 7" STEC strains and the EHEC serotype O104:H4 (2.1.1.2) to determine if salmocins exhibit a similar interspecies antimicrobial activity spectrum as colicins (
Figure 39). Salmocin-containing plant extracts were analyzed for their antimicrobial activities using soft agar overlay assay as described in 3.4.3.1.

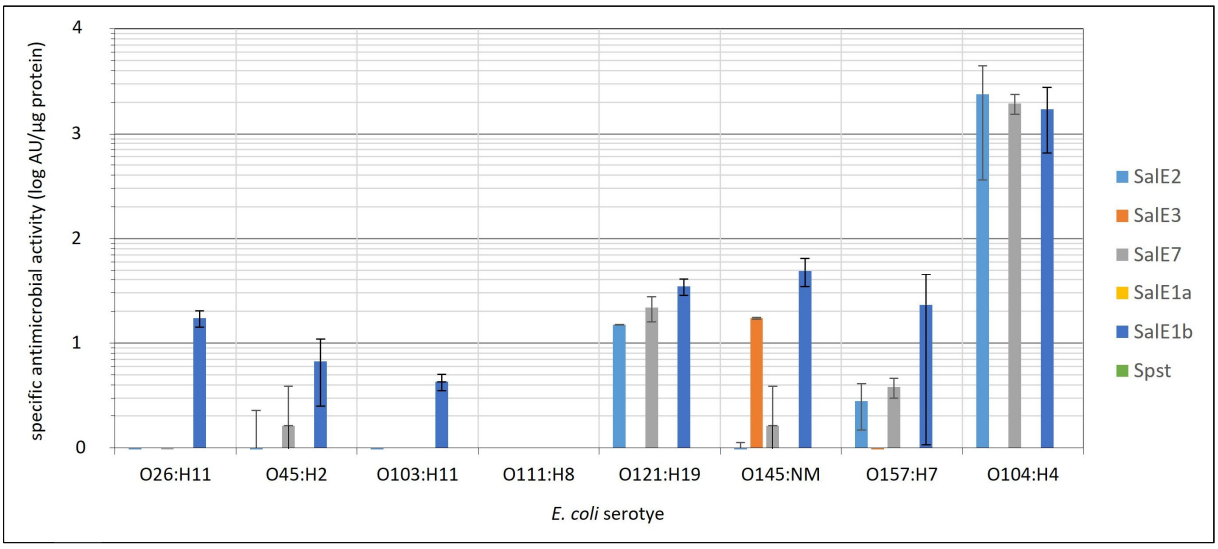


Figure 39: Specific antimicrobial activity of the salmocins against the "Big 7" STEC strains and STEC serotype O104:H4 (2.1.1.2).
Assessment of antimicrobial activity by soft agar overlay assay (2.8.1) using salmocin-containing plant extracts (2.6.2) as described in (3.4.3.1). Average standard deviation of 2 independent experiments.

The antimicrobial activity of salmocins on the tested STEC strains was low compared to that of colicins (Schulz et al., 2015), which aligns with the typical intraspecific specificity of bacteriocins. For 3 of the salmocins, SalE2, SalE7, and SalE1b, an activity below 100 AU/µg protein was observed. SalE3 was only active against one strain. The only serotype showing a higher susceptibility to salmocins was O104:H4. This coincides with its susceptibility to colicins which was also much higher than for the other STEC strains (Schulz et al., 2015). *E. coli* DH10B (pICH73311) was susceptible to the same 3 salmocins but at a much higher rate: SalE2 had an antimicrobial activity of 34,000 AU/µg protein, SalE7 had 150,000 AU/µg protein, and SalE1b - 220,000 AU/µg.

3.4.4 Potency of salmocins and salmocin-colicin mixtures in comparison to kanamycin

The antimicrobial activity of salmocins on 36 *Salmonella* strains was successfully shown by soft agar overlay assay. For a more precise quantitative evaluation of the salmocin´s antimicrobial activity at low concentrations, the bacterial cell reduction of a *Salmonella* strain mixture in broth culture upon being treated with individual salmocins, a salmocin-colicin mixture, and the antibiotic kanamycin were analyzed (2.8.2).

Briefly, the bacteriocins and the antibiotic were tested on a mixture of the 2 *Salmonella* strains: *S. enterica* ssp. *enterica*, serotype Enteritidis (ATCC®13076™*) and *S. enterica* ssp. *enterica*, serotype Typhimurium (ATCC®14028™*). According to CDC data, the serotypes Enteritidis and Typhimurium caused the most human infections in 2012 (CDC, 2014) (2.1.1.3).

The salmocins (3.4.3.1) and colicins (Schneider et al., 2018) with the highest antimicrobial activities on *Salmonella* (as found in previous experiments) were examined. Salmocins SalE1a, SalE1b and SalE7 applied in the concentrations 1.0, and 0.1 µg/ml were tested individually. In parallel, a salmocin-colicin mixture combining SalE1b (0.03µg/ml), ColM (0.01 µg/ml) and ColIb (0.01 µg/ml) was analyzed. As a comparison, a kanamycin treatment (5 µg/ml) was used.

The bacteriocins for this experiment were affinity purified (2.7.4). The cultures were inoculated with the mixture of 2 Salmonella strains in a ratio of 1:1 at an OD_{600}=0.0001 (~1.5x104 CFU/ml) and subsequently supplemented with the bacteriocins or antibiotic mentioned above. For each treatment type, 3 biological replicates were analyzed. The bacterial cultures were then incubated at 37°C (

Figure 40) (optimal growing temperature) and at 10°C (

Figure 41) (suboptimal growing temperature) for 2, 4 and 24 h.

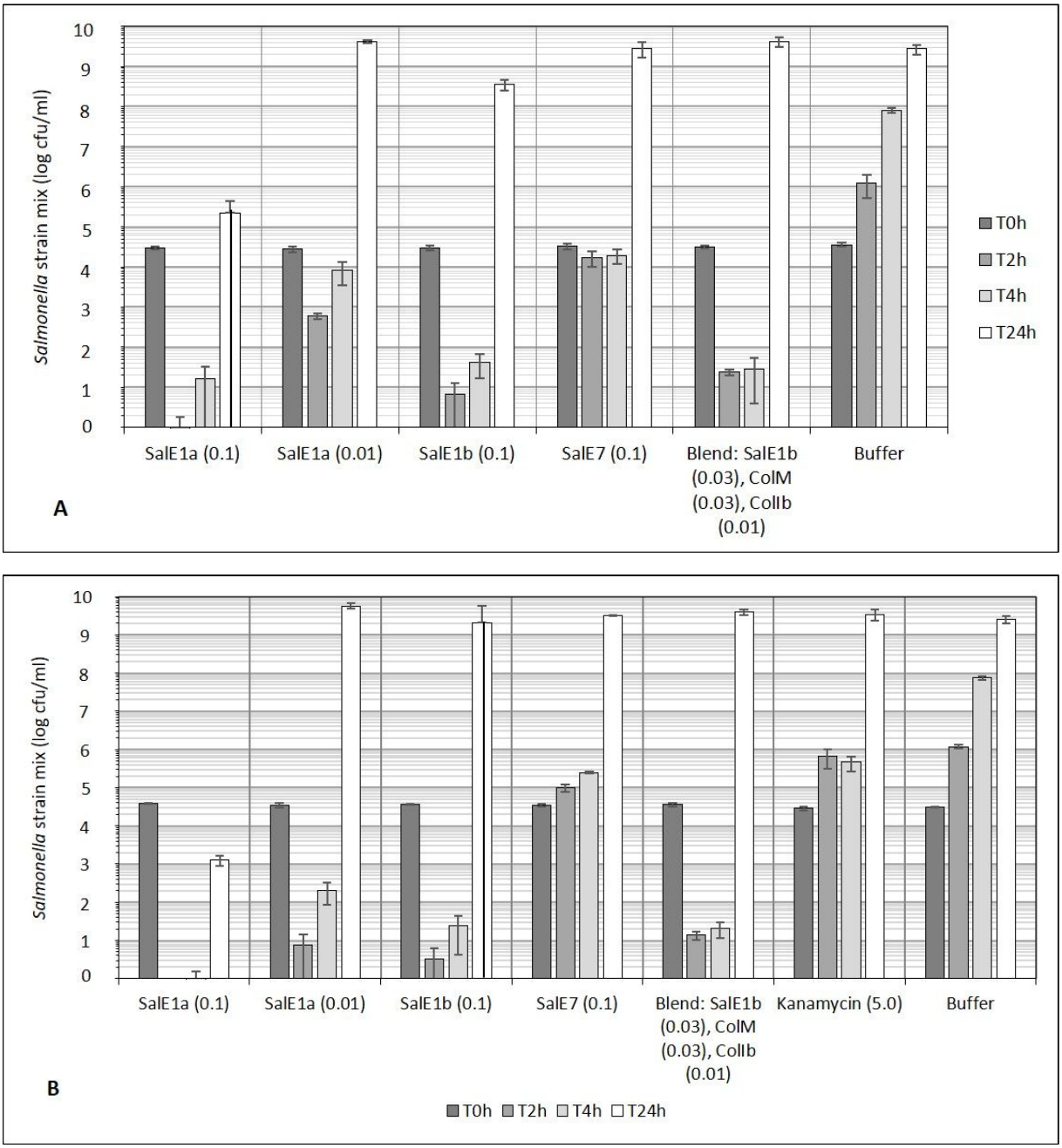


Figure 40: Assessment of the activity of individual salmocins, a salmocin-colicin blend, and kanamycin on the mix of 2 *Salmonella* serovars: Enteritidis (strain ATCC® 13076™*) and Typhimurium (strain ATCC® 14028™*) in broth culture at 37°C.
Two independent experiments are shown in (A) and (B). The bacterial enumeration by dilution plating (CFU/ml) was assessed at the beginning of the experiment (T=0 h) and at 3 further time points (T=2 h, 4 h, 24 h). The concentrations of the antimicrobials are given in brackets (µg/ml). The antibiotic kanamycin was only tested in B. Error bars show mean CFU/ml and correspond to SD of 3 biological replicates.

The statistical analysis for

Figure 40 A: Comparing **SalE1a (0.1 µg/ml)-** with carrier-treatment yielded p-values of 0.1062, 0.0395, 0.0002, and 0.0032 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE1a (0.01 µg/ml)**- with carrier-treatment yielded p-values of 0.0987, 0.0396, 0.0002 and

0.0495 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE1b (0.1 µg/ml)**- with carrier-treatment yielded p-values of 0.0187, 0.0008, <0.0001 and <0.0001 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE7 (0.1 µg/ml)**- with carrier-treatment yielded p-values of 0.4486, 0.0412, 0.0002 and 0.9092 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. **Salmocin-colicin blend** - with carrier-treatment yielded p-values of 0.2446, 0.0395, 0.0002, and 0.1525 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively.

For

Figure 40 **B**: Comparing **SalE1a (0.1 µg/ml)**- with carrier-treatment yielded p-values of <0.0001, <0.0001, <0.0001 and 0.0013 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE1a (0.01 µg/ml)**- with carrier-treatment yielded p-values of 0.2756, <0.0001, <0.0001 and 0.0079 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE1b (0.1 µg/ml)**- with carrier-treatment yielded p-values of 0.9499, <0.0001, <0.0001 and 0.2044 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE7 (0.1 µg/ml)**- with carrier-treatment yielded p-values of 0.1062, <0.0001, <0.0001 and 0.1054 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. **Salmocin-colicin blend** - with carrier-treatment yielded p-values of >0.0913, <0.0001, <0.0001, and 0.0494 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing kanamycin (5.0 µg/ml) with carrier-treatment yielded p-values of >0.3486, 0.0644, <0.0001 and 0.2829 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively.

The criterion for the null hypothesis rejection was $p<0.05$. The statistical differences were assessed in relation to carrier-treated treated samples by unpaired parametric t-test with GraphPad Prism v. 6.01.

SalE1a (0.1 µg/ml) and SalE1b (0.1 µg/ml), as well as the salmocin-colicin blend, all showed a high short-term reduction of *Salmonella* CFU counts at 37°C incubation (

Figure 40 A and B). SalE1a is the most potent salmocin: at a concentration of 0.1 µg/ml, it reduced the bacterial populations below the detection limit after 2 h incubation, followed by a regrowth occurring between 4 and 24 h. SalE1a is as potent at lower concentrations (0.01 µg/ml) as SalE1b at a concentration of 0.1 µg/ml. SalE7 treatment (0.1 µg/ml) only led to a retardation of bacterial growth compared to the carrier (buffer) control. The blend of SalE1b, ColM, and ColIb showed a similar bacterial reduction as the SalE1b treatment. The antibiotic kanamycin was tested in one experiment only (

Figure 40 B) and, similar to SalE7, caused a slight delay of bacterial growth.

The main difference between both experiments was that experiment 1 (

Figure 40 A) had a higher variation for the efficacy of the SalE1a (0.1 µg/ml) treatment at 24 h. In contrast, in experiment 2 (

Figure 40 B), there was a higher variation for SalE1b (0.1 µg/ml) treatment at the same time point. For these 2 treatments, some of the replicates had much higher CFU counts than others. The observed deviation between the replicates (n=3) may have been caused by "jackpot cultures," where some cells become resistant to the salmocin at an earlier stage of the growth phase and can then proliferate stronger than in cultures where such a mutation happens at a later stage of the growth phase or does not happen at all (Luria & Delbruck, 1943).

The potency of the antimicrobial activity of salmocins against *Salmonella* in broth cultures incubated at 10°C can be seen in

Figure 41.

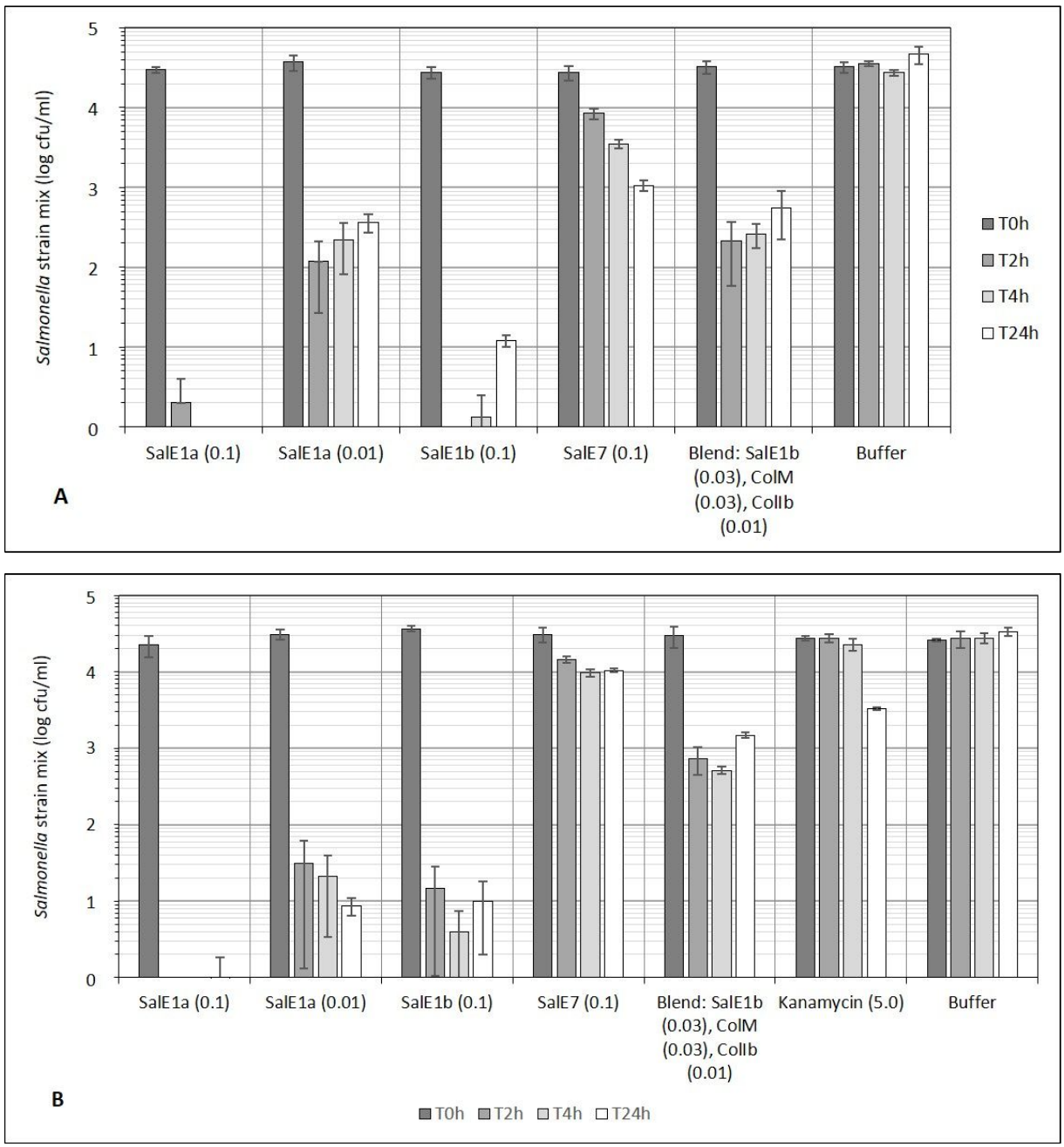


Figure 41: Assessment of the activity of individual salmocins, a salmocin-colicin blend and kanamycin on the mix of 2 *Salmonella* serovars: Enteritidis (strain ATCC® 13076™*) and Typhimurium (strain ATCC® 14028™*) in broth culture at 10°C.
2 independent experiments are shown in (A) and (B). The bacterial enumeration by dilution plating (CFU/ml) was assessed at the beginning of the experiment (T=0 h) and at 3 other time points (T=2 h, 4 h, 24 h). The concentrations of the antimicrobials are given in brackets (µg/ml). The antibiotic kanamycin was only tested in B. Error bars show mean CFU/ml and correspond to the standard deviation of 3 biological replicates.

The statistical analysis of the results presented in

Figure 41 are as follows: For

Figure 41 B: Comparing **SalE1a (0.1 µg/ml)-** with carrier-treatment yielded p-values of 0.437, <0.0001, <0.0001 and 0.0022 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing

SalE1a (0.01 µg/ml)- with carrier-treatment yielded p-values of 0.4098, <0.0001, <0.0001 and 0.0023 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE1b (0.1 µg/ml)-** with carrier-treatment yielded p-values of 0.0482, 0.0791, 0.0515 and 0.096 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE7 (0.1 µg/ml)-** with carrier-treatment yielded p-values of 0.3065, <0.0001, <0.0001 and 0.0024 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. **Salmocin-colicin blend -** with carrier-treatment yielded p-values of >0.9999, <0.0001, <0.0001, and 0.0023 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively.

For

Figure 41 B: Comparing **SalE1a (0.1 µg/ml)-** with carrier-treatment yielded p-values of 0.4082, 0.0022, 0.0003 and 0.0001 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE1a (0.01 µg/ml)-** with carrier-treatment yielded p-values of 0.1647, 0.0023, 0.0003 and 0.0001 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE1b (0.1 µg/ml)-** with carrier-treatment yielded p-values of 0.5213, 0.0820, 0.0515 and 0.0960 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **SalE7 (0.1 µg/ml)-** with carrier-treatment yielded p-values of 0.2879, <0.0338, <0.0018 and 0.0006 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. **Salmocin-colicin blend -** with carrier-treatment yielded p-values of 0.5249, 0.0025, 0.0003, and 0.0002 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively. Comparing **kanamycin (5.0 µg/ml)** with carrier-treatment yielded p-values of 0.3739, 0.9425, 0.2043 and 0.0002 at 0 h, 2 h, 4 h, and 24 h post-treatment, respectively.

The criterion for the null hypothesis rejection was $p<0.05$. The statistical differences were assessed in relation to carrier-treated samples by unpaired parametric t-test with GraphPad Prism v. 6.01.

The results showed that the treatment of the *Salmonella* cultures with SalE1a (0.1 µg/ml) had sterilizing effects with no regrowth at incubation temperatures of 10°C (

Figure 41). The treatment with SalE1a (0.01 µg/ml) and SalE1b (0.1 µg/ml) led to a 3 log reduction with barely any regrowth. SalE7 and kanamycin treatment led to a minor bacterial reduction compared to the carrier control. Applying the salmocin-colicin blend (SalE1b, ColM, ColIb) led to a ~1.5 log reduction in CFU/ml. The antimicrobial effect for the blend was much smaller than for the SalE1b (0.1 µg/ml) treatment.

In this experiment, the salmocins showed excellent antimicrobial activity on *Salmonella* at very low concentrations. Cell reductions were high at optimal and suboptimal bacterial growth temperatures.

3.4.5 **Reduction of *Salmonella* contamination on food using salmocins**

3.4.5.1 Analysis of the antimicrobial activity of salmocins on *Salmonella* contaminated chicken breast

Salmonella is most prevalent in poultry, and controlling it in the production and processing of foods such as chicken is highly important for the food industry, which may use several decontamination strategies, including chemical, physical and biological intervention procedures (1.2.4). Alarmingly, *Salmonella* strains throughout the food chain are showing increasing resistance to commonly used antibiotics (Bridier et al., 2019). Applying salmocins to meat matrices could be used as a new biological intervention strategy to decontaminate them from *Salmonella*.

Salmonella-contaminated chicken breast pieces were treated with salmocins that exhibited the best activity profiles against several of the most prevalent *Salmonella* strains. The experimental procedure is explained in 2.8.3.1. In short, the skinless chicken breast fillet was cut up into pieces and contaminated with a mixture of 7 pathogenic *S. enterica* ssp. *enterica* strains (2.1.1.3) ($1x10^3$-$1x10^4$ CFU/g food) that are known to cause the majority of salmonellosis cases in humans (CDC, 2014). The meat matrix was then treated with an individual salmocin or a blend of salmocins. SalE1a showing the highest and broadest antimicrobial activity in prior experiments, was used individually at a dose of 3 mg/kg meat. The blend consisted of the 4 salmocins, SalE1a, SalE1b, SalE2, and SalE7, at a concentration of 3, 1, 1, and 1 mg/kg meat, respectively, or 10 times lower. The salmocin solutions and a carrier control (plant extract of uninfiltrated plant material) were applied by spraying and subsequent hand mixing of the meat. The meat samples were then packed into bags and sealed, with 4 replicates for each sample. After this, the bags were stored at 10°C, and bacterial populations were assessed at 1 h, 24 h, 48 h, and 72 h post-treatment by dilution plating and bacterial enumeration (
Figure 42).

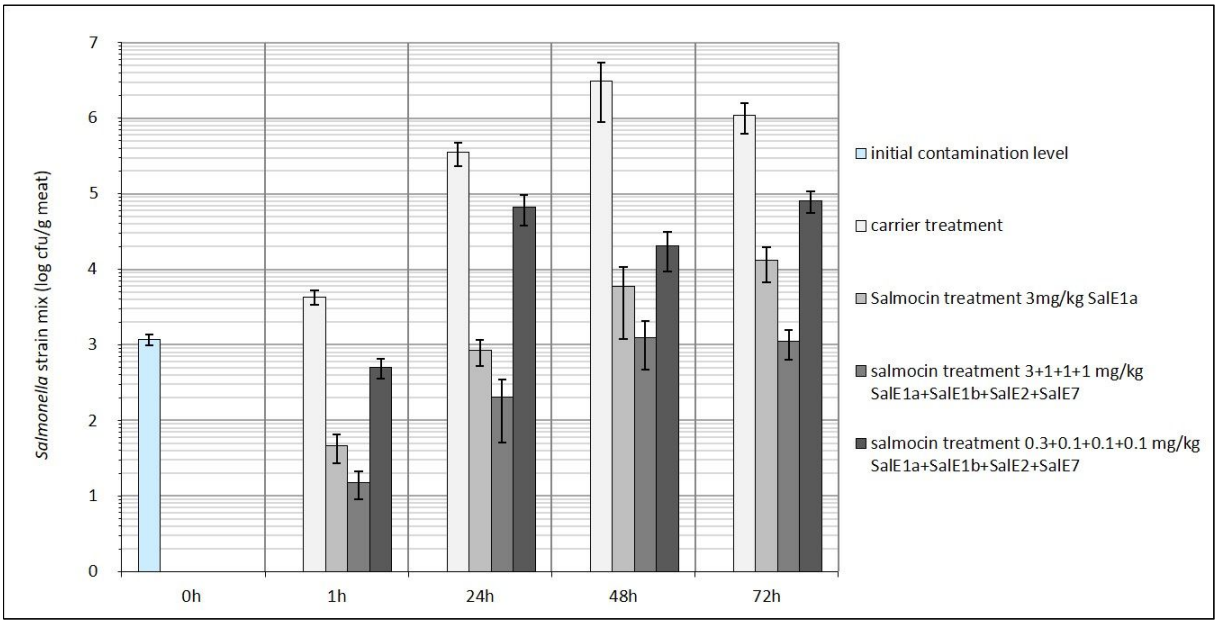


Figure 42: Reduction of *Salmonella* contamination on chicken breast meat by salmocins (Schneider et al., 2018). Skinless chicken breast fillet was contaminated with *Salmonella* strains treated with SalE1a or 2 different doses of a salmocin blend. Bacterial populations recovered from meat upon storage for 0, 1, 24, 48, and 72 h at 10°C (2.8.3.1). Error bars show the standard deviation of biological replicates, N = 4. Statistically significant reductions in bacterial contamination were achieved. Comparing individual SalE1a at an application rate of 3 mg/kg meat with carrier yielded p-values of <0.0001, 0.0012, 0.0137, and 0.0037 at 1 h, 24 h, 48 h, and 72 h post-treatment, respectively. The high dose salmocin blend consisting of SalE1a + SalE1b + SalE2 + SalE7 at an application rate of 3 + 1 + 1 + 1 mg/ kg meat compared to carrier yielded p-values of <0.0001, 0.0012, 0.0136 and 0.0035 at 1 h, 24 h, 48 h and 72 h post-treatment, respectively. The low-dose salmocin blend consisting of SalE1a + SalE1b + SalE2 + SalE7 at an application rate of 0.3 + 0.1 + 0.1 + 0.1 mg/kg meat compared to carrier control yielded p-values of 0.0002, 0.0040, 0.0139 and 0.0050 at 1 h, 24 h, 48 h and 72 h post-treatment, respectively. The criterion for the null hypothesis rejection was p<0.05. The statistical differences were assessed in relation to the salmocin-free carrier-treated meat samples by unpaired parametric t-test with GraphPad Prism v. 6.01.

Statistically significant bacterial reductions of ~2–3 logs CFU/g meat were found compared to the carrier control for all treatment types. The highest reductions for all treatments were found at 48h of storage. The high-dose salmocin blend (6 mg/kg meat total salmocin) showed the highest bacterial reduction level, a 3.39 mean log reduction vs. carrier treatment after 48 h of storage, which corresponds to a 99.96 mean percent reduction of the bacteria. SalE1a, administered individually at 3 mg/kg meat (2.72 mean log CFU reduction or 98.81 mean percent reduction at 48 h), controlled the *Salmonella* contamination on meat almost as efficiently as the 4-salmocin blend, applied at double the concentration (6 mg/kg meat total salmocin). The low-dosed blend (0.6 mg/kg meat) showed statistically significant bacterial reductions of about 2.19 mean log (99.54 mean percent CFU reduction) for up to 48 h of storage.

All samples that showed a significant reduction in bacterial growth due to salmocin treatment also demonstrated regrowth of viable bacteria after 48 h, indicating a limited technical effect of salmocins. Thus, bacteriocin activity (technical effect) should be absent or negligible when

salmocin-treated food products reach the consumer. Three further independent experiments were performed, which all confirmed the results (
Figure 42).

3.4.5.2 Analysis of the antimicrobial activity of salmocins against *Salmonella* on contaminated beef, tuna, and egg

Salmocin-based *Salmonella* decontamination on beef and tuna and in whole egg was also tested, as these food matrices offer a variety of surface textures that can potentially influence the antimicrobial activity of the salmocins. The experimental procedure is explained in 2.8.3.2 and is comparable to 2.8.3.1.

In short, the meat was cut into pieces, and the egg white and yolk were homogenized (whole egg). Afterward, the food was contaminated with a mixture of 2 *Salmonella* strains Enteritidis and Typhimurium (2.1.1.3) at $1x10^3$-$1x10^4$ CFU/g food. The meat was then treated with affinity-purified SalE1b at an application rate of 5 mg/kg meat (experiment 1) or with a lower SalE1b dose of 0.5 mg/kg meat (experiments 2 and 3). Salmocin solutions and the carrier control were applied by spraying and subsequent mixing of the meat. The meat samples were packed into bags and sealed, with 4 replicates for each sample. The bags were stored at 10°C. Bacterial populations were sampled at 1 h and 48 h post-treatment and enumerated upon dilution plating. The statistical differences were assessed in relation to salmocin-free carrier-treated meat samples by unpaired parametric t-test with GraphPad Prism v. 6.01. The criterion for the null hypothesis rejection was $p<0.05$

The results of SalE1b activity on *Salmonella*-contaminated beef cuts are shown in Figure 43.

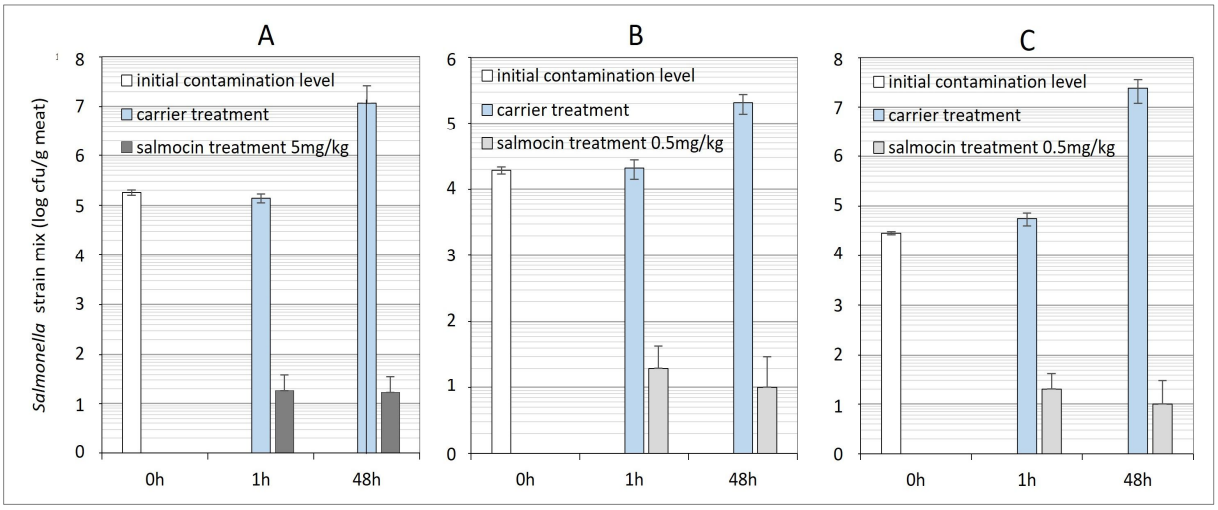


Figure 43: Reduction of *Salmonella* contamination on beef cuts with purified SalE1b.
Beef cuts were contaminated with *Salmonella* (0 h, initial contamination level) and treated with a single application of affinity-purified SalE1b, stored at 10°C, and sampled for bacteria enumeration at 1 h and 48 h of storage. The panels A, B, and C show the results from 3 independent experiments: averaged contamination in CFU/g of 4 biological replicates with error bars (SD). The experiments differed in the initial contamination level and the salmocin application rate. Panel A shows the results of an experiment with a high bacterial inoculum of $1.81x\ 10^4$ CFU/g beef (initial contamination level) and a SalE1b application of 5 mg/kg (10 ml/kg). Panel B and C show the results of an experiment with a moderate bacterial inoculum (encompassing 1.93×10^3 CFU/g and 2.85×10^3 CFU/g) and a SalE1b application of 0.5 mg/kg (10 ml/kg).

The SalE1b treatment led to a very dramatic reduction of *Salmonella* for both contamination levels and both application rates after 1 h, which was maintained and even slightly lowered after 48 h. Strong reductions in bacterial counts of up to 6 logs CFU/g on beef were achieved by SalE1b application at both the high dose (5 mg SalE1b/kg meat) (A) and the low dose (0.5mg SalE1b/kg) (B and C) at 48h post-treatment. Using 5 mg SalE1b/kg meat (Figure 43 A), mean CFU reductions of 3.0 logs (99.96% reduction, $p<0.0001$) at 1h to 6.4 logs (almost 100% reduction, $p=0.1575$, statistically not significant due to high variability) at 48h were achieved. For the lower dosed application rate (0.5 mg SalE1b/kg meat) (Figure 43 B and C), CFU reductions were similar, with statistically significant p-values ranging from 0.0009 to 0.0075. The results of SalE1b activity on *Salmonella*-contaminated raw tuna are shown in Figure 44.

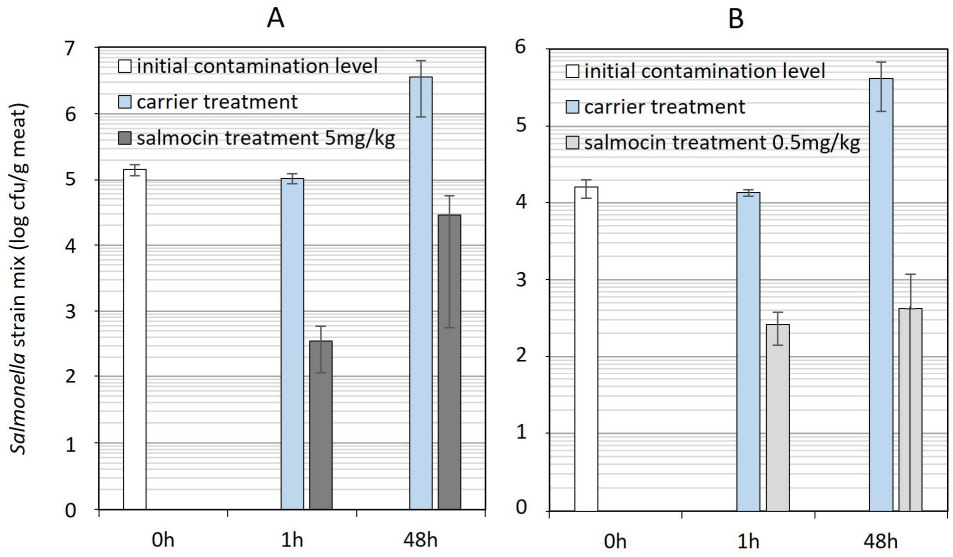


Figure 44: Reduction of *Salmonella* contamination on raw tuna by affinity-purified SalE1b.
Raw tuna filet cuts were contaminated with *Salmonella* (0 h, initial contamination level), treated with a single application of affinity-purified SalE1b, stored at 10°C and sampled for bacteria enumeration at 1 h and 48 h of storage. The panels A and B show the results of 2 independent experiments: averaged contamination in CFU/g of 4 biological replicates with error bars (SD). In panel A, the initial contamination level of raw tuna meat with a mixture of two *S. enterica* strains was 1.44×10^4 CFU/g. SalE1b solution was applied at a rate of 5 mg/kg (10 ml/kg). In panel B, the initial contamination level with a mixture of two *S. enterica* strains was 1.6×10^3 CFU/g, and a lower dose of 0.5 mg/kg salmocin SalE1b was applied to the tuna (10 ml/kg).

Although 3 experiments were conducted, only 2 provided statistically significant results (Figure 44 A and B). The reductions in bacterial counts achieved by SalE1b application on tuna were

less strong than for beef or whole egg. All reductions were statistically significant relative to the carrier control. In Figure 44 A, after 1 h incubation at 10°C, salmocin treatment reduced viable bacteria by 2.5 logs, which corresponds to a 99.66% mean CFU reduction ($p<0.0001$) relative to the carrier control. Upon 48 h incubation, the difference between treated samples and carrier was 2.1 logs CFU/g (99.21% mean CFU reduction, $p=0.0381$).

In Figure 44 B the differential reduction of viable bacteria in total was 1.7 logs CFU/kg at 1 h of incubation, a 98.06% mean CFU reduction ($p<0.0001$) relative to the carrier control. At 48 h, the differential reduction was 3.0 logs CFU/g (99.90% mean CFU reduction, $p=0.0189$) relative to the carrier control.

The results of SalE1b activity on *Salmonella*-contaminated whole egg are represented in Figure 45.

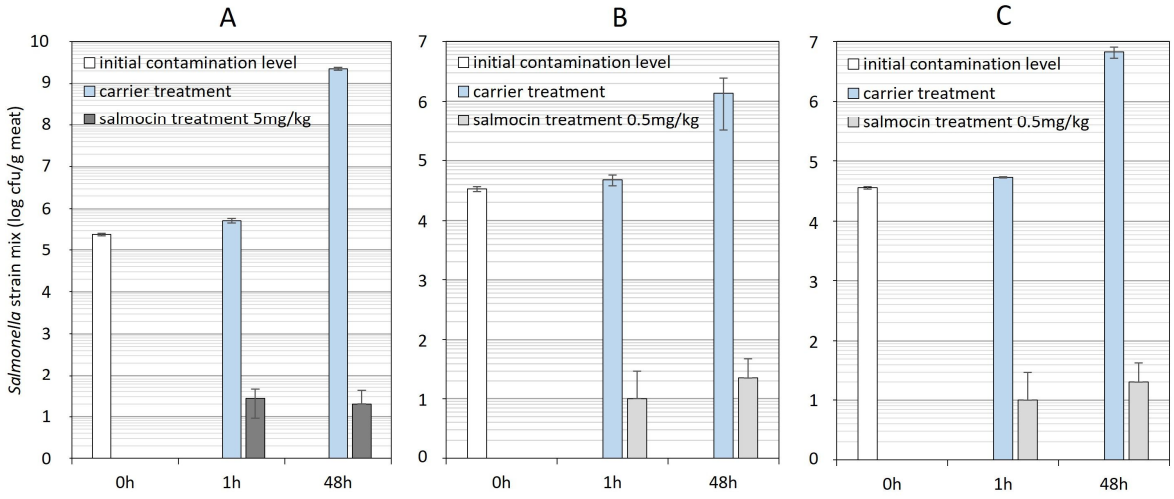


Figure 45: Reduction of *Salmonella* contamination in whole egg by affinity-purified SalE1b.
Whole egg was contaminated with *Salmonella* (0 h, initial contamination level), treated with a single application of affinity-purified SalE1b, stored at 10°C, and sampled for bacteria enumeration at 1 h and 48 h. The panels A, B, and C show the results from 3 independent experiments: averaged contamination in CFU/g whole egg of 4 biological replicates with error bars (SD). In the experiment shown in panel A, an initial *Salmonella* contamination level of 2.43 x 10^4 CFU/g egg and a salmocin application of 5 mg/kg egg (10 ml/kg) were used. In Panels B and C, the initial *Salmonella* levels were 3.36 x 10^3 and 3.59 x 10^3 CFU/g egg, respectively. The SalE1b application rate was 0.5 mg/kg egg (10 ml/kg) for both experiments.

The reductions in bacterial counts in whole egg achieved by salmocin E1b application were stronger than the reductions on beef. In experiment 1 (Figure 45 A), the 1 h sampling point showed a difference of 4.3 logs CFU/g whole egg relative to carrier (99.99% mean CTU reduction, $p<0.0001$), and at 48 h, a reduction of 8.1 logs CFU/g was achieved (100% mean CFU reduction, $p<0.0001$).

In experiment 2 (Figure 45 B), the reduction in viable bacteria counts was 3.7 logs CFU/g (99.98% mean CFU reduction, $p<0.0001$) at 1 h post-treatment. The bacterial reduction at 48 h was 4.8 logs CFU/g (100% mean CFU reduction, $p=0.0401$) compared to the carrier treatment.

In experiment 3 (Figure 45 C), the CFU reduction values for 1 h and 48 h were 3.7 logs CFU/g (99.98% mean CFU reduction, $p<0.0001$) and 5.5 $\log_{10}$ CFU/g (100% mean CFU reduction, $p<0.0001$), respectively, relative to control treatment.

These results show that salmocins have high activity not only on chicken meat; they also have a great potential for the decontamination of beef, tuna, and whole egg products from pathogenic *Salmonella*.

4. Discussion and outlook

4.1 Colicin mixtures drastically reduce the emergence of resistance

4.1.1 Colicins have high efficacy on *E. coli* DH10B

At Nomad Bioscience GmbH, the use of certain colicin mixtures had synergistic/additive effects for the antimicrobial activity on STEC strains (Schulz et al., 2015). The synergistic effects are usually more prominent if the antimicrobials use different modes of entry and killing (Brochado et al., 2018; Cotter et al., 2005; Diez-Gonzalez, 2007; Kumariya et al., 2019; Zharkova et al., 2019). Synergistic effects of antimicrobial mixtures have been documented for antimicrobials of the same class of molecules (Bush, 2015) or from different classes (Chi & Holo, 2018; Tyers & Wright, 2019). Therefore, 4 colicins with good potency against STEC strains (Schulz et al., 2015) that use different receptors and translocation pathways were chosen (3.1.1).

The MIC was assessed for the determination of the mutation rate of *E. coli* ElectroMAX™ DH10B™ to individual colicins and mixtures thereof (3.1.2). Additionally, the MIC indicates how effectively the bacteriocin inhibits the microorganism´s growth (Bouttefroy & Milliere, 2000; Kumariya et al., 2019).

For the purified colicins used for MIC determination, a 100% purity was assumed. Nevertheless, these colicins do not have a purity of 100% after one chromatography step. (Stephan et al., 2017).

Compared to commonly used antibiotics, the MIC values obtained for colicins on ElectroMAX™ DH10B™ were all very low, indicating their high effectiveness (Table 11).

The MICs of colicins on ElectroMAX™ DH10B™ were 100,000x lower than the value obtained for rifampicin (25 ng/µl MIC). Additionally, colleagues at Nomad Bioscience GmbH evaluated the MIC of kanamycin for ElectroMAX™ DH10B™ and obtained a MIC of 3 ng/µl with the broth dilution method, which is about 40,000x higher compared to the MICs obtained for ColM, ColU or ColK (Nomad Bioscience internal data). Even though the MIC was determined by the broth dilution method, the values are comparable as MICs obtained from either method are typically within one two-fold dilution from another (Burns et al., 2000; Luber, Bartelt, Genschow, Wagner, & Hahn, 2003).

The protocol for MIC determination was optimized to stabilize the antimicrobial proteins in the agar. BSA was added to the colicin solutions and the gel, as it is known to aid in ColM stabilization (Schaller et al., 1981). It could also act as a protease “decoy” and thereby possibly

help maintain their antimicrobial activity. Additionally, the agar concentration in Mueller Hinton Agar medium was reduced to 0.75% (w/w) (Cabo et al., 1999). These adaptations were used in all MIC and mutation rate determination experiments. By this approach, the MIC of ColM, was reduced 8-fold to 0.00024 ng/µl. For ColK, the MIC was lowered 2-fold, and for ColIa and ColU, there was no noticeable effect.

In a later experiment at Nomad Bioscience GmbH, researchers confirmed the strong positive effect of these alterations for colicin MIC reduction (Nomad Bioscience internal data).

Literature on MICs of colicins is scarce and mainly covers pathogenic strains but not DH10B. Therefore, the MIC values obtained in our experiments can only partially be compared to other studies focussing on either colicins or DH10B.

Bratu et al. 2008 obtained a MIC of 8 ng/µl for ampicillin on ElectroMAX™ DH10B™ by agar dilution method (Bratu et al., 2007). This value is ~16,000x times higher than the MICs for colicins obtained in this study.

Lojewska et al. 2019 used purified ColM in the liquid broth dilution method on *E. coli* DH5α, with MIC values ranging from 3.9 to 7.8 ng/µl (Łojewska et al., 2019). This value is ~10,000 times higher than the value obtained in our experiments. This could be due to the different strains used. Furthermore, the protein extraction method (phenol extraction) and the protein concentration determination method (microplate spectrophotometer) differed. The lack of ColM stabilizing agents or protease "decoys" (e.g., BSA) in the agar could also have lowered antimicrobial activity.

When MICs of common antibiotics are compared to the MICs of colicins, it becomes evident that colicins are more effective in inhibiting bacterial growth, as much less colicin is required to achieve the same effect. Colicins also have a much higher molecular weight than antibiotics, so it seems like much fewer molecules of colicin are needed than of an antibiotic to kill a cell. This could help to underline the single-hit-kinetics theory, which states that 1 colicin protein is sufficient to kill a bacterial cell (Cascales et al., 2007; J. Smarda, Damborsky, J, 1991).

Although the MICs to pathogenic strains will differ, the MIC against a common non-pathogenic *E. coli* should have indications for MICs of pathogenic *E. coli*. Therefore, it is probable that colicins will also require lower concentrations than common antibiotics to inhibit pathogenic *E. coli*.

The 4 colicins chosen for MIC and mutation rate determination used 4 different receptors, both translocation pathways and 2 of the 3 possible modes of activity. Due to their potential synergy and an established method of simple and inexpensive protein purification (Stephan et al., 2017), these 4 colicins should also be an ideal commercial mixture against the Shiga-toxin-producing

E. coli STEC. Specifically, Nomad Bioscience GmbH recommends colicin M (3 mg/kg); colicin Ia/Ib (1 mg/kg); colicin K (1 mg/kg), and colicin U (1 mg/kg), in a mixture of total 6 mg colicins/kg, to control STEC on food matrices (Stephan et al., 2017).

No significant synergistic effects could be witnessed. Overall, the MICs (3.1.2 Table 11) did not differ much, and the mixing of colicins did not show synergistic effects in the form of a significant MIC reduction. A reason for this may be that the colicins in the mixtures were only combined at an equal ratio (1:1:1:1).

The results indicate potential synergistic effects when considering that the concentration of each colicin was halved for each additional colicin added to the mixture. The MIC for ColM individually was 0,00024 ng/µl. In the 4-colicin blend, however, the ColM concentration was 0,00012 ng/µl of the 0.00046 ng/µl of total colicins.

Assessing different combination ratios might have illuminated synergistic effects and what factor each individual colicin plays in the growth inhibition potency of the total mixture.

This could be done in the future by conducting the checkerboard assay that assesses the fractional inhibitory concentration index (FICI), a method to determine synergistic, antagonistic, or indifferent effects between 2 antimicrobials (Meletiades 2010, Tyers 2019) (Zharkova et al., 2019). As the main focus of this work was the determination of the mutation rates by using a pre-determined MIC, this index was not assessed.

4.1.2 The occurrence of resistance against colicin mixtures in *E. coli* DH10B is exceptionally rare

In this study, the mutation rate of resistance to individual colicins and mixtures thereof in *E. coli* ElectroMAX™ DH10B™ was determined by fluctuation assays (3.1.3 Table 12 and 3.1.4 Table 15).

Overall, the mutation rates of resistance ot the individual group B colicins (ColM, ColIa) are higher than those of the individual group A colicins (ColU and ColK).

From the individual colicins, ColU had the lowest mutation rate. ColU has been found to be active on just one of the STEC strains tested but has shown the highest overall activity against 69 multi-resistant clinical *E. coli* isolates (Nomad Bioscience internal data) (Schulz et al., 2015).

All mutation rates for individual colicins were higher than for rifampicin. Rifampicin resistance, however, can only occur by mutations in the rpoB gene that encodes the vital ß subunit of the

RNA-dependent RNA polymerase (Durfee et al., 2008). Therefore, non-lethal mutations in this gene are very rare events.

Theoretically, if the mutations of resistance development to 2 drugs A and B are independent events, the probability of spontaneous resistance to a combination of the 2 drugs should be the product of both rates (e.g., 1 x 10^{-6} x 1 x 10^{-7} = 1x 10^{-13}) (Coates et al., 2020; Fischbach & Walsh, 2009).

Practically, in our experiments, the mutation rates decreased significantly when combinations of colicins were used, although not as strongly as Fischbach and Walsh 2009 suggest. Nevertheless, the results confirm that resistance against various antimicrobial agents is harder to come by than against individual agents (Chi & Holo, 2018; Schmelcher et al., 2012; Tyers & Wright, 2019; Xu et al., 2018).

In a 2005 study, Schamberger & Diez-Gonzalez came to a similar conclusion, while the resistance of *E. coli* O157:H7 to individual colicinogenic strains was observed, it was only rarely seen in the presence of multiple colicinogenic strains that produced several different colicins (Schamberger & Diez-Gonzalez, 2005).

While different combinations of the 4 colicins with each other were not tested in this work, further studies at Nomad Bioscience GmbH showed that a combination of colicins from distinct groups (group A and group B) lowered the mutation rate much stronger than a combination of colicins from the same group (Nomad Bioscience internal data). Therefore, antimicrobial mixtures simultaneously targeting both translocation pathways seem to have a synergistic effect and are advisable. A mutation in one translocation pathway should leave the other pathway functional. For resistance to such a mixture of colicins, at least 1 independent mutational event in each of the pathways should be necessary.

4.1.2.1 Mutants with resistance to individual colicins could be obtained, whereas multiresistant mutants could not

In each fluctuation assay experiment conducted to determine the mutation rates of *E. coli* ElectroMAX™ DH10B™ to an individual colicin, 30 resistant clones were stored for further analysis. The majority of these clones (93.3% of the ColM resistant clones, 90.0% of the ColK resistant clones, 96,6% of the ColIa resistant clones, and 66.6% of the ColU resistant clones) had acquired a significantly decreased sensitivity to the colicin they had been selected with (3.1.4, Table 13). They were able to grow at concentrations >100x higher than the WT. Only a small proportion of these clones had not acquired any insensitivity to the colicin they were

selected with: 2 out of 30 clones (6.6%) for ColM and ColK and 0 out of 30 clones (0%) for ColIa and ColU.

This small number of "survivors" could be explained by potential differences in antimicrobial concentrations and cell densities in the agar during the fluctuation assay experiments. Antimicrobial concentration differences may have locally led to sublethal concentrations, allowing non-resistant cells to survive. Additionally, in areas of high cell density, cells may have taken up more colicin molecules, leaving some cells with a non-lethal dose.

Different results were obtained for mutant clones from fluctuation assays with selection to multiple colicins. For colicins selected on the mixture of ColM and ColK, 96.6% of the clones had a strong insensitivity to ColM but only 2 of the clones showed a very minor insensitivity to ColK (3.1.4, Table 14). It seems that it was sufficient for the clones to develop an insensitivity to ColM, to survive the 10x MIC mixture of both colicins. In turn, this shows that the inhibitory activity of the mixture was mainly due to ColM activity and not ColK activity. Therefore, it would be important for future experiments to examine different ratios of the colicins in addition to a 1:1 mixture, similar to the determination of the FICI in the checkerboard assay described in 4.1.1. That way, it might be easier to assess the individual MICs for each of the colicins when used in a mixture.

Initially, the obtained mutation rate was 5.78x 10^{-8} (Table 12). Upon testing their sensitivity to ColK and ColM individually, only 2 mutants showed a slight insensitivity to ColK in addition to a ColM insensitivity. Therefore the mutation rate was recalculated, resulting in a rate of 6.99 x 10^{-9} (3.1.4 Table 15).

For comparison, Calcuttawala et al. 2015 assessed the mutation rate for *E. coli* DH5α when exposed to combinations of ColE2, ColE3, and ColE8. They obtained mutation rates of 2.51 x 10^{-8} for a combination of ColE3 and ColE8 and 5.52 x 10^{-8} for a combination of ColE2 and ColE3. The resistance-conferring mutations, mostly insertion sequence elements, were found in the BtuB receptor (Calcuttawala, Hariharan, Pazhani, Ghosh, & Ramamurthy, 2015) that all 3 colicins use for cell entry (Feldgarden & Riley, 1998).

Theoretically, if the mutation rate to 2 drugs A and B are independent events, then the spontaneous resistance probability to a combination of both should be the product of both rates (1 x 10-6 x 1 x 10-7 = 1x 10-13) (Coates et al., 2020; Fischbach & Walsh, 2009).

However, for our experiments, this was not the case. This could be due to mutations that do not affect the proteins used for cell entry or the target of the activity domain but occur at the level of cellular metabolism. An upregulation of protease production, e.g., could cause resistance to

a large number of colicin-like bacteriocins. However, such resistances should come at a high fitness cost for the cell.

Similar to the clones selected with 2 colicins, the clones selected with 3- or 4-colicin mixtures did not show an insensitivity to all of the colicins they were selected with. Only half of the clones selected with 3 colicins had resistance to ColM with no resistance towards ColK and ColIa.

None of the clones selected with the 4 colicin mixture had any insensitivity to the colicins they were selected with. By having very low individual concentrations of each colicin in the mixture, the actual MIC for each individual colicin might have barely been attained. This means that slight concentration fluctuations across the plate may have led to the survival of some non-resistant clones.

Therefore the actual mutation rate was below the lowest mutation rate that can be calculated with the program and the formula used (Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) calculation method (Radchenko et al., 2018)). The program cannot calculate a mutation rate when 0 actual mutants were found.

As a prevention against attaining non-resistant mutants because of slight concentration variations within the plate, a 10x MIC was used to obtain resistant mutants in fluctuation assays which is higher than in most studies that usually use a 4x MIC. Other studies used 2-4x MIC (Pope et al., 2008), 2x and 8x, and 16x MIC (Cortes, Pinas, Albarracin Orio, & Echenique, 2008), 2-4x MIC (Gravesen, Jydegaard Axelsen, Mendes da Silva, Hansen, & Knochel, 2002) and 4x MIC (L. L. Ling et al., 2015).

A limitation of calculating the mutation rate by fluctuation assays is that they use the MIC to determine the spontaneous mutation of a bacterium. However, this does not take into account resistance formation at sublethal antimicrobial concentrations, which may be more dominated by stress-induced directed mutations than by spontaneous mutation. Selection at sublethal antimicrobial concentrations plays an important role in the population dynamics of the natural environment and clinical settings (Frenoy & Bonhoeffer, 2018).

Another interesting parameter that one could determine for new antimicrobials is the mutant prevention concentration (MPC), which lies above the MIC and is the minimum concentration necessary to inhibit the growth of the least susceptible single-step mutant (Blondeau & Fitch, 2019; Liu et al., 2013; Sindelar et al., 2000). A cell would have to acquire a minimum of 2 concurrent mutations for resistance development at this concentration (Liu et al., 2013).

Overall, the determination of a mutation rate offers a simple and comparably metric that can indicate how quickly antimicrobial resistance could occur (Radchenko et al., 2018; Ragheb et al., 2019; Rosche & Foster, 2000; Windels et al., 2019). Nevertheless, there are other important factors that affect microbial resistance formation in clinical settings and the natural environment. These include, e.g., the number of resistance genes that are already available in the population. How easily do the resistance genes spread among the population, and at what fitness cost are they maintained (Andersson, 2015; Hughes & Andersson, 2017).

Two studies from Feldgarden and Riley, in particular, found ubiquitous colicin resistance in *E. coli* and non-*E. coli* isolates. Over 75% of the sampled *E. coli* isolates had a resistance to one or more colicins, with the number in non-*E. coli* isolates being even higher. The studies also emphasized that the resistance in one translocational pathway could render a cell resistant to all colicins using that pathway. However, they found significant fitness costs associated with colicin resistance, comparable to antibiotic resistance for *in vitro* growth performance. Most importantly, while resistance in non-Diarrheagenic *E. coli* (DEC - *E. coli* associated with diarrhea, e.g., STEC) strains was high, it was very rare in DEC strains. While the DEC strains had acquired resistance against multiple antibiotics, they had not acquired resistance against colicins (Feldgarden & Riley, 1998, 1999).

Our data from this study and later studies done at Nomad Bioscience, however, does not show that the mutation rate in *E. coli* ElectroMAX™ DH10B™ to colicins is significantly higher than the mutation rate to antibiotics (Nomad Bioscience internal data).

This study found that the addition of BSA as an additional target for proteases was able to reduce the MIC of colicins needed for *E. coli* ElectroMAX™ DH10B™. Further studies at Nomad Bioscience confirmed this data and further concluded that the addition of BSA significantly lowered the development of resistance (mutation rate) to colicins in *E. coli* ElectroMAX™ DH10B™ (Nomad Bioscience internal data).

There are now experiments being done *in vivo* (in animal efficacy models) that already show promising results due to high efficacy of reducing *E. coli* in blood with no obvious formation of resistance (Nomad Bioscience internal data).

The engineering of active chimeric bacteriocins that combine cytotoxic domains with different mechanisms of cell entry provides another theoretical way of controlling mutations of resistance, namely the use of mixtures of natural bacteriocins with chimeras that employ different machinery for cell entry than the natural bacteriocin.

4.1.2.2 Cross resistances for group A colicins are more likely

If mutant clones showed a cross-resistance to a colicin from the same colicin group (e.g. ColM and ColIa are both group A colicins) that they were not selected with, this indicated a mutation in the translocation pathway (Table 10).

Overall TonA-dependent colicin mutants show much more cross-resistance (ColK mutants - 40%, ColU mutants - 60%) than TolB dependent colicins (ColM mutants - 3%, ColIa mutants - 13%). But when selecting with colicins ColM + ColK, 5 clones had a cross-resistance to ColIa, which is 17% of the mutants. These results could hint at a greater likelihood for colicin resistance-conferring mutations in the TolA pathway than in the TonB pathway.

For ColK mutants, several showed a cross-resistance to ColU, but 5 mutants were also resistant to ColIa. This could hint at a type of resistance that prevents the insertion of a wider range of pore-forming activity domains into the inner membrane (e.g., PacB (Protection against colicin B) character). PacB is a gene product expressed by a group of non-colicinogenic plasmids (IncH) conferring resistance to all known channel-forming colicins (Alonso, Gomes, Gonzalez, & Rodriguez Lemoine, 2000; Rodriguez Lemoine, 1992).

In the end, colicins are vulnerable to resistance, and a single mutation can cause resistance to multiple colicins. For example, mutations in the BtuB receptor can cause resistance to all nine "E" colicins (Feldgarden & Riley, 1998). Mutations in the translocation pathway can also lead to resistance to several or all colicins using this pathway (Davies & Reeves, 1975a, 1975b; Feldgarden & Riley, 1998).

4.1.2.3 Receptor and the translocation machinery seem equally involved in conferring ColM resistance

In total, 10/28 mutants had a mutation in the receptor gene *fhuA*, while 11/30 mutants had a mutation in the translocation machinery genes, mostly in *exbB*, which is an equal distribution of mutations. The gene *exbB* seems to be the gene of the translocation machinery, most prone to mutation. For 7 of the mutants, no mutation could be found in the receptor or translocation genes responsible for ColM cell entry. There might have been second-site mutations that have caused the resistance to ColM

There are also proteins inside the targeted cell that play a crucial role in ColM activity and are not required for its uptake. The presence or absence of these proteins can confer ColM resistance. The periplasmatic FkpA chaperone, for example, is essential for the correct folding of the ColM after import into the cell and is therefore vital for its toxicity (Volkmar Braun &

Patzer, 2013; Hullmann, Patzer, Romer, Hantke, & Braun, 2008). Likewise, the presence of CbrA in the targeted cell can give resistance to ColM and osmotic shock (Helbig, Hantke, Ammelburg, & Braun, 2012).

There seems to be a correlation between the degree of sensitivity and the resistance type, as receptor mutations lead to a higher degree of resistance than a translocation machinery mutation.

Nevertheless, only the translocation machinery genes of the mutants with no mutation in the receptor gene were analyzed. Therefore a mutant may exist where both were mutated, although this is unlikely.

4.2 Legocins

With the widespread threat of antibiotic resistance and a dwindling pipeline of new antimicrobials, broadening the antimicrobial arsenal is of utmost importance. This can be done by discovering new antimicrobial compounds or diversifying known drugs (O'Connell et al., 2013).

Colicin-like bacteriocins have a modular structure that could offer many rearrangement opportunities to customize them towards their specific application. One objective of swapping bacteriocin domains was to create a hybrid antimicrobial with significant advantages over the 2 parental molecules. Another objective was to create hybrid antimicrobials with antimicrobial activity similar to the parental molecules that could broaden the antimicrobial arsenal. It could, e.g., be used as an alternative in case of resistance formation against the parental molecule.

4.2.1 Legocins with a ColIb or ColM activity domains were not successful

These legocins were a combination of the TR-modules from colicins with high antimicrobial activity using diverse methods of cell entry, with the 2 most broadly and highly active A-modules. Unfortunately, no promising new colicin chimeras were obtained. Legocins with a ColIb activity domain showed activity against DH10B (pICH73311), but it was much lower than that of the corresponding parental molecules. Legocins with a ColM activity domain showed no activity at all. In conclusion, the hybrid agents offered no significant advantage over the individual parental agents and could not replace them.

The low or non-existent activity may have been due to incorrect annotation of activity domains. As the annotations for the TR-modules worked with other A-modules of ColU and SalE1b, the

error is most likely in the chosen annotation of the ColIb and ColM A-module. The ColIb annotation was not found in literature and was therefore predicted from ColIa alignments due to its high homology (Jakes, 2017) (7.3). It was unclear if the amino acids NMV are the C-terminal end of the receptor domain or if they form the N´ terminal end of the activity domain. This could be tested with a new set of ColIa A-module legocins.

The ColM activity domain was annotated using structural data from Helbig et al. 2011 (Helbig & Braun, 2011). Zeth et al. 2008 (Zeth, Romer, Patzer, & Braun, 2008) had also annotated the activity domain 3 years before, with a 2 aa shift of the domain annotation compared to Helbig et al. 2011 (7.3). Nevertheless, Helbig acknowledged Zeth as a helpful discussion partner in his acknowledgments, so the more recent domain annotation published by Helbig et al. 2011 was chosen. Both publications state that ColM is the most challenging colicin for domain prediction due to its compact structure and distinctive folding, unique among colicins and all known proteins (Helbig & Braun, 2011; Zeth et al., 2008). Due to its uniqueness, the chimeric modules fused to the ColM activity domain might have caused structural perturbations that led to the protein's misfolding and destabilization (Chen, Zaro, & Shen, 2013). However, significant destabilization is unlikely as the legocins were expressed well.

The ColM is also distinctive in its size (271 aa) and its activity. The activity domain inhibits the murein and O-antigen biosynthesis and presumably needs to be translocated into the proximity of the peptidoglycan layer (Cascales et al., 2007; Cherier et al., 2021). The translocation domains of all other colicins probably translocate their activity domains with pore-forming or nuclease activity into proximity of the inner membrane or even across the inner membrane (Cascales et al., 2007; El Ghachi et al., 2006). This could be another reason why the ColM A-module legocins had no activity.

In general chimeras combining domains from different classes of colicins (e.g. porin-type and peptidoglycan inhibiting-class, or colicins of A and B), were are all nonfunctional. This could be due to all the reasons stated above, or it could mean that the combination of TR-modules with A-modules from different groups or activity classes doesn’t function as well.

Alternative domain annotations should be tested for future legocins. Additionally, C-terminal parts of the ColM receptor domain could be added to the activity domain.

4.2.2 **Legocins with a ColU activity domain have a good potential**

The legocins with a ColU activity domain were well expressed and active. Due to cloning artifacts, 1 or 2 aa linkers were left between the TR- and the A-module of the legocins.

As mentioned in (3.2.2), the antimicrobial activities are presented in AU/mg FW of plant biomass, not in specific activity (AU/µg recombinant protein). Therefore, the values can be significantly affected by colicin expression levels and can only serve as a rough estimate for the antimicrobial activities.

A significant difference in antimicrobial activity between the 1 aa linker (lysin - basic, positive charge, long aliphatic side chain) and the 2 aa linker (glycine – neutral charge, very short side chain + serine – polar/uncharged, short side chain) was only observed in the TR_{ColN}-A_{ColU} legocin (Table 19). Here the 1 aa linker led to a 64x higher antimicrobial activity (AU/ mg FW) compared to the 2 aa linker. Flexible linkers for chimeric proteins are often composed of non-polar amino acids, e.g., glycine, and polar amino acids, e.g., serine (Chen et al., 2013). Therefore, the Gly-Ser linker, although very short, might have acted as a flexible linker, decreasing the antimicrobial activity of the legocin compared to the lysine linker.

The TR_{ColK}-A_{ColU} legocin was engineered with and without a 60 bp linker between the modules. This linker was composed of the C-terminal end of the ColK receptor domain (30 aa) and the C-terminal end of the ColU translocation domain (30 aa). This approach had no influence on the legocins activity compared to the non-linker legocin. Therefore, this linker did not seem to contain any vital sequences for correct protein folding or sequences significant for cross-domain interactions that make the ColK TR-module and the ColU A-module function properly. Typically, natural fusion protein linkers are between 5 and 21 amino acid residues long, so the 60 bp linker, it was within this range (Chen et al., 2013).

The TR_{ColIa}-$A_{GS\text{-}ColU}$ legocin combined a group A colicin with a group B colicin. The TolA translocation machinery successfully translocated the ColU activity domain, usually transported by the TonB machinery. However, it was one of the legocins with the weakest activity and was much less active than the parental molecules (32 times less active than ColIa and 1024x less active than ColU). This may have been due to inefficient translocation. But it is important to keep in mind that the expression level of this legocin was very low compared to the other legocins Figure 12.

The TR_{ColE1}-$A_{K\text{-}ColU/GS\text{-}ColU}$ legocins had a 128x higher antimicrobial activity than ColE1. This was the only TR-module that showed a substantial alteration of activity when combined with the ColU activity domain. This could be due to a higher stability than ColE1. But ColE1 expression is also very cytotoxic to the plant and visible necrosis appears as early as 3 days post-inoculation (Nomad Bioscience internal data). Combining it with a far less cytotoxic activity domain presumably allowed for the attainment of a much higher recombinant protein yield as this legocin was harvested 8 dpi with little to no signs of plant tissue necrosis.

Legocins with a ColU activity domain were expressed at high levels in *N. benthamiana*. Especially the legocin with the TR-module from ColK was expressed strongly, which coincides with the known high expression level of ColK (Nomad Bioscience internal data).
Overall, a new set of promising hybrid antimicrobials were created. They all have an antimicrobial activity similar to the parental molecules and could be employed to broaden the antimicrobial arsenal in case of resistance formation against the parental molecule.
In future experiments, the specific antimicrobial activity (AU/µg recombinant protein) for these legocins should be assessed so more precise comparisons of antimicrobial activity can be made.

4.2.3 **Interspecies legocins with activity on both *E. coli* and *Salmonella***

The legocins with a SalE1b activity domain and TR-modules derived from colicins were all expressed and had similar antimicrobial activity to the parental molecules. SalE1b has 70% homology with ColE1, and the interspecies domain swapping worked well.
While SalE1b has activity on DH10B (pICH73311) (3.2.3, Figure 15) and very low activity on 2 of the pathogenic STEC strains (Figure 17), all legocins had an increased and broadened activity on the STEC strains.
This outcome was expected as the receptor and translocation domains usually define the target-cell specificity of a colicin-like bacteriocin (Volkmar Braun & Patzer, 2013; S. C. Yang et al., 2014). In contrast, the activity domains and their mechanisms of activity are presumably much less specific as their targets exist in multiple bacterial species.
One could expect that the SalE1b activity domain, if combined with TR-modules from highly active colicins, would successfully exert its pore-forming activity on the strains resistant to full-length SalE1b but sensitive to those colicins.
However, all legocins showed a lower activity on DH10B (pICH73311) and the STEC strains than the parental colicin molecules. Explanations for this could be that the SalEb activity domain is inadequately transported to the inner membrane or that differences in the inner membrane of *E. coli* partially inhibit pore formation.
Overall, all legocins had lower activity on *Salmonella* than SalE1b. It seems as if the SalE1b activity domain is more effectively transported to the inner membrane by its original TR-module than it is by the TR-modules of the colicins.
In conclusion, compared to the SalE1b parental molecule, the legocins with a SalE1b activity domain have a broader activity range to include most of the STEC strains. However, they did not offer higher and broader activity ranges over the individual agents of origin (4.2.4).

4.2.4 **A future outlook for legocins**

The mining of novel molecules continues, but the total number may not be infinite. Among the already characterized colicin-like bacteriocins cloned from genomes of Gram-negative bacteria, only a dozen or so demonstrate high and broad efficacy, usually within the same species or the same genus (Behrens, Six, Walker, & Kleanthous, 2017; Denkovskiene et al., 2019; Diez-Gonzalez, 2007; Klein et al., 2016; Schulz et al., 2015; Soltani et al., 2021).

Colicin-like bacteriocins have a modular structure consisting of domains that have been shown to be functionally independent (Cascales et al., 2007). Therefore, they are ideal candidates for domain rearrangement, which could create new combinations of existing cell entry and cytotoxicity mechanisms (V. Braun et al., 2002; Riley & Wertz, 2002). This could broaden the antimicrobial arsenal against resistant pathogens, and depending on the requirements, legocins could be customized towards their specific application.

We limited the scope of our engineering by working mainly with porin- and peptidoglycan-inhibiting colicins (except for ColE3) and by testing only combinations in which receptor and translocator domains of the parental molecule were kept together. We also limited our study by using only cytotoxicity domains of the most potent colicins (ColM, ColIb, ColU) and the highly active domain of salmocin E1b (SalE1b).

With relatively simple measures, we successfully expressed 25 novel bacteriocin chimeras, with 14 of them showing significant activity on the tested *E. coli* DH10B strain. None of these legocins surpassed their original bacteriocins in breadth and potency of activity. Nevertheless, SalE1b activity domain was able to form pores in *E. coli* STEC strains.

In general, the use of activity domains from other bacterial species, could bypass immunity proteins present in the species (Lukacik et al., 2012; Paskevicius et al., 2022). In experiments with *Pseudomonas* bacteriocin chimeras conducted in the Lithuanian subsidiary of Nomad Bioscience GmbH, some of the chimeras demonstrated a broader spectrum of activity than parental molecules due to lack of recognition of the new cytotoxicity domain by the immunity protein (Paskevicius et al., 2022).

In further experiments, researchers at Nomad Bioscience discovered that some mixtures of natural bacteriocins with chimeras that employ different machinery for cell entry than the A-module parental molecule have lower rates of resistance mutations than the latter (Nomad Bioscience internal data).

One disadvantage of legocins is that they will not be as easy to implement into the food market as natural bacteriocins such as colicins. They are not natural proteins and thus have no safety record of regular human exposure. Therefore, it is unlikely they would qualify for the GRAS approval pathway (1.3.2.4) without extensive toxicology studies (Hahn-Lobmann et al., 2019).

Nevertheless, there is still great potential for new domain combinations, where efficient TR-modules, prone to low resistance development, can be combined with the most active A-modules, potentially even altering the stability of the bacteriocin. Like most bacteriocins, due to their narrow target range, they avoid selective pressure on commensal bacteria, reducing the formation of resistance and the alteration of important human and animal microbiomes (Cotter et al., 2013; Hols et al., 2019; Kleanthous, 2010).
In future experiments, 4 or 5-domain colicin could also be engineered to test if additional receptor, translocation, or activity domains increase or change the activity spectrum. If this works, having different methods of cell entry or activity within one molecule could strongly inhibit the formation of resistance.
In this study, the translocation and receptor domains of a parental molecule were always kept together. In future experiments, these domains could be used separately to create a more diverse set of molecules. To restore or optimize the functionality of chimeras, a broader understanding of the colicin domain interactions and potential structural conformation changes would help. Along with a deeper understanding of the domain "borders" to understand, e.g., functional overlap. This would further expand the potential of finding promising chimeric colicin-like bacteriocins and widen the antimicrobial arsenal as a potential alternative for antibiotics

4.3 Analysis of colicin activity in piglets

Nomad Bioscience GmbH conducted the piglet study to evaluate if colicin mixtures in 2 different colicin formulations could be used to cure or alleviate PWD in piglets, making it a possible treatment alternative to antibiotics. As many countries are severely restricting the use of antibiotics (regulation (EU) 2019/4 and 2019/6), the need for alternatives is growing.
The actual study was performed at the Central Veterinary Institute (CVI) of the Wageningen University in the Netherlands. However, the study was terminated early due to health problems in the test subjects, which resulted in too many confounding factors. In the end, the study did not yield the full package of data we expected, and the results can only be used as an indication for the effectiveness of using a colicin treatment for PWD in an animal model.

The 4 colicins chosen for this experiment, except for ColU, belong to the group of the most active colicins (M, E2, E6, E7, Ia, K, and 5) against the 7 most important Shiga toxin-producing *E. coli* (STEC) strains (STEC "Big 7") and DH10B (pICH73311) (Schulz et al., 2015). However, their activity on CVI-1000 was considerably higher.

The proportion of ColM and ColU in the mix was higher than that of ColK and ColIb as ColM had the highest activity on CVI-1000, and ColU, a group A counterpart, had shown good activity against multiple antibiotic-resistant clinical isolates of *E. coli* (Nomad Bioscience GmbH internal data).

The main reason this study was terminated earlier was that an inadequate oral capsule administration tool (dosing gun) was utilized. For this study, 2 different types of dosing guns were used (Figure 46).

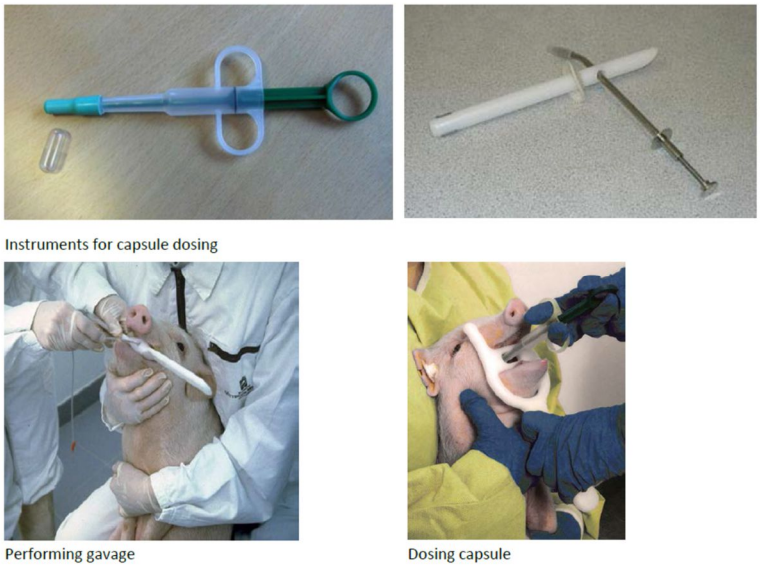


Figure 46: The 2 different dosing guns used in the piglet experiment.
From day -3 to -1 of the study, the metal dosing gun was employed (right). From day 0 to 6 of the study, the silicone tube dosing gun was utilized (left). (picture from (Adrian Zeltner, 2013)).

The metal dosing gun (Figure 46) is recommended for administrating capsules to mini pigs in animal trials (Adrian Zeltner, 2013). It is also used for piglets 6 weeks and older. This device was only used on the first 3 days of the study (-3 to 0) because soon after the first gavaging procedures, the veterinary technicians reported having difficulty getting the capsules into the back of the oral cavity properly. Therefore, from study day 0 onwards, a dosing gun with a silicone tube was applied, making the capsule positioning for oral gavage easier and more manageable.

However, after a few days of gavage, some piglets started getting sick. By Day +1, two piglets had a swollen neck and showed heavy breathing with strong mucus production. Similar but milder symptoms were observed across all groups. By day +2, three piglets had to be euthanized due to severe sickness. Over the first weekend (day +3 and 4), 4 more piglets had to be euthanized. By the beginning of the second week (day +5), almost all piglets showed some form of the sickness, including swelling in the ventral neck area, coughing, respiratory distress, strong mucus production, reduced feed uptake, and some degree of lethargy.

On day +6 , the study was terminated 3 days earlier. This decision was made due to animal welfare concerns and substantial confounding factors for the study's integrity.

The first 2 unplanned necroscopies on day +2 revealed perforations and lesions in the back of the piglets´ throats. These perforations connected the oral cavity with the body cavity, which had caused severe inflammation throughout the entire body cavity. This inflammation also affected the lungs and other organs and explained many of the observed sickness symptoms of the piglets.

How widespread these pathological findings were was revealed in the final necroscopy, where it became evident that almost all piglets, with few exceptions, showed the same physical damage and inflammation. Thus, the technicians concluded that the wounds and the coherent misplacement of capsules had occurred within the first 3 days of oral gavage when the metal dosing gun was used.

The most probable explanation for the piglets' injuries was that during gavage, the end of the metal dosing gun had been mispositioned at the esophagus entrance before the swallowing was induced by stroking the piglets´ throat. For a more in-depth discussion on the cause of the piglets´ injuries and the consequent inflammation, see 7.4.

In the end, the number of capsules that actually reached their intestinal target varied significantly between the piglets. Some metal balls were found in the throat tissue and the body cavity, while some had even remained in the stomach. In almost all piglets, varying numbers of metal balls could be found in the intestine and the caecum.

The number of balls found in the intestine and the caecum were not counted for all piglets, allowing no reasonable estimate of the number of capsules that had arrived at the area of infection. However, one can assume that across all test groups, similar numbers of capsules must have arrived there.

The piglets only showed a diarrhea phenotype at the beginning of the study. Then the stool consistency gradually solidified. The feed intake was lower than usual due to the illness. Fecal dry matter and blood samples were not analyzed as planned due to the outcome of the study.

Another parameter that was not concluded (post-mortem) was the receptor status of the intestinal epithelium of the piglets in the study because none of the piglets fulfilled the criteria for remaining in the study (having less than 4 capsules in the stomach or no trauma in the neck area). The presence of the F4 and/or F18 receptor/s is significant for the susceptibility of a piglet to CVI-1000. However, according to Nguyen et al. 2016, flemish pigs (including TOPIG) often have these receptors, making them susceptible to F4 and F18 positive ETEC like CVI-1000 (Fairbrother et al., 2005; Nguyen et al., 2017).

Another confounding factor was premature death by euthanization before the end of the study. The piglets that died first might have been the weaker /and or more CVI-1000 infected piglets, leaving the stronger and possibly less sick piglets to survive and skew the data. Although all piglets showed significant shedding of CVI-1000 after infection, the ones that were more immune to the disease or did not significantly fall ill might have survived the longest and might have contributed to a drop of shedded bacteria in the group´s mean. However, the control group and the lower dose treatment group also had piglets dying prematurely, and the fecal shedding of ETEC was not reduced as strongly as in the high-dose treatment groups.

Even though no dose-effect could be calculated, an antimicrobial effect of the colicin treatment groups compared to the control group was observable.

In another study that examined the effect of ColE7 on O157:H7 in cattle, colicin E7-producing *E. coli* were fed to cattle which slightly lowered the bacterial count of O157:H7 in fecal shedding and the intestinal tissue (Schamberger & Diez-Gonzalez, 2005). This shows that colicins could have promising potential.

Therefore, it would be essential to conduct a new animal model study that uses colicins to reduce pathogenic *E. coli*. Various improvements should be implemented for a follow-up study, which unfortunately could not be conducted during the time of this thesis due to prohibitive costs. Generally, the colicin formulations could be optimized. One formulation was made of colicin-containing dried leaf material from *N. benthamiana*, as its production, downstream processing, and storage requirements are very cost-effective. However, prolonged treatment with this formulation would not be acceptable in the food industry due to its alkaloid content, especially nicotine and anabasine. Therefore, tobacco seeds of transgenic plants could be used for recombinant protein expression as the seeds contain much fewer alkaloids than the leaves (Abdoh & Pirelahi, 1964; Boothe et al., 2010; Prado et al., 2019). This option is currently being implemented at Nomad Bioscience GmbH.

Furthermore, bacteriocin expression optimization in edible plants could make edible plants a possible expression host in the future. As stated earlier, the colicin expression in spinach was not feasible for the protein amount needed for this study. Another alternative would be to lower or inhibit the alkaloid production in the host plant. This could be done by knocking out essential genes in the synthesis pathway of *Nicotiana* or reducing their expression by RNA interference (Hung et al., 2013; Shoji & Hashimoto, 2013). Alkaloid-free interspecific *Nicotiana* hybrids are also being developed (H. Y. Ling et al., 2012).

Purified colicin protein was used in the other formulation, as purification is an effective way to remove most of the alkaloids (Stephan et al., 2017). Even though Nomad Bioscience GmbH has developed a cost-effective purification protocol, purification is still a significant contributor to the protein´s final market price accounting for 50-80% of the production costs (Giritch et al., 2017). Therefore, reducing the purification procedure's cost is vital to make it a feasible antibacterial treatment for the animal industry (Hahn-Lobmann et al., 2019). This can undoubtedly be achieved partly by upscaling and/or finding more cost-efficient methods of removing nicotine and anabasine.

For future experiments, alternative administration methods should be applied. Even though the flexible silicon dosing gun most likely did not harm the piglets, the gavaging is stressful and potentially dangerous. This can be avoided if the colicins are incorporated into the piglets' feed. According to the CVI´s internal data, the pH in a 4-week-old piglet's stomach pH is at pH 4.5 because of constant feed intake, so even if the protein is not protected, some of it could pass through the stomach while retaining its activity. This relatively high pH value in the stomachs of piglets is also supported by literature (Knarreborg, Miquel, Granli, & Jensen, 2002). Nomad Bioscience GmbH only tested colicins' gastric degradation at a pH of 2.5 and found it to be thorough (Schulz et al., 2015).

Alternative approaches for gastric protection of proteins compatible with direct feeding include microencapsulation or micro granulation. In both cases, tiny compact spheres of the active ingredient coated with gastric protective outer layers are produced (Ma, 2014; Solanki et al., 2013). They could be formed by wet or dry granulation or freeze-drying (Ishwarya, Anandharamakrishnan, & Stapley, 2015; Rajam & Anandharamakrishnan, 2015; Virdi et al., 2019). If colicins were expressed inside crop seeds or possibly tobacco, the entire seeds could be fed and could potentially be a suitable natural protection against the acidic stomach environment for the colicins. This approach has been successfully tested in piglets for the administration of antibodies that target PWD-causing ETEC strains (Virdi et al., 2013; Virdi et al., 2019) and is currently being implemented by Nomad Bioscience GmbH as discussed above.

4.4 Salmocins

4.4.1 Overview of the new salmocins

In an NCBI database search, researchers at Nomad Bioscience identified that all of the colicin-targeted receptors (BtuB, OmpF, OmpW, OmpA, Tsx, Cir, FhuA, FepA, IutA) exist in *Salmonella*.

However, the translocation machinery in *Salmonella*, especially the TolQRA region of serovar Typhimurium that is required for translocation of group A colicins, most likely functions differently than in *E. coli* with different levels of expression and mechanisms of regulation (Lahiri, Ananthalakshmi, Nagarajan, Ray, & Chakravortty, 2011; Prouty, Van Velkinburgh, & Gunn, 2002).

ColS4 was the only group A colicin to show antimicrobial activity against *Salmonella*. ColS4 import is known to depend on the TolA translocation system. However, TonB, ExbB, and ExbD, mutants show a reduced sensitivity towards ColS4. ColS4 is also unique in that it has a duplicated receptor domain (Arnold, Zeth, & Linke, 2009).

At Nomad Bioscience GmbH, researchers predicted 6 promising colicin-like bacteriocins in *Salmonella* (SalE2, SalE3, SalE7, SalE1a, SalE1b, and SpSt) that represented 4 different activity groups (DNase, RNase, pore-forming, degradation of murein) (Table 24). As the designation of these proteins did not follow a conclusive nomenclature, the researchers changed the names to distinguish the colicin-like bacteriocins from *Salmonella* from those of *E. coli*. They were called salmocins or Sal followed by the colicin designation, with which they shared the highest homology. The activities of the salmocins were predicted based on sequence homology to colicins with known activity domain function. This work was all done outside of the work presented in this thesis.

Some of the salmocins used in this thesis may have received some attention before. The colicin-like bacteriocin in *Salmonella,* referred to as colicin E1 in literature may be similar to SalE1a and SalE1b in this thesis. To our knowledge, for colicin E1 (from *Salmonella*), the producing serovar was differentiated (Barker, 1980; Campos & Hofer, 1988), and to some extent, its inhibition activity on *E. coli* was tested (Kaldhone et al., 2019). The colicin-like bacteriocin in *Salmonella* referred to as colicin E2 in literature may be similar to SalE2 in this thesis. To our knowledge, for colicin E2 (from *Salmonella*), only the producing serovar was differentiated (Barker, 1980; Campos & Hofer, 1988).

4.4.2 Salmocin expression and yield characteristics

The expression of bacteriocins in plants can have various toxic effects on the plant tissue; therefore, the optimal harvesting time point can vary. Nevertheless, salmocins, apart from SalE1b, had low cytotoxicity on plant cells, similar to colicins (Schulz et al., 2015).

They were also very similar to colicins in their high yields in *N. benthamiana* leaves. All tested salmocins were expressed at levels of up to 1.2 - 1.8 mg/g FW (up to 18 - 47% of TSP) in syringe infiltration experiments (3.4.2.2, Table 26) (Schneider et al., 2018; Schulz et al., 2015).

When plants are inoculated by vacuum (Figure 28) and spraying (Figure 29), the cost-effectiveness and expression levels should be feasible for industrial production. However, there is room for optimization for, e.g., inoculation techniques, viral vectors, or surfactants used. As expected from the experience with colicin plant expression, the optimal harvesting time point is earliest when using syringe infiltration and is slightly later for vacuum and spraying infiltration.

Companies like Kentucky BioProcessing, Inc. (KBP) (Owensboro, KY, USA) and Medicago Inc. (Québec city QC, Canada) are already using protein plant expression in *N. benthamiana* on an industrial scale and are further working on its optimization.

The salmocins were also expressed in *Spinacia oleracea* upon agroinfiltration with syringe (3.4.2.3, Figure 30). The optimal harvesting time point was similar to *N. benthamiana*. Salmocin expression in edible plants could be an alternative to *N. benthamiana* expression in the future, allowing manufacturers to skip the costly purification step (Stephan et al., 2017).

The salmocins were also analyzed by mass spectrometry-based sequencing revealing that they are correctly expressed *in planta* and identical to their bacterial counterparts (3.4.2.5) (Schneider et al., 2018).

4.4.3 Four of the salmocins are very potent and show broad activity on 36 *Salmonella* strains

This high and broad antimicrobial activity of salmocins was unexpected because it greatly surpassed the activity range and potency of colicins vs. *E. coli*.

Salmocin antimicrobial activity was tested by soft agar overlay (2.8.1) on 36 *Salmonella* strains that encompass the most significant serotypes responsible for food poisoning in recent years (2.1.1.3).

The salmocins E1a and E1b (group 1) were active against 100% and 99%, respectively, of all tested strains (

Figure 31). They also had the highest activity of all the salmocins, with an average of 10^7 AU/µg protein (Figure 32). Therefore, it would be advisable for future salmocin mixtures to use one of the two as the main component. Due to the better purification characteristics and higher stability of SalE1b, Nomad Bioscience GmbH has mainly chosen to work with SalE1b (Nomad Bioscience internal data).

SalE1a and SalE1b are 69% identical to each other and have the highest homology with ColE1 (7.2), probably using the same translocation machinery and activity but a different receptor for cell entry (Schneider et al., 2018). This shows in the different antimicrobial potency on the serotype Kintambo: while SalE1a has good activity on it, SalE1b has no activity. Furthermore, SalE1b, out of all salmocins, has the highest antimicrobial activity on the STEC strains tested - SalE1a, on the other hand, shows no activity (Schneider et al., 2018).

SalE2 and SalE7 (group 2) were active against 94% and 95% of all strains tested, respectively, with an average activity of 10^5 AU/µg protein.

SalE3 (group 3) is active against 70% of all strains tested at an average activity of 10^2 AU/µg protein.

Spst (group 4) was only active against a few strains at an activity below 10^2 AU/µg protein under the conditions tested.

Pesticin in *E. coli* and *Yersinea* has a murein hydrolysis activity which leads to cell lysis, the activity domain very closely resembling a T4-like lysozyme (Patzer, Albrecht, Braun, & Zeth, 2012). Additionally, it only consists of 2 different domains, the N-terminal domain functioning as receptor and translocation domain (V. Braun, Helbig, Patzer, Pramanik, & Romer, 2015). As the quantity of a particular receptor type in the outer membrane can depend on nutrient availability and physiological conditions (Sibinelli-Sousa et al., 2022). It is important to note that the expression of the receptor which *E. coli* pesticin uses (FyuA) is known to be temperature-dependent (Jacobi, Gregor, Rakin, & Heesemann, 2001). These differences of pesticin to the other chosen salmocins could have all factored into its lack of antimicrobial activity on *Salmonella* in these experiments.

Nevertheless, the identities (only ~34% (7.2)) in a sequence alignment between the *Salmonella* pesticin and *E. coli* pesticin are not very high. Therefore Spst could use a different receptor and translocation machinery. Even the catalytic domain could have a different activity.

In a subsequent salmocin activity test (n=1), researchers at Nomad Bioscience GmbH tested the salmocins on 73 newly acquired *Salmonella* strains. The salmocins showed a very similar breadth and strength of activity to the 36 strains tested in this study (Schneider et al., 2018).

One explanation for the difference in activity between the individual salmocins may be found in the uniqueness of their translocation domain compared to the corresponding colicin. The translocation machinery in *Salmonella* has different levels of expression and mechanisms of regulation. Therefore, it most likely functions differently from its corresponding region in *E. coli* (Lahiri et al., 2011; Prouty et al., 2002). To ensure sufficient specificity of the novel salmocins to *Salmonella*, their translocation domains needed to have a ≤80% identity to the colicin translocation domains. However, SalE3 was an exception to this rule as its translocation domain has 95% identity to the ColE3 translocation domain. This could be an important reason for its relatively low antimicrobial activity on *Salmonella* (Schneider et al., 2018).

4.4.4 Salmocins are more potent and have a wider breadth of antimicrobial activity on *Salmonella* than colicins on *E. coli*

Salmocins, like all other colicin-like bacteriocins, have an activity spectrum mostly limited to their own species. However, salmocins had a much higher (+2-3 logs) and broader activity on *Salmonella* than colicins do on *E. coli*. Colicins have < 10^3 AU/µg average antimicrobial activity on the Big 7 STEC serotypes. Only the colicin´s activity on serotype O104:H4 (>10^5 AU/µg) was higher. This strain was responsible for a significant outbreak in Europe in 2011. Furthermore, only ColIa (92%) and ColIb (90%) offered a comparable breadth of activity on the STEC strains as the group 1 and 2 salmocins had on *Salmonella*. Therefore, to inhibit all 7 STEC strains, mixtures of 2 or more colicins should be used (Schulz et al., 2015). Data from pyocins against *Pseudomonas* show a similar range of activity as for colicins against *E. coli*. The most broadly active pyocin P5 (pore-forming) was active against 40% of tested clinical isolates (Paskevicius et al., 2017).

The salmocins described in the dissertation are the most broadly and highly active bacteriocins known today, with a single salmocin providing full control of all tested pathovars of the *Salmonella enterica* (Schneider et al., 2018) (4.4.8).

4.4.5 Salmocins are very potent in comparison to kanamycin

For a more precise quantitative evaluation of the antimicrobial activity of salmocins at low concentrations, the bacterial cell reduction of a *Salmonella* strain mixture in broth culture after treatment with individual salmocins, a salmocin-colicin mixture, and the antibiotic kanamycin were analyzed (3.4.4). Additionally, the experiment showed how the temperature and, therefore, the growth of the bacteria influences the antimicrobial activity.

At optimal growth temperature for the bacteria, 37°C, SalE1a and SalE1b were very active at low concentrations (
Figure 40). SalE1a at the high dose (0.1 µg/ml) had a nearly sterilizing effect. At a lower dose (0.01 µg/ml), it reduced cell count by ~3 logs. SalE1b reduced the cell count by more than 3 logs. SalE7 only had bacteriostatic activity compared to buffer samples.

The antimicrobial activity of the blend was very similar to the SalE1b 0.1 µg/ml treatment.

This effect can be explained by two hypotheses. The first hypothesis is that the lower concentration of SalE1b (0.03µg/ml) in the blend had an equal antimicrobial activity to the higher concentrated SalE1b treatment (0.1 µg/ml) used individually. The colicins in the blend (ColM 0.03 µg/ml, ColIb 0.01 µg/ml), therefore, had little to no effect on the activity of SalE1b or on the bacteria. The second hypothesis is that the lower concentration of SalE1b did have a smaller antimicrobial activity than the higher dosed-treatment. However, this was compensated by the synergistic or additive antimicrobial effects of the colicins in the blend.

Further experiments would be needed to evaluate the synergies and the optimal concentrations for a salmocin-colicin blend to target *Salmonella*. This could be done by conducting the checkerboard assay that assesses the fractional inhibitory concentration index (FICI), a method to determine synergistic, additive, indifferent, or antagonistic effects between 2 antimicrobials (Meletiades 2010, Tyers 2019).

At 10°C, which only allowed for very little growth of the bacteria, SalE1a and SalE1b showed very good bactericidal activity (
Figure 41). SalE1a and SalE1b most likely are pore-forming bacteriocins; hence their killing activity on the bacterial cell should also work when the cells are not or barely proliferating (Heidary et al., 2018; Mascio, Alder, & Silverman, 2007).

Similar colicin potency experiments at Nomad Bioscience GmbH at 10°C showed mostly bacteriostatic activity on STEC with very little bacteriocidal activity. For these experiments, ColM, ColE7, ColB, and Col7 were used individually or in mixtures and applied to broth cultures of O157:H7 (ATCC 35150). While the bacteriocidal effects at 37°C and 22°C, when *E. coli* cells proliferate, were strong, they were little to non-existent in cultures stored at 10°C (Nomad Bioscience internal data).

SalE7 most likely has nuclease activity and only slowly killed off bacteria at 10°C. Here the activity seems more dependent on cell proliferation.

Due to the assumptions discussed above, the killing effect of the blend at 10°C was most likely due to the pore-forming bacteriocins SalE1b and ColIb (although to a lesser degree). ColM, with its peptidoglycan degrading activity, most likely has higher activity on proliferating bacteria.

Nevertheless, the salmocin-colicin blend was able to reduce the bacterial cell count by almost 2 logs. The low dose of SalE1b (0.03 µg/ml) and the ColIb (0.01 µg/ml), both with porin activity, in a mixture with ColM (0.03 µg/ml) that it most likely not active against non-growing cells, probably made this blend not so effective at 10°C.

This experiment shows that salmocins and colicins have differing bacteriocidal effectivity on *Salmonella* at 10°C. This should be taken into account when using bacteriocin mixtures for food contamination during low-temperature processing and storage.

Kanamycin had little effect on *Salmonella*. It was used at a concentration 10x lower than the concentration used to select cells on agar. Nevertheless, the concentration used (5 µg/ml) was 50-500x higher than the salmocin concentrations on a molar basis, which highlights the potency of salmocins at very low concentrations.

The data indicate that even SalE7, the weakest broad-spectrum salmocin tested here, is about 50x more potent than kanamycin.

The weak activity of kanamycin at 10°C is probably because the blockage of protein synthesis only affects actively growing cells (Greulich, Scott, Evans, & Allen, 2015).

When these values are compared, it becomes evident that salmocins are more effective in inhibiting bacterial growth. Salmocins also have a much higher molecular weight than antibiotics. Therefore, one molecule of salmocin should be much more potent at killing a bacterial cell than one antibiotic molecule. This could help to underline the single-hit-kinetics theory, which states that 1 colicin protein is sufficient to kill a bacterial cell (Cascales et al., 2007; J. Smarda, Damborsky, J, 1991).

The activity of salmocins on *Salmonella* with a reduced growth rate and metabolism (e.g., at 10°C) is promising. Bacteria in biofilms are usually in this exact metabolic condition and are, therefore, difficult for antimicrobials to target (Nelson et al., 2012). Biofilms are a major problem in food decontamination and healthcare (Marin, Hernandiz, & Lainez, 2009; Rohatgi & Gupta, 2021). Marin et al. concluded that in poultry farms, 50% of *Salmonella* strains found were able to produce a biofilm (Marin et al., 2009).

4.4.6 **Potential applications for salmocins**

Salmocin treatment of chicken breast meat, which is among the most significant sources for salmonellosis, was very effective. This experiment demonstrated that a mixture of 7 *Salmonella* serovars could be controlled efficiently with one individual salmocin or a mixture of salmocins

with broad activity. SalE1a, SalE1b, SalE2, and SalE7 showed the most promising activity spectra.

With the salmocin treatment, bacterial reductions of ~2.2–3.4 logs CFU/g meat compared to the carrier control were observed (3.4.5.1.

Figure 42).

The most effective treatment was the low-dosed (0.6 mg/kg meat) 4-salmocin blend (SalE1a, SalE1b, SalE2, and SalE7), which achieved a reduction in CFU numbers of 2.19 logs after 48 h storage, underlining that bacteriocin mixtures can reduce the amount of salmocin needed for treatment.

The most substantial reduction (3.39 logs after 48 h storage) was achieved with the high dose salmocin blend (6 mg/kg meat). Overall, a mean CFU reduction of 98.81 to 99.96 % could be achieved with a single application of salmocins to the chicken breast meat

All treatments, except for the high dose salmocin blend, showed a limited temporal technical effect which was essential for FDA registration as a food processing aid and minimized regulatory requirements (1.3.2.4) (Sohaib et al., 2016). However, the high dose blend showed regrowth between 48 to 72h in the other independent experiments performed, indicating that it also has a limited technical effect. The limited technical effect might be a consequence of salmocin degradation or inactivation on the meat (proteolytic enzymes) (Devlieghere, Vermeiren, & Debevere, 2004) and a resulting regrowth of surviving bacteria.

There is also the possibility that the regrowth is partially due to bacteria that have developed resistance against the treatment. To verify this, further studies would be required.

Another experiment testing SalE1b treatment of contaminated skin-on chicken breast, conducted by scientists at Nomad Bioscience GmbH, showed very similar results to the experiment from this thesis (Hahn-Lobmann et al., 2019).

Beef, fish, and eggs are very important sources of salmonellosis. In our experiments, the decontamination of these food products with SalE1b was also very successful. Even at the lowest tested one-time application rate of 0.5 mg/kg (0.5 ppm), the activity of SalE1b on raw beef contaminated with *S. enterica* serotypes Enteritidis and Typhimurium had a nearly sterilizing effect when incubated at a suboptimal but permissive bacterial growth temperature of 10°C for 1-48 h (Hahn-Lobmann et al., 2019).

Salmonella is also known to grow well on seafood as it provides optimal environmental conditions (Kumar, Datta, & Lalitha, 2015). On raw tuna filet cuts, SalE1b applied at 0.5 mg/kg (0.5 ppm) reduced the viable bacteria counts by 1.7 to 3 log CFU/g showing a slightly lower activity than on beef and whole egg. Tuna and beef meats have a similar texture, but fish is

known to break down and spoil much more quickly, mainly due to different sets of enzymes and microbiota. Because of this, the cold chain for seafood is more tightly regulated than for other food products, and the average processing and handling temperatures are lower than the 10°C tested here (Odeyemi, Burke, Bolch, & Stanley, 2018).

Salmonella contamination of egg products is well known (De Cesare, 2018; McDermott et al., 2018). Similar to beef, the antimicrobial activity of SalE1b in whole egg was nearly sterilizing at the lowest application rate of 0.5 mg/kg (0.5 ppm).

These results show that salmocins can control *Salmonella* contaminations on a wide range of food products, including different physiochemical model matrices ranging from avian meat and whole egg to beef and even seafood products under market and supply chain relevant food processing and storage conditions.

Based on our studies, foods could be sprayed or temporarily dipped with salmocin solutions at application rates ranging from 0.5 to 3.0 mg/kg treated food as a postharvest intervention strategy in the food industry. Furthermore, the first indications suggest that the application rates and modalities should be cost-efficient enough for use in the highly competitive food processing industry for relevant processing times and temperatures (Hahn-Lobmann et al., 2019).

The salmocins can be applied individually or in mixtures. As shown for the salmocin treatment of chicken breast meat, mixtures were able to lower the amount of salmocin necessary for effective decontamination and could reduce the formation of bacteriocin resistance (1.3.2.4), similar to the results for the mutation rates of *E. coli* DH10B to colicins (3.1.3).

4.4.7 **The technical effect of salmocins on food matrices**

All experiments where salmocins were used for food decontamination showed that the antimicrobial effect of salmocins occurred quickly, persisted for a minimum of 48h, and then reduced until 72 h when regrowth occurred. Therefore, similar to the colicins tested at Nomad Bioscience GmbH previously, the technical effect of the salmocins was temporary with no prolonged effect on the food (Schulz et al., 2015). This and other factors were important for the salmocin microbial decontamination treatment to be classified as a food processing aid by the FDA. For this, the technical effect of a bacteriocin used for microbial decontamination must be undetectable in the final food product when it reaches the consumer (1.3.2.4). If this is the case, there is no requirement to mention food processing aids on the product label.

The use of bacteriocins in health and food should therefore produce much less unwanted resistances or secondary effects in organisms and the environment as their environmental

resilience is much lower than that of antibiotics, which are known to be very persistent (Kirchhelle, 2018; Koluman & Dikici, 2013).

4.4.8 **A possible outlook for salmocins**

Antibiotics are not suitable for food treatment to control foodborne pathogens as they enhance the selection for resistant bacteria on the foods, which are then transferred to humans (Bai et al., 2016; Verraes et al., 2013). Currently, there is a significant lack of effective treatments available to the food industry (1.2.4).

Even though some salmocins have been analyzed before, this is the first in-depth assessment of these particular recombinant salmocins (1.3.3.2). Their antimicrobial activities on pathogenic *Salmonella* were tested individually and in mixtures on agar, in broth, and on different relevant food products. Overall, the broad activity spectrum and potency of some salmocins surpassed all expectations.

This was also the first study to highlight the potential of recombinantly produced salmocins in plants. With this cost-effective method of production and their high efficiency, salmocins could play a vital role in the future treatment of MDR-*Salmonella* infections as an antibiotic alternative.

SalE1a and SalE1b had antimicrobial activity on all of the strains tested and are, therefore, the most broadly and highly active bacteriocins known today. SalE2 and E7 had activity on ~90% of those strains. The tested strains included all major enteropathogenic serotypes of *Salmonella enterica* ssp. *enterica* (2.1.1.3). The potency was much higher than for colicins against STEC strains (Schulz et al., 2015) and showed a very high antimicrobial efficiency at very low doses.

In fact, all other bacteriocins known today (colicins, klebicins, pyocins, etc.) have much lower average activity and provide lower coverage of pathogenic strains within their target species/genus (Behrens et al., 2017; Denkovskiene et al., 2019; Diez-Gonzalez, 2007; Klein et al., 2016; Schulz et al., 2015; Soltani et al., 2021).

This study further highlights a high potential for salmocins in topical *Salmonella* decontamination of meats such as chicken, beef and fish, and whole egg products (3.4.5).

The findings have been independently verified as novel and practically important by patent agencies (USPTO, WPO, EPO) that have issued broad patents in response to applications essentially based on dissertation data (AU2018239310; US11161886B2); and the U.S. regulatory agencies CFSAN/FDA and FSIS/USDA have granted a GRAS (Generally

Recognized As Safe) status for the salmocins as ‘food processing aids’ (GRN 824, 2019; FSIS Directive 7120.1, 2020).

From our data, SalE1a and SalE3 do not seem to have activity on *E. coli*. However, for SalE2, SalE7, and SalE1b, the activity on STEC strains compared to *Salmonella* was much lower. Additionally, these salmocins could also be used against some pathogenic *E. coli* (3.4.3.2).

A disadvantage of colicin-like bacteriocins to antibiotics is their lower environmental resilience, but just like purified freeze-dried colicins (Hahn-Lobmann et al., 2019), researchers at Nomad Bioscience found that purified freeze-dried salmocins maintain their activity over long storage periods (Nomad Bioscience internal data).

The full spectrum of activity of salmocins on different bacterial genera is yet to be analyzed. Nevertheless, the potentially high specificity to *Salmonella* and some *E. coli* could be seen as a disadvantage for medical applications because the pathogen must be identified for treatment. However, this characteristic also makes them much less disruptive to the gut microbiome and less likely to cause general collateral resistance (Hols et al., 2019). Broad-spectrum antibiotics therapy can significantly alter the microbiome´s composition. Mounting evidence suggests that the human microbiome, especially the gut microbiome, affects our immune system's overall function, ability to resist infection, and food processing capabilities. Disruptions in the ecology of the human microbiome, e.g., after antibiotic therapy, have been linked to diseases such as malnutrition, obesity, diabetes, and *Clostridium dificile* infections (Francino, 2015; Langdon, Crook, & Dantas, 2016).

The narrow activity spectrum of most bacteriocins can be used to precisely target the pathogenic population, minimizing collateral damage to the microbiome's homeostasis (Hols et al., 2019). Deep sequencing and metagenomics have made the identification of disease-causing pathogens faster and cheaper. Therefore, bacteriocins such as salmocins could play an essential role in future personalized medical solutions, used as a “surgical strike” targeting only the relevant pathogenic populations (Hols et al., 2019).

There are already promising developments in the field of antibiotic alternatives in human medicine. Bacteriophage or endolysin treatments are being developed and already successfully commercialized by companies such as Micreos (Netherlands) and ContraFect Corporation (US) for the treatment of skin-based *Staphylococcus* infections (e.g., eczema and acne). While some are already commercially available, others are in promising stages of clinical trials.

ContraFect even has an endolysin in phase 3 clinical trials to treat bacteremia (bloodstream infection) caused by *S. aureus*. Micreos is working on a similar product for the treatment of bloodstream infections. The approval of endolysin treatments in clinical settings should

positively impact potential future approval and use of bacteriocins. Endolysins are already successfully used against gram-positive bacteria, whereas colicin-like bacteriocins could be used against gram-negative bacteria such as *E. coli* and *Salmonella* in human medicine.

5. Summary

Resistance mutations to colicins

The overall aim of this thesis was to address the ever-increasing need for developing new antimicrobials due to the accelerated formation of antimicrobial resistance combined with a lack of new drug development.

The practical value of new antimicrobials for the treatment of pathogenic bacteria depends on the target pathogen's ability to develop resistance against them. Therefore, the calculation of the minimum inhibitory concentrations with a consecutive determination of the resistance mutation rates are essential data required to assess the potential of any said antimicrobial protein as a medicine or a food antimicrobial.

In our study, the obtained MIC for individual colicins and mixtures thereof on *E. coli* DH10B are much lower than for the antibiotic rifampicin, especially when considering the molecular weight and, therefore the number of antimicrobial molecules needed to kill a single cell. This underlines the efficiency and the single-hit kinetics of colicin molecules.

The dissertation research devoted to analyses of mutations of resistance is the first in-depth study of mixtures of colicins. As expected, mutations of resistance do occur, and the mutation rates are around or slightly higher than the mutation rates of resistance to the standard antibiotic rifampicin (10^{-7} -10^{-8} versus 10^{-9} per cell generation). Our work also demonstrates that the mutations of resistance can be drastically reduced (to well below 10^{-12} per cell generation) by employing mixtures of colicins.

Resistance to ColM was caused at equal rates by mutations in the receptor and translocation machinery genes. This knowledge is important for future studies that will broaden our understanding of the primary and secondary machineries employed by bacteriocins to gain entry into the cell periplasm.

The other study within the dissertation describes the engineering of active chimeric bacteriocins that combine cytotoxic domains with different mechanisms of cell entry. Demonstrated functionality of multiple chimeras generated provides another theoretical way of controlling mutations of resistance, namely the use of mixtures of natural bacteriocins with chimeras that employ different machinery for cell entry than the natural bacteriocin.

Domain swapping and bacteriocin chimeras

The mining of novel antimicrobial molecules continues, but the total number may not be infinite. Among the already characterized colicin-like bacteriocins cloned from genomes of

Gram-negative bacteria, only a dozen or so demonstrate high and broad efficacy, usually within the same species or the same genus.

Furthermore, only two types of bacteriocins, namely pore-forming and peptidoglycan-inhibiting bacteriocins, show the best properties, whereas bacteriocins employing RNAse or DNAse cytotoxicity mode of action turned out to be very narrowly specific. Given a similar molecular architecture of colicin-like bacteriocins, namely their modular design that comprises receptor-binding, translocation, and cytotoxicity domains, the simple other way of producing novel bacteriocins would be engineering by swapping domains coming from different natural bacteriocins.

The theoretical number of combinations of chimeras containing domains of natural bacteriocins easily exceeds the number of natural molecules by at least an order of magnitude as it includes at least 6 types of receptor, at least 12 translocator, and over 20 cytotoxicity domains. Our study of engineered colicin and salmocin chimeras is the most extensive attempt to generate and analyze colicin-like protein chimeras through domain swapping to date. We have limited the scope of our engineering by working mainly with porin- and peptidoglycan-inhibiting colicins (except for ColE3) and by testing only combinations in which receptor and translocator domains of the parental molecule were kept together. We also limited our study by using only cytotoxicity domains of the most potent colicins (ColM, ColIb, ColU) and the highly active domain of salmocin E1b (SalE1b). Engineering novel molecules by attaching receptor/translocation coming from ColA, ColE1, ColE3, ColIa, ColK, ColM, or ColN, we successfully expressed 25 novel bacteriocin chimeras, with 14 of them showing significant activity on the tested *E. coli* DH10B strain.

Several observations and conclusions have been made from this essentially pioneering research. First, many chimeric proteins are fully functional, although the best of them are less active and more narrowly active than the parental form that contributed the cytotoxicity domain. Surprisingly, several intergeneric chimeras with salmocin SalE1b cytotoxicity domain turned out to be functional.

In further experiments, researchers at Nomad Bioscience discovered that some mixtures of natural bacteriocins with chimeras that employ different machinery for cell entry than the natural bacteriocin have lower rates of resistance mutations. Furthermore, in experiments with *Pseudomonas* bacteriocin chimeras conducted in the Lithuanian subsidiary of Nomad Bioscience GmbH, some of the chimeras demonstrated a broader spectrum of activity than parental molecules due to lack of recognition of the new cytotoxicity domain by the immunity protein.

Obviously, experiments with chimeras are promising and should be pursued further, probably in a high throughput format, with much more attention to the area of domain fusion. Also, chimeras combining heterologous receptor and translocation domains should be investigated.

Colicin activity against Post-Weaning Diarrhea (PWD) in an animal study

One of these economically significant infections in animal husbandry is post-weaning diarrhea (PWD), an enteric disease that significantly impacts the growth performance of piglets and can be lethal.

The use of antibiotics in animal husbandry has been almost completely banned for all routine use in the EU. Therefore, alternative antimicrobials for PWD treatment are urgently needed.

While colicins have shown great *in vitro* capabilities, they have only seldomly been tested *in vivo*. Thus, the feasibility of colicin mixtures for the prevention of PWD was tested in an animal study.

The study was performed at the Central Veterinary Institute (CVI) of the Wageningen University in the Netherlands, where the colicins were administered orally by capsule to piglets challenged with a PWD-causing ETEC strain. However, the study was terminated early due to health problems in the test subjects, which resulted in too many confounding factors. In the end, the study did not yield the full package of data we expected, and the results can only be used as an indication for the effectiveness of using a colicin treatment for PWD in an animal model.

***Salmonella* bacteriocins salmocins**

Salmonella enterica causes an estimated 1 million foodborne illnesses each year in the United States alone, resulting in 19,000 hospitalizations, is the leading cause of death (380 deaths per year), and is one of the four major global causes of diarrhoeal diseases.

The use of antibiotics for prophylactic and growth-promoting purposes in animal husbandry has been banned in many countries. Additionally, antibiotics are not suitable for food treatment to control foodborne pathogens as they enhance the selection for resistant bacteria on the foods, which are then transferred to humans. Thus, there is a significant lack of effective treatments available to the food industry.

Much attention has been given to colicins, natural non-antibiotic proteins of the bacteriocin class, to control the related pathogen *Escherichia coli*. We have searched *Salmonella* genomic databases for colicin analogs and cloned, expressed in plants at very high levels, and characterized six such proteins, which we named salmocins.

Among those, salmocins SalE1a and SalE1b were found to possess broad antimicrobial activity against all 99 major *Salmonella* pathovars. Each of the two salmocins also showed remarkably high average potency (>10^6 AU/μg recombinant protein, or >10^3 higher than the best colicins) against major pathogenic target strains. In fact, salmocins described in the dissertation are the most broadly and highly active bacteriocins known today, with a single salmocin providing full control of all tested pathovars of the *Salmonella enterica*. All other bacteriocins known today (colicins, klebicins, pyocins, etc.) have much lower average activity and provide lower coverage of pathogenic strains within its target species/genus. Treatment of poultry meat matrices contaminated with seven major pathogenic serovars confirmed the efficacy of salmocins as a food safety intervention against *Salmonella*.

Novelty and scientific, as well as practical relevance of the dissertation chapter dedicated to the discovery and characterization of Salmonella bacteriocins, have been confirmed by the publication of the results by a highly rated peer-reviewed journal Nature Scientific Reports; the findings have been independently verified as novel and practically important by patent agencies (USPTO, WPO, EPO) that have issued broad patents in response to applications essentially based on dissertation data (AU2018239310; US11161886B2); and the U.S. regulatory agencies CFSAN/FDA and FSIS/USDA have granted a GRAS (Generally Recognized As Safe) status for the salmocins as ‘food processing aids’ (GRN 824, 2019; FSIS Directive 7120.1, 2020).

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7. Appendix

7.1 Plasmids and oligonucleotides

7.1.1 Plasmids

7.1.1.1 Colicin expression plasmids

Used for the amplification of the legocin-modules 3.2, for assessment of colicin activity on the strain CVI-1000 3.3.2, and for colicin production for the animal study 3.3. The colicins were expressed in TMV-based constructs, whereas immunity proteins were expressed in PVX-based constructs (1.5, Figure 2).

Table 28: Plant expression constructs for colicins

plant expression constructs for colicins			
plasmid pNMD	for expression of	use	optimal dpi
15511	ColE2	Expression constructs for colicins (TMV-based) and immunity proteins (PVX-based) in *N. benthamiana*	6
15521	ColE3		5
25880	ColE5		6
16121	ColE6		6
8802	ColE7		5
25891	ColE8		5
25901	ColE9		6
19162	ColD		7
15351	DF13		5
25831	ColA		6
3680	ColN		5
25856	ColS4		5
15252	ColK		7
15311	Col5		8
25848	Col10		7
15271	ColU		8
25813	ColR		6
25871	Col28b		5
25826	ColY		5
15291	ColB		8
19141	ColIa		6
25861	ColIb		5
10221	ColM		6
15231	ImmE2		6
25911	ImmE5		6

16141	ImmE6		6
9060	ImmE7		5
25921	ImmE8		5
25931	ImmE9		6
19183	ImmD		7
15371	ImmDF13		5

7.1.1.2 Salmocin expression plasmids

Table 29: Plant expression constructs for salmocins
Nuclease salmocins were synthesized with a Cat1 intron (from *Ricinus communis* cat1 gene for catalase CAT1 (GenBank #D21161.1, base pairs 679-867)) The nucleotide sequences for the salmocins and their respective immunity proteins were codon-optimized for *N. benthamiana* and synthesized by Thermo Fischer Scientific.

Plant expression constructs for the salmocins		
plasmid	characteristics	use
pNMD28150	SalE3-cat1 (G554) (GenBank: GAS18013.1) from S. enterica with ATG; Cloning: pICH29912 (v, BsaI) (kanamycin), pNMD0337 (i, BsaI) (amp)	TMV-based vector for *Agrobacterium* mediated expression of SalE3
pNMD28160	SalE2-cat1 (V510) (GenBank: KTM78572.1) from *S. enterica* with ATG; Cloning: pICH29912 (v, BsaI) (kanamycin), pNMD0338 (i, BsaI) (amp)	TMV-based vector for *Agrobacterium* mediated expression of SalE2
pNMD28170	SallE7-cat1 (V532) (GenBank: KSU39545.1) from *S. enterica* with ATG; Cloning: pICH29912 (v, BsaI) (kanamycin), pNMD0339 (i, BsaI) (amp)	TMV-based vector for *Agrobacterium* mediated expression of SalE7
pNMD28180	Spst (GenBank: ESF65298.1) from *S. enterica* with ATG. Cloning; pICH29912 (v, BsaI) (kanamycin), pNMD0340 (i, BsaI) (amp)	TMV-based vector for *Agrobacterium* mediated expression of Spst
pNMD28190	SalE1a (GenBank: KRG27003.1) from *S. enterica* with ATG. Cloning; pICH29912 (v, BsaI) (kanamycin), pNMD0341 (i, BsaI) (amp)	TMV-based vector for *Agrobacterium* mediated expression of SalE1a
pNMD28200	SalE1b (GenBank: KRG25604.1) from *S. enterica* with ATG; Cloning: pICH29912 (v, BsaI) (kanamycin), pNMD0342 (i, BsaI) (amp)	TMV-based vector for *Agrobacterium* mediated expression of SalE1b

Table 30: Plant expression constructs for salmocins

Plant expression constructs for the Salmocin immunity proteins		
plasmid	characteristics	use
pNMD28210	SImmE3 (GenBank: GAS18012.1) from *S. enterica* with ATG; Cloning: pNMD0674 (v, BsaI) (kanamycin), pNMD0343 (i, BsaI) (amp)	PVX-based vector for *Agrobacterium* mediated expression of SImmE3
pNMD28220	SImmE2 (GenBank: KTM78571.1) from *S. enterica* with ATG; Cloning: pNMD0674 (v, BsaI) (kanamycin), pNMD0343 (i, BsaI) (amp)	PVX-based vector for *Agrobacterium* mediated expression of SImmE3

pNMD28230	SImmE7 (GenBank: KSU39546.1) from *S. enterica* with ATG; Cloning: pNMD0674 (v, BsaI) (kanamycin), pNMD0343 (i, BsaI) (amp)	PVX-based vector for *Agrobacterium* mediated expression of SImmE3

7.1.1.3 Legocin expression plasmids

Table 31: Intermediate vectors to assemble legocin plant expression constructs with ColIb and ColM activity domain

Intermediate vectors to assemble legocin plant expression constructs with ColIb and ColM activity domains		
plasmid	characteristics	use
pNMD45710	aa1-aa337 (T+R domain) of ColE1 (Genbank: AAA87379.1) for cloning with activity domain of ColIb (GenBank: AAA23188.1) in pNMD45850; PCR colE1_Ib_fwd/colE1_Ib_rev (1038bp) on pNMD086 (i), pIch41021 (v, SmaI)	intermediate vectors for legocin expression construct cloning
pNMD45720	aa1-aa337 (T+R domain) of ColE1 (Genbank: AAA87379.1) for cloning with activity domain of ColM (GenBank: AAA23589.1) in pNMD45770; PCR colE1_M_fwd/colE1_M_rev (1038bp) on pNMD086 (i); pIch41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45730	aa1-aa182 (T+R domain) of ColN (UniProtKB/Swiss-Prot: P08083.1) for cloning with activity domain of ColIb (GenBank: AAA23188.1) in pNMD45850; PCR colN_Ib_fwd/colN_Ib_rev (573bp) on pNMD089 (i); pIch41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45740	aa1-aa182 (T+R domain) of ColN (UniProtKB/Swiss-Prot: P08083.1) for cloning with activity domain of ColM (GenBank: AAA23589.1) in pNMD45770; PCR colN_M_fwd/colN_M_rev (573bp) on pNMD089 (i); pIch41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45750	aa1-aa123 (T+R domain) of ColM (GenBank: AAA23589.1) for cloning with activity domain of ColIb (GenBank: AAA23188.1) in pNMD45850; PCR colM_Ib_fwd/colM_Ib_rev (586bp) on pNMD0134 (i); pIch41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45770	aa124-aa271 (activity domain) of ColM (GenBank: AAA23589.1); PCR colM_act_fwd/colM_act_rev (473bp) on pNMD0134 (i); pIch41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45780	aa1-aa368 (T+R domain) of ColK (UniProtKB/Swiss-Prot: Q47502.1) for cloning with activity domain of ColIb (GenBank: AAA23188.1) in pNMD45850; PCR colK_Ib_fwd/colK_Ib_rev (1131bp) on pNMD0196 (i); pIch41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45790	aa1-aa368 (T+R domain) of ColK (UniProtKB/Swiss-Prot: Q47502.1) for cloning with activity domain of ColM (GenBank: AAA23589.1) in pNMD45770; PCR colK_M_fwd/colK_M_rev (1131bp) on pNMD0196 (i); pIch41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45800	aa1-aa368 (T+R domain) of ColK (UniProtKB/Swiss-Prot: Q47502.1) for cloning with activity domain of SalEIb (GenBank: KRG25604.1) in pNMD45860; PCR colE1_Ib_fwd/colE1_Ib_rev (1038bp) on pNMD086 (i), pIch41021 (v, SmaI) (v) (Antibiotica)	intermediate vectors for legocin expression construct cloning

pNMD45810	aa1-aa449 (T+R domain) of ColIa (NCBI ref seq: WP_001283344.1) for cloning with activity domain of ColM (GenBank: AAA23589.1) in pNMD45770; PCR colIa_M_fwd/colIa_M_rev (1374bp) on pNMD0263 (i); pICh41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45830	aa1-aa388 (T+R domain) of ColA (UniProtKB/SwissProt: P04480.1) for cloning with activity domain of ColIb (GenBank: AAA23188.1); PCR colA_Ib_fwd/colA_Ib_rev (1191bp) on pNMD0299 (i); pICh41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45840	aa1-aa388 (T+R domain) of ColA (UniProtKB/SwissProt: P04480.1) for cloning with activity domain of ColM (GenBank: AAA23589.1) in pNMD45770; PCR colA_M_fwd/colA_M_rev (1191bp) on pNMD0299 (i); pICh41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45850	aa450-aa626 (activity domain) of ColIb (GenBank: AAA23188.1); PCR colIb_act_fwd/colIb_act_rev (560bp) on pNMD0299 (i); pICh41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45870	aa1-aa450 (T+R domain) of ColE3 (GenBank: AAA88416.1) for cloning with with (activity domain) of ColIb (GenBank: AAA23188.1) in pNMD45850; PCR colE3_Ib_fwd/colE3_Ib_rev (1377bp) on pNMD4541 (i); pICh41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning
pNMD45880	aa1-aa450 (T+R domain) of ColE3 (GenBank: AAA88416.1) for cloning with with aa124-aa271 (activity domain) of ColM (GenBank: AAA23589.1) in pNMD45770; PCR colE3_M_fwd/colE3_M_rev (1377bp) on pNMD4541 (i); pICh41021 (v, SmaI) (Antibiotica)	intermediate vectors for legocin expression construct cloning

Table 32: Plant expression constructs for legocins with ColIb and ColM activity domains

Plant expression constructs for legocins with ColIb and ColM activity domains		
plasmid	characteristics	use
pNMD45900	aa1-aa337 (T+R domain) of colicin E1 (Genbank: AAA87379.1) with aa450-aa626 (activity domain) of colicin Ib (GenBank: AAA23188.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45710 (i), pNMD45850 (i)	Plant expression contruct for legocin (ColE1-ColIb)
pNMD45910	aa1-aa337 (T+R domain) of colicin E1 (Genbank: AAA87379.1) with aa124-aa271 (activity domain) of colicin M (GenBank: AAA23589.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45720 (i), pNMD45770 (i)	Plant expression contruct for legocin (ColE1-ColM)
pNMD45920	aa1-aa182 (T+R domain) of colicin N (UniProtKB/Swiss-Prot: P08083.1) with aa450-aa626 (activity domain) of colicin Ib (GenBank: AAA23188.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45730 (i), pNMD45850 (i)	Plant expression contruct for legocin (ColN-ColIb)
pNMD45930	aa1-aa182 (T+R domain) of colicin N (UniProtKB/Swiss-Prot: P08083.1) with aa124-aa271 (activity domain) of colicin M (GenBank: AAA23589.1) in assembled TMV vector (Cytosol);MoClo (BsaI): pICH29912 (v), pNMD45740 (i), pNMD45770 (i)	Plant expression contruct for legocin (ColN-ColM)

pNMD45940	aa1-aa123 (T+R domain) of colicin M (GenBank: AAA23589.1) with aa450-aa626 (activity domain) of colicin Ib (GenBank: AAA23188.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45750 (i), pNMD45850 (i)	Plant expression contruct for legocin (ColM-ColIb)
pNMD45960	aa1-aa368 T+R domain) of colicin K (UniProtKB/Swiss-Prot: Q47502.1) with aa450-aa626 (activity domain) of colicin Ib (GenBank: AAA23188.1) in assembled TMV vector (Cytosol);MoClo (BsaI): pICH29912 (v), pNMD45780 (i), pNMD45850 (i)	Plant expression contruct for legocin (ColK-ColIb)
pNMD45970	aa1-aa368 (T+R domain) of colicin K (UniProtKB/Swiss-Prot: Q47502.1) with aa124-aa271 (activity domain) of colicin M (GenBank: AAA23589.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45790 (i), pNMD45770 (i)	Plant expression contruct for legocin (ColK-ColM)
pNMD45990	aa1-aa449 (T+R domain) of colicin Ia (NCBI ref seq: WP_001283344.1) with aa124-aa271 (activity domain) of colicin M (GenBank: AAA23589.1) in assembled TMV vector (Cytosol);MoClo (BsaI): pICH29912 (v), pNMD45810 (i), pNMD45770 (i)	Plant expression contruct for legocin (ColIa-ColM)
pNMD46010	aa1-aa388 (T+R domain) of colicin A (UniProtKB/SwissProt: P04480.1) with aa450-aa626 (activity domain) of colicin Ib (GenBank: AAA23188.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45830 (i), pNMD45850 (i)	Plant expression contruct for legocin (ColA-ColIb)
pNMD46020	aa1-aa388 (T+R domain) of colicin A (UniProtKB/SwissProt: P04480.1) with aa124-aa271 (activity domain) of colicin M (GenBank: AAA23589.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45840 (i), pNMD45770 (i)	Plant expression contruct for legocin (ColA-ColM)
pNMD46030	aa1-aa450 (T+R domain) of colicin E3 (GenBank: AAA88416.1) with aa450-aa626 (activity domain) of colicin Ib (GenBank: AAA23188.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45870 (i), pNMD45850 (i)	Plant expression contruct for legocin (ColE3-ColIb)
pNMD46040	aa1-aa450 (T+R domain) of colicin E3 (GenBank: AAA88416.1) with aa124-aa271 (activity domain) of colicin M (GenBank: AAA23589.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45880 (i), pNMD45770 (i)	Plant expression contruct for legocin (ColE3-ColM)

Table 33: Intermediate vectors to assemble legocin plant expression constructs with the ColU activity domain

Intermediate vectors to assemble legocin plant expression constructs with the ColU activity domain		
plasmid	characteristics	use
pNMD47290	aa417-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) for cloning together with colicin T+R domains, adding 1 Lysin in between to fit with T+R domains originally amplified for colicin Ib activity domain fusion; PCR colU_act_fwd/colU_act_rev (641bp) on pNMD0197 (i); pIch41021 (SmaI) (v)	intermediate vectors for lecocin expression construct cloning
pNMD47300	aa417-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) for cloning together with colicin T+R domains adding 1 Lysin and 1 Serine in between to fit with T+R domains originally amplified for colicin M activity domain fusion; PCR colU_M_act_fwd/colU_M_act_rev (644bp) on pNMD0197 (i); pIch41021 (SmaI) (v)	intermediate vectors for lecocin expression construct cloning

pNMD47310	aa387-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) for BsaI cloning together with colicin K T+R domains (+30aa of ColU receptor domain); PCR ColU_K_act_fwd/colU_M_act_rev (728bp) on pNMD0197 (i); pIch41021 (SmaI) (v)	intermediate vectors for lecocin expression construct cloning
pNMD47320	aa1-aa398 (T+R domain +30aa of activity domain) of colicin K (UniProtKB/Swiss-Prot: Q47502.1) for cloning together with Colicin U activity domain (+30 last aa of ColU receptor domain); PCR colK_Ib_fwd/ColK_U_rev (1221bp) on pNMD0196 (i); pIch41021 (SmaI) (v)	intermediate vectors for lecocin expression construct cloning

Table 34: Plant expression constructs for legocins with the ColU activity domain

Plant expression constructs for legocins with the ColU activity domain		
plasmid	characteristics	use
pNMD48101	aa1-aa337 (T+R domain) of colicin E1 (Genbank: AAA87379.1) with aa417-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45710 (i), pNMD47290(i)	Plant expression contruct for legocin (ColE1-ColU)
pNMD48111	aa1-aa182 (T+R domain) of colicin N (UniProtKB/Swiss-Prot: P08083.1) with aa417-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45730 (i), pNMD47290 (i)	Plant expression contruct for legocin (ColN-ColU)
pNMD48121	aa1-aa368 (T+R domain) of colicin K (UniProtKB/Swiss-Prot: Q47502.1) with aa417-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45780 (i), pNMD45290 (i)	Plant expression contruct for legocin (ColK-ColU)
pNMD48131	aa1-aa388 (T+R domain) of colicin A (UniProtKB/SwissProt: P04480.1) with aa417-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) in assembled TMV vector (Cytosol);MoClo (BsaI): pICH29912 (v), pNMD45830 (i), pNMD47290 (i)	Plant expression contruct for legocin (ColA-ColU)
pNMD48141	aa1-aa337 (T+R domain) of colicin E1 (Genbank: AAA87379.1) with aa417-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45720 (i), pNMD47300 (i)	Plant expression contruct for legocin (ColE1-ColU)
pNMD48151	aa1-aa182 (T+R domain) of colicin N (UniProtKB/Swiss-Prot: P08083.1) with aa417-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45740 (i), pNMD47300 (i)	Plant expression contruct for legocin (ColN-ColU)
pNMD48161	aa1-aa368 (T+R domain) of colicin K (UniProtKB/Swiss-Prot: Q47502.1) with aa417-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45790 (i), pNMD47300 (i)	Plant expression contruct for legocin (ColK-ColU)
pNMD48172	aa1-aa449 (T+R domain) of colicin Ia (NCBI ref seq: WP_001283344.1) with aa417-aa619 (activity domain) of Colicin U (GenBank: CAA72509.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45810 (i), pNMD47300 (i)	Plant expression contruct for legocin (ColIa-ColU)
pNMD48182	aa1-aa388 (T+R domain) of colicin A (UniProtKB/SwissProt: P04480.1) with aa417-aa619 (activity domain) of colicin U (GenBank: CAA72509.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45840 (i), pNMD47300 (i)	Plant expression contruct for legocin (ColA-ColU)

pNMD48250	aa1-aa398 (T+R domain + 30aa of activity domain) of colicin K (UniProtKB/Swiss-Prot: Q47502.1) with aa387-aa619 (activity domain + 30 last aa of receptor domain) of colicin U (GenBank: CAA72509.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD47320 (i), pNMD47310 (i)	Plant expression contruct for legocin (ColK+30-ColU-30)

Table 35: Intermediate vectors to assemble legocin plant expression constructs with the SalE1b activity domain

Intermediate vectors to assemble legocin plant expression constructs with the SalE1b activity domain		
plasmid	characteristics	use
pNMD45760	aa1-aa123 (T+R domain) of colicin M (GenBank: AAA23589.1) for cloning with with activity domain of salmocin EIb (GenBank: KRG25604.1) in pNMD45860; PCR colM_salE1b_fwd/colM_salE1b_rev (586bp) on pNMD0134 (i); pIch41021 (SmaI) (v)	intermediate vectors for lecocin expression construct cloning
pNMD45800	aa1-aa368 (T+R domain) of colicin K (UniProtKB/Swiss-Prot: Q47502.1) for cloning with with activity domain of salmocin EIb (GenBank: KRG25604.1) in pNMD45860; PCR colK_salE1b_fwd/colK_salE1b_rev (1131bp) on pNMD0196 (i); pIch41021 (SmaI) (v)	intermediate vectors for lecocin expression construct cloning
pNMD45820	aa1-aa449 (T+R domain) of colicin Ia (NCBI ref seq: WP_001283344.1) for cloning with with activity domain of salmocin EIb (GenBank: KRG25604.1) in pNMD45860; PCR colIa_salE1b_fwd/colIa_salE1b_rev (1374bp) on pNMD0263 (i); pICH41021 (SmaI) (v)	intermediate vectors for lecocin expression construct cloning
pNMD45860	aa343-aa527 activity domain of salmocin EIb (GenBank: KRG25604.1); PCR SalmEIb_act_fwd/SalmEIb_act_rev (584bp) on pNMD0342 (i); pICH41021 (SmaI) (v)	intermediate vectors for lecocin expression construct cloning
pNMD45890	aa1-aa450 (T+R domain) of colicin E3 (GenBank: AAA88416.1) for cloning with with activity domain of salmocin EIb (GenBank: KRG25604.1) in pNMD45860; PCR colE3_salE1b_fwd/colE3_salE1b_rev (1377bp) on pNMD4541 (i); pICH41021 (SmaI) (v)	intermediate vectors for lecocin expression construct cloning
pNMD48270	aa1-aa416 (T+R domain) of colicin U (GenBank: CAA72509.1) for cloning with with activity domain of salmocin EIb (GenBank: KRG25604.1) in pNMD45860; PCR colU_E1b_fwd/colU_E1b_rev (1275bp) on pNMD0197(i); pICH41021 (SmaI) (v)	intermediate vectors for lecocin expression construct cloning

Table 36: Plant expression constructs for legocins with the SalE1b activity domain

Plant expression constructs for legocins with the SalE1b activity domain		
plasmid	characteristics	use
pNMD48280	aa1-aa416 (T+R domain) of colicin U (GenBank: CAA72509.1) with aa387-aa619 (activity domain) of Salmocin E1b (GenBank: KRG25604.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD48270 (i), pNMD45860 (i)	Plant expression contruct for legocin (ColU-SalE1b)
pNMD45980	aa1-aa368 (T+R domain) of colicin K (UniProtKB/Swiss-Prot: Q47502.1) with aa343-aa527 (activity domain) of salmocin EIb (GenBank: KRG25604.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45800 (i), pNMD45860 (i)	Plant expression contruct for legocin (ColK-SalE1b)

pNMD46000	aa1-aa449 (T+R domain) of colicin Ia (NCBI ref seq: WP_001283344.1) with aa343-aa527 (activity domain) of salmocin EIb (GenBank: KRG25604.1) in assembled TMV vector (Cytosol); MoClo (BsaI): pICH29912 (v), pNMD45820 (i), pNMD45860 (i)	Plant expression contruct for legocin (ColIa-SalE1b)

7.1.1.4 Other plasmids

pNMD0319:

Vector for constitutive (intermediate strength promoter) expression in *E. coli*

pSF-OXB12-derivative (Oxford Genetics/Sigma)

OBX12 promoter – RBS – SV40 polyA/RrnG terminator

pNMD41440

fhuA expression plasmid

pNMD26041 (a derivative of pNMD0319) (BpiI, v)

OBX12 promoter – RBS – *fhuA* ORF (*E. coli* DH10B) – SV40 polyA/RrnG terminator

pICH41021

Intermediate cloning vector for blunt-ended cloning

ampicillin-resistance, lacZ Gen

7.1.2 **Oligonucleotides**

Table 37: Oligonucleotides

	No.	Name	Sequence (5´ - 3´)	Description
Sequencing of DH10B colicin resistant mutants to verify identity (3.1.4)	1	fP2	ACGGCTACCTTGTTACGACTT	degenerated primer for prokaryote 16S PCR amplification (fwd)
	2	fD1	AGAGTTTGATCCTGGCTCAG	degenerated primer for prokaryote 16S PCR amplification (rev)
	3	EUB800	CCAGGGTATCTAATCCTG	degenerated primer for prokaryotes 16S PCR amplificate sequencing (fwd)
	4	Eco1392	GTGACGGGCGGTGTGTAC	degenerated primer for prokaryotes 16S PCR amplificate sequencing (rev)
	5	K12L	TTCCCACGGACATGAAGACTACA	degenerated primer for prokaryotes 16S PCR amplificate analysis by nested PCR (fwd)
	6	K12R	ACTCTGCGCACCAATCAACAA	degenerated primer for prokaryotes 16S PCR amplificate analysis by nested PCR (fwd)
legocin TR and A-	7	colE1_Ib_fwd	TTTTGGTCTCTCATGGAAACCGCAGTTGCCTATTATAAGG	ColE1 TR-module amplification for legocin cloning with ColIb A-module using BsaI

module amplification	8	colE1_Ib_rev	TTTTGGTCTCACCTTCTGGGCTTTCTTCAGATTTTCCTC	ColE1 TR-module amplification for legocin cloning with ColIb A-module using BsaI
	9	colE1_M_fwd	TTTTGGTCTCTCATGGAAACCGCAGTTGCCTATTATAAGG	ColE1 TR-module amplification for legocin cloning with ColM A-module using BsaI
	10	colE1_M_rev	TTTTGGTCTCATACCCTGGGCTTTCTTCAGATTTTCCTCTGCC	ColE1 TR-module amplification for legocin cloning with ColM A-module using BsaI
	11	colE3_Ib_fwd	TTTTGGTCTCTCATGTCAGGTGGGGATGGGAGAGGCC	ColE3 TR-module amplification for legocin cloning with ColIb A-module using BsaI
	12	colE3_Ib_rev	TTTTGGTCTCACCTTCTTCTCGTCATTCAAGTTGTTCTCTGC	ColE3 TR-module amplification for legocin cloning with ColIb A-module using BsaI
	13	colE3_M_fwd	TTTTGGTCTCTCATGTCAGGTGGGGATGGGAGAGGCC	ColE3 TR-module amplification for legocin cloning with ColM A-module using BsaI
	14	colE3_M_rev	TTTTGGTCTCATACCCTTCTCGTCATTCAAGTTGTTCTCTGC	ColE3 TR-module amplification for legocin cloning with ColM A-module using BsaI
	15	colA_Ib_fwd	TTTTGGTCTCTCATGCCTGGTTTCAACTACGGTGGTAAGG	ColA TR-module amplification for legocin cloning with ColIb A-module using BsaI
	16	colA_Ib_rev	TTTTGGTCTCACCTTTTCCATAGCTTGCCTCTGTCTCTCAGC	ColA TR-module amplification for legocin cloning with ColIb A-module using BsaI
	17	colA_M_fwd	TTTTGGTCTCTCATGCCTGGTTTCAACTACGGTGGTAAGGG	ColA TR-module amplification for legocin cloning with ColM A-module using BsaI
	18	colA_M_rev	TTTTGGTCTCATACCTTCCATAGCTTGCCTCTGTCTCTCAGCC	ColA TR-module amplification for legocin cloning with ColM A-module using BsaI
	19	colN_Ib_fwd	TTTTGGTCTCTCATGGGATCCAACGGTGCCGATAACG	ColN TR-module amplification for legocin cloning with ColIb A-module using BsaI
	20	colN_Ib_rev	TTTTGGTCTCACCTTTAATAGAAATTGAGAAACAAATCGC	ColN TR-module amplification for legocin cloning with ColIb A-module using BsaI
	21	colN_M_fwd	TTTTGGTCTCTCATGGGATCCAACGGTGCCGATAACGC	ColN TR-module amplification for legocin cloning with ColIb M-module using BsaI
	22	colN_M_rev	TTTTGGTCTCATACCTAATAGAAATTGAGAAACAAATCGC	ColN TR-module amplification for legocin cloning with ColIb M-module using BsaI
	23	colK_Ib_fwd	TTTTGGTCTCTCATGGCTAAAGAGCTGTCTGGTTACGGTCC	ColK TR-module amplification for legocin cloning with ColIb A-module using BsaI
	24	colK_Ib_rev	TTTTGGTCTCACCTTCTTCTCAAGAGCATCCTGAGCCTC	ColK TR-module amplification for legocin cloning with ColIb A-module using BsaI
	25	colK_M_fwd	TTTTGGTCTCTCATGGCTAAAGAGCTGTCTGGTTACGG	ColK TR-module amplification for legocin cloning with ColM A-module using BsaI
	26	colK_M_rev	TTTTGGTCTCATACCCTTCTCAAGAGCATCCTGAGCCTC	ColK TR-module amplification for legocin cloning with ColM A-module using BsaI
	27	colIa_Ib_fwd	TTTTGGTCTCTCATGAGTGACCCGGTACGCATTACTAATCC	ColIa TR-module amplification for legocin cloning with ColIb A-module using BsaI
	28	colIa_Ib_rev	TTTTGGTCTCACCTTTGTCGCTTTTAACTCATCCTGTTTCC	ColIa TR-module amplification for legocin cloning with ColIb A-module using BsaI
	29	colIa_M_fwd	TTTTGGTCTCTCATGAGTGACCCGGTACGCATTACTAATCCC	ColIa TR-module amplification for legocin cloning with ColM A-module using BsaI
	30	colIa_M_rev	TTTTGGTCTCATACCTGTCGCTTTTAACTCATCCTGTTTCC	ColIa TR-module amplification for legocin cloning with ColM A-module using BsaI

	31	colM_Ib_fwd	TTTTGGTCTCTCATGGAAACCCTTACTGTGCACGCTCC	ColM TR-module amplification for legocin cloning with ColIb A-module using BsaI
	32	colM_Ib_rev	TTTTGGTCTCACCTTGCTCATCTGCTTCATGTTCATGGATCT	ColM TR-module amplification for legocin cloning with ColIb A-module using BsaI
	33	colM_act_rev	TTTTGGTCTCTGGTAACGTGACCACCCCTATTGTGGC	colicin domain amplification for legocin cloning with BsaI
	34	colM_act_fwd	TTTTGGTCTCAAAGCTTACCTCTTACCAGACTCTTTGATGTGG	colicin domain amplification for legocin cloning with BsaI
	35	colIb_act_fwd	TTTTGGTCTCTAAGGATGCTATCAAGCTGACCAGC	colicin domain amplification for legocin cloning with BsaI
	36	colIb_act_rev	TTTTGGTCTCAAAGCTTAGATACCGATAAGCTTGTTCACC	colicin domain amplification for legocin cloning with BsaI
	37	SalmEIb_act_fwd	TTTTGGTCTCTGATAACCTGAACAGCAGCCAGATGAAGAACG	salmocin domain amplification for legocin cloning with BsaI
	38	SalmEIb_act_rev	TTTTGGTCTCAAAGCTTAGATTCCCAGTGCCTCGTTCAGC	salmocin domain amplification for legocin cloning with BsaI
	39	colE3_salE1b_fwd	TTTTGGTCTCTCATGTCAGGTGGGGATGGGAGAGGCCAC	ColE3 TR-module amplification for legocin cloning with SalE1b A-module using BsaI
	40	colE3_salE1b_rev	TTTTGGTCTCATATCCTTCTCGTCATTCAAGTTGTTCTCTGC	ColE3 TR-module amplification for legocin cloning with SalE1b A-module using BsaI
	41	colK_salE1b_fwd	TTTTGGTCTCTCATGGCTAAAGAGCTGTCTGGTTACG	ColK TR-module amplification for legocin cloning with SalE1b A-module using BsaI
	42	colK_salE1b_rev	TTTTGGTCTCATATCCTTCTCAAGAGCATCCTGAGCCTC	ColK TR-module amplification for legocin cloning with SalE1b A-module using BsaI
	43	colIa_salE1b_fwd	TTTTGGTCTCTCATGAGTGACCCGGTACGCATTACTAATCC	ColIa TR-module amplification for legocin cloning with SalE1b A-module using BsaI
	44	colIa_salE1b_rev	TTTTGGTCTCATATCTGTCGCTTTTAACTCATCCTGTTTCC	ColIa TR-module amplification for legocin cloning with SalE1b A-module using BsaI
	45	colM_salE1b_fwd	TTTTGGTCTCTCATGGAAACCCTTACTGTGCACGCTCC	ColM TR-module amplification for legocin cloning with SalE1b A-module using BsaI
	46	colM_salE1b_rev	TTTTGGTCTCATATCGCTCATCTGCTTCATGTTCATGGATC	ColM TR-module amplification for legocin cloning with SalE1b A-module using BsaI
	47	colU_act_fwd	TTTTGGTCTCTAAGGAAGAGAAGGCAAACGATGAGAAGGCAGTGC	ColU A-module amplification for legocin cloning with ColIb A-module using BsaI
	48	colU_act_rev	TTTTGGTCTCAAAGCTTAGTAAGCAGGCCTGATAATCTCGTTGTTCAGC	ColU A-module amplification for legocin cloning with ColIb A-module using BsaI
	49	colU_M_act_fwd	TTTT GGTCTC TGGTA GC GAAGAGAAGGCAAACGATGAGAAGGCAGTGC	ColU A-module amplification for legocin cloning with ColM A-module using BsaI
	50	colU_M_act_rev	TTTTGGTCTCAAAGCTTAGTAAGCAGGCCTGATAATCTCGTTGTTCAGC	ColU A-module amplification for legocin cloning with ColM A-module using BsaI
	51	colK_U_rev	TTTTGGTCTCACCTTCTGAGCAATCTTAGCGTACTTCTCACC	ColK TR-module amplification +30aa of activity domain, for legocin cloning with ColU A-module (-30aa) using BsaI
	52	colU_act_K_fwd	TTTTGGTCTCTAAGGCTGATGCTGCTGCTAAGGCAGCTGC	ColU A-module amplification +30 last aa of receptor domain (-30aa), for legocin cloning with ColK (+30aa) TR-module using BsaI
	53	colU_salE1b_fwd	TTTTGGTCTCTCATGCCTGGTTTCAACTACGGTGGTCACGG	ColU A-module amplification for legocin cloning with SalE1b A-module using BsaI
	54	colU_salE1b_rev	TTTTGGTCTCATATCAGCCTCTTTCCTAGCCTTCTCAGCC	ColU A-module amplification for legocin cloning with SalE1b A-module using BsaI

For ColM resistant mutants: amplification and sequencing of the genes responsible for expression of proteins targeted by ColM for cell entry (receptor and translocation proteins) (3.1.5)	55	fhuA-k1-fwd	CGTCTAAAGGTAAGCAGTATGAAGTCG	Further sequencing of inserts in fhuA ORF of ColM *fhuA* mutant clones generated by fluctuation assays
	56	fhuA-k1-rev	ACGCCACGTGCGCGGATCTCGCCACC	sequencing DH10B *fhuA*, clone-specific sequencing
	57	fhuA-k6-fwd	CTGCTGACCGGTGTCGACTTTATGC	sequencing DH10B *fhuA*, clone-specific sequencing
	58	fhuA-k6-rev	GTTGGTTTTAGTGAGATTATACACG	sequencing DH10B *fhuA*, clone-specific sequencing
	59	fhuA-k7-fwd	GATTTTTACCAAAATCATTAGG	sequencing DH10B *fhuA*, clone-specific sequencing
	60	fhuA-k7-rev	CGATCTTCCGGTACATATTTCACG	sequencing DH10B *fhuA*, clone-specific sequencing
	61	FhuA r4	GTTTGTTTCTGTTTATTCAGAATGC	sequencing DH10B *fhuA*
	62	FhuA-140 f	CATCTGGTTGTTTATTAACCCTTCAGG	amplification of FhuA from *E. coli* DNA, sequencing FhuA PCR product
	63	FhuA f1	TCGGTAAAAGAAGCGCTTAGCTACACG	sequencing DH10B *fhuA*
	64	FhuA f2	CGTTATGCTATTGCACCGGCGTTCACC	sequencing DH10B *fhuA*
	65	FhuA f3	CCAGTTGCAGAGCAAGTTTGCCACTGG	sequencing DH10B *fhuA*
	66	FhuA f4	ACCTTCTTCGCAAGTTGGGAAGGATGG	sequencing DH10B *fhuA*
	67	FhuA f5	GTGATCCGGCTAACTCCTTTAAAGTGG	sequencing DH10B *fhuA*
	68	FhuA150 r	AGGTTAACGACAGCGGATGCAAAAGCG	amplification of FhuA from *E. coli* DNA, sequencing FhuA PCR product
	69	TonB-156 f	AAGATGATGTCTTTGTTAAGGCCATGC	amplification of TonB from *E. coli* DNA, sequencing TolB PCR product
	70	TonB f1	GGTCATTGAAAAGCCGAAGCCGAAACC	sequencing DH10B t*onB*
	71	TonB177 r	GTCAGCATTAATATTGAAGTGTGGGTG	amplification of TonB from *E. coli* DNA, sequencing TolB PCR product
	72	ExbB-122 f	ACGCAAGGAAAAGAAAGCAAAGGTACG	sequencing DH10B *exbB*
	73	ExbB f1	GATAACGAAGGTATTAAAGAACGTACC	sequencing DH10B *exbB*
	74	ExbB148 r	TCACCTTCACATCTACCGTCGCTAACG	sequencing DH10B *exbB*
	75	ExbD-130 f2	GCTGGGTGATGTTGCAGCGCAGGTATTGTTGC	amplification of *ExbD* from *E. coli* DNA, sequencing *TolB* PCR product
	76	ExbD-151 r2	GTAGCGCATCAGGCATTGAGCACCTAATGC	amplification of ExbD from *E. coli* DNA, sequencing TolB PCR product
Analysis of correct colicin cat1 intron splicing in plants (3.4.1)	77	ScolE3 f	ATTACCAGGCAGAACGAGATCAACC	amplification of the salmocin expression sequence in pNMD 28151 and sequencing of the SalE3 PCR product
	78	ScolE3 r	ATTACCAGGCAGAACGAGATCAACC	
	79	ScolE2 f	ATGTGGCAAATGGCTGGTCTTAAGG	amplification of the salmocin expression sequence in pNMD 28161 and sequencing of the SalE2 PCR product
	80	ScolE2 r	ATGTGGCAAATGGCTGGTCTTAAGG	
	81	ScolE7 f	ATGCTGATGCAGCTCTGAACGCTGC	amplification of the salmocin expression sequence in pNMD 28172 and sequencing of the SalE7 PCR product
	82	ScolE7 r	CTATGAATATCGATGTGCCTCTTAGG	

7.2 Salmocin genetic sequences

Table 38: Salmocin sequences and homologies
NCBI database search for homologs colicins from *E. coli* in *Salmonella enterica* was done using the blastp suite (BLASTP 2.6.1+ program (Altschul et al., 1997).

Nomad name	Genbank name	Genbank number Salmocin	most homology to *E. coli* bacteriocin (identities)	Genbank number *E. coli* bacteriocin
Salmocin E2	colicin	KTM78572.1	ColE2 (68%)	AAA23068.1
Salmocin E3	colicin-E3	GAS18013.1	ColE3 (75%)	AAA88416.1

Salmocin E7	colicin	KSU39545.1	ColE7 (71%)	AAA98054.1
Salmocin E1a	colicin-10	OIN35410.1	ColE1 (60%), Col10 (57%)	AAA87379.1 CAA57998.1
Salmocin E1b	colicin-10	OIN32443.1	ColE1 (70%)	AAA87379.1
Spst	Pesticin	ESF65298.1	pesticin (34%)	EHV67255.1

7.3 Legocins

Table 39: Legocin protein sequences for the ColIb and ColM A-module legocins

	TR-Module			A-module		
plasmid	colicin	aa	accession No. (UniProtKB)	colicin	aa	accession No. (UniProtKB)
pNMD45900	E1	1-337	P02978	Ib	450-626	P04479
pNMD45910	E1	1-337	P02978	M	124-271	P05820
pNMD45920	N	1-182	P08083	Ib	450-626	P04479
pNMD45930	N	1-182	P08083	M	124-271	P05820
pNMD45940	M	1-123	P05820	Ib	450-626	P04479
pNMD45960	K	1-368	Q47502	Ib	450-626	P04479
pNMD45970	K	1-368	Q47502	M	124-271	P05820
pNMD45990	Ia	1-449	P06716	M	124-271	P05820
pNMD46010	A	1-388	Q47108	Ib	450-626	P04479
pNMD46020	A	1-388	Q47108	M	124-271	P05820
pNMD46030	E3	1-450	P00646	Ib	450-626	P04479
pNMD46040	E3	1-450	P00646	M	124-271	P05820

Table 40: Legocin protein sequences for the ColU A-module legocins

	TR-Module			linker (aa)	A-module		
plasmid	colicin	aa	accession No. (UniProtKB)		colicin	aa	accession No. (NCBI)
pNMD48101	E1	1-337	P02978	K	U	417-619	CAA72509.1
pNMD48111	N	1-182	P08083	K	U	417-619	CAA72509.1
pNMD48121	K	1-368	Q47502	K	U	417-619	CAA72509.1
pNMD48131	A	1-388	Q47108	K	U	417-619	CAA72509.1
pNMD48141	E1	1-337	P02978	GS	U	417-619	CAA72509.1
pNMD48151	N	1-182	P08083	GS	U	417-619	CAA72509.1
pNMD48161	K	1-368	Q47502	GS	U	417-619	CAA72509.1
pNMD48172	Ia	1-449	P06716	GS	U	417-619	CAA72509.1
pNMD48182	A	1-388	Q47108	GS	U	417-619	CAA72509.1
pNMD48250	K	1-398	Q47502		U	387-619	CAA72509.1

Table 41: Legocin protein sequences for the SalE1b A-module legocins

	TR-Module			A-module		
plasmid	colicin	aa	Accession No.	salmocin	aa	Accession No.
pNMD48280	U	1-416	CAA72509.1	E1b	343-527	OIN32443.1
pNMD45980	K	1-368	Q47502	E1b	343-527	OIN32443.1

pNMD46000	Ia	1-449	P06716	E1b	343-527	OIN32443.1

The annotation of the domains was done with the stated literature (see below) and/or predicted via sequence alignment to homologous colicins.

Colicin E1 (Jakes, 2017)
Colicin E3 (Jakes, 2017; Soelaiman, Jakes, Wu, Li, & Shoham, 2001)
Colicin A (Penfold, Li, Zhang, Vankemmelbeke, & James, 2012)
Colicin N (L. J. Evans, Labeit, Cooper, Bond, & Lakey, 1996)
Colicin K (Pilsl & Braun, 1995)
Colicin Ia (Ghosh, Mel, & Stroud, 1994)
Colicin M (Helbig & Braun, 2011; Zeth et al., 2008),

7.4 Piglet experiment

Further details on the cause of the dosing gun-inflicted sickness:
The pressure applied on the dosing gun to hold it in the back of the throat against the piglet's resistance, in combination with the act of swallowing, must have created enough force to penetrate the soft tissue at the back of the piglet´s throat and inject the capsules into body tissues and the ventral body cavity. During the pilot study, this complication had not occurred because an endoscope had been used in conjunction with the metal dosing gun to test and observe the feasibility of positioning a capsule in the piglet's throat for swallowing. At the time, it was unclear that mispositioning of the dosing gun during gavaging could lead to these severe consequences. The circumstance that 4-week-old piglets have less space in the back of their mouth and most likely have more vulnerable throat tissue than mini-pigs, and 6-week-old piglets was underestimated.
The wounds inflicted by the gavaging created a connection between the mouth cavity with its open microbial environment and the body cavity with a very confined microbial environment. For the body cavity, the intrusion of large foreign objects and microorganisms from the mouth into this space can cause peritonitis, an inflammation of the peritoneum (mesothelial membrane) that lines the body cavity. This is a life-threatening infection that can lead to sepsis and organ failure and requires immediate medical attention (Capobianco, Cottone, Monno, Manfredi, & Rovere-Querini, 2017; Culp & Holt, 2010; Park et al., 2019).

Statement of Originality

I hereby confirm that I have written the accompanying thesis by myself, without contributions from any sources other than those cited in the text, the references, and the acknowledgments. This also applies explicitly to all graphics, drawings, maps, and images included in the thesis. This thesis has not been submitted to any other institution or published.

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine weiteren außer den angegebenen Quellen und Hilfsmittel verwendet habe. Diese Erklärung bezieht sich explizit auch auf die in der Arbeit enthaltenen Graphiken, Zeichnungen, Kartenskizzen und bildliche Darstellungen.
Diese Arbeit wurde an keiner anderen Einrichtung vorgelegt oder veröffentlicht.

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Place, date
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- Hahn-Lobmann, S., et al. (2019). “Colicins and Salmocins - New Classes of Plant-Made Non-antibiotic Food Antibacterials.” Front Plant Sci **10**(437): 437.
  DOI: 10.3389/fpls.2019.00437

- Schneider, T., et al. (2018). "Plant-made Salmonella bacteriocins salmocins for control of Salmonella pathovars." Sci Rep **8**(1): 4078.
  DOI: 10.1038/s41598-018-22465-9

Thesis:

- Diploma thesis Biology: “Phenotypic Characterisation of point mutations in the Chromo-Shadow-Domain of the HP1-Homologues HcpA and HcpB of *D. discoideum”* (translated from the German title), at the department of genetics of the University of Kassel, Supervisor: Prof. Dr. Wolfgang Nellen (german grade: 1.7 – GPA: 3.3)
- Diploma thesis Economics: “Standardization Potential of Customer Loyalty Programs in the European Area illustrated by the Example of Bayer CropScience’s National Offices in Poland and England” (translated from the German title), at the FHDW Bergisch Gladbach, Supervisor: Prof. Dr. Michael Bernecker
- Pre-diploma thesis Economics: “The Change Management Process of a Sales organization when implementing new CRM tools illustrated by the example of a specific measure (Weather-Fax)” (translated from the German title), at the FHDW Bergisch Gladbach, Supervisor: Prof. Dr. Thomas Zacher; Prof. Dr. Klaus Eckrich

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2014	Diploma (old equivalent to MSc) in Biology at the Universität Kassel (german grade: 1.7 – GPA: 3.3)
2011	Pre-Diploma (Vordiplom) (old equivalent to a BSc) in Biology at the Universität Kassel (grade: 1.3 – GPA 3.7)
2008	Biology studies at the Universität Kassel
2007	Diploma (FH) in Economics (European Business Management) (Fachhochschule der Wirtschaft Bergisch Gladbach)
2004	Studies in Economics (European Business Management) at the Fachhochschule der Wirtschaft, Bergisch Gladbach
2004	German Abitur from the „Freiherr-vom-Stein-Gymnasium“, Leverkusen
2001	1 year Rotary International School Exchange program in Coatzacoalcos, Mexico
1995-2001	Secondary school “Gymnasium Leichlingen”
1994	Elementary School “Grundschule Flamerscheid” in Leichlingen, Germany
1991-1994	“Indian Valley Elementary School” in Kansas City, USA

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Place, date Signature