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Breeding for Enterotoxigenic *Escherichia coli* F4ab/ac resistant pigs: reduction of antibiotic use by genetic, nutritional and immunological methods

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List of abbreviations

PWD	Post-weaning diarrhoea
ETEC	Enterotoxigenic *Escherichia coli*
LT	Heat-labile toxin
ST	Heat-stable toxin
F4acR	ETEC F4ac receptor gene
F4bcR	ETEC F4ab/F4ac receptor gene
SSC13	*Sus scrofa* chromosome 13
LD	Linkage disequilibrium
SLW	Swiss Large White pigs
IMF	Intramuscular fat content
INF	Infected piglets
NINF	Non-infected piglets
MAT	Microscopic adhesion test
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
HT	Hydrolysable tannins
R	*F4acR* resistant allele
S	*F4acR* susceptible allele
CO	Control standard diet
TA	Diet with tannins
SLW	Swiss Large White pigs
HTE	Hydrolysable chestnut-tannin extract
AM	Antimicrobials
SD	Standard deviation
FI	Feed intake
ADG	Average daily gain
BW	Body weight

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Summary

Enterotoxigenic *Escherichia (E.) coli* (ETEC) with F4 fimbriae is one of the major cause of diarrhoea and death among neonatal and post-weaning piglets. It is the most prevalent strain worldwide and has three antigenic variants – F4ab, F4ac and F4ad – that vary in their receptor specificities. ETEC F4ab/ac seem to adhere to the same receptor *F4bcR* where as ETEC F4ad adhere to a separate *F4adR*. Adherence to specific F4 receptors (F4R) on the brush borders of enterocytes by adhesive F4 fimbriae, and the subsequently production of heat-labile enterotoxin (LT) and in some cases heat-stable (STa or STb) enterotoxins cause water loss into the intestinal lumen leading eventually to diarrhoea. Pigs that do not express F4R in the small intestine are resistant (RR) to ETEC F4 adhesion and in consequence will not develop diarrhoea after infection. The expression of F4R receptors is a monogenic inherited trait, the susceptible (*S*) allele being dominant over the resistant allele (*R*) (susceptible genotypes S- (SS, SR)). Breeding for ETEC F4 resistant pigs is one of the most promising methods to diminish the adverse effects of ETEC F4 diarrhoea. Disease resistant pigs could increase animal welfare, diminish the severity of illness, decrease pathogen proliferation and shedding and infection pressures. The farmers could profit from disease resistant pigs with lower animal loss, higher performance and better product quality compared to susceptible pigs. Antibiotics usage and antibiotic resistant pathogens would decrease during rearing. Indeed, consumers prefer meat produced with less antibiotics and the pig breeding sector will have a better reputation.

Previous studies revealed two markers *CHCF1* and *ALGA0106330* with a high predictive power to identify resistant and susceptible pigs in an experimental herd. The candidate causative region of *F4bcR* is localised on pig chromosome 13 q41-q44 (135’265’806-135’403’999). The first part of this project (Chapter 2) was to evaluate the effectiveness of these markers in the active Swiss breeding populations. Among 2034 Swiss Large White (SLW) pigs, only 2 recombinants (0.1%) occurred where *CHCF1* and *ALGA0106330* predicted different phenotypes. In Duroc, 21 of 99, in Swiss Landace 12 of 72 and in Piétrain 14 of 69 boars for artificial insemination (AI) were recombinant. Three recombinant AI bores per breed and two recombinant SLW were genotyped with additional markers of the candidate region and phenotyped using the microscopic adhesion test. In this test only *ALGA0072075*, *CHCF1* and *CHCF3* predicted the phenotype correctly, whereas two markers in the *MUC13* gene (*MUC13*g.15376, *ALGA0106330*) were incorrect. The frequency of the resistant allele of marker *CHCF1* varied from 0.49 to 0.96 in four pig breeds. In SLW, the most important breed in Switzerland the frequency was 0.60, which is advantageous for implementation of a breeding program for selecting disease resistant pigs. Another prerequisite for a successful selection program is the effect of the resistant allele on important production traits. In 530 pigs from national test station, a significant but small difference was observed among *F4bcR* genotypes in the intramuscular fat content (IMF) of the *longissimus dorsi* muscle, whereas 16 other important economic traits were not influenced by the resistance allele in SLW pigs.

Breeding for disease resistant without loss of genetic diversity pigs cannot be accomplished in one move. There are plenty of susceptible piglets in the population while selecting for resistant pigs. The second part of the project is determining the effectiveness of food additives, as a nutritional strategy, to protect weaner pigs from ETEC F4 diarrhoea and reduce the usage of antibiotics (Chapters 3 & 4). The potential effects of hydrolysable chestnut tannin extracts (HTE) and salicylic acid (SA) on reducing severity of diarrhoea in SLW pigs were investigated. An ETEC-infection model was developed using a native ETEC F4ac strain (LT^+, STb^+ STa^-) resistant to sulfamethoxazole (smx^R) isolated from a weaned piglet with diarrhoea at the piggery of the research station. A Rifampicin resistant sub-strain was selected for better identification. All 144 piglets for the challenging test were susceptible, according to the *CHCF1* marker (SS/S-). Piglets were challenged with a single dose of $5x10^8$ colony forming units of the test strain. Faecal scores, percentage of days in diarrhoea, ETEC shedding by culturing and qPCR were recorded. Polyphenols might be a potent inhibitor for the toxicity of LT produced by ETEC F4 fimbriae. To identify the effects on preventing from the prevalence of ETEC F4 diarrhoea, 1% and 2% HTE were used as food additives in diet comparison. Diet with 2% HTE showed positive effects on growth performance and prevention of ETEC F4 diarrhoea (faecal scores, numbers of colony growing, qPCR of the LT gene) while diet with 1% HTE presented no significant difference compared to control diet. Besides, SA was used as supplementation in diet to decrease pathogen infective inflammation, which manifest in loss of appetite and fever. However, SA did not indicate significantly positive effects on growth performance and reduction of severity of diarrhoea.

The last part of the project is related to vaccination (Chapter 5). Vaccination is a practical method to decrease the loss of suckling pigs due to ETEC diarrhoea. Vaccination of sows and immunoglobulin uptake of pigs might mask the effect of ETEC F4 susceptible/resistant genotype. Particularly susceptible piglets from litters of SS boars x RR sows) will profit as these offspring are all susceptible and due to the lack of naturally occurring antibodies in the colostrum of their resistant mothers. The immune response to vaccination of genetically susceptible and resistant pregnant gilts were compared. Significant lower antibody titres (15%) against ETEC F4ab and F4ac were measured in the colostrum of ETEC F4 resistant sows, while there was no difference of LT antibody titres in when compared to susceptible gilts. Although the resistant gilts showed a significantly reduced immune response to F4ab/ac fimbriae, the differences in colostrum antibody concentration were probably not of practical relevance. The analysis of over 380 litters of vaccinated resistant and vaccinated susceptible sows revealed the highest survival rate from birth to weaning in litters of homozygous susceptible boars and resistant vaccinated sows. This might indirectly indicate that reduced immune response in resistant gilts, did not have negative effects on the development of suckling piglets. An alternative to vaccination could be to start the selection in the sire line avoiding “risky” mating.

In conclusion, in this thesis complementary genetic, nutritional and immunological tools were developed to successfully introduce a breeding program for ETEC F4ac disease resistant pigs in the active breeding population.

Zusammemfassung

Enterotoxigene *Escherichia (E.) col*i (ETEC) Bakterien mit F4 Fimbrien sind für die meisten Coli-Durchfälle bei Saug- und Absetzferkeln weltweit verantwortlich. Drei antigene Varianten wurden beschrieben – F4ab, F4ac, F4ad, die sich in unterschiedlichen Bindungseigenschaften zu ihren Rezeptoren unterscheiden. ETEC Fab/ac scheinen am gleichen Rezeptor F4bcR zu binden, während ETEC F4ad an einen eigenen F4adR bindet. Die Bindung ETEC F4 an einen spezifischen F4R auf dem Bürstensaum des Dünndarms und die Ausschüttung des hitze-labilen Enterotoxins (in einigen Fällen auch die Enterotoxine STa oder Stb) führen zu Wasserverlust ins Darmlumen und schliesslich zu Durchfall. Schweine, die keine F4-Rezeptoren im Dünndarm ausbilden sind resistent (RR) gegenüber der Besiedelung durch ETEC F4Bakterien und entwickeln keinen Durchfall.

Die Expression von F4R unterliegt einem monogenen Erbgang, wobei das Allel für Empfänglichkeit (*S*) dominant über das Resistenzallel (*R*) ist. Die Zucht auf ETEC F4 resistente Schweine ist ein vielversprechender Ansatz die schädlichen Auswirkungen von ETEC F4 bedingten Durchfällen zu verhindern. Resistente Schweine erhöhen das Tierwohl in dem sie nicht erkranken, die pathogenen Keime vermehren sich weniger schnell, weniger Erreger werden ausgeschüttet und der Infektionsdruck nimmt ab. Bauern profitieren von resistenten Schweinen weil die Tierverluste kleiner sind, die Tiere eine bessere Leistung zeigen und die Qualität der Produkte höher ist, verglichen mit anfälligen Tieren. Der Antbiotikaverbrauch und der Anteil antibiotika-resistenter Keime sinken während der Aufzucht. Schliesslich mögen Konsumenten Fleisch mehr, wenn sie wissen, dass es ohne Antibiotikaeinsatz produziert wurde, was der Schweinebranche zu einem besseren Ruf verhilft.

In Früheren Studien mit Versuchsschweinen konnten wir zwei genetische Marker finden (*CHCF1*, *ALGA0106330*), die mit einer sehr grossen Sicherheit empfängliche und resistente Schweine gegenüber ETEC F4-Besiedelung voneinander unterscheiden können. Die Kandidatenregion des ETEC F4ab/ac Rezeptorgens (*F4bcR*) liegt auf den Schweinechromosom 13q41-q44 (135'265’806-135'403'999). Im ersten Teil dieses Projektes (Kapitel 2) überprüften wir die Brauchbarkeit dieser Marker in den aktiven Zuchtpopulationen der Schweiz. Unter 2034 Edelschweinen fanden wir nur 2 rekombinante Schweine (0.1%) wo die beiden Marker unterschiedliche Phänotypen voraussagten. Bei Duroc waren es 21 von 99, der Landrasse 12 von 72 und beim Piétrain 14 von 69 untersuchten Zuchteber. Von den rekombinanten Ebern wurden je drei pro Rasse zusammen mit den 2 rekombinanten Edelschweine mit zusätzlichen Markern aus der Zielregion untersucht und der richtige Phänotyp mittels mikroskopischem Adhäsionstest bestimmt. Nur die Marker *ALGA0072075*, *CHCF1* und *CHCF3* sagten den richtigen Phänotyp voraus, während zwei Marker im *MUC13* Gen (*MUC13.g*15376, *ALGA01063309*) falsch lagen. Die Allelfrequenz des Resistenzallels von *CHCF1* lag zwischen 0.49 und 0.96 in den 4 Rassen. Beim Edelschwein, der wichtigsten Rasse in der Schweiz, war die Frequenz 0.60, was vorteilhaft für die Zucht auf resistente Schweine ist. Eine andere Voraussetzung eines erfolgreichen

Selektionsprogrammes ist der Alleleffekt des Resistenzallels auf wichtige Produktionseigenschaften. Bei 530 Schweinen aus der Stationsprüfung (MLP Sempach) wurde ein signifikanter aber kleiner Effekt auf den intramuskulären Fettgehalt festgestellt (*M. longissimus dorsi*), während bei den übrige 16 untersuchten Eigenschaften kein negativer Effekt gefunden wurde.

Im 2. Teil der Arbeit (Kapitel 3 und 4) geht es um den Einsatz von Futtermittelzusätzen zur Reduktion von ETEC F4-Durchfällen bei Saugferkeln. Um bei der Resistenzzucht einen Verlust an genetischer Vielfalt zu vermeiden darf die Selektion nicht zu schnell erfolgen. Darum sind am Anfang der Selektion noch viele empfängliche Ferkel vorhanden. Wir haben Tannin-Extrakte aus Kastanien und Salizylsäure als Futtermittelzusätze bei der Fütterung von Absetzferkel eingesetzt und die Wirkung in einem ETEC F4-Infektionsmodell überprüft. Ein ETEC F4ac Infektionsstamm (LT^+, STb^+ STa^-) mit einer Sulfamethoxazol (smx^R) Resistenz wurde aus dem Kot eines Ferkels mit Durchfall isoliert. Zur besseren Identification wurde ein Rifampicin resistenter Sub-Stamm zur Infektion eingesetzt. Alle 144 Ferkel wurden im *CHCF1* Marker empfänglich getestet (SS oder SR). Die Ferkel erhielten eine einmalige Gabe von $5x10^8$ CFU (colony forming units) des Infektionsstamms. Der Schweregrad des Durchfalls, Anzahl Tage mit Durchfall, Zahl der Ausgeschiedenen Infektionskeime und die DNA Menge des LT-Gens wurde mittels quantitativer PCR im Kot erhoben. Infizierte Ferkel mit 2% Tannin-Extrakten sind schneller gewachsen, zeigten weniger schwere Durchfälle, und haben weniger Keime ausgeschieden als infizierte Kontrollen ohne Tannin-Extrakte im Futter. Tannin-Konzentrationen von 1% im Futter waren nicht wirksam.

Im letzten Teil des Projektes wurden resistente und empfängliche Erstlingssauen mit einem kommerziellen Impfstoff geimpft und die Antikörper Titer gegen Fimbrien F4ab, F4ac und LT-Toxin bestimmt (Kapitel 5). Impfungen von Muttersauen sind bewährte Methoden, um Saugferkelverluste wegen Durchfall zu vermindern. Dabei nehmen die Ferkel die von der Mutter gebildete Immunglobuline über das Kolostrum auf und sind vor Infektionen mehr oder weniger geschützt. Vor allem empfängliche Ferkel von resistenten Sauen profitieren von diesen Impfungen, weil resistente Sauen kaum mit ETEC F4 in Kontakt kommen und daher im Kolostrum natürlicherweise sehr wenig schützende Antikörper gebildet werden. Wir konnten zeigen, dass geimpfte resistente Erstlingssauen 15% weniger Antikörper gegenüber F4ab und F4ac bildeten als empfängliche Erstlingssauen. Die Antikörper Titer gegen das LT-Toxin waren in den beiden Gruppen gleich.

Ob die Ferkel von geimpften resistenten Mutterschweinen gleich gut gegenüber ETEC F4 Infektionen geschützt sind, haben wir indirekt über eine Analyse von 380 Würfen vom Versuchsbetrieb Posieux bestimmt. Die Genotypen der Eltern waren bekannt und alle Muttersauen wurden alle geimpft. Es hat sich gezeigt, dass die Aufzuchtrate von resistenten Sauen die mit homozygot empfänglichen Ebern gepaart wurden höher war als bei den übrigen Paarungen.

Eine Alternative zur Impfung der Muttersauen könnte der Beginn der Selektion in den Vater-Linien sein, um die «riskanten» Paarungen zu vermeiden.

In dieser Arbeit wurden komplementäre genetische, nutritive und immunologische Methoden verwendet um eine erfolgreiche Zucht auf resistente Schweine in der Praxis umzusetzen.

Chapter 1: General introduction

1.1. Diarrhoea general information

Diarrhoea is defined as malabsorption of water and electrolytes in the lumen of the small intestine, a condition with the water content in faeces exceeding 80% (Makinde *et al.* 1996) or the frequent passage of soft or watery faeces (Liebler-Tenorio *et al.* 1999). Infectious diarrhoea disease is a leading cause of death in developing countries where more than 21% of all deaths in children under the age of 5 years old (Kosek *et al.* 2003). Collectively, pathogenic diarrhoeas are estimated to cause approximately 1~2.5 million deaths of children annually (Kosek *et al.* 2003; Boschi-Pinto *et al.* 2008).

Diarrhoea is a common problem in the farm animal production worldwide, especially in modern pig production, which can cause large economic losses due to poor health, high morbidity, increasing costs of the use of colibacillosis antibiotics, increased resistance to antibiotics and high mortality (Amezcua *et al.* 2002; Johansen *et al.* 2004). For example, compared to a high health farm in the United States, $32 more were needed to produce a 100 kg pig in a conventional herd, which indicated the economic losses caused by diarrhoea (Batista & Pijoan 2002). The incidence of diarrhoea in piglets was estimated to be between 6.8% and 22% in the period of 1989 to 1992 in Denmark (Svensmark *et al.* 1989a; Svensmark *et al.* 1989b; Christensen *et al.* 1994), and the mortality rate accounted for diarrhoea is between 11.5% - 29.5% of death in China (Li *et al.* 2007).

Diarrhoea is a multi-factorial disease, where management and housing routines, the pathogen and the immunity of the pigs play key roles in the pathogenesis of the disease (Cagienard *et al.* 2005; Weber *et al.* 2015). The infective agents of diarrhoea in piglets can be viruses, parasites and bacteria; however, infection with pathogenic *Escherichia coli* (*E. coli*) contributes to the major part of diarrhoeal disease incidences.

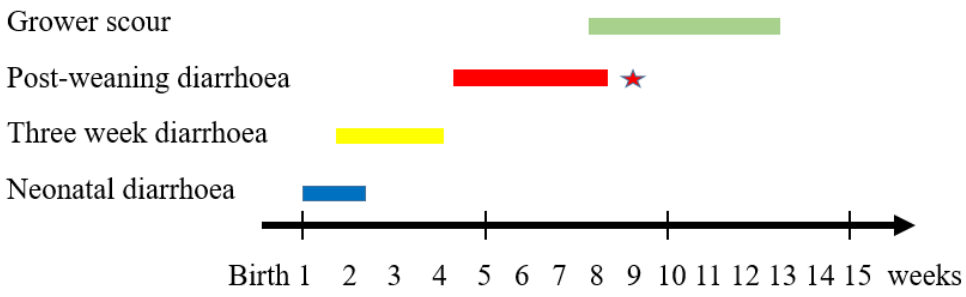

Figure 1: Appearance of diarrhoea in pig during rearing.

In general, pigs suffer from diarrhoea problems throughout their lifetime (Figure 1, modified from Jacobson (2003)). Piglets are born without any antibodies, and are completely dependent on the colostral transfer of immunoglobulin G across the epitheliochorial placenta of the sow to obtain sufficient immunity (Schnulle & Hurley 2003). Neonatal diarrhoea (at 4-5 days after birth) is particularly hazardous for piglets (Kohler 1974), which is associated with *E. coli* and *Clostridiun perfringens* type

C infection (Morin *et al.* 1983). From two weeks onwards, own antibody production of the piglets slowly increases; however, it is not fully developed yet to protect piglets from a "three-week-diarrhoea", which is presumably caused by the intestinal parasite *Isospora suis* or by rotavirus (Bourne 1976; Nilsson *et al.* 1984). Post-weaning diarrhoea (PWD) (a star in Figure 1) occurs at approximately 4 weeks of age after weaning, and it is one of the most significant diarrhoeal diseases in pigs worldwide. It is mainly associated with pathogenic *E. coli*, *Salmonella*, and *Lawsonia intracellularis* (Moxley & Duhamel 1999). In recent years, another type of diarrhoeal disease referred to grower scour or colitis that occurred due to *Brachyspira pilosicoli* or *Lawsonia intracellularis* infections. Grower scour or colitis was often not diagnosed due to the high mortality and morbidity rates of PWD (Duhamel 1996; Morris *et al.* 2002). Furthermore, medications, malabsorption, and other non-pathogen-related reasons may lead to diarrhoea or death in pigs during rearing.

Based on the similarities of diarrhoea morbidity and enteric zoonosis disease due to *E. coli* infections between humans and pigs, increasing concern about human health occurs. In recent years, increasing attention has been paid to antibiotic overuse, leading to increasing antibiotic resistance of pathogens, food-borne pathogens, and zoonosis ingrained, which finally influence human health.

1.2. Pathogenic *E. coli* in pigs

E. coli, a common type of gram-negative bacterium from the Enterobacteriaceae family found in the lower intestine in most mammalian species, is a globally significant pathogen responsible for diarrhoea in neonatal and post-weaning piglets. *E. coli* strains possess fimbrial antigens that allow the bacteria to adhere to the enterocytes and colonize the intestine (Mackinnon 1998) (Figure 2).

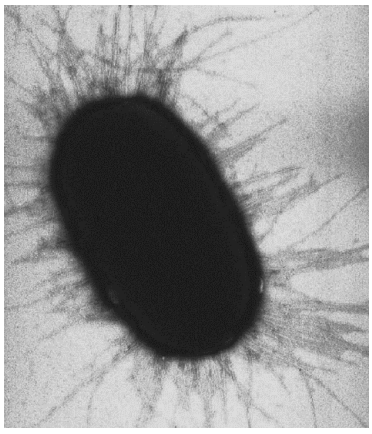

Figure 2: *E.coli* bacterium with fimbriae (Gross 2006).

Diarrhoeal disease of piglets is frequently due to infection by one or other pathotypes of *E. coli*: enterotoxigenic (ETEC), necrotoxigenic (NTEC), enteropathogenic (EPEC), vero- or Shiga-like toxin producing (VTEC or STEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC), and enteroaggregative (EAggEC) (Nagy & Fekete 2005). In Switzerland, pigs suffering from diarrhoea are mostly caused by infection with ETEC (24.8%) and EPEC (7.1%) (Schneeberger *et al.* 2017). Among

all the pathotypes, ETEC is a significant and worldwide cause of severe; watery diarrhoea in the offspring of neonatal/suckling calves and suckling/weaned pigs. In contrast, ETEC is very rare or almost non-existent in other farm animals, such as rabbits or chickens (Nagy & Fekete 2005).

Animals affected with pathogenic *E. coli* show a decreased growth rate and an increased morbidity and mortality rate, resulting in a negative economic effect in the swine industry (Gyles 1994). In the western world, pig breeders use various measurements, such as vaccination of the sow, colostrum from the first day and not mixing animals from different farms to prevent diarrhoea in pigs. However, in some developing countries where the use of vaccines is not prevalent or not all the measurements are applied, for instance, in Vietnam, the morbidity of diarrhoea in all born litters is approximately 71.5% and 43% diarrhoea cases in neonatal pigs and 23.9% diarrhoea cases until the age of weaning (Do *et al.* 2006). Pathogenic *E. coli* are responsible for 50% of all diarrhoea cases in post-weaning pigs (Schneeberger *et al.* 2017).

1.2.1. ETEC infections and enterotoxins in pig

ETEC adhere to the small intestinal epithelial cells, thereby colonizing the gut and secreting heat-stable (STa, STb) and/or heat-labile (LT) enterotoxins, which stimulate the small intestine for, increased water and electrolyte secretion and/or decreased fluid absorption (Nataro & Kaper 1998; Loos *et al.* 2012).

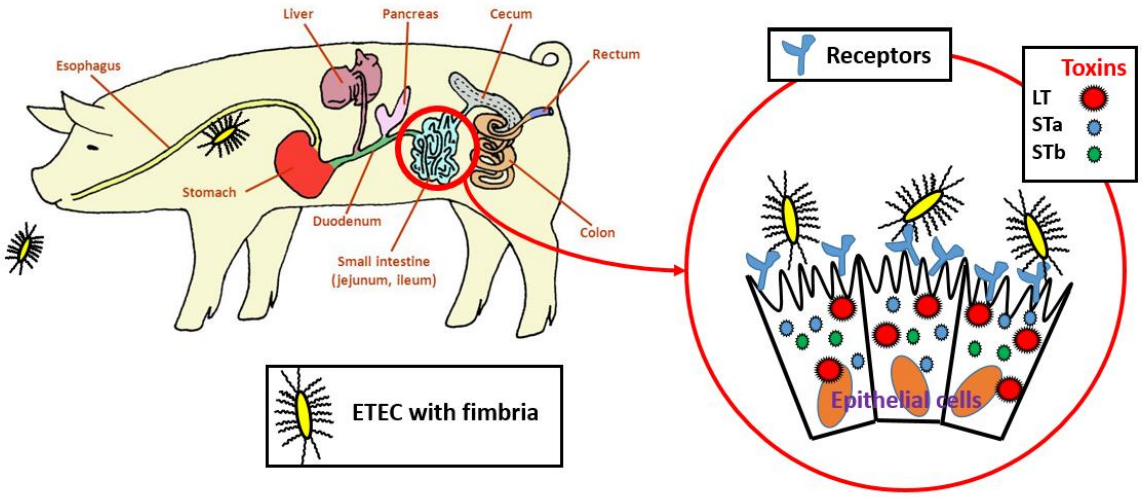

Figure 3: The pathogenesis of ETEC in pigs.

The bacteria are ingested by the pig, and enter the intestinal tract. The fimbriae located on the surface of the bacteria adhere to specific receptors displayed on the surface of the intestine epithelial cells; and begin to secrete toxins (LT, STa, STb), which are endocytosed and translocated through the cell. The illustration is modified from (Fairbrother *et al.* 2005).

Figure 3 illustrates how ETEC infects pigs and causes diarrhoea. Enterotoxins are extracellular proteins or peptides, that are able to exert their actions on the intestinal epithelium. Large molecular weight LT toxins have good antigenicity, while ST toxins do not. LT is used synonymously with the subtype LT-

I, which is found in humans and pigs, and subtype LT-II is antigenically and biologically distinct from LT-I, lacking enterotoxic activity and is not associated with diseases in animals (Nagy & Fekete 1999; Fairbrother *et al.* 2005). The LT toxin is a heterohexameric molecule composed of a B-subunit pentamer and a single A-subunit. Toxin activity resides in the A-subunit, while the B-subunit is largely responsible for host cell binding. The subunit B binds predominantly to the monosialotetrahexosylganglioside (GM1) receptor on the cell surface. The A-subunit is comprised of two principal domains, the A1 domain, responsible for the toxicity, and A2, which non-covalently anchors the A-subunit in the centre of the B pentamer (Sixma *et al.* 1991). A1 translocates to the endoplasmic reticulum and activate the adenylate cyclase system to increase cellular cyclic adenosine monophosphate (cAMP), inducing increased fluid and electrolyte secretion and decreasing their absorption. The effect of LT is irreversible (Sixma *et al.* 1991; O'brien & Holmes 1996; Fujinaga *et al.* 2003).

Two variants of ST toxins (STa and STb) occur in pigs. STa is soluble in methanol and induces small intestinal fluid secretion in newborn but not in weaned pigs, all in contrast to STb, which can induce fluid secretion in both newborn and weaned pigs (Whipp 1991; Nagy & Fekete 2005). LT and ST enterotoxins do not produce pathological lesions or morphological alterations on the mucosa (Nagy & Fekete 1999). They merely stimulate an increased secretion of chloride (Cl^-), an elevation of the calcium (Ca^{2+}) concentration, an inhibition of sodium (Na^+) absorption and a concomitant decrease in fluid absorption in the gut (Nataro & Kaper 1998; Nagy & Fekete 1999). These processes result in substantial ion secretion, leading to diarrhoea dehydration and acidosis.

1.2.2. Fimbriae

Fimbriae are long proteinaceous and filamentous surface structures of bacteria that play key roles in infections (Westerlund-Wikström & Korhonen 2005). Although the types of fimbriae vary with different types of bacteria, all allow bacteria to adhere tightly to cells, colonize host cells, and subsequently survive and persist in the localized host environment to cause disease. Attachment occurs by specific interactions between fimbriae and their receptors on the host cell (Xia *et al.* 2015). For animal ETEC, the most common adhesions are the fimbriae on the surface: F4(K88), F5(K99), F6(987P), F17(Fy), F18, F41, F42 and F165 (Nagy & Fekete 2005) (Table 1). F4 (K88), F5 (K99), F6 (987P), F18 (F107) and F41 are responsible for ETEC infection in pigs worldwide. Fimbriae F6, F18 and F41 have a carbohydrate-binding protein site at the tip serving as a ligand to the intestinal receptor (Moon 1997). ETEC expressing F6, F18, and F41 fimbria are found mainly in the neonatal period and ETEC F4 are found in both neonatal and post-weaning periods (Fairbrother *et al.* 2005; Nagy & Fekete 2005). Current studies show that F4 and F18 are the most frequent fimbriae in ETEC strains and are prominent worldwide (Frydendahl 2002; Zhang *et al.* 2007). The prevalence of ETEC F4 and F18 fimbria isolated from pigs with diarrhoea is geographically highly diverse ranging from 4% to 64.6%.

Table 1: Fimbrial and enterotoxins of enterotoxigenic *E. coli* (ETEC) of animals (Nagy & Fekete 2005).

Virulent factors			Age of outbreak	Localisation	Action
Fimbriae /variants	Enterotoxin /receptor	Molecular size			
F4(K88)/ab, ac, ad F5(K99) F6(987P) F17(Fy) F18/ab, ac F41 F42 F165	LT-I/GM1 LT-II/GD1a, GD1b	28 kDa 11.5 kDa	Newborn and/or Post-weaning	Colonization of the small intestine (epithelial cells, enterocytes)	Hypersecretion/malabsorption: stimulation of adenylate-cyclase system
	STa/pGCc	2 kDa			Hypersecretion/malabsorption: stimulation of guanylate-cyclase system
	STb/Lipid	5 kDa			Secretion/malabsorption: opening G-protein linked Ca^{2+} plasma membrane channel
	EAST1/pGCc	5 kDa			The same as STa

Fimbriae F18

The highest prevalence of F18 fimbria causing PWD in piglets is 35% in Slovakia, 34.3% in the US, 26.3% in Japan and 26.25% in China (Cheng *et al.* 2006; Katsuda *et al.* 2006; Khac *et al.* 2006; Zhang *et al.* 2007). F18 fimbriae are long flexible appendages that show a characteristic zigzag pattern and have two antigenic variants, F18ab and F18ac (Nagy *et al.* 1997) (Table 1). Neither F18ab nor F18ac strains adhered to brush borders from newborn pigs (Wittig *et al.* 1995; Nagy *et al.* 1997). F18 fimbriae strains adhered to intestinal loops of weaned pigs (over two weeks of age).

F18-positive ETEC strains frequently produce STa, STb, with or without Stx2e (Shiga toxin type 2e) and EAST1 (enteroaggregative *E. coli* heat-stable enterotoxin 1), and infrequently produce LT (Rippinger *et al.* 1995; Francis 2002) (Table 1). Enterotoxin Stx2e is known to cause oedema disease in pigs, while the significance of EAST1 in porcine diarrhoeal disease is unclear even if EAST1 has predicted structural similarity to STa molecules (Fairbrother *et al.* 2005).

Two α(1,2) fucosyltransferase genes (*FUT1* and *FUT2*) contribute to the formation of blood group antigen structures, which are present on cell membranes and in secretions. In pigs, they localized on SSC6q11 as candidate genes for the F18 fimbriated *Escherichia coli* (*ECF18*) receptor and the inhibitor *S* loci (Meijerink *et al.* 1997). The *FUT1* polymorphism was found at residue 307 ($M307^G$ and $M307^A$) with either a guanine (G) or an adenine (A), which resulted in an amino acid substitution at position 103 (Ala to Thr, GCG to ACG). The *FUT1* $M307^{AA}$ genotype was resistant to *ECF18* infection while the *FUT1* $M307^{GA}$ and *FUT1* $M307^{GG}$ genotypes were susceptible to *ECF18* colonization (Meijerink *et al.* 2000). Elimination of the susceptible allele for ETEC F18 from the porcine population is currently carried out in Switzerland (Luther *et al.* 2009).

Fimbriae F4

The F4 fimbriae are divided into three antigenic variants: F4ab, F4ac and F4ad (previously known as K88ab, K88ac, and K88ad). The fimbria of these three serological variants share similarities in structures including the major subunit, FaeG (27.5 Kda), several minor subunits (FaeF, FacC, probably FaeI, and FaeJ), all of which are controlled by a single gene cluster (from *faeA* to *faeJ*) (Figure 4) (Westerlund-Wikström & Korhonen 2005; Xia *et al.* 2015). FaeG of F4 fimbria is both a major subunit, and the adhesion, which is related to amino acid substitutions, causes different three binding antigens (red box in Figure 5) (Bakker *et al.* 1992; Van Den Broeck *et al.* 2000).

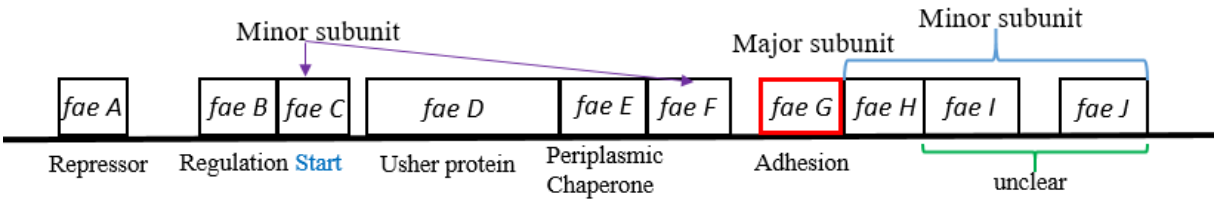

Figure 4: The F4 gene cluster and the function of different subunits (Van Den Broeck *et al.* 2000).

Among the three F4 fimbriae variants in pigs with diarrhoea, F4ab and F4ac are the most predominant. Investigations in European and US pig breeds, such as Large White and Landrace, indicated that the susceptibility rate ranges from 40% to 88% (Baker *et al.* 1997; Li *et al.* 2007; Yan *et al.* 2009). In contrast to the high prevalence of susceptibility to ETEC F4ab/ac, Chinese breeds, such as Meishan and Tibetan pigs, have been reported to be resistant or low susceptible to ETEC F4ab/ac (Li *et al.* 2007; Yan *et al.* 2009). Minzu and Songliao Black breeds have a low susceptibility, from 8% to 28% to F4ab/ac (Li *et al.* 2007). It is possible that Chinese breeds have been isolated from the rest of the world centuries ago which are similar to wild pigs naturally resistant to ETEC F4.

1.3. ETEC F4 receptor phenotypes in pigs

The F4 fimbria variants share a common antigen (a) and express a specific antigen (b, c, or d) (Van Den Broeck *et al.* 2000). The F4 receptor is inherited as a dominant Mendelian trait, where the dominant allele *S* represents susceptibility to ETEC infection while the recessive allele *s* presents resistance (Gibbons *et al.* 1977).

Bijlsma *et al.* (1982) identified five patterns of adherence, and these phenotypes were designated as A, B, C, D and E, which were prevalent in pigs. In phenotype A, the three F4 fimbrial variants bind to the brush borders (A1 in Table 2), and in phenotype E, none of the variants binds (Table 2). Baker *et al.* (1997) confirmed these 5 phenotypes and identified a sixth phenotype F that binds only F4ab to the brush borders. Nguyen *et al.* (2016) identified six F4 ETEC adhesion phenotypes mentioned above and identified the seventh phenotype G, which binds only F4ac to the brush borders. Phenotype H, which binds F4ac and F4ad to the brush borders, observed by Li *et al.* (2007) is mainly in Chinese indigenous

pigs or eastern breeds (Yan *et al.* 2009). However, phenotypes F, G and H were rarely detected in European breeds.

Pigs that were susceptible to F4ac were always susceptible to F4ab, and therefore, the two receptors were suggested to be encoded by a single locus. Rapacz and Hasler-rapacz (1986) hypotheses that one gene for the F4ab/F4ac receptor locus (*F4bcR*) or two closely linked loci coding for both the F4ab and F4ac receptors, respectively. Pigs susceptible to one F4 variant also show adhesion to two other variants. There exists a high correlation between F4ac and F4ab adhesion, but a moderate correlation between F4ac and F4ad adhesion and F4ab and F4ad adhesion (Nguyen *et al.* 2016).

Table 2: Phenotypes observed in pigs according to the binding of ETEC variants.
The table is modified from Rampoldi *et al.* (2014). The presence of the receptor is marked with ●.

	F4abR[1]			F4adR[2]	
Phenotype	FA	PA	F4acR[1]	FA	PA
A1	●		●	●	(●)[3]
A2	●		●		●
B	●		●		
C1		●		●	(●)[3]
C2		●			●
D1				●	(●)[3]
D2					●
E					

FA = fully adhesive (>85% adhesive enterocytes); PA = partially adhesive (>0% to 85%).
[1]For the detection of F4abR and F4acR only one intestinal segment was examined.
[2]Four segments examined for F4adR.
[3]The F4adR^{FA} phenotype masks the expression of the F4adR^{PA} phenotype.

There are two explanations for the high correlation between F4ac and F4ab adhesion. One explanation reveals that high similarities of FaeG structure between F4ac and F4ab lead to common function in the interactions of F4ab and F4ac fimbriae with their intestinal receptors. Another explanation is that the loci controlling *F4abR* and *F4acR* genes are identical or real close to each other (Python *et al.* 2002; Python *et al.* 2005). The fimbrial variant F4ad has received little attention so far, and the inheritance of receptors for fimbriae F4ad (*F4adR*) is not well understood. Bijlsma and Bouw (1987) suggested a dominant receptor locus, *F4adR*, that is inherited independently; but is closely linked to *F4bcR*. Peelman (1999) demonstrated that *F4adR* is not localized on porcine chromosome 13.

Targeted mating allowed the discrimination between fully adhesive receptor (F4abR^{FA} and F4adR^{FA}) expressed on all enterocytes and at all small intestinal sites, and partially adhesive receptor (F4abR^{PA} and F4adR^{PA}) variably expressed at different sites and often leading to partial bacterial adhesion (Rampoldi *et al.* 2014). These observations led to three additional receptor phenotypes: A2 (F4abR^{FA}/F4acR^{+}/F4adR^{PA}), phenotype C2 (F4abR^{PA}/F4aCR^{-}/F4adR^{PA}) and D2 (F4abR^{-}/F4acR^{-}

/F4adR^{PA}) (Table 2). In pigs with both types of F4ad receptors, F4adR^{FA} masks F4adR^{PA}. It postulated that at least two complementary or epistatic dominant genes control F4adR^{FA}, whereas F4adR^{PA} was inherited as a monogenetic dominant trait.

1.4. Investigated candidate genes for *F4bcR*

The specific location of *F4bcR* was identified on SSC13 in the 69 cM region by linkage analysis using a European wild boar x Swedish Large White sow three generation pedigree (Edfors-Lilja *et al.* 1995). The *F4bcR* was refined by fine mapping in a 6 cM interval between microsatellites *SW207* and *SW225* (Python 2003).

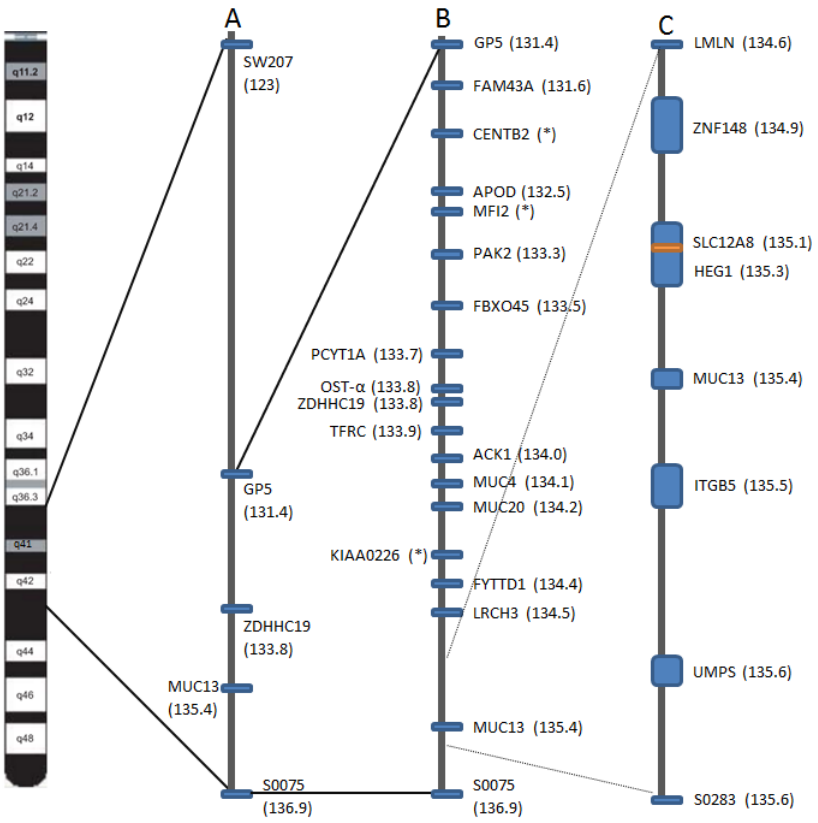

Figure 5: Localization of the candidate region for *F4bcR* at SSC13.

The approximate scales and gene annotations are deduced from the porcine map in NCBI (Sscrofa11.1). A) The candidate region identified by linkage studies (Joller *et al*. 2009). B) The refined candidate region identified by haplotype sharing containing 18 annotated genes (*: annotated genes mapped in Sscrofa9 and are not found in Sscrofa11.1) (Jacobsen *et al*. 2010). C) The further refined candidate region identified by recombination events containing 7 annotated genes (modified from Sscrofa10.2 to Sscrofa11.1) (Rampoldi *et al.* 2011). It is modified according to the latest porcine map in NCBI (Sscrofa11.1).

A combination of pedigree data between the Swedish and Swiss material study identified linkage to a 10 Mb interval (5.7 cM) between microsatellites *SW207* and *S0075* (Figure 5A) (Joller *et al.* 2009). Jacobsen *et al.* (2010) further refined the *F4bcR* locus to a 3.1 Mb interval through haplotype mapping

containing 18 annotated genes between *ZDHHC19* and microsatellite S0075 (Figure 5B). A recombination event in a Large White boar from a Swiss experimental herd was noticed by Rampoldi *et al.* (2011). They further refined the candidate region between the porcine *LMLN* gene and microsatellite *S0283* which contains 7 annotated genes (Figure 5C).

MUC4 encoding an extremely large transmembrane mucin on SSC13q41 was identified with strong associations with susceptibility to ETEC F4ab/ac in a White Duroc X Erhualian cross (Peng *et al.* 2007). The most extensively studied polymorphism in relation to F4ab/ac susceptibility is the SNP at position 8227 in intron at *MUC4*, with the *G* allele associated with susceptibility dominating the resistant *C* allele (Jörgensen *et al.* 2010). However, some studies showed that the mutation in the *MUC4* gene was not in complete linkage disequilibrium (Jacobsen *et al.* 2010; Rampoldi *et al.* 2011). Rampoldi *et al.* (2011) found recombinants between the *MUC4-8227* polymorphism and the phenotype; and suggested the causative mutation downstream of the chromosome and possibly located around the region of *MUC13.*

MUC13, a transmembrane mucin, *HEG1* (heart of glass homologue 1) and *ITGB5* (Integrin beta-5) genes are highly expressed on the apical surface of intestinal epithelia and are thought to be candidate genes for controlling the expression of the receptor for ETEC (Fu *et al.* 2012; Zhou *et al.* 2013). *ITGB5* might regulate the expression of mucin genes, including *MUC13.* The *MUC13* gene maps between 135426700 and 135446300 bp to SSC13 on the reference sequence 11.1. Ren *et al.* (2012) showed two isoforms of *MUC13* (*MUC13A* and *MUC13B*) in pigs and suggested that the possible causative region is proximal to the *MUC13* gene in a tandem repeated 2.3 Mb region. The *MUC13A* allele was completely associated with the F4ab/ac non-adhesion phenotype, while all susceptible pigs carried at least one *MUC13B* allele. This tandem repeat region in exon 2 of *MUC13* is the most likely location for the *F4bcR* locus in Ren's research. However, in the study of Goetstouwers *et al.* (2014), in none of the conditions, *MUC13* antibodies recognized the F4-specific high molecular weight glycoproteins, and the immune-precipitated *MUC13* was not recognised by F4ac fimbriae. They concluded that the two *MUC13* variants are probably not responsible for ETEC F4ab/ac susceptibility. Therefore, the exact causative mutation in this region is still unknown due to sequence uncertainty or technical problems.

1.5. Antibiotic resistance

To control PWD and optimize growth performance, antibiotics and minerals, especially ZnO and $CuSO_4$, are often included in the diets for weaned pigs. Antibiotics are used in food animals for four main purposes: therapeutic use to treat diseased animals; a short-term medication to treat sick animals; prophylaxis use to prevent infections; and growth promotion to improve feed utilization and production (Mathew *et al.* 2007). Antibiotic utilization to improve growth promotion had not been allowed in Switzerland since 1999 and in the European Union since 2006. Antibiotic-resistant bacteria increase rapidly in the environment due to natural selection and spread to humans via unwell-handled meat

products and contaminated crops. Increased public and media awareness has put increased pressure on farmers and veterinarians to reduce antimicrobial use.

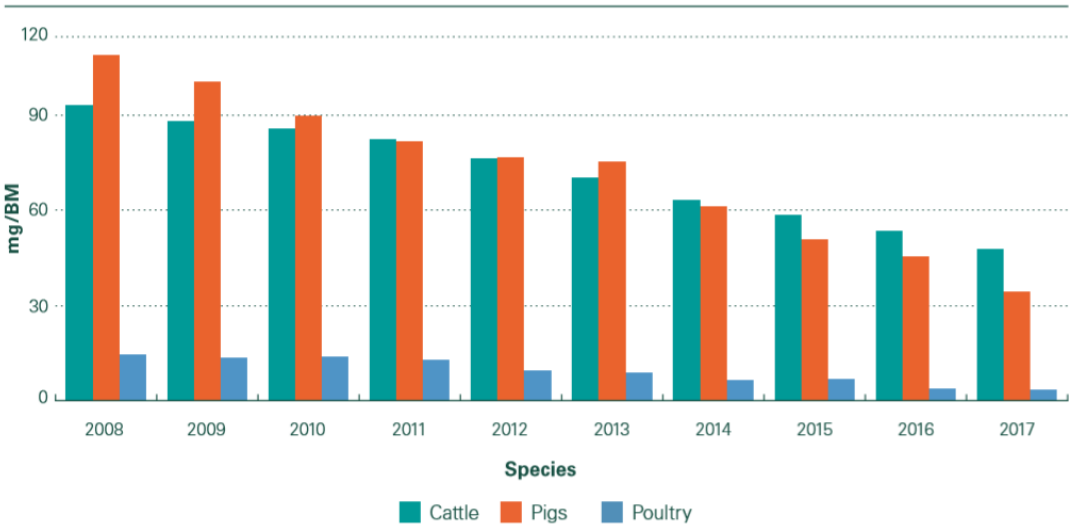

Figure 6: Longitudinal Study Extrapolation of veterinary antibiotic sales among the main livestock species in Switzerland (2008 - 2017) (mg/BM: mg of active ingredient per kg of body mass) (FOPH 2018).

In Switzerland, the total quantity of veterinary antimicrobial products sold for use in all animal categories; reached a peak in 2008 and decreased since 2009 (Arnold *et al.* 2016). The total amount sold in 2013 represents a reduction of 26% in total Swiss antimicrobial sales compared to 2008. Veterinary antibiotic sales decreased substantially from 2008 to 2017 (FOPH 2018). Figure 6 provided an estimate of the distribution among the main livestock species- cattle, pigs and poultry in Switzerland from 2008 to 2017. During the considered the time period, antibiotic consumption in the pig sector was from 109.4 to 34.7 mg/BM (Figure 6). Although the exact proportion of antimicrobials used in pigs in Switzerland is not known, pigs and cattle were estimated to account for the majority of the veterinary antimicrobial use from 2008 to 2017 (Carmo *et al.* 2015; FOPH 2018) (Figure 6). Swine in Switzerland have a high health status; however, antimicrobial use in Switzerland is still relatively high compared to several other European countries (Grave *et al.* 2010). Eighty percent of the total amount of antimicrobials was frequently administered prophylactically. However, prophylactic antimicrobial use has not been shown to decrease mortality; or to reduce the number of therapeutic treatments while causing other negative effects (Arnold *et al.* 2016).

Colistin, a polymyxin antibiotic produced by *Paenibacillus polymyxa var. colistinus,* is widely used for PWD control in pigs (Wang *et al.* 2015). In humans, this antibiotic is considered the last therapeutic option for the treatment of infections caused by multidrug-resistant gram-negative bacteria (MDR-GNB) (Liu *et al.* 2016). On the other hand, studies have reported the isolation of colistin-resistant *E. coli* from pigs, reaching a 35% proportion in some countries in the last several years (Harada *et al.* 2005; Boyen *et al.* 2010; Morales *et al.* 2012). Food-producing animals, especially pigs, have been singled out as the

most potential reservoirs for the spread and amplification of colistin resistance (Nordmann & Poirel 2016). Regulatory agencies such as the European Medicine Agency have recommended reducing the use of colistin in animal production and restricting its use to the treatment of sick animals as a "last resort". To maintain antibiotic efficacy, to avoid the dissemination of resistant bacteria and the exposure of humans to resistant food germs; and to reduce oral prophylactic treatments for *F4bcR*-resistant pigs can be helpful.

The excessive and inappropriate use of antibiotics in animal production has caused ever more bacteria to become resistant to antibiotics and finally influence human health. Antibiotic resistance is responsible for increased morbidity and mortality and adds significant health care costs. Antibiotic resistance is a worldwide problem and threatens public health and the achievements of modern medicine on a global scale. One Health approach is an integrative approach in which national and international cooperation among various disciplines is intended to achieve the best possible effect on human and animal health and the environment. StAR (The Swiss Antibiotic Resistance Strategy) following the One Health approach is carried out which was adopted by the Federal Council on 18 November 2015 in Switzerland (https://www.star.admin.ch/star/en/home/star/strategie-star.html). The major goal of StAR is to maintain the efficacy of antibiotics for humans and animals for the long term. StAR was developed through close cooperation among different offices as followed: the Federal Food Safety and Veterinary Office (FSVO), the Federal Office for the Environment (FOEN), the Federal Office of Public Health (FOPH), the Federal Office for Agriculture (FOAG) and the Swiss Conference of Cantonal Minister of Public Health (CMPH). Eight areas: prevention, prudent use of antibiotics, regulatory and political environment, information and education, cooperation, research and development, monitoring and combating resistance, were involved in StAR against antibiotic resistance. To support the producers or companies in optimizing animal health and antibiotic use, a Swiss Pig Health Service (SGD) project is established. An MMA project to dedicate the problem of milk fever in the mother sow and examine the influence of antibiotic treatment of the mother sow on the intestinal flora. SuisSano (the innovation in pig health, https://www.suisag.ch/gesundheit/suissano-gesundheitsprogramm) is a new health programme from SUISAG, Sempach, Switzerland, to optimize and reduce the use of antibiotics in Swiss pig farming. SuisSano is an electronic treatment journal to record all the treatments the farmers used which might be used for genetic analysis for breeding disease resistance later.

In addition, there are also concerns about the environmental accumulation of minerals resulting from high dietary levels of inorganic zinc and copper. Market, society and consumers currently demand high-quality, healthy and natural foods. Reducing the use of antibiotics is a practical method to meet the requirements mentioned above in the pig industry.

1.6. Food additives

Food additives can be used instead of antibiotics in pig production to control PWD. Feed supplements such as organic acids, zinc oxide, pre-probiotics, antimicrobial peptides, dehydrated porcine plasma, specific egg yolk and bacteriophages (Heo *et al.* 2013; Wang *et al.* 2016) have been used in weaned pigs to enhance growth, feed efficiency to reduce PWD (Rhouma *et al.* 2017). Over the years, plants and plant extracts have been used as traditional medicine to treat various gastrointestinal aliments including diarrhoea in human and animal production (Dubreuil 2013). Antidiarrheal activities of plant products on ETEC are related with the interference function with ETEC enterotoxins activity upon the intestinal epithelium and the inhibitory function on bacterial growth or viability (Dubreuil 2013).

1.6.1. Tannins

Polyphenols are generally believed to have antibacterial activity due to their ability to precipitate protein. Polyphenols from plant extracts are widely used additives in various feed (and food) products, which have recently been suggested to be a potent inhibitor for CT (*Vibrio cholera*) *in vitro* (Morinaga *et al.* 2005). LT is similar regarding physiologic, structural and antigenic capabilities to the toxin produced by CT, with approximately 77% identity at the nucleotide level, 83% amino acid sequence homology and has a comparable mode of action (Fairbrother *et al.* 2005; Verhelst *et al.* 2014). These enterotoxins bind to the oligosaccharide part of the GM1 ganglioside (Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1ceramide) in epithelium of the small intestine and activate the cAMP (cyclic adenosine monophosphate) pathway, which initiates metabolic cascades characterized by net fluid and electrolytes secretions into the intestinal lumen, causing secretory diarrhoea (Turner *et al.* 2006) (Figure 7). The accumulation of cAMP within the cell activates protein kinase A, which phosphorylates the cystic fibrosis transmembrane regulator (CFTR) (Rappuoli *et al.* 1999). STa binds to guanlylate cyclase C (GC-C) leading to the activation of its intracellular catalytic domain, which converts GTP to cyclic guanosine monophosphate (cGMP) and activate cGMP-dependent protein kinase II. This kinase acts on the CFTR is responsible for secretion of chloride and carbonate ions (Figure 7). STb binds to sulfatide, an acidic glycosphingolipid present on intestinal epithelia cells, inside the cell, a G protein is stimulated resulting in calcium ions increase (Dreyfus *et al.* 1993; Rousset *et al.* 1998).

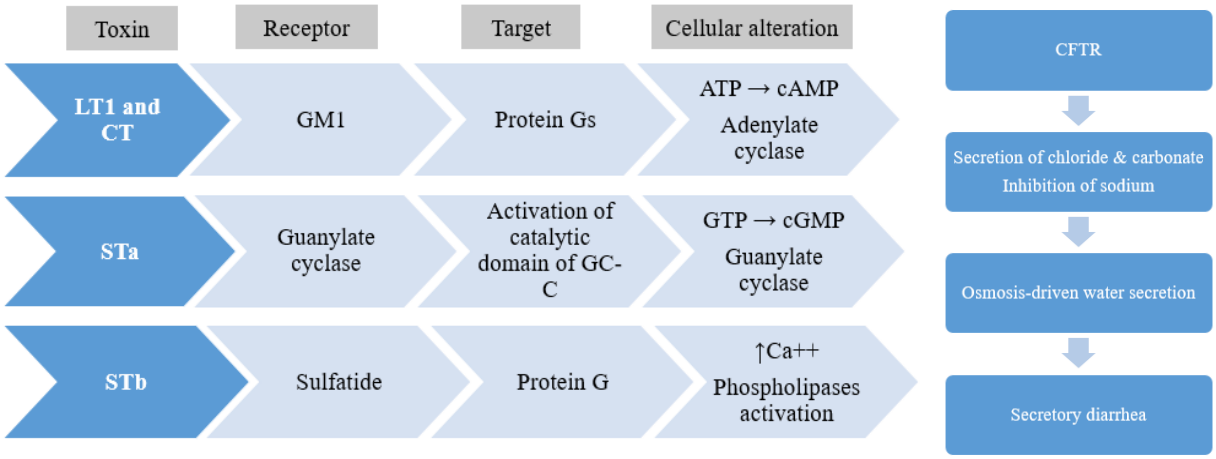

Figure 7: Mechanism of action of ETEC and *V. cholera* enterotoxins leading to secretory diarrhoea. Plant products can inhibit enterotoxins activity at different steps from toxin binding to receptor, uptake and the cellular alterations they provoke (Dubreuil 2013).

Tannins are natural polyphenolic that are found in many vegetable feedstuffs and can be extract from the wood of several trees (Kumar & Vaithiyanathan 1990). However, only a few tannins are able to reduce ETEC diarrhoea due to specific interactions among bacteria toxins (Dubreuil 2013; Verhelst *et al.* 2014). Tannin compounds differ in their antibacterial activity (Akiyama *et al.* 2001; Puupponen - Pimiä *et al.* 2005) and the composition of tannins is influenced by plant of origin and method of extraction. Chestnut tannins extract might be a good candidate to decrease PWD since it possesses already *in vitro* bacterial activity on *Clostridium perfringens* (Elizondo et al. 2010).

Tannins are classified into two broad groups: the hydrolysable and the condensed or non-hydrolysable tannins (Akiyama *et al.* 2001). The hydrolysable tannins are usually compounds containing a central core of glucose or other polyhydric alcohols esterified with gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins) (Graziani *et al.* 2006). The consumption of hydrolysable tannins results in the precipitation of a protein/tannin complex which forms a thin layer of insoluble proteins on the surface of the digestive tract. This thin layer can reduce the absorption of toxic substance and protect the mucous membrane, which consequently having less fluid elimination in the digestive tract and reducing the danger of dehydration. Hydrolysable tannins have antimicrobial properties because of an affinity for membrane proteins and therefore they may have an undefined effect on the flora of the digestive tract. Tannins can reduce digestibility of dietary protein (Mariscal-Landín *et al.* 2004) due to their ability to form insoluble complexes with both dietary protein and digestive enzymes (Jansman *et al.* 1994). Besides their anti-nutritional properties, tannins also display beneficial antibacterial (Ahn *et al.* 1998; Min *et al.* 2007) and antidiarrheal effects (Palombo 2006).

Although tannins are often reported as anti-nutritional products due to the negatively effect on protein digestion (Butler 1992), some authors observed that feeding pigs with feedstuffs high in tannins such as field beans and carob powder did not affect animal growth (Flis *et al.* 1999; Lizardo *et al.* 2002). Tannic

acids also seem to have a negative impact on the growth performance of weaned piglets (Lee *et al.* 2010), the negative effects of tannins remain controversial. However, many studies has produced evidence that feeding weaned piglets with a tannin-rich wood extract can result in improved feed efficiency and reduction of intestinal bacterial proteolytic reactions (Biagi *et al.* 2010). Dietary supplementation with tannins might be beneficial to reduce the incidence of diarrhoea in piglets.

1.6.2. Salicylate

Previous studies showed that aspirin can inhibit cyclooxygenase (COX) and the formation of prostaglandins in humans and animals, and in certain bacteria such as *E. coli* and *Cholera Vibris* causing diarrhoea in pigs (Xu *et al.* 1990). Aspirin fed at low levels (125 to 250 ppm) in a starter diet increased growth performance and reduced scouring of weaning pigs (Xu *et al.* 1990). Acetylsalicylic acid, also known as aspirin, is known to directly inhibit COX-1 and COX-2 via acetylating serine residues in the active site of the enzymes, and hence eventually inhibits the production of prostaglandin E2 (PGE2) (Vilalta *et al.* 2012). Inflammatory responses caused by pathogen infections manifest in loss of appetite and fever, aspirin can be used to smooth the inflammatory responses which might influence the growth performance and severity of pathogenic diarrhoea. Salicylate is an analogue of acetylsalicylic acid and is naturally present in some plants like in sage (*Salvia sp.*) and in sweet birch (*Betula lenta*). Salicylates have been claimed to against cholera toxin (Farris *et al.* 1976) possibly by reducing cAMP concentrations through blockade of prostaglandin synthesis. A previous report on a non-steroid anti-inflammatory agent, flunixin meglumine, suggested that salicylates reduced the severity of diarrhoea in calves (Jones *et al.* 1977). Although the mechanism was unclear, salicylates might be effective in reducing the incidence of diarrhoea in pigs (Bywater 1983).

1.7. Vaccine and Vaccination

Despite four decades of ETEC vaccine development effort, there is at present no vaccine against ETEC, which has demonstrated sustained, broad-based protection in any of the target populations at risk. Nowadays, vaccines against bacterial diseases consist of live-attenuated microorganisms, purified microbial components, inactivated bacteria, recombinant proteins or DNA, and polysaccharide-carrier protein conjugates. However, not all these vaccines are available for use in pigs. For protection against ETEC diarrhoea, commonly used commercially available vaccines are given parentally or orally and may be inactivate whole-cell bacterins or purified fimbrial subunit vaccines (Sellwood 1984; Haesebrouck *et al.* 2004). Some vaccines also contain LT enterotoxin. An experimental vaccine containing a non-toxic form of LT enterotoxin conjugated with the non-immunogenic STa induced antibodies against both toxins (Haesebrouck *et al.* 2004). Enterotoxin neutralising antibodies might be less effective due to release of toxins directly on to the surface of the host cell membrane by adhering bacteria based on the lack of antibodies against fimbrial adhesions (Ofek *et al.* 1990).

The vaccination of pregnant sows with *E. coli* antigens can stimulate production of serum antibodies in the colostrum, however, it does not stimulate local immunity in the gut and does therefore not increase IgA titers in milk. Subsequently, commercial vaccines are only protecting suckling piglets from neonatal *E. coli* diarrhoea and it is a common and efficient method that already used for many years (Haesebrouck *et al.* 2004; Kosorok & Kastelic 2008). These vaccines are not very effective against young pig diarrhoea and PWD. Unlike vaccines for pet animals, there are very few polyvalent pig vaccines available on the EU market. In 1996 (Ivanov 2007), Intervet International B.V. company has developed Porcilis Porcoli DF, which is an inactivated vaccine recommended for the passive immunisation of sow/piglets to reduce mortality and diarrhoea due to neonatal enterotoxicosis caused by *E. coli* F4ab/ac strains. Commercial available vaccines are given parenterally at about 6 and 3 weeks prior to prior to parturition (Ivanov 2007).

In pigs, the six cell layers of the placental structure between the mother and the fetuses in the sow prevent the transfer of maternal antibodies to the fetuses before birth (Matías *et al.* 2017). Newborn piglets can receive antibodies only postnataly from the maternal colostrum and milk (Sellwood 1982). There is no efficient materno-fetal transfer of immunoglobulins (IgG, IgM, IgA) via placenta. Piglets require the immunoglobulins from colostrum to obtain sufficient immunity during early postnatal development (Schnulle & Hurley 2003). Therefore, sow milk provides the chief nutrient source during suckling period of the piglets, it is reasonable to assume that the composition and quantity of milk produced by sows is an important factor in successful piglet production (Klobasa *et al.* 1987). Milk composition differs among breeds and the moment of lactation (Matías *et al.* 2017). The concentration of mother's antibodies is higher in the first days of lactation and is decreased throughout lactation (Rooke & Bland 2002; Hanson *et al.* 2003). The decrease of antibody concentration throughout lactation is connected with predominant isotype changes. A highly pigmented and viscous colostral secretion (colostrum) is accumulated in the mammary gland during the final hours of gestation (Klobasa *et al.* 1987). IgG is the predominant immunoglobulin in sow colostrum while IgA in the mature milk.

Nowadays, ETEC infections of suckling pigs can be best prevented by maternal immunisation and by early supply of immune colostrum (Nagy & Fekete 2005). Several maternal vaccines are available on the market mainly for parenteral application in pregnant sows. As a result, specific colostral antibodies can be supplied for the neonatal piglets as early as the first few hours after birth, thereby blocking the virulence factors and propagation of ETEC bacteria in the intestine. Nowadays, for protection against weaned and post-weaning diarrhoea, safe and effective vaccines are not yet available.

1.8. Objectives and hypotheses of the thesis

The goal of this project is to implement a breeding programme for ETEC F4-resistant pigs and reduce the amount of antibiotic usage in pig production through the breeding programme. The feasibility of

breeding disease-esistant Swiss Large White pigs was tested in genetic, nutritional and immunological aspects.

The following hypotheses were developed and tested:

1) Are *CHCF1* and *ALGA0106330* suitable markers to distinguish ETEC F4ac susceptible/resistant genotypes in Swiss Large White pigs? Is it beneficial to breeding for ETEC F4 resistant pigs? Does breeding for disease resistant pigs influence important production traits? (Chapter 2)
2) Is the established experimental ETEC F4 model effective for testing the effects of food additives on reducing the severity of PWD? Does one percent of hydrolysable chestnut tannins reduce the morbidity and severity of diarrhoea? (Chapter 3) Does two percent hydrolysable chestnut tannins decrease the severity of PWD caused by ETEC F4 with measurements of faecal score, ETEC F4 shedding by plate culturing and qPCR? (Chapter 4)
3) Does salicylate acid supplement improve performance and reduce diarrhoea? (Chapter 4)
4) Is it necessary to vaccinate resistant pregnant gilts? What's the difference of antibody titres in serum, milk and colostrum between susceptible and resistant gilts? (Chapter 5)

Chapter 2: Effective genetic markers for identifying the *Escherichia coli* F4ac receptor status of pigs

This chapter is modified from " Hu, D., Rampoldi, A., Bratus-Neuenschwander, A., Hofer, A., Bertschinger, H. U., Vögeli, P., & Neuenschwander, S. (2019). Effective genetic markers for identifying the Escherichia coli F4ac receptor status of pigs. *Animal genetics*".

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2.1. Abstract

The F4ac receptor gene (*F4acR*), which encodes susceptibility or resistance to *E. coli* diarrhoea, is inherited as an autosomal recessive monogenetic trait. *F4acR* is localized on pig chromosome 13 (SSC13q41-q44) near the *MUC13* gene. Two flanking markers (*CHCF1*, *ALGA0106330*) with high linkage disequilibrium (LD) to *F4acR* were found to be effective for the genetic identification of F4ac-resistant pigs in Swiss Large White breed (one recombinant out of 2034 genotyped pigs). Three recombinant boars, one each from the Duroc, Swiss Landrace and Piétrain breeds, were genotyped with seven different markers and phenotyped by means of a microscopic adhesion test. Only *ALGA0072075*, *CHCF1* and *CHCF3* indicated the correct phenotype. To test the effect of the resistant allele on production trait, 530 Swiss Large White pigs from the national test station were investigated. A significance difference existed among the *F4acR* genotypes in the intramuscular fat content (IMF) of the *longissimus dorsi* muscle, whereas no other production traits were influenced by the resistant allele. The allele frequency of the *CHCF1-C* and *ALGA0106330-A* alleles associated with resistance in the Swiss Large White population was 60%, which is advantageous for implementing this trait in a breeding program to select for *E. coli* F4ac-resistant animals. The selection of resistant pigs should start on the male side due to the inability of resistant sows to produce sufficient amounts of protecting antibodies in the colostrum. Selection of genetically F4ac-resistant pigs is a sustainable and suitable alternative to decrease animal loss and antibiotic use due to diarrhoea.

Keywords: Enterotoxingenic *Escherichia coli,* F4ac receptor, selection for disease resistance, pigs

2.2. Introduction

Enterotoxigenic *Escherichia (E.) coli* (ETEC) with fimbriae of the F4 family are frequently associated with diarrhoea in neonatal and weaned pigs (Moon *et al.* 1999; Osek 1999). In the studies of Kjaersgaard *et al.* (2002) and Li *et al.* (2007), mortality because of pre-weaning diarrhoea has been reported to be 10% and can be up to 25% if not treated. F18 fimbria are typically associated with oedema and/or diarrhoea in pigs over two weeks of age, whereas F4 fimbria are involved in diarrhoea in both neonatal piglets and weaned pigs. To cause diarrhoea, ETEC F4 or ETEC F18 needs to adhere their fimbriae to specific receptors (Bijlsma *et al.* 1982) on the brush borders of the enterocytes in the piglet intestine (Figure 3, Chapter 1). The colonizing bacteria produce enterotoxins that stimulate the secretion of water and electrolytes into the lumen of the small intestine and lead to diarrhoea (Moon *et al.* 1999). In the United States, as well as in Switzerland, fimbriae F18 and F4 are detected in nearly all ETEC isolated (Zhang *et al.* 2007; Schneeberger *et al.* 2017). The receptor of ETEC F18 is controlled by the *FUT1* gene, and the mutation underlying the difference in susceptibility has been identified as a *FUT1c*.307 A>G transition (Meijerink *et al.* 1997; Meijerink *et al.* 2000). Selection for the F18 resistance *FUT1c*.307 *A* allele has been conducted since 2006 in Switzerland and is extremely effective (Luther *et al.* 2009). The genes encoding the F4 receptors have not yet been identified (Rampoldi *et al.* 2011; Rampoldi *et al.* 2014). Three antigenic variants of F4 have been described: F4ab, F4ac and F4ad (Guinee & Jansen 1979). The variant F4ac predominates in the US (Westerman *et al.* 1988), in Central Europe (Alexa *et al.* 2001; Holoda *et al.* 2005) and in Switzerland (Sarrazin *et al.* 2000). Gautschi and Schwörer (1988) reported that 54% and 47% of the Swiss Large White (SLW) and the Swiss Landrace (SL) pigs, respectively, had the F4ac susceptible phenotype. Five patterns of adherence were identified by Bijlsma *et al.* (1982): these phenotypes were designated A (binds F4ab, F4ac and F4ad), B (F4ab and F4ac), C (F4ab and F4ad), D (F4ad) and E (binds no F4 variant). Baker *et al.* (1997) confirmed these five phenotypes and identified a sixth: the phenotype F (binds F4ab only). Targeted mating allowed for the discrimination between fully adhesive receptors ($F4abR^{FA}$ and $F4adR^{FA}$) expressed on all enterocytes and at all sites in the small intestine and partially adhesive receptors ($F4abR^{PA}$ and $F4adR^{PA}$) variably expressed at different sites, often leading to partially bacterial adhesion (Rampoldi *et al.* 2014). These observations led to three additional receptor phenotypes: A2 ($F4abR^{FA}/F4acR^{+}/F4adR^{PA}$), phenotype C2 ($F4abR^{PA}/F4acR^{-}/F4adR^{PA}$) and D2 ($F4abR^{-}/F4acR^{-}/F4adR^{PA}$) (Table 2, Chapter 1).

Sellwood (1979) found evidence that adherence is a dominant trait, inherited in a Mendelian manner with the two alleles: *S* (adhesion, susceptible) and *s* (non-adhesive, resistant). The loci encoding the F4ab and F4ac receptors were assigned to SSC13 by linkage analysis (Edfors-Lilja *et al.* 1995) (More details about genetic mapping can be found in Chapter 1.4.). Guerin *et al.* (1993) reported that the inheritance of *F4bcR* are under the control of two closely linked loci. Python *et al.* (2002) and Python *et al.* (2005) reported the absence of the $F4ab^{-}/F4ac^{+}$ phenotype. Pigs that were susceptible to F4ac were always susceptible to F4ab, and therefore, the two receptors were suggested to be encoded by a single

locus. Rampoldi *et al.* (2014) proposed that the fully expressed F4adR^{FA} phenotype is controlled by two genes that have an epistatic component, where the partially expressed F4adR^{PA} phenotype is controlled by a dominant gene.

The most extensively studied polymorphism in relation to F4ac susceptibility is the SNP at position 8227 in the intron of mucin 4 (*MUC4*), with the *G* allele associated with susceptibility and dominant to the resistant *C* allele (Jörgensen *et al.* 2010). This polymorphism was found to be in complete linkage disequilibrium with the susceptibility phenotype for ETEC-F4ac and is used as a genetic test in the Danish pig breeding industry. Later, Rampoldi *et al.* (2011) found recombinants between the *MUC4*-8227 polymorphism and the phenotype. They suggested the causative mutation downstream of the chromosome and possible located around the region of *MUC13.* Ren *et al.* (2012) performed genome-wide mapping using 39720 informative SNPs. This study revealed that the most significant markers are proximal to the *MUC13* gene in a 2.3 Mb region. They described two *MUC13* alleles. The *MUC13 A* allele was completely associated with the F4ac non-adhesion phenotype, while all susceptible pigs carried at least one *MUC13 B* allele. *MUC13 B* has a unique O-glycosylation region that forms the binding site for the bacteria, while *MUC13 A* does not. However, the exact causative mutation in this region is still unknown, and a diagnostic test using genomic DNA as a template could not be established due to sequence uncertainty or technical problems.

Rampoldi (2013) found that the markers *CHCF1* and *ALGA0106330* predicted the adhesion phenotype correctly in an experimental herd consisting of more than 400 related SLW pigs. Furthermore, 40 randomly selected SLW and 6 SLW pigs that were recombinant in the *MUC4-8227* marker were phenotyped and genotyped using *CHCF1* and *ALGA0106330* with no recombination between these two markers and the *F4acR* phenotype. The aim of the present study was to test the suitability of these markers in SLW, Duroc, Swiss Landrace and Piétrain breeding herds. Recombinant pigs between *CHCF1* and *ALGA0106330* from Duroc, Swiss Landrace and Piétrain pigs were selected and genotyped with the additional markers *MUC4-8227*, *ALGA0072075*, *CHCF3*, *MUC13g*.15376, and *MUC13-226*, which are located around the *MUC13* gene (Figure 8). These pigs were also phenotyped in a microscopic adhesion test (MAT). Furthermore, the possible effects of the resistance allele on production traits were analysed in the Swiss Large White pig, which is the dominating breed in Switzerland.

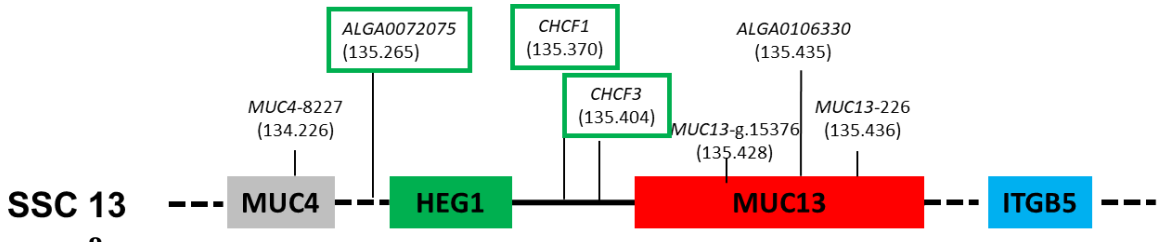

Figure 8:
Markers used for the genotyping of F4acR. Marker positions (Mbp) are according to the reference genome Sscrofa 11.1.

2.3. Materials and methods

2.3.1. Animals, sample collection and screening for genetic polymorphism

This study was conducted in accordance with the ethics guidelines of the Swiss Animal Welfare Act. We collected ear biopsies of 1429 SLW pigs (530 pigs from the Pig Testing Station [SUISAG], Sempach, and 899 pigs of the Swiss Federal Research Station for Animal Production [Agroscope], Posieux, Switzerland). A KASP assay was established (LGC, Teddington, Middlesex, UK) to test the concordance between *CHCF1* and *ALGA0106330* and to estimate the allele effects on production traits in the SLW population (Details can be found in Appendix 1). Additionally, 845 boars (605 SLW, 99 Duroc, 72 SL, and 69 Piétrain) of the artificial insemination centre (SUISAG) in Knutwil, Switzerland, were routinely genotyped in the van Haering Laboratory (Wageningen) using these two markers (Table 3). Nine boars representing recombinants between *CHCF1* and *ALGA0106330* (3 SL, 3 Duroc and 3 Piétrain) of Table 3 were genotyped with the five additional markers *MUC4-8227, ALGA0072075, CHCF3, MUC13G.*15376, and *MUC13-226.* Genotypes were determined by sequencing the PCR fragments using the primers in Table 4. These recombinant boars were also phenotypes for F4ac receptors by MAT (Table 5) when they were slaughtered (electric stunning and exsanguination) in an accredited abattoir after their regular service at the artificial insemination station.

2.3.2. Microscopic adhesion test

For the MAT, the small intestine was separated from the mesentery, and at 4 sites, a 10 to 20 cm empty segment of jejunum was taken (Details can be found in Appendix 2). The site A segment was taken 2 m distal from the cranial mesenteric artery; site D was 2 m proximal to the ileocaecal valve; and sites B and C were 1/3 and 2/3 of the distance between sites A and D. All segments were opened longitudinally, placed in PBS-EDTA and stored at 4°C until further processing. The enterocytes were prepared, and MAT was performed according to (Vögeli *et al.* 1996; Python *et al.* 2002; Rampoldi *et al.* 2014).

2.3.3. Statistical analyses and pigs for estimation of the genotype effect on production traits

Swiss Large White pigs from the pig testing station were selected to estimate the effects of the resistance allele on 17 production traits. Per litter, one castrated male and one female pig per litter were performance tested between 35 and 110 kg live weight. Pigs were fed ad libitum in pens of 9 to 10 animals and feed intake was recorded for each individual pig using feeders equipped with radio-frequency identification (RFID) ear tag readers. Animals to be genotyped were chosen by randomly selecting tested litters that finished their test between July 2013 and March 2014. The requirements for genotyping sire family was 3 to 5 litters, i.e., 6 to 10 progeny per sire to ensure a minimal number of progeny per sire. In total 530 pigs of 265 litters and 75 sires were genotyped. The performance traits provided by SUISAG were pre-corrected for the effect of sex and weight at end of the test by SUISAG

correction factors. Additionally, 1551 contemporarily tested pigs of the same breed as well as 400 crosses with Swiss Landrace, were included in the analysis to more accurately estimate and account for the contemporary group effects acting on production traits. Contemporary groups were defined according to the barn where the pig was tested and the time period of the test. In total, 2081 pigs (530 with known and 1531 with unknown genotypes), descending from 238 sires of 1142 litters and grouped into 55 contemporary groups, were analysed. The mixed model included the fixed effects of the contemporary group, breed and the genotype effect. The pigs without a genotype were assigned to a separate level ("unknown") of genotype effect. The random effects included a sire and litter effect to model the variance-covariance structure among the observations by accounting for polygenic and permanent environmental effects on littermates. Data were analysed by the lme package in R (R Core Team 2014). To check the concordance of the genetic variance with the literature, a rough estimate of heritability as h^2=4 x sire variance/phenotypic variance was calculated. The significance of the genotype effect was tested using an F-test. If significant, the contrasts between the genotypes were tested by a t-test.

2.4. Result

2.4.1. Genotyping of *CHCF1* and *ALGA0106630* marker loci for prediction of *F4acR* genotypes in SLW, Duroc, SL and Piétrain pigs

From a total of 2274 genotyped pigs, we identified 48 pigs with a recombination in the interval between *CHCF1* and *ALGA0106330* (Table 3). The frequency was lowest in SLW (0.05%) followed by SL (16.6%), Piétrain (20.2%) and Duroc (21.2%).

Table 3: Number of genotyped pigs and recombinants between the markers *CHCF1* and *ALGA0106330.*

Breed	Number of pigs genotyped	Number of recombinant pigs	% recombinant pigs
Swiss Large White	2034	1	0.05
Duroc	99	21	21.2
Landrace	72	12	16.6
Piétrain	69	14	20.2
Total	2274	48	-

Table 5 contains the results of 9 recombinant boars from 3 different breeds (3 Duroc, 3 Swiss Landrace and 3 Piétrain) with the genotypes of seven markers and the phenotype. All boars were resistant. They did not show bacterial adhesion in the MAT.

The recombinant pigs provide information for the location of *F4acR* in the interval *MUC4-MUC13.* Landrace boars 8318JS3 and 844JS4 were recombinant between *MUC*-8227 and *ALGA0072075* and *CHCF3* and *MUC13*g.15376, respectively. All other boars showed only recombination between the markers *CHCF3* and *MUC13*g.15376 (Table 5). The Landrace boar 5676NO3 showed recombination on the same chromosomes between the two markers. Our analysis indicates that in Swiss Landrace, Durco and Piétrain pigs, *ALGA0072075*, *CHCF1* and *CHCF3* are better markers to predict the F4ac phenotype than *MUC4*-8227, *MUC13*g.15376, *ALGA0106330* and *MUC13*-226. Obviously, a boundary between the upstream and downstream markers of *MUC13* confirm the hypothesis that the region proximal to *MUC13* is most likely the cause of ETEC F4ac susceptibility in pigs.

The *CHCF1* and *ALGA0106330* allele frequencies are summarized in Table 6. The *CHCF1-C* and *ALGA0106330-A* alleles associated with the *F4acR* resistant allele vary from 0.39 to 0.96. The frequency of the resistance allele in lowest in the SL, followed by SLW and Piétrain and is highest in the Duroc breed.

Table 4: Description of the SNPs.

SNP name	Sequence (5'-3')	Annealing temperature (°C)	Amplicon length (bp)	SNP position alleles (*R/S*)[a]
MUC4-8227[b]	F: CACTCTGCCGTTCTCTTTCC	56	367	216 bp
	R: GTGCCTTGGGTGAGAGGTTA			C/G
ALGA0072075[b]	F: ATCACCTCCTGGAACCACAG	57	693	413 bp
	R: AAAGCTGCGGACAGTGAGAT			C/T
CHCF1[b,c]	F: AACATTTTGGAGTCAAATCGTG	52	540	216 bp
	R: GGGTAACATCCATCACCACA			C/A
CHCF3[b]	F: CAAAGTGATTCGGTTTCTCATC	52	463	298 bp
	R: GTGGGGGAGGAATAAATTAGG			C/A
*MUC13*g.15376[b]	F: ACCATGTGTGTAAGTCGCTGAG	55	361	230 bp
	R: ACGTTTCCCCCTCTTTGTAGTT			G/A
ALGA0106330[b,c]	F: CGATCAAGTTCAAGATCTCTTCTG	55	250	167 bp
	R: TGACTGTCACATCTTCCTTATCTT			A/G
MUC13-226[b]	F: TGAGCAAGATGAGTGCCCCAGT	58	536	205 bp
	R: TAGCCAGGCAGGCACAAGCA			G/A

[a]*R* alleles are associated with resistance and *S* are associated with susceptible haplotypes.
[b]SNPs from Rampoldi (2013). The primers were used to genotype the recombinant boars by sequencing.
[c]These loci were also typed by a KASP assay

Table 5: Haplotype results at 7 loci in three recombinant Duroc, Landrace and Piétrain boars and two recombinant Swiss Large white pigs.

R/R represents the resistant, *R/S* represents the heterozygous and *S/S* represents the homozygous susceptible genotype. The resistant phenotype (R) was determined by means of a microscopic adhesion test (MAT).

Breed	**Animal ID**	***MUC4*-8227**	***ALGA 0072075***	***CHCF1***	***CHCF3***	***MUC13* g.15376**	***ALGA 0106330***	***MUC13*-226**	**Phenotype**
Duroc	1221HK	*R/R*	*R/R*	*R/R*	*R/R*	*R/S*	*R/S*	*R/S*	R
Duroc	1259HK	*R/R*	*R/R*	*R/R*	*R/R*	*R/S*	*R/S*	*R/S*	R
Duroc	1352HK	*R/R*	*R/R*	*R/R*	*R/R*	*R/S*	*R/S*	*R/S*	R
Landrace	8318JS3	*R/S*	*R/R*	*R/R*	*R/R*	*R/S*	*R/S*	*R/S*	R
Landrace	844JS4	*R/S*	*R/R*	*R/R*	*R/R*	*R/S*	*R/S*	*R/S*	R
Landrace	5676NO3	*R/R*	*R/R*	*R/R*	*R/R*	*S/S*	*S/S*	*S/S*	R
Piétrain	660HVE	*R/R*	*R/R*	*R/R*	*R/R*	*R/S*	*R/S*	*R/S*	R
Piétrain	946HVE	*R/R*	*R/R*	*R/R*	*R/R*	*R/S*	*R/S*	*R/S*	R
Piétrain	8649WAV	*R/R*	*R/R*	*R/R*	*R/R*	*R/S*	*R/S*	*R/S*	R
SLW *	5.8250.JR2	*R/R*	*R/R*	*R/R*	*R/R*	*R/S*	*R/S*	*R/S*	R
SLW **	3100.CF5	*R/R*	*R/R*	*R/R*	*R/R*	*R/S*	*R/S*	*R/S*	R

The nucleotides for the resistance (*R*) and susceptibility (*S*) alleles are listed in Table 3.

*: 5.8250.JR2 is the recombinant pig mentioned in this chapter in Swiss Large White.

**: 3100.CF5 is another recombinant pig found in SUISAG breeding system after the submission of these chapter.

Table 6 : Allele frequencies of *CHCF1* and *ALGA0106330.*

Breed	[a]*CHCF1-R*	*CHCF1-R*	[a]*ALGA0106330-S*	*ALGA0106330-S*
[b]Swiss Large White	0.60	0.40	0.60	0.40
[b]Duroc	0.96	0.04	0.85	0.15
[b]Landrance	0.49	0.51	0.39	0.61
[b]Piétrain	0.80	0.20	0.69	0.31

[a]*CHCF1-R* and *ALGA0106330-R* alleles are associated with the *F4acR* resistant allele.

[b]Number of pigs are given in Table 3.

2.4.2. Effect of *CHCF1* genotypes on production traits in SLW pigs

The estimates for heritability in Table 7 are concordant with the literature, except for most traits related to the leanness of the carcass where our estimates are lower than expected. The *S/S* genotype at *CHCF1* was associated with higher intramuscular fat content (IMF) compared to the *R*/*R* and *R*/*S* pigs (Tables 7 and 8). The *R* allele seems to be dominant over the *S* allele. Although significant, the difference in IMF between *S*/*S* and the other genotypes is relatively small (Table 8). No other important production traits were influenced by the resistance allele (Table 7). The heritability value for IMF is 0.58. This high value enables keeping the IMF% constant through conventional breeding.

Table 7: Effect of the CHCF1 genotype on 17 production traits in Swiss Large White pigs (N=530).

Trait[a]	Heritability h^2	P-value(F-test)[b]
ADG	0.24	0.151
TDG	0.25	0.758
Fcons	0.30	0.211
Fconv	0.37	0.704
LMC	0.20	0.211
FlFl	0.47	0.233
FeFl	0.36	0.470
FlFeV	0.24	0.423
SpB	0.24	0.899
IMF	0.58	0.030*
DL	0.12	0.306
pH1K	0.19	0.798
pH1S	0.22	0.952
pH30K	0.09	0.557
pH30S	0.17	0.374
Pigm	0.24	0.208
L	0.10	0.227

[a]Abbreviations: ADG, average daily gain birth to slaughter; TDG, average daily gain per day of test; Fcons, feed consumption per day of test; Fconv, feed conversion ratio (feed consumed/weight gain); LMC, lean meat content of carcass; FlFl, loin area (surface of the m. longissimus dorsi (mld)); FeFl, fat area (surface of fat area over the

mld); FlFeV, ratio of meat to fat area (mld); SpB, back fat thickness; IMF, intra muscular fat content (mld); DL drip loss (mld); pH1K, pH 1.5 h post mortem (pm) in loin; pH1S, pH 1.5 h pm in ham; pH30K, pH 24 h pm in loin; pH30S, pH 24 h pm in ham; Pigm, pigmentation of meat in mld; L, degree of lightness of meat in mld (Minolta L).

[b]*P-value <0.05 was considered significantly different.

Table 8: Effect of *CHCF1* genotypes on intramuscular fat content (IMF%) in the mld of Swiss Large White pigs.

Differences between genotypes			
RR-SS		*SR*-0.5*RR/SS*	
Estimate	P-value (t-test)	Estimate	P-value (t-test)
-0.19%	0.031*	-0.13%	0.023*

*P<0.05 was considered significantly different.

2.5. Discussion

The DNA marker-based test with the KASP assay is a suitable and effective method to determine the genotypes at the two markers, *CHCF1* and *ALGA0106330* for *F4acR* in pigs. In Swiss Landrace, Duroc and Piétrain pigs, *ALGA0072075*, *CHCF1* and *CHCF3* were better markers to predict the *E. coli* F4acR phenotype than *MUC4*-8227, *MUC13*g.15376, *MUC13-226* and *ALGA0106330.*

The allele frequency of the *CHCF1-C* allele was 0.96 in the Duroc breed. Therefore, more than 90% of the Duroc pigs were assumed to be resistant. The allele frequencies of the *CHCF1-C* allele in the SL, SLW and Piétrain pigs were between 0.49 and 0.80, which is advantageous for implementing a breeding programme to select for *E. coli* F4ac resistant pigs in these breeds.

A significant but small difference was observed among *F4acR* genotypes in the intramuscular fat content (IMF) of the *longissimus dorsi* muscle, whereas no other important economic traits were influenced by the resistance allele. Only one recombinant was present between *CHCF1* and *ALGA0106330* of 2034 genotyped SLW pigs (Table 3). Therefore, the allele effect of both markers in the same. Häfliger (2016) reported a negative effect of the resistant allele on the rearing ration. Indeed, *R/R* (sow) x *S/S* (boar) mating resulted in the lowest rearing rate compared to other mating types (A. Hofer 2017, Suisag internal reviews). Therefore, if breeding for resistance to *E. coli* F4ac becomes applicable, the immunity component should be considered regarding a breeding strategy. Virtually no transfer of immunoglobulins occurs across the placenta in the sow. Piglets born to susceptible sows are protected by *E. coli* F4ac antibodies present in the colostrum if the mother had previous contact with this pathogen. Resistant sows devoid of F4ac receptors do not produce antibodies in the colostrum, and piglets are not passively immune (Sellwood 1982, 1984). Thus, the selective advantage of the resistant genotype is reduced, as the susceptible sows become immune. Sellwood (1979) showed that resistant sows mated with homozygous susceptible boars have the highest incidence (32%) of diarrhoea in the offspring, while there is lower incidence of diarrhoea in the offspring of susceptible sows. Consequently, breeding for

resistant *E. coli* F4ac pigs should start on the male side or resistant gilts should be vaccinated when mated to susceptible boars (Riising *et al.* 2005).

The data from Edfors-Lilja *et al.* (1986) indicated that the presence of the F4ac receptor resulted in poorer daily gain during the first weeks of life but had a beneficial influence on the daily lean growth during the fattening period. No such influence could be observed in the present study. The estimated genotype effect on IMF was small. We have no biological explanation and the significant test result could be a false positive due to multiple testings.

The results of the nine phenotyped boars confirmed that the *MUC4*-8227 SNP is associated with F4ac ETEC susceptibility (Jorgensen *et al.* 2004). Nevertheless, the genotypes, especially *R/R*, were not completely consistent with the results of the *in vitro* MAT. This result confirms the earlier findings that the *MUC4*-8227 SNP is a marker but not the causative mutation (Rasschaert *et al.* 2007; Goetstouwers *et al.* 2014). Recently, *MUC13* was suggested as the causal gene for F4ac susceptibility (Ren *et al.* 2012). In the study of Goetstouwers *et al.* (2014), *MUC13* antibodies did not recognize the F4-specific high molecular weight (MW) glycoproteins under any conditions. Furthermore, the immunoprecipitated *MUC13* was not recognized by the F4ac fimbriae. From these results, Goetstouwers *et al.* (2014) excluded *MUC13* as one of the F4-binding high MW glycoproteins, and F4 fimbriae do not bind specifically to *MUC13.* They conclude that the two *MUC13* variants (*MUC13A* and *MUC13B*) are probably not responsible for susceptibility towards F4ac ETEC. Our candidate region of (SSC13: *ALGA0072075-CHCF3*; 135’265’806-135’403’999) is in complete linkage with the F4acR phenotype. In this region, only *HEG1* is annotated based on the available reference genome sequence (Sscrofa 11.1). According to Rampoldi (2013), the markers found in *HEG1* by RNAseq were not associated with *F4acR*. We hypothesize that the interval *HEG1-MUC13* is the most likely candidate region, although it lacks annotated genes.

2.6. Acknowledgements

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Appendix I

1. Kompetitive Allele Specific PCR (KASP) assay

The Kompetitive Allele Specific PCR (KASP™, LGC, Teddington, Middlesex, UK) genotyping system is a homogeneous, fluorescent endpoint genotyping technology. KASP genotyping technology utiliszs a unique form of competitive allele-specific PCR (polymerase chain reaction) that enables highly accurate biallelic scoring of SNPs (single nucleotide polymorphisms) and InDels (insertions and deletions) at specific loci across a wide range of genomic DNA samples, including those of complex genomes. In this study we tested the concordance between *CHCF1* and *ALGA0106330*. KASP assays with *CHCF1* and *ALGA0106330* were used for genotyping ETEC susceptible/resistant pigs.

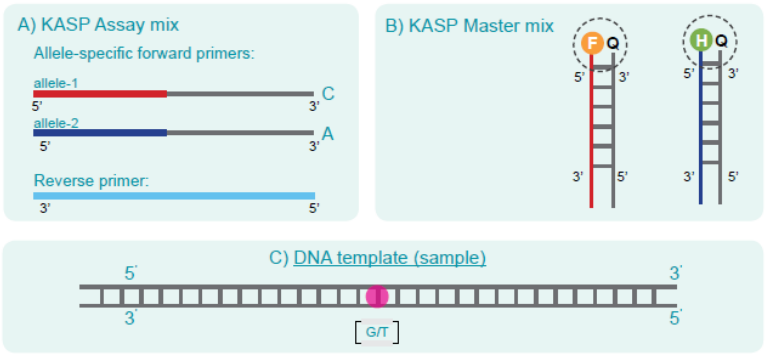

Figure 9: Assay components.

KASP uses three components: A) KASP Assay mix containing two different, allele-specific, competing forward primers with unique tail sequences (Red and Blue tails); B) KASP Master mix containing FRET cassette plus Taq polymerase in an optimized buffer solution (Orange FAM dye and Green HEX dye); C) test DNA with the SNP of interest (Pink SNP) (modified from KASP brochure). The picture is taken from https://www.lgcgroup.com/products/kasp-genotyping-chemistry.

KASP genotyping reactions are run by KASP assay mix (two allele-specific forward primers and one common reverse primer), KASP master mix and sample DNA (Figure 9). KASP by Design (KBD) was purchased in this study which was designed *in-silico* and shipped to our lab to determine SNP genotypes (allele-specific primer in master mix is for each SNP allele, unknown due to business secret). The KASP primer sequences were unknown.

Table 9: Constituent reagent volumes for making KASP genotyping mix.
*DNA sample diluted to final concentration of 5 – 50 ng per reaction.

	KASP genotyping mix assembly	
Components	Wet DNA method (µL)	Dry DNA method (µL)
DNA*	5	N/A
2x Master mix	5	5
Primer mix	0.14	0.14
H_2O	N/A	5
Total reaction volume	10	10

All reagents should be briefly vortex-mixed prior to use.

In our study, SNP genotyping was carried out using a total reaction volume of 10 µL for 96-well plate (Wet DNA method in Table 9). Fifty micrograms of high-quality DNA per reaction were used for the KASP assay. The mechanism of KASP PCR is illustrated in detail in Figure 10. Fluorescence for a specific target allele will be quenched depending on the test samples. Figure 10 explains how PCR works when the test DNA samples are homozygous for allele-1 and releasing the FAM fluorophore in the end.

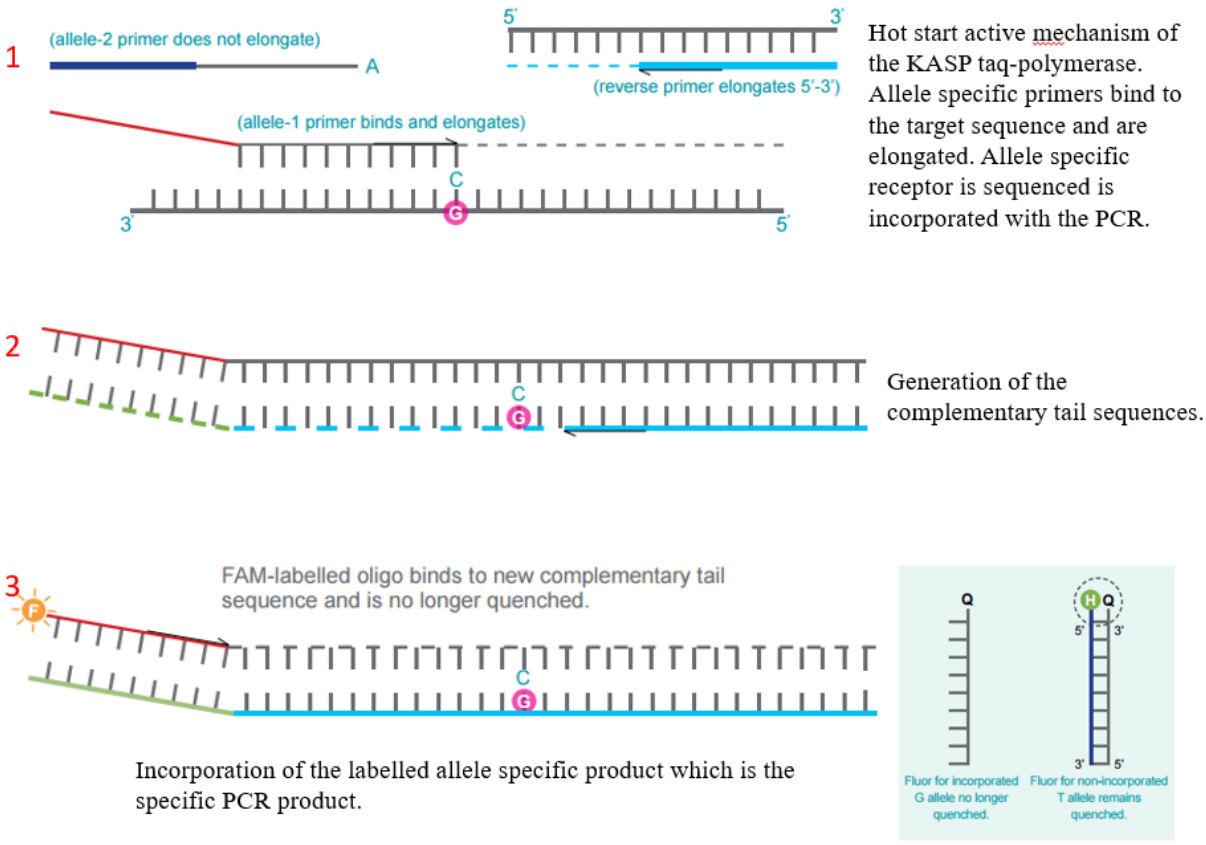

Figure 10: PCR mechanism using KASP assay.

1) In the first round of PCR, one of the allele-specific primers matches the target SNP and amplifies the target region with the common reverse primer. 2) To complete allele-specific tail sequence generation by binding reverse primer and elongating a complementary copy of allele-1 tail. 3) In the third round of PCR, levels of allele-specific tail increase. The flour labelled part of the FRET cassette is complementary to new tail sequences and binds, releasing the flourophore from the quencher to generate a fluorescent signal, which will be caught by the machine. The picture is taken from KASP brochure.

KASP PCRs were generally performed in a 10 µl reaction volume based on Table 9. Amplification was carried out in 96-well plates on a Bio-Rad CFX96 Connect™ Real-Time PCR Detection System. After hot-start activation at 94°C for 15 min, the samples were cycled 10 times as follows (round 1): denaturation at 94°C for 20 s and annealing at 61-55°C for 60 s with 0.6°C dropping per cycle. At the end, the samples were cycled 26-30 times as follows (round 2): denaturation at 94°C for 20 s and elongation at 55°C for 60 s. A fluorescence reading step after the final cycling step at 37°C for one minute was added. If no clear genotyping clusters have been obtained, the plate should be thermally cycle 2-3 times (round 2) further and fluorescence read again.

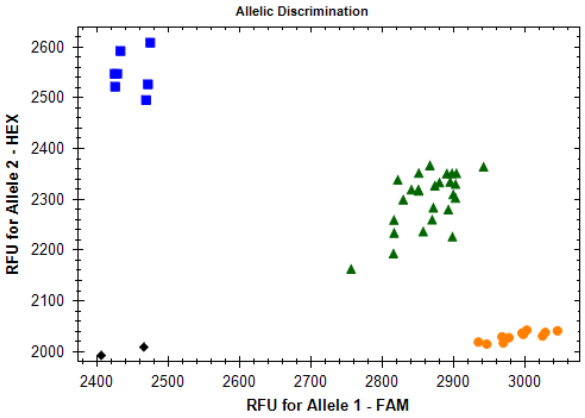

Figure 11: Fluorescence plot after the KASP assay plate reading.

Blue squares indicate that the sample is homozygous for the HEX allele. Green triangles indicate that the sample is heterozygous and contains both the FAM allele and the HEX allele. Orange dots indicate that the sample is homozygous for the FAM allele. Black squares represent negative controls. RUF: relative fluorescent units.

After completion of the KASP PCR, reaction plates were read and the data analysis was performed using CFX manager 3 software. The detected signals are plotted as a graph, with samples of same genotypes clustering together (details can be found in Figure 11). For *CHCF1*, orange dots represent the resistant genotype. For *ALGA0106330*, blue dots represent the resistant genotype.

2. Microscopic adhesion test (MAT)

The phenotype (susceptible or resistant to ETEC F4) was established by MAT using three *E.coli* test strains E68I (O 141: K85ab: F4ab), G4 (O 45: K(E65): F4ac) and Guinée (O 8: K 87: F4ad), which were procured from the Veterinary Laboratories Agency Weybridge, Surrey GB (Thorns *et al.* 1987). The expression of the fimbriae was checked serologically, and the variant specificity was determined by PCR (Alexa *et al.* 2001). Adhesive and non-adhesive enterocytes were conserved frozen and used as controls in each MAT. Tested pigs were slaughtered and phenotyped at 7 months of age. Founder and the offspring that were used for breeding were tested when they were eliminated from breeding. Occasionally, we slaughtered weaner pigs at 2 to 3 months of age. Usually, feed was withheld for 16 h before the pigs were slaughtered. Water and straw were always offered (Rampoldi *et al.* 2014).

The interval between slaughtering and separation of the small intestine from the mesentery from the upper mesentery root following duodenum should be kept as short as possible to avoid degeneration of the epithelial intestinal cells. Four sites of 10 to 20 cm empty segment of jejunum were collected, opened longitudinally and immersed immediately into 80 ml cold PBS-EDTA buffer stored at 4 °C until further processing. The interval between killing and sampling of intestinal tissue was not more than 20 to 40 min to avoid degeneration of the epithelial intestinal cells. Site A segment was taken 2 m distal from the cranial mesenteric artery; site D segment was taken approximately 2 m proximal to the ileocecal valve; site B and C segments were taken at 1/3 and 2/3 of the distance between sites A and D. The enterocytes

were prepared, and the MAT was performed according to Vögeli *et al.* (1996), Python *et al.* (2002) and updated version from Rampoldi *et al.* (2014). Epithelial cells were removed by scraping the mucosal surface with a microscopic slide and collected in PBS containing 2% formaldehyde. Gross particles were pieced by scissors, and the supernatant was cleansed twice by centrifuging 10 min at 200 x g, resuspended in 10 ml PBS and vortexed at +4°C. The final suspension was diluted in PBS containing 2% mannose. A 1 ml aliquot of enterocyte suspension was mixed with 1 ml of undiluted broth culture in wells of a 6 well microtitre plate and statically incubated for 30 min at 37°C. After incubation, 20 µl mix solution was placed on a slide, covered with a cover-slip and examined by light microscopy (Figure 12). Bacteria adhere to the brush border of enterocytes from slaughtered pigs with a susceptible phenotype, otherwise, with a resistant phenotype.

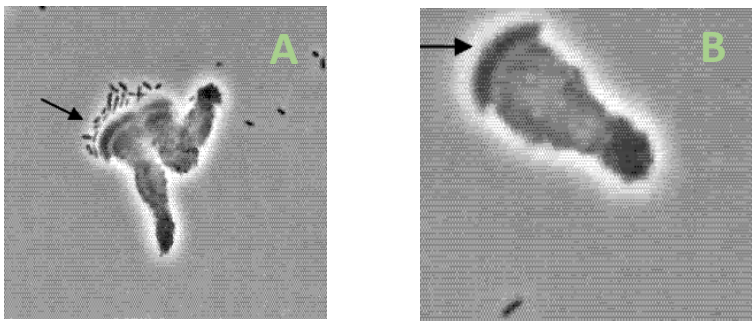

Figure 12: Microscopic enterocyte adhesion test with ETEC F4 bacteria.
A: Bacteria adhere to the brush border of enterocytes from slaughtered pigs with a susceptible phenotype.
B: Bacteria do not adhere to the brush border of enterocytes with a resistant phenotype (Python 2003).

Twenty well-separated enterocytes were selected per segment per strain and scored under an optical microscope. An enterocyte was classified as adhesive if more than five bacteria adhered to the breush border. When more than 0%, but less than 30% of the cells show adhesion of > 5 bacteria, 40 cells must be examined. In the case with 0% adhesive enterocytes at all four sites was considered as resistant; more than 85% of adhesive enterocytes proposed as fully adhesive; for the case between 0% and 85%, adhesive enterocytes at all sites were proposed as partially adhesive (Table 2).

3. Sequence with seven markers

The GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) was used to extract DNA from ear biopsies according to the manufacturer's instructions. All extracted DNAs were genotyped with KASP assay with markers *CHCF1* and *ALGA0106330*. When the genotyping results point to different F4ac phenotypes, the piglet is assumed to be a recombinant. Additionally, five markers, *MUC4-8827*, *ALGA0072075*, *MUC13*g.15376, *MUC13*-226 and *CHCF3*, were selected for further sequencing to distinguish genotypes. The F4ac phenotype of these recombinant pigs was determined by MAT.

JumpSmart™ Taq DNA polymerase (D9307, Sigma-Aldrich) was used for PCR with these seven markers (Table 4). Reagent volumes per reaction in total 25 µl were as follows: 2.5 µl of 10X reaction buffer containing 15 mM $MgCl_2$, 4 ml of dNTPs (1.25 mM), 1 ml of each forward and reverse primer (20 µM), 0.5 ml DNA Taq polymerase (2.5 U/µl), 14.5 µl water and 1.5 µl DNA. The thermal cycling conditions for all these primers in Table 4 were 94˚C for 15 min followed by 40 cycles at 94˚C for 30 s, YY˚C for 30 s, and 72˚C for 45 s. Then, 72˚C for 5 min and 4˚C until termination. YY indicates the annealing temperature of these seven markers. PCR products were placed in 1.2% agarose gels. The QIAquick Gel Extraction Kit (28704, Qiagen) was used to extract and purify DNA from agarose gels.

Barcode Economy Run from Microsynth AG, Switzerland was used for sequencing. DNA samples and sequencing primers were sent pre-mixed (within one tube). Each DNA sample should have a volume of 12 µl (Table 10). Three microliters of sequencing primer solution (forward or reverse, 10 µM) is included. Except for *CHCF3*, six other markers were used forward primer to sequence.

Table 10: Sample amounts per sequencing reaction and concentration.

DNA Template	Concentration	Effective Amount (12 µl)	Pipetting scheme for pre-mixed Option
PCR	18 ng per 100 bases in a volume of 12 µl		12 µl DNA template solution + 3 µl sequencing primer solution
PCR (200bp)	3.0 ng/µl	36 ng	
PCR (300bp)	4.5 ng/µl	54 ng	
PCR (400bp)	6.0 ng/µl	72 ng	
PCR (>400bp)	Etc.	Etc.	

Chapter 3: Hydrolysable chestnut tannins for reduction of post-weaning diarrhoea: efficacy on an experimental ETEC F4 model

This chapter is modified from "Girard, M., Thanner, S., Pradervand, N., Hu, D., Ollagnier, C. and Bee, G., 2018. Hydrolysable chestnut tannins for reduction of postweaning diarrhea: Efficacy on an experimental ETEC F4 model. *PloS one*, *13*(5), p.e0197878.

All experimental procedures were in compliance with Swiss animal welfare guidelines and were approved (No. 2014_54_FR) by the Cantonal Veterinary Office of Fribourg (Switzerland). This study was performed at the piggery of the research station Agroscrope-Posieux (Switzerland).

3.1. Abstract

An experimental model for post-weaning diarrhoea (PWD) with enterotoxigenic Escherichia coli F4 (ETEC F4) was set up in piglets, and the efficacy of 1% chestnut-tannin extract in preventing diarrhea was subsequently assessed. In a first trial (infection model), 32 Swiss Large White piglets (age: 24 days; average BW: 7.8 ± 0.8 kg) were randomly assigned to two experimental groups (infected [INF], non-infected [NINF]).

In a subsequent trial, 72 Swiss Large White piglets (age: 26 days; average BW: 7.4 ± 1.5 kg; genotype: susceptible) were blocked by bodyweight and assigned within block to four experimental groups:

NINF-CO: not infected and fed a standard control starter diet (CO);
INF-CO: infected and fed the CO diet;
NINF-TA: not infected and fed the CO diet supplemented with 1% chestnut extract containing 54% of hydrolysable tannins (TA);
INF-TA: infected and fed the TA diet.

Both diets (TA and CO) were formulated to be isocaloric and isoproteic and to meet or surpass the nutritional requirements. In both trials, four days after weaning, piglets assigned to the INF group received an oral suspension of ETEC F4. Faecal score, ETEC shedding in faeces (only in trial 2), and growth performance traits were measured for the following 14 days post infection. In both trials, more than 50% of the INF piglets developed diarrhoea within six days post infection. Tannins reduced ($P < 0.05$) the average faecal score, the percentage of piglets in diarrhoea, and the duration of diarrhoea, whereas feed intake and the average daily gain were unaffected.

3.2. Introduction

Antimicrobials (AM) have revolutionized medicine in many respects, but have sole and over usage led to rapid appearance of resistant mechanisms (Organization 2014). The prevalence of resistant microbes increase with the selection pressure applied by AM utilization in human and veterinary medicines. The majority of AM (weight based) is used in industrial food animal production (Silbergeld *et al.* 2008). Prophylactic and metaphylactic measures, consisting of treating entire groups of mixed healthy and diseased animals, drastically enhance microbes' exposure to AM. While targeting pathogens, AM also affect natural microflora, and especially the gut microflora. Selection and dissemination of antimicrobial resistant microbes or plasmids frequently occur in the gastrointestinal tract of livestock. In addition, up to 90% of AM administered orally retain their antimicrobial activity in faeces and potentially alter the soil microbial ecosystem after application of manure (Kumar *et al.* 2005). It is suggested that the use of AM in livestock might increase the prevalence of resistant microbes in the human gastrointestinal tract (Dutil *et al.* 2010) and therefore may increase the risk of human infections with resistant pathogens.

One of the production disease for which AM are commonly used in pig production is post-weaning diarrhoea (PWD). Numerous stress factors are associated with weaning, including social, environmental and dietary changes. These stressors can alter the homeostasis of intestinal microflora (Melin *et al.* 2004) rendering young piglets more inclined to gastrointestinal tract infections. The etiology of PWD is multifactorial, although it is commonly associated with the proliferation of beta-hemolytic ETEC, sometimes in association with rotavirus infections (Melin *et al.* 2004). Fimbria ETEC adhere to enterocytes specific receptors and secrete enterotoxins (LT, STa and STb), causing electrolytes and net fluid losses (Heo *et al.* 2013). This results in dehydration, weigh loss for the piglet, and sometimes death (Fairbrother *et al.* 2005).

Thus, managing the period around weaning is challenging for the piglets and the farmers. With the increasing occurrence of antimicrobial resistance, there is an urgent need to reduce AM used by identifying dietary alternatives for alleviating PWD in pig production (Biagi *et al.* 2006; Turner *et al.* 2006; Adeola & Cowieson 2011; Halas & Nochta 2012). Polyphenols, such as tannins from oak (*Cortex quercus*), were used to treat diarrhoeal disease in the pre-antibiotic era. Plant polyphenols are known to have antimicrobial properties (Cowan 1999) and inhibitory effects on bacterial toxins (Morinaga *et al.* 2005). However, interactions with bacterial toxins seems to be specific (Dubreuil 2013), as only a few tannins are able to reduce ETEC diarrhoea (Verhelst *et al.* 2014). Chestnut-tannin extract may be a good candidate for decreasing PWD, because it already possesses in vitro bactericidal activity on several bacteria (Graziani *et al.* 2006; Elizondo *et al.* 2010). The main objective of this study was to assess the effect of hydrolysable chestnut-tannin extract on the prevalence of diarrhoea using an ETEC-infection model with weaned piglets.

3.3. Materials and Methods

3.3.1. Harbouring the infectious ETEC strain

The native ETEC strain used in this study was isolated from a weaned piglet at the piggery of the research station Agroscope-Posieux (Switzerland); the piglet exhibited acute PWD. The strain was resistant to sulfamethoxazole (smx^R) and harbored the F4 fimbriae gene (K88ac+ subvariant), the heat-labile toxin gene (LT+), and the heat-stable toxin gene (STb+) but not the STa gene, as determined by polymerase chain reaction (PCR; primers are listed in Table 11). In order to obtain a convenient selection marker to retrieve the ETEC strain from faces at the output of the infection model, a spontaneous mutant resistant to rifampicin was searched for and isolated. To do so, the strain was cultured overnight in Luria-Bertani broth (Becton Dickinson, UK) at 37˚C with 180 revolutions per minute (rpm) in a shaker incubator, and 100 µL were transferred on several Eosin-Methylene Blue (EMB) agar plates (Oxoid CM0069, UK) supplemented with 50 µg/ml rifampicin (rif50). A colony growing on one of these plates

was isolated, purified, and checked again by PCR for the presence of K88ac, LT toxin, and STb genes. This strain would serve as the ETEC used in the infection model described below.

Table 11: Primers used to characterize the ETEC F4 strain for infection.

Primer name	Sequence (5'-3')	Amplification length (bp)	Reference
K88ac Fwd	TTTGCTACGCCAGTAACTG	436	(Alexa *et al.* 2001)
K88ac Rev	TTTCCCTGTAAGAACCTGC		
K88ab Fwd	TTGCTACGCCAGTAAGTGGT	296	
K88ab Rev	CGAAACAGTCGTCGTCAAA		
K88ad Fwd	GGCACTAAAGTTGGTTCA	169	
K88ad Rev	CACCCTTGAGTTCAGAATT		
LT Fwd	TAGAGACCGGTATTACAGAAATCTGA	282	(Mohlatlole *et al.* 2013)
LT Rev	TCATCCCGAATTCTGTTATATATGTC		
STa Fwd	TCTTTCCCCTCTTTAGTCAG	166	(Osek 2001)
STa Rev	ACAGGCCGGATTACAACAAAG		
STb Fwd	GCCTATGCATCTACACAATC	278	
STb Rev	TGAGAAATGGACAATGTCCG		
LT qPCR Fwd	GGCGTTACTATCCTCTCTAT	272	(Boerlin *et al.* 2005)
LT qPCR Rev	TGGTCTCGGTCAGATATGT		

Inoculi were prepared by growing overnight the strain in sterile Luria-Bertani broth, as described above. The culture was centrifuged 10 minutes at 6000 rpm in order to get rid of the toxin-ladden supernatant. The bacterial pellet was then resuspended in 1X phosphate buffered saline (PBS) and adjusted to a final concentration corresponding to 10^8 CFU/ml (using the optical density at 600nm absorbance, Biowave II WPA, LABGENE Scientific SA, Châtel-Saint-Denis, Switzerland).

3.3.2. Animals and rearing conditions

Two weeks before the expected day of farrowing, sows were vaccinated with Porcilis® Porcoli DF® (ad us. vet., MSD Animal Health GmbH, Lucerne, Switzerland). A suspension for injection that contains deactivated fimbria adhesions of *E. coli* F4ab, F4ac, F5, F6, and the LT-toxoid. Within 7 days after birth, an ear sample 2 mm in diameter was taken from each piglet with special plier, and its DNA analysed to determine whether the animal would be susceptible or resistant to ETEC F4 infection (i.e., harbouring a genetic variant of the marker for F4ac/ab) or resistant to ETEC F4 infection (i.e., harbouring a genetic variant of the marker for F4ac/ab receptor that makes piglets resistant) (Hu *et al.* 2017). The DNA analyse was done by Animal Genetic Group, ETHZ, Zurich, Switzerland.

In both trials, piglets were reared in pairs in individual pen (1.6 m^2 concrete floor and 1 m^2 slatted metal floor) and had access to a wooden box with straw placed underneath a heating lamp. Each pen was equipped with nipple drinkers, giving *ad libitum* access to clean fresh water. An electrolytes (NaCl hypertonic) solution was also available for drinking. From weaning to 18 days post-weaning (which marked the end of the two trails), ambient temperature was maintained at around 28°C.

3.3.3. Experimental designs

Trial 1: To set up and validate the ETEC infection model of PWD, 32 Swiss Large White piglets ([average ± standard deviation] 24 ± 1 days of age and 7.8 ± 0.8 kg BW [body weight]) were included. On the day of weaning (day -4), they were blocked within littermates by BW and assigned equally to either the infected (INF) or non-infected (NINF) group. Directly at weaning, piglets had ad libitum access to a standard starter diet formulated to meet or surpass the nutritional requirements (Table 12). Four days post-weaning (day 0), piglets were offered 5 ml of the previously described ETEC suspension containing 108 CFU/ml (INF) or 5 ml of PBS (NINF) by oral administration with a syringe. Of these 32 piglets, two INF and one NINF were determined to be resistant to ETEC F4.

Trail 2: The objective of trail 2 was to assess the effects of dietary hydrolysable tannin (HT) supplementation on the incidence of PWD in INF and NINF piglets. Piglets were weaned at 26 ± 2 days of age with a weaning weight of 7.4 ± 1.5 kg BW. The trial was arranged according to a 2 × 2 factorial design with two levels of infection (INF vs. NINF) and two diets (unsupplemented control starter diet [CO; Table 12] and the CO diet supplemented with 1% of chestnut tannin extract [TA; Table 12]), resulting in four experimental groups (INF-CO, NINF-CO, INF-TA, NINF-TA) of 18 piglets each. The two diets were formulated according to current Swiss recommendations for pigs (Agroscope 2017). The commercial hydrolysable chestnut-tannin extract (HTE; Silvafeed Nutri P/ENC for Swine, Silvateam, Italy) used in the trial contained 45% gallotannins, 9% ellagitannins, and 3.7% gallic acid. Rearing condition, infection procedure, and feed access were equal to those used in trial 1. Three INF-CO, 2 INF-TA, 1 NINF-CO, and 1 NINF-TA (out of 72 piglets) were genotyped as resistant to ETEC F4.

Table 12: Ingredient composition and chemical content (g/kg) of the experimental CO diets[a]

Ingredient	CO	TA
Barley, ground	33.39	33.39
Oat flakes	2.00	2.00
Corn, ground	20.00	20.00
Wheat, ground	9.20	9.20
Wheat meal	0.38	0.38
Whey permeate	5.00	5.00
Rapeseed oil	3.36	3.36
Potato protein	6.47	6.47
Soybean meal	9.88	9.88
Wheat bran	2.42	1.42
Apple pomace, dried	4.00	4.00
L-lysine-HCl (79%)	0.40	0.40
L-threonine (99%)	0.01	0.01
Dicalcium phosphate	1.37	1.37
Sodium chloride	0.20	0.20
Calcium formate	1.00	1.00
Pellan[b]	0.30	0.30
Vitamin–mineral premix without Fe[c]	0.40	0.40
Luctarom[d]	0.01	0.01
Greencab-70-C[e]	0.20	0.20
Natuphos 5000 G[f]	0.01	0.01
HTE[g]	-	1.00
Nutrient and digestible energy content (expressed per kg as fed)		
Dry matter, g	889	890
Crude protein, g	166.7	164.3
Crude fat, g	55.9	51.9
Crude fiber, g	32.6	40.5
Fe, mg	100.6	100.6
DE, MJ[i]	14.0	13.9

[a] CO = control diet; TA = control diet supplemented with 1% hydrolysable chestnut tannin extract
[b] Pellet binding aid: Pellan, Mikro-Technik, Bürgstadt, Germany
[c] Supplied per kg of diet: vitamin A, 8000 IU; vitamin D_3, 1000 IU; vitamin E, 25mg; menadione, 3 mg; thiamine, 2 mg; riboflavin, 5 mg; biotin, 0.1 mg; niacin, 20 mg; pantothenic acid, 15 mg; iodine, 0.15 mg as calcium iodate; copper, 6 mg as copper sulfate; manganese, 10 mg as manganese oxide; zinc, 75 mg as zinc oxide; selenium, 0.2 mg as sodium selenite;
[d] Luctarom, Lucta, Montornès del Vallès, Spain
[e] Coated calcium butyrate: Greencab 70-c, Brenntag, Denmark
[f] Phytase supplemented at 500 units of aspergillus niger phytase/kg diet
[g] Hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Silvateam, Italy)
[i] DE = digestible energy content estimated according to the Swiss (Agroscope, 2017) database taking into account the relative amount of each feed ingredient in the diet.

3.3.4. Clinical parameters and laboratory analysis

From the day of infection (day 0, i.e., four days after weaning) to 14 days post-infection (day 14), faecal scores were regularly assessed using the score scheme previously proposed (Madec *et al.* 2000): 1 = dry, pelleted faeces; 2 = molded faeces; 3 = moist, cow-dung appearance; 4 = diarrhoea; 5 = watery diarrhoea.

Piglets were considered as having diarrhoea when faecal score was 4 or above. Individual bodyweight (BW) and feed intake (FI) per pen were determined weekly and daily, respectively. General health status was daily monitored throughout the trial.

In trial 2, faecal samples were collected directly from the rectum on day 0 (before infection) and day4. These samples were used to determine the presence of the ETEC strain applied with the suspension. The presence of the ETEC strain was detected by quantitative real-time PCR (qPCR). The DNA of the dried faeces samples was extracted using QIAamp® Fast DNA stool Mini Kit (Qiagen GmbH) according to the manufacturer's instructions. The qPCR test performed using primers targeting the heat-labile (LT) toxin gene (Table 11) (Boerlin *et al.* 2005). A Bio-Rad CFX96 Touch PCR and a KAPA SYBR® FAST qPCR universal kit was used (Kapabiosystem, USA). The DNA of the infective ETEC F4ac strain was used as the standard curve. The DNA concentration of standard 1 was 3.1 ng/µl, and serial 1:10 dilutions were performed for standards 2 to 7. A total of 15 ng DNA of each faeces sample was used for qPCR. Thermal cycling conditions were 95˚C for 3 min followed by 40 cycles at 95˚C for 10 s, 58˚C for 30 s, and 72˚C for 30 s. Melting curve analysis confirmed primer specificities with the following thermal cycling conditions: 95˚C for 10 s and increments of 0.5˚C per 5 s from 65 to 95˚C. In addition, in trial 2, when a piglet developed watery diarrhoea (i.e., a faecal score of 5), an additional rectal swab sample was collected to check for the presence of other bacterial and viral pathogens causing PWD. Analyses were performed by a commercial laboratory (Idexx Diavet Labor AG, 8806 Bäch SZ, Switzerland).

3.3.5. Data analysis and statistics

In trial one, data for growth performances, incident days of diarrhoea and faecal score were analysed using PROC MIXED of SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA), where the effect of infection, run and time were considered as fixed effects and the piglet and litter as random effects. The percentage of piglets with diarrhoea, following a binomial distribution, was analysed with the GLIMMIX procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA). In addition, data for faecal score and percentage of piglets with diarrhoea were analysed using the repeated statement.

In trial 2, data for feed intake per pen (FI)m weight of the piglets, average daily gain (ADG), and feed efficiency (FE, per pen) were analysed with linear mixed models using R software (R Core Team 2014). Discrete dependent variables were modelled using R: counts for days in diarrhoea as quasi-Poisson, ordinal faecal scores as proportional odds logistic regression using generalized estimating equations, and dichotomous responses for percentage of diarrhoea as binary generalized linear mixed model. The initial models included the effects of infection, feed, run, time (weeks or days), genotype (susceptible or resistant), and sex and the first-order interactions infection × feed, infection × time, and feed × time as fixed effects, and they included pairs of piglets (for feed intake) or piglets and litter as random effects. In general, the models were reduced by stepwise exclusion of non-significant interactions and factors (except feed and infection) on a P-level of 0.10. Least-squares means of the response variables and

Tukey-Kramer pairwise comparisons were computed, and differences were considered significant if $P < 0.05$ and considered a tendency if $P \leq 0.10$. Because of the non-normality of the data, ETEC shedding was converted in $\log_{10}$ (1+N) and analysed with a nonparametric Wilcoxon test with the NPAR1WAY procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC, USA).

3.4. Results

3.4.1. Trial one

Age at weaning, BW at weaning and at day 38 of age and consequently ADG did not differ ($P \geq 0.10$) between INF and NINF piglets (Table 13). In accordance, no differences ($P > 0.20$) in average daily FI per pen were observed between INF and NINF piglets during experimental days 0 to 7, and 8 to 14. In the first week post-infection, diarrhoea lasted on average 2 days longer ($P = 0.002$) in INF piglets than in NINF susceptible piglets.

Table 13: Growth performance and days on diarrhoea of infected (SR-INF, n = 16) and non-infected piglets (SR-NINF, n = 16)[a]

	INF	NINF	SEM[b]	P-value
Age at weaning, d	24	24	0.1	1
BW at weaning, kg	7.85	7.74	0.198	0.71
BW 18d after weaning (14d post-infection), kg	11.46	11.28	0.438	0.78
Average daily gain[c], g/d	200	197	16.9	0.87
Days in diarrhoea[d]	3.6	1.6	0.43	0.002

[a] Four days post-weaning, INF piglets were infected orally with 5 ml of the ETEC suspension containing 10^8 CFU/ml (INF) whereas NINF piglets received orally 5 ml of PBS.
[b] SEM = pooled standard error of mean
[c] Average daily gain was calculated over the period between weaning and 18d after weaning.
[d] Days in diarrhoea (i.e. with fecal score ≥4) were determined in the first experimental week

The faecal score was greater ($P < 0.001$) in INF than in NINF piglets for the entire duration of the study (Figure 13). The impact of ETEC F4 infection was more evident in the first days after infection, when the faecal score is increased markedly in INF piglets compared to NINF piglets, whereas it leveled out between the two treatments at the end of the 14-day trial (infection x time interaction: $P < 0.001$; Figure 13).

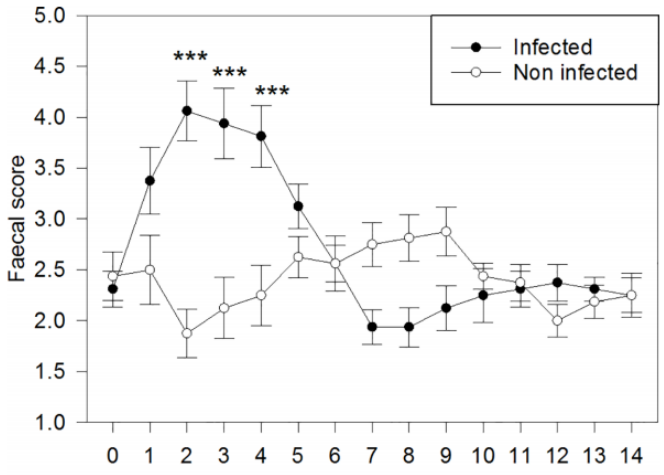

Figure 13: Average faecal score (± standard error) of infected and non-infected piglets monitored during 14 d post-infection.

P-values for the main factors: infection: $P < 0.001$; days: $P < 0.001$; infection x days: $P < 0.001$. *** indicates differences between infected and non-infected piglets at $P < 0.001$ within the same day. Four days post weaning (day 0), infected piglets were orally administered 5 ml of the ETEC F4 suspension containing 10^8 CFU/ml, whereas NINF piglets received 5 ml of PBS orally.

The percentage of INF piglets developing diarrhoea was significantly greater ($P = 0.01$; maximum 80% on day 4 post-infection) than for the NINF piglets (25% on the same day) (Figure 14).

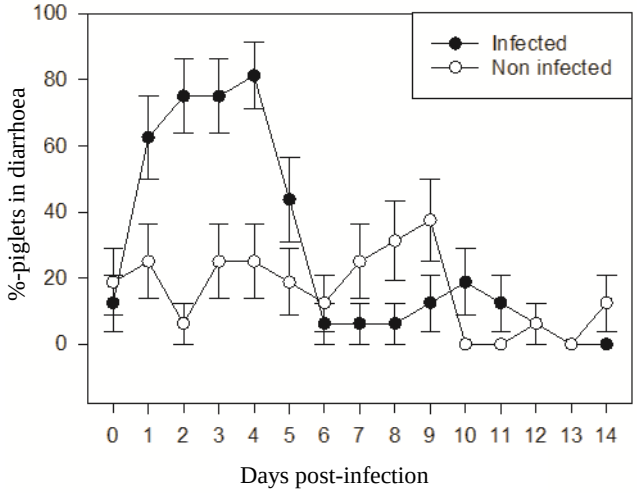

Figure 14: Percentage of piglets displaying diarrhoea (i.e. faecal score ≥ 4) in the infected and non-infected susceptible groups.

P-values for the main factors: infection: P = 0.01; days: P = 0.42; infection × days: P = 0.07. At four days post weaning (day 0), infected piglets were orally administered 5 ml of the ETEC F4 suspension containing 10^8 CFU/ml, whereas NINF piglets received 5 ml of PBS orally.

There was a tendency of infection x time interaction ($P = 0.07$), due mainly to the greater development of diarrhoea at day 2, 3, and 4 in INF piglets compared to NINF piglets. Interestingly, 10 out of 16 INF piglets had diarrhoea at least once in the second week (day 7 to day 14 post-infection) compared to 4 out of 16 INF piglets. No mortality occurred amongst the 32 piglets.

3.4.2. Trial two

Over the entire course of the experiment, NINF piglets tended to be heavier (P = 0.08) than INF piglets (8.93 and 8.40 kg for NINF and INF piglets, respectively) (Table 14). Regardless of whether piglets received orally the ETEC F4 solution or PBS, dietary HTE supply had no ($P \geq 0.10$) effect on ADG, average daily FI, feed efficiency (Table 14) compared to CO diet. There was a significant difference of infection × days interaction (P= 0.03) for ADG, which probably due to a compensatory growth of the INF piglets who reached the same ADG as NINF piglets in the second week post-infection.

Table 14: Growth performance of infected (INF) and non-infected piglets (NINF) fed either a control standard starter diet or the control starter diet (CO) supplemented with 1% chestnut extract (TA)[a,b]

Infection	NINF		INF		SEM	P-values[c]			
Diet[d]	CO	TA	CO	TA		I	D	W	I × W
BW (post-infection), kg									
at D 0	7.35	7.80	7.57	7.69					
at D 7	8.54	8.75	8.06	8.38	0.521	0.08	0.49	<0.001	0.57
at D 14	9.99	10.07	9.41	10.27					
Daily feed intake, g/d									
D 0-7	551	555	488	517	81.8	0.35	0.30	<0.001	0.91
D 8-14	1001	1059	946	1043					
D 0-14	776	807	717	780	45.3	0.35	0.31	-	-
Average daily gain, g/d									
D 0-7	135	122	84	104	24.4	0.45	0.46	<0.001	0.03
D 8-14	208	188	192	270					
D 0-14	171	155	138	187	20.9	0.97	0.44	-	-
Gain-to-feed, g/g									
D 0-7	0.51	0.42	0.37	0.42	0.077	0.89	0.92	0.86	0.18
D 8-14	0.43	0.36	0.44	0.53					
D 0-14	0.44	0.38	0.38	0.46	0.053	0.88	0.82	-	-

[a] Four days post-weaning, INF piglets were infected orally with 5 ml of the ETEC suspension containing 10^8 CFU/ml (INF) whereas NINF piglets received orally 5 ml of PBS.
[b] Results are presented as least square of means and pooled standard error of means (SEM).
[c] P-values for the main factors infection (I), diet (D), W (week) and infection × week interaction (I × W)
[d] The commercial chestnut tannin extract (HTE; Silvafeed Nutri P/ENC for Swine, Silvateam, Italy) contained 45% gallotannins, 9% ellagitannins, and 3.7% gallic acid

Surprisingly, infection had no effect (P = 0.19) on faecal score (Table 15). However, dietary THE supplementation lowered (P < 0.001) the average faecal score monitored for 14 d in both INF and NINF piglets by approximately 0.5 units (2.58 vs. 3.07). Accordingly, the number of days in diarrhoea was lower (P = 0.008) in TA (INF-TA and NINF-TA) than in CO piglets (INF-CO and NINF-CO; Figure 15) but was unaffected (P = 0.67) by the extent of infection to which piglets were subjected. Despite not

being significant, it is noteworthy that from day 1 to 5 post infection, the percentage of piglets exhibiting diarrhoea was lower in the NINF than in the INF group (P = 0.55; Table 16).

Table 15: Average faecal score of INF-CO, INF-TA, NINF-CO and NINF-TA piglets[a, b] from d 0-7 post-infection (daily) and at d 14.

INFECTION	**NINF**		**INF**		**Pooled SE**	***P*-values[c]**			
Diet	**CO**	**TA**	**CO**	**TA**		**I**	**D**	**days**	**D x days**
Days									
0	2.7	1.7	2.3	2.1	0.12				
1	3.2	2.3	3.7	3.2	0.17				
2	3.2	2.9	3.4	3.2	0.17				
3	2.9	2.7	3.7	3.2	0.18				
4	3.2	2.2	3.6	2.9	0.16	0.19	< 0.001	< 0.001	< 0.001
5	2.9	2.3	3.1	2.8	0.11				
6	3.2	2.5	2.9	2.4	0.11				
7	2.8	2.7	2.4	2.2	0.12				
14	2.2	1.7	1.9	1.7	0.08				

[a] At four days post weaning, INF-CO and INF-TA piglets were infected orally with 5 ml of the ETEC suspension containing 108 CFU/ml (INF) and fed either an unsupplemented standard control starter diet or a control standard diet supplemented with 1% chestnut-tannin extract from weaning (day -4) for 18 days, respectively. Piglets in the NINF-CO and NINF-TA groups were fed the same diets for the same time span as previously described but received 5 ml of PBS orally.

[b] Results are presented as means and pooled standard error (SE).

[c] P-values for the main factors infection (I), diet (D), days, and diet × days interaction (D × days).

Table 16: Percentage of piglets in the INF-CO, INF-TA, NINF-CO, and NINF-TA groups exhibiting signs of diarrhoea (i.e., faecal score ≥ 4)[a,b].

INFECTION	**NINF**		**INF**		**Pooled SEM**	***P*-values[c]**			
Diet	**CO**	**TA**	**CO**	**TA**		**I**	**D**	**days**	**I x days**
Days									
0	28	6	17	11	4.3				
1	44	28	67	33	5.9				
2	50	39	61	39	5.9				
3	44	44	56	44	5.9				
4	44	17	50	39	5.7	0.55	0.007	< 0.001	0.01
5	33	11	44	28	5.4				
6	56	22	28	11	5.4				
7	39	28	22	17	5.2				
14	6	0	6	0	2				

[a] At four days post weaning, INF-CO and INF-TA piglets were infected orally with 5 ml of the ETEC suspension containing 108 CFU/ml (INF) and fed either an unsupplemented standard control starter diet or a control standard diet supplemented with 1% chestnut-tannin extract from weaning (day -4) for 18 days, respectively. Piglets in the NINF-CO and NINF-TA groups were fed the same diets for the same time span as previously described but received 5 ml of PBS orally.

[b] Results are presented as last square of means and pooled standard error of means (SEM).

[c] *P*-values for the main factors infection (I), diet (D), days, and infection × days interaction (I × days).

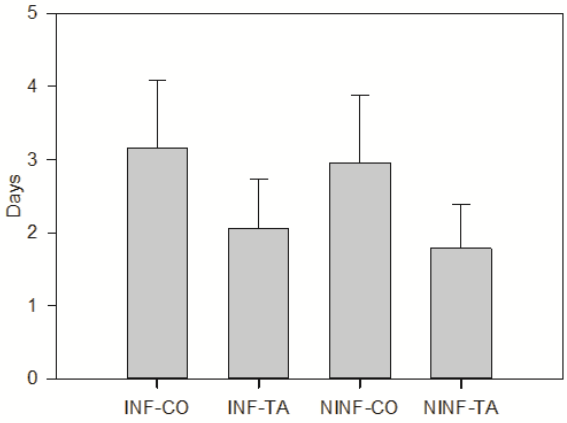

Figure 15: The number of days in diarrhoea (i.e. faecal score ≥ 3) of piglets from the INF-CO, INF-TA, NINF-CO and NINF-TA group.

P-values for the main factors: infection: P = 0.67; diet: P = 0.008. Piglets of the INF-CO and INF-TA group were infected orally with 5 ml of the ETEC F4 suspension containing 10^8 CFU/ml four days post-weaning and fed either an unsupplemented standard control starter diet or a control standard diet supplemented with 1% chestnut tannin extract from weaning (day -4) for 18 days, respectively. The piglets of the NINF-CO and NINF-TA group were fed the same diets for the same time span as previously described but received orally 5 ml of PBS.

There was a significant infection × days interaction (P = 0.01) due to the development of diarrhoea in the NINF-CO group at day 6 and to a lesser extent at day 7. Over the 14-d post-infection period, the addition of HTE decreased (P = 0.007) by half the percentage of piglets with diarrhoea (43% and 26% for CO and TA groups, respectively).

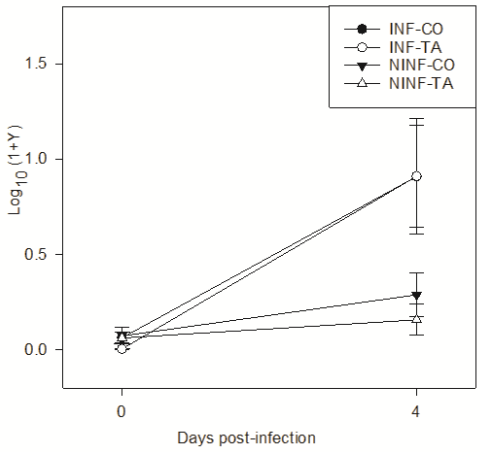

Figure 16: The LT gene abundance determined by qPCR in the faeces at day 0 and 4 post-infection of INF-CO, INF-TA, NINF-CO and NINF-TA piglets.

Data are expressed as Log10 (1+Y) where Y represents the number of LT gene DNA copy per g faeces. The P-values of the Wilcoxon test for the main factors: at day 0: infection: P = 1.00; Feed: P = 0.26 and at day 4: infection: P = 0.009; diet: P = 0.34.

Infection had no effect (P = 1.00) on the excreted quantity of ETEC F4 in the faeces, as determined by qPCR at day 0 (i.e., before infection) (Figure 16). However, four days after infection, INF piglets excreted more ETEC F4 (P < 0.05) than did NINF piglets. On the other hand, feed had no effect (P >

0.10) on ETEC F4 excretion on days 0 and 4. Of the 32 swabs sampled for laboratory analyses (pathogen analyses), 20 contained rotaviruses (8 INF/12 NINF), and the main isolated bacteria was *E. coli*.

3.5. Discussion

The ETEC F4 infectious strain was quite representative of the strains commonly found in PWD worldwide, as it harbored adhesine F4ac and toxins, LT+ and STb+ which might be caustive reason for PWD incidence (Fairbrother *et al.* 2005). Previous studies on artificial ETEC infection models reported incidence of diarrhea ranging from 50 to 70% (Madec *et al.* 2000; Bruins *et al.* 2011) during the first two weeks post-weaning. In the present study, greater occurrence of diarrhoea were observed in INF piglets in trial one as prevalence was 60% to 80% from day 1 to 4. Duration of diarrhoea was also longer, with 3.6 days on average in the current study compared to 1.7 days in the study of Madec *et al.* (2000). These greater prevalence and duration of diarrhoea were achieved while administering the lowest infectious dose of the one proposd by Madec *et al.* (2000). This low infective dose allowed to induce diarrhoea, but wasn't severe enough to impact growth performance traits, even if there was a tendency to decrease BW within the two weeks post-infection. Furthermore, INF piglets shed more ETEC F4 than did NINF piglets. The infectious inocula was administered only once, compared to administration of the inoculum for up to eight consecutive days in other studies (Madec *et al.* 2000; Gao *et al.* 2013). Timing of infection (i.e., 4 days after weaning) was chosen based on outcomes from other studies (Madec *et al.* 2000; Bruins *et al.* 2011) and in order to target the highest susceptibility possibilities after weaning.

The infectious model was repeatable, as the average fecal score, days in diarrhoea, and percentage of piglets in diarrhoea were within the same range in trials 1 and 2. The prevealence of diarrhoea in NINF (Trial 1), NINF-CO, and NINF-TA (Trial 2) was unexpectedly great. This contrasts with the findings of the study of Madec *et al.* (2000), in which none of the non-infected piglets developed diarrhoea within the three weeks after weaning. However, the Madec study was performed in a high-disease-security experimental facility within Specific Pathogen Free piglets, not in an experimental and conventional piggery as in the current study. Furthermore, the ETEC infective strain used here was already part of the enverionmental flora of our piggery. In the present study, two processes may explain the relatively high incidence of diarrhoea in NINF piglets: the presence of rotaviruses in the environment, as confirmed by the laboratory analyses performed in trial 2, and potential cross contamination between INF and NINF piglets. Cross contamination could explian the rise in diarrhoea incidence observed in NINF piglets after day 6 in both trials. Although Rotavirus is not regarded as primary cause of PWD, it favors ETEC colonisation by modifying the gut environment (Lecce *et al.* 1982). In their PWD induction protocol, Niewold *et al.* (2007) inoculated in addition rotaviruses before the artifical infection with ETEC in weaned piglets.

For trials 1 and 2, a control starter diet with low crude protien and minimal iron supplementation was formulated to minimize the risk of PWD (Kim *et al.* 2011; NRC 2012; Heo *et al.* 2015). Iron is an

essential nutrient ofr basic bacterial metabolic pathways, but also an essential mineral for mammals (Rantzer *et al.* 2010). The diet was formulated to meet the minimum iron physiological requirements (NRC 2012). In trial 2, the 1% chestnut extract in the TA diet repalce 1% of wheat bran in the CO diet (as fiber source), knowing that the latter has been shown to reduce prevalence of PWD (Molist *et al.* 2010). In the present study, the THE supplement combined with wheat bran exceeded the effect of the wheat bran alone, as all INF-TA and NINF-TA piglets had lower prevalence of diarrhoea, lower faecal score, and fewer days in diarrhoea compared to INF-CO and NINF-CO piglets.

Previous studies (Verhelst *et al.* 2010; Verhelst *et al.* 2014) have evaluated the efficacy of polyphenols in preventing PWD caused by ETEC. Several mechanisms have been proposed to be involved in the antimicrobial property of polyphenols, including cell wall adherence, membrane integrity disturbance, and cell growth inhibition. One mode of action of the hypothesized polyphenols is linked to their capacity to bind proteins, implying that they could inactivate microbial adhesins, extracellular microbial enzymes, and envelope transport proteins (Cowan 1999). Numerous polyphenols have a proven capacity to inhibit ETEC adhesion to intestinal epithelium (Jass & Reid 2009; Verhelst *et al.* 2010), and a few polyphenols are able to inactivate in vitro enterotoxins (Morinaga *et al.* 2005; Verhelst *et al.* 2010). Indeed, tannins are able to bind to a variety of substrates. For instance, hydrolysable tannins contained in Terminalia chebula fruits were able to bind to and inhibit the bacterial efflux pumps that are often involved in multidrug resistances (Bag & Chattopadhyay 2014). Polyphenols may also deprive bacteria of essential substrates for growth (Scalbert 1991). By capturing iron, tannic acids (Afsana *et al.* 2004) reduce iron absorption in laboratory rats and deprive iron-requiring enteric pathogens such as *E. coli* (Chung *et al.* 1998), thus reducing coliform faecal count. Tannins are often reported as antinutritional products because they may negatively affect protein digestion (Butler 1992). Tannic acids also seem to negatively impact the growth performance of weaned piglets (Lee *et al.* 2010). Similarly, black tea extract, rich in polyphenols, seems to reduce growth performance (Bruins *et al.* 2011), most likely by impacting diet palatability through its bitter and astringent taste. However, the reduction in feed intake and growth performance was not observed in this study. The antinutritive effects of tannins remain controversial. The present results are in accordance with a previous study that assessed the common antinutritive effects in pigs (Myrie *et al.* 2008). Although not statistically significant, the supplementation of HTE seems to improve feed intake and ADG in TA piglets compared to CO piglets. Other studies have confirmed the beneficial effects of tannins on feed efficiency, growth performance and concentration of beneficial Lactobacillus bacteria in weaned piglets (Biagi *et al.* 2010) and broilers (Sarica & Urkmez 2016). The antinutritive effect may be a question of dose and/or type of tannins.

The supplementation of 1% HTE in the diet reduced only the severity and duration of PWD and was not able to completely prevent the occurrence of PWD and to reduce ETEC shedding in the feces. This can be explained partly by the concentration of HTE in the chymus. Although not measured in this study, it has been suggested that tannins are already partly degraded by host or microbial enzymes when reaching

the distal regions of the small intestine (Van Parys *et al.* 2010). In general, hydrolysable tannins, such as the HTE used in the current study, are more susceptible to hydrolysis than condensed tannins (Goel *et al.* 2005). As for other polyphenols, tannins are very reactive, and their effects seem not to be restricted to one type of molecule. Indeed, they can bind various constituents present in the gut, like iron or dietary proteins. For instance, the inhibition effect of HTE on E. coli toxins is suppressed when proteins are added to the medium (Verhelst *et al.* 2010). The addition of iron in a medium partly reversed the positive effect of polyphenols on ETEC growth (Bruins *et al.* 2011). These components compete with microbial toxins or adhesins for tannins' binding sites.

3.6. Conclusion

The infectious model was repeatable, as the average faecal score, days in diarrhoea, and percentage of piglets in diarrhoea were within the same range in trials 1 and 2. Thus, the present infection model was suitable for studying approaches to preventing PWD using chestnut tannins. Adding 1% chestnut-tannin extract successfully decreased incidence and diarrhoea severity but was not sufficient to reduce ETEC shedding. Increasing the dose of chestnut-tannin extract may improve the tannin efficiency, but care should be taken to stay within the "therapeutic window," using a dose that does not induce anti-nutritional effects on protein digestion or feed palatability.

3.7. Acknowledgements

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Chapter 4: Hydrolysable tannins but not sodium salicylate decrease the severity of diarrhoea and enterotoxigenic *Escherichia coli* F4 shedding in artificially infected piglets

This chapter is under review.

All procedures for this study were conducted in accordance with the Swiss guidelines for animal welfare and approved by the Swiss cantonal veterinary office of Fribourg (approval number: 2016_14_FR).

4.1. Abstract

Development of alternatives to antibiotics is crucial to limit the apparition of antimicrobial resistances, especially in prophylactic and metaphylactic use to treat PWD. Feed additives such as bioactive compounds could be promising alternative. This study aimed to test two bioactive compounds, sodium salicylate and an extract containing hydrolysable tannins (HT) on the occurrence of PWD. At weaning, piglets were assigned to four treatments which combine two factors: diets (control; CO or with 2% of tannins extract; TA) and supplementation (with 35 mg/kg BW of sodium salicylate; SA or without; NSA).

Four days after weaning (D0), all piglets were infected with a suspension at 10^8 CFU/ml of enterotoxigenic *Escherichia coli* (ETEC F4ac) while NINF group received a same amount of 1X PBS solution. Each piglet had free access to an electrolytes solution containing or not. The SA supplementation was administered for five days *i.e.* from the day of infection (D0) to 4 days post-infection (D4). During the two weeks post-infection, supplementation with SA had no effect ($P > 0.05$) on growth performances nor on faecal score. A significant SA × time interaction ($P < 0.01$) for faecal score and the morbidity of diarrhoea indicated that piglets with SA did not recover faster and did have a second episode of diarrhoea. By contrast to the SA treatment, inclusion of TA increased ($P < 0.05$) growth performances and feed intake. In the first week post-infection, TA decreased ($P<0.001$) overall faecal score, percentage of piglets with diarrhoea, days in diarrhoea and ETEC shedding in the faeces. There was a SA × diet interaction ($P< 0.05$) for ETEC shedding, suggesting a negative effect of combining SA with TA. This study highlighted that, contrary to SA, TA could be used immediately after weaning as a promising alternative to antibiotics to improve growth performance and to reduce PWD.

Keywords: Bioactive compounds, post-weaning diarrhoea, infection, nutrition, pig

4.2. Implications

The increased occurrence of antimicrobial resistances is a worldwide issue for veterinary and human medicine. Especially during weaning when post-weaning diarrhoea problems occur and cause substantial economic losses, pig production has to fall back on antimicrobials. The present study proposes an efficient nutritional solution as a possible alternative to antimicrobials to help piglets and farmers to cope with post-weaning diarrhoea problems. This sustainable solution could be employed for organic farming as well.

4.3. Introduction

Post-weaning diarrhoea (PWD) is a major enteric disease in pig production occurring mainly during the first two weeks after weaning. PWD causes substantial economic losses due to high rates of mortality and morbidity, to depression of feed intake and growth of the piglet and to increased costs of medication (Fairbrother *et al.* 2005). Apart from the multifactorial etiology owing to several stressors such as nutritional social and environmental changes, PWD is often related to specific pathogen infection.

Enterotoxigenic *Escherichia coli* (ETEC) is one of the prevalent pathogen involved in PWD (Fairbrother *et al.* 2005). For instance, in Switzerland, 42.5% weaned pigs with diarrhoea were detected positive to ETEC (Schubnell *et al.* 2016). Those bacteria possess fimbriae that adhere to receptors located on the apical side of the enterocytes and secrete enterotoxins such as heat-labile toxin (LT) and heat-stable toxin (STb), which results in fluid secretion into the intestinal lumen leading to dehydration and acidosis, which ultimately results in diarrhoea (Nataro & Kaper 1998; Nagy & Fekete 1999).

Antibiotics are usually used to treat this type of infection. However, the widespread use of antimicrobials, especially in prophylactic and metaphylatic practices has led to the increase in microbial resistances. A recent survey in Switzerland reported that 53.3% of *E. coli* isolated from pig farms are resistant to one or more antimicrobials. The One Health approach promotes a multidisciplinary collaboration to limit the recrudescence of antimicrobial resistances (FOPH 2016). The One Health approach promotes a multidisciplinary collaboration to limit the recrudescence of antimicrobial resistances. The field of animal nutrition can contribute to this approach by exploring nutritional alternatives to antibiotics in order to prevent microbial infections. Some plants are known as nutraceuticals because they exhibit antimicrobial properties for both susceptible and multiresistant-drug bacteria (Valle Jr *et al.* 2015). It was demonstrated *in vitro* that polyphenols-rich plants could disrupt ETEC adhesion and inhibit some enterotoxins, including LT from ETEC (Morinaga *et al.* 2005; Verhelst *et al.* 2010). Among polyphenols, hydrolysable tannins (HT) from pomegranate and chestnut extract have also been shown to impair microbial growth of bacteria *in vitro* such as *E. coli, Listeria monocytogenes, Staphylococcus aureus* and *Yersinia enterocolitica, Salmonella enteritidis* or *Clostridium perfringens* (Graziani *et al.* 2006; Al-Zoreky 2009).

Pathogen infections provoke dotory responses which manifest in loss of appetite and fever. Previous studies reported an improvement of performance and a reduction of diarrhoea in weaned pigs receiving acetylsalicylic acid also known as aspirin (Xu *et al.* 1990; Kim *et al.* 2016). Salicylate is an analogue of acetylsalicylic acid and is naturally present in some plants like in sage (*Salvia sp.*) and in sweet birch (*Betula lenta*). The prime mode of action of salicylate is to inhibit cyclooxygenase and reduce prostaglandin E2 production, which ultimately reduces the extent of inflammation and stimulate appetite. In addition, salicylate has anti-secretory properties (Wise *et al.* 1983).

Thus, the main purpose was to examine the consequence of a supplementation with HT from chestnut combined or not with sodium salicylate (SA), on growth performances, severity of diarrhoea and on bacterial load using a previously established ETEC F4 infection model (Chapter 3) (Thanner & Gutzwiller 2018). An additional goal was to compare two quantitative methods, plate culture counting and quantitative real-time polymerase chain reaction (qPCR), for measuring ETEC shedding in the faeces. Owing to their antimicrobial and anti-secretory properties, we hypothesize that the inclusion of HT from chestnut and SA will reduce the severity of diarrhoea, with an additive effect when the two compounds are combined.

4.4. Material and methods

4.4.1. Bacterial strain

The ETEC F4 strain was isolated from a piglet suffering from acute PWD at the Institute of Livestock Science, Posieux, Switzerland. This strain was found to carry the genes for fimbriae F4 (K88ac) and enterotoxins STb and LT and to grow on the Eosin-Methylene Blue (EMB) agar (Oxoid CM0069, UK) medium supplemented with 50 µg/ml rifampicin (rif50). This isolate was stored at -80 °C. The day prior infection, this isolate was incubated at 37 °C in Luria-Bertani broth with orbital shaking (170 rpm) overnight. Subsequently, the ETEC F4 inoculum was centrifuged at 6000 rpm for 10 min at room temperature and resuspended in a phosphate buffered saline (PBS) solution to contain approximatively 1×10^8 CFU/ml using the optical density at 600 nm absorbance (Biowave II WPA, LABGENE Scientific SA, Chatel-Saint-Denis, Switzerland).

4.4.2. Animals and housing

To determine their susceptibility or resistance to ETEC F4ac, genotyping was performed on ear biopsies week-old piglets (Hu *et al.* 2017)(details in Chapter 2). Seventy-two large White male and female piglets susceptible to ETEC F4ac were then selected for experiment. Piglets were weaned at 26 ± 1 days (mean ± SD, standard deviation) of age and weighed on average 7.2 ± 1.3 kg (mean ± SD). The animals were housed in pairs in pens of 2.6 m^2 divided in a concrete floor and a galvanized steel floor. Each pen was equipped with wooden box with straw bed beneath infrared lamps, with a single feeder, with nipple

drinker and with drinking through. During the whole experiment, piglets were kept at ambient temperature (> 25 °C) and had free access to the feed, to clean water and to a daily prepared solution of electrolytes (NaCl hypertonic) in the drinking troughs.

4.4.3. Experimental design, diets and infection challenge

The study was set up according to a completely randomized 2 x 2 factorial design. The main factors were the experimental diets (standard [CO] vs. tannins [TA]), salicylate supplementation (none [NSA] vs. sodium salicylate [SA]) and the one-way interaction. The resulting four experimental groups were 1) NSA-CO, piglets receiving the CO diet without supplementation; 2) NSA-TA, piglets receiving the TA diet without supplementation; 3) SA-CO, piglets receiving the CO diet supplemented with sodium salicylate; and 4) SA-TA, piglets receiving the TA diet supplemented with sodium salicylate.

A total of three farrowing series were necessary to obtain the 72 susceptible piglets (24 piglets each). In each farrowing serie, on the day of weaning, piglets were assigned to one of the four treatments by balancing littermates and weaning body weight. From the day of weaning to the end of the trial, 36 piglets had access to the CO and 36 to the TA diet. In the TA diet, wheat straw was substituted for with 2% (20 g/kg) of chestnut tannin extract: the commercial hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Silvateam, Italy) used contained 45% gallotannins, 9% ellagitannins and 3.7% gallic acid, hence a total of 54% of hydrolysable tannins. Diets were formulated to contain 18% crude protein and 14 MJ /kg digestible energy, according to the Swiss feed recommendations for pigs (Agroscope 2017). The composition and the nutrient analyses of the diets are reported in Table 17.

To gradually adapt piglets to the feed, they were given access to a trough with either CO or TA feed and 100 ml of milk from the day of weaning to one day before infection. Four days after weaning i.e. on the infection day (D0), all piglets were orally challenged with an ETEC solution of 5 ml diluted in 50 ml of milk. To ensure that each piglet received the required amount, piglets were kept individually for 10 min. After this time span, piglets which did not ingest the total solution were offered the remaining solution orally via a syringe. Within each feeding group, one-half of the piglets (i.e. 18 fed diet CO and 18 fed diet TA) received from D0 to D4 after infection a daily dose of sodium salicylate (SA, Sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). The daily dose of SA was dispensed via the electrolytes solution and was calculated according to the total weaning weight of the two piglets in the pen (35 mg/kg BW per day). To maximize the chance of ingestion, the daily doses were offered in a small volume of 500 ml. based on daily observations, both piglets drank from the solution and in the following mornings, the dispensers were always empty. However, in this setup it was impossible to ensure that each individual piglet ingested the planned dose.Piglets in the NSA groups received a dose of 15 ml of tap water in the electrolytes solution.

Table 17: Composition (%) and gross chemical content (g/kg as fed) of the experimental diets[a]

Ingredients (%)	CO	TA
Wheat, ground	39.6	39.6
Corn, ground	20.0	20.0
Potato protein	11.6	11.6
Oat flakes	7.0	7.0
Whey permeate	5.0	5.0
Apple pomace, dried	4.0	4.0
Wheat bran	3.1	3.1
Rapeseed oil	2.5	2.5
Dicalcium phosphate	2.1	2.1
Calcium formate	1.0	1.0
Wheat meal	0.40	0.39
Vitamin-mineral premix without Fe[b]	0.40	0.40
L-lysine-HCI (79%)	0.35	0.35
Pellan[c]	0.30	0.30
Soybean meal	0.22	0.22
Sodium chloride	0.21	0.21
Greencab-70-C[d]	0.20	0.20
Luctarom[e]	0.01	0.01
Natuphos 5000 G[f]	0.01	0.01
L-Tryptophan	0.004	0.008
Wheat straw, ground	2.0	
Hydrolysable tannins extract[g]		2.0
Gross chemical composition (g/kg as fed)		
Dry matter	901	901
Crude protein	189	182
Fat	51.4	50.0
Crude fibre	32.7	25.2
NDF	130	150
ADF	41.6	49.8
ADL	5.4	6.7
Ash	49.6	50.9
Ca	10.1	10.8
Cu (mg/kg)	9.7	9.4
Fe (my/kg)	132.2	130.1
K	5.4	5.2
Mg	1.2	1.2
Mn (mg/kg)	34.2	36.4
Na	1.5	1.7
P	7.2	7.1
Zn (mg/kg)	79.6	84.5
Digestible energy (MJ/kg)[h]	14.0	14.0

[a] Each diet was formulated according to the Swiss feeding recommendations for pigs (Agroscope 2017) and was analyzed in triplicate to determine chemical composition. CO: control standard starter diet. TA: In the tannin-supplemented diet, wheat straw was substitute by 2% of a hydrolysable chestnut tannin extract.

[b] Supplied per kg of diet: vitamin A, 8000 IU; vitamin D_3, 1000 IU; vitamin E, 25mg; menadione, 3 mg; thiamine, 2 mg; riboflavin, 5 mg; biotin, 0.1 mg; niacin, 20 mg; pantothenic acid, 15 mg; iodine, 0.15 mg as calcium iodate; copper, 6 mg as copper sulfate; manganese, 10 mg as manganese oxide; zinc, 75 mg as zinc oxide; selenium, 0.2 mg as sodium selenite

[c] Pellet binding aid: Pellan, Mikro-Technik, Bürgstadt, Germany

[d] Coated calcium butyrate: Greencab 70-c, Brenntag, Denmark

[e] Luctaron, Lucta, Montornès del Vallès, Spain

[f] Phytase; 500 units aspergillus niger phytase/kg diet; one phytase unit corresponds to the amount of enzyme that release 1 µmol P from 5 mM phytate/min at pH 5.5 and 37°C.

[g] Hydrolysable chestnut tannin extract provided by Silvateam (Silvafeed Nutri P/ENC for Swine, Italy)

[h] Digestible energy content estimated according to the Swiss (Agroscope, 2017) databases taking into account the relative amount of each feed ingredient in the diet.

4.4.4. Measurements, sample collection and analysis

Feed intake per pen, weight and faecal score

Feed intake per pen was recorded daily in the first week (D0 to D7) and weekly in the second week post-infection (D8 to D14). Individual piglet BW was recorded on D-4 (day of weaning), D0, D7 and D14. Health status of each piglet was closely monitored throughout the experiment. piglets were observed visually twice a day before infection (D-4 ~ D-1), then three times a day during the 5 days after infection (D0 ~ D4) and then twice a day from D5 ~ D14. The apathetic behaviour was closely monitored and antimicrobial treatments (according to the veterinarian recommendations) were planned if a piglet showed such behaviour. However, in the present study, it did not occur and none of the piglets received antimicrobial treatments.

From D0 to D14, individual faecal score was visually evaluated based on the consistency of the faeces using the following scores: 1 = dry, molded orpelleted faeces; 2 = creamy, sloppy, cow-dung appearance; 3 = liquid diarrhoea; 4 = watery diarrhoea. Piglets were considered to have diarrhoea when the faecal score was ≥ 3. In addition, fresh faecal samples were collected directly in the rectum from each animal at D0, D1, D2, D3, D4 and D7. These samples were used to detect the challenge strain using microbial culture and qPCR techniques. Samples for microbial culture were directly plated whilst samples for qPCR were frozen at -80°C for further analysis.

ETEC shedding in the faeces by microbial culture and by quantitative PCR after DNA extraction

After collection, faecal swab samples were homogenized in 500 µl sterile PBS for 2 h at room temperature before serial dilutions in sterile PBS from 10^0 to 10^{-7}. For each dilution, 4 µl were deposited as a droplet on an EMB rif50 agar plate. Plates were incubated overnight at 37 °C and colonies were counted on each droplet the next morning using binoculars (Thomas *et al.* 2015). The mean of the count from two dilutions was used in the results. In addition, those colonies were restricted on a nutrient agar rif50 plate (Becton Dickinson, UK) and cultivated overnight at 37 °C in order to test the presence of the LT toxin gene by PCR to ensure that the growing strains in the faeces were the same as the infection strain form the inoculum,.

Faeces initially frozen were then freeze dried. DNA of the dried faeces sample was extracted using QIAamp® Fast DNA stool Mini Kit (Qiagen GmbH) according to the manufacturer's instructions. The qPCR test was performed (Appendix). Briefly, the following LT primers (F: 5'-GGCGTTACTATCCTCTCTAT-3'; R: 5'-TGGTCTCGGTCAGATATGT-3') were used, resulting in a PCR fragment of 272 bp (Boerlin *et al.* 2005). A Bio-Rad CFX96 Touch PCR machine and KAPA SYBR® FAST qPCR universal kit (KAPA Biosystem) were used. The DNA of the infective ETEC F4ac strain was used as standard curve. The DNA concentration of standard 1 was 3.1 ng/µl and serial 1:10 dilutions were done for standards 2 to 7. A total of 15 ng DNA of each faeces sample was used for qPCR. Thermal cycling conditions were 95℃ for 3 min followed by 40 cycles at 95℃ for 10 s, 58℃ for 30 s and 72℃ for 30 s. Melting curve analysis confirmed primer specificities with the following thermal cycling conditions: 95℃ for 10 s and increment 0.5℃ per 5 s from 65 to 95℃.

Statistical analysis

Data for feed intake per pen, weight of the piglets, average daily gain and ETEC shedding determined by plate counting were analysed with linear mixed models using R software (R Core Team 2014). Before analysis, data for ETEC shedding determined by plate counting were expressed as Log_{10}. Discrete dependent variables were modelled using R: counts for days in diarrhoea as quasi-Poisson, ordinal faecal scores as proportional odds logistic regression using generalized estimating equations, and dichotomous responses for percentage of diarrhoea as binary generalized linear mixed model.

Because of non-normality of the data, ETEC shedding determined by qPCR was ranked and the ranks were analysed by the MIXED procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA). In the figures, those data are presented as $\log_{10}(1+Y)$ where Y represent the ng LT-DNA per g of faeces dry matter.

All the models included the effects of SA, diet, farrowing series, time (weeks or days) and the first-order interactions SA x diet, SA x time, and diet x time as fixed effects and pairs of piglets (for feed intake) or piglets, litter and pen as random effects. In general, models were reduced by stepwise exclusion of non-significant interactions and factors (except diet and salicylate) at a $P \leq 0.10$. Least squares means of the response variables and Tukey-Kramer pairwise comparisons were computed and differences were considered as significant if $P \leq 0.05$ and as a tendency if $P \leq 0.10$. Pearson correction coefficients between faecal score, ETEC shedding determined by plate counting and ETEC shedding determined by qPCR were calculated with the CORR procedure of SAS (version 9.2). A list of the codes for the different models used is presented in the Appendix I.

4.5. Results

4.5.1. Growth performance and feed intake

Feed intake per pen and growth performances are presented in Table 18. Piglets ingested more ($P < 0.001$) feed and grew faster in the second week post-infection than in the first (Table 18). Piglets of the SA group had similar performances ($P > 0.10$) compared with piglets of the NSA group ($P > 0.10$). In contrast, intake per pen in the TA groups were on average 27% greater ($P = 0.02$) than in the CO groups. Piglets fed the TA diet tended ($P = 0.10$) to be heavier and had on average a growth rate 40 g/d greater ($P = 0.01$) than that of the CO piglets. Accordingly, the increase in BW in the first and second weeks was more distinct in the TA group than in the CO group (first week: 0.95 vs. 0.60 kg; second week: 1.92 vs. 1.63 kg, respectively [Diet x Time interaction: $P = 0.001$]). Nevertheless, feed efficiency was not ($P > 0.10$) affected by the diet.

Table 18: Feed intake (g/d × pen^{-1}), body weight (kg), average daily gain (g/d) and feed efficiency (g/g × pen^{-1}) from day 0 (D0) to day 7 (D7), day 8 to day 14 (D14) as well from D0 to D14 post-infection.

Salicylate[1]	NSA		SA			P-values[3]			
Diet[2]	CO	TA	CO	TA	SEM	S	D	T	D × T
Body weight (kg)									
at D0	7.30	7.53	7.26	7.31					
at D7	8.01	8.57	7.76	8.18	0.365	0.36	0.10	< 0.001	0.001
at D14	9.67	10.47	9.37	10.14					
Feed intake per pen (g/d)									
D 0-7	415	488	353	458	61.7	0.22	0.02	< 0.001	0.77
D 8-14	827	979	811	887					
Average daily gain (g/d)									
D 0-7	101	149	70	124	21.5	0.35	0.004	< 0.001	0.74
D 8-14	238	273	230	280					
Gain-to-feed per pen (g/g)									
D 0-7	0.52	0.60	0.28	0.51	0.111	0.22	0.35	0.11	0.26
D 8-14	0.62	0.59	0.59	0.59					

[1] SA: Pigs (N = 36) were offered from D0 to D4 a daily dose of sodium salicylate (35 mg/kg BW per day; sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). NSA: Pigs (N = 36) were offered from D0 to D4 a daily dose of tap water.
[2] CO: Pigs (N = 36) had ad libitum access to the control standard starter diet, which was formulated according to the Swiss feed recommendations for pigs (Agroscope 2017). TA: Pigs (N = 36) had ad libitum to the tannin supplemented diet where wheat straw in the CO diet was substituted by 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine; Italy).
[3] P-Values for the main effect of salicylate supply (S), Diet (D), Time (T) and D × T interaction

4.5.2. Consistency of the faeces and percentage of piglets with diarrhoea

The consistency of the faeces given by the faecal score varied ($P < 0.001$) over days with a maximal score the second or third day post-infection (Figure 17). Supplementing piglets with SA for 5 days post-infection had no improvement ($P = 0.70$) on the faecal score, whereas the addition of tannins reduced ($P < 0.001$) the faecal score.

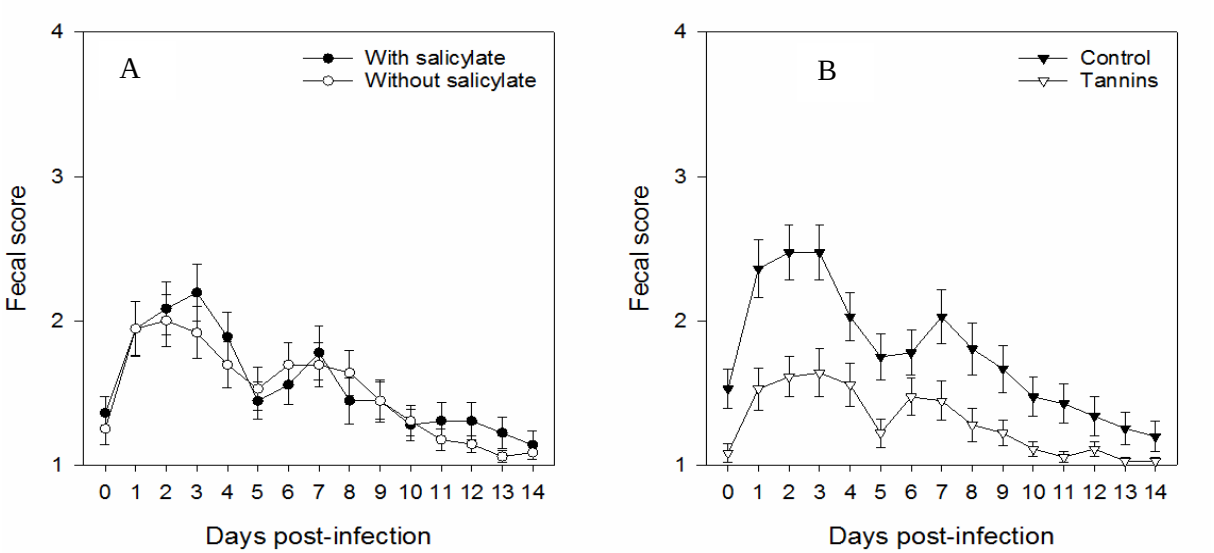

Figure 17: Effects of salicylate supply (A) and 2% dietary tannin content (B) on the faecal score assessed from d 0 to 14 post-infection.

The faecal score was assessed according to the following score scale: 1 = dry pelleted faeces, 2 = cow-dung appearance, 3 = liquid diarrhoea and 4 = watery diarrhoea during 14 days post-infection. Pigs (N = 36) of the SA group were offered from d 0 to d 4 a daily dose of sodium salicylate (35 mg/kg BW × d-1; sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Pigs (N = 36) of the NSA group were offered from D0 to D4 a daily dose of tap water. Pigs (N = 36) of the CO group had ad libitum access to a control standard starter diet, which was formulated according to the Swiss feed recommendations for pigs (Agroscope 2017). Pigs (N = 36) of the TA group had ad libitum to the tannin supplemented diet where wheat straw in the CO diet was substituted by 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy). P-values for the main effects: Salicylate: $P = 0.70$; Diet: $P < 0.001$; Time: $P < 0.001$; Salicylate × Time: $P = 0.01$

In the first week, an increase in faecal score from Day 5 to Day 7 post-infection was observed in SA piglets while the faecal score of NSA piglets decreased progressively over the period (SA x Time interaction: $P = 0.01$).

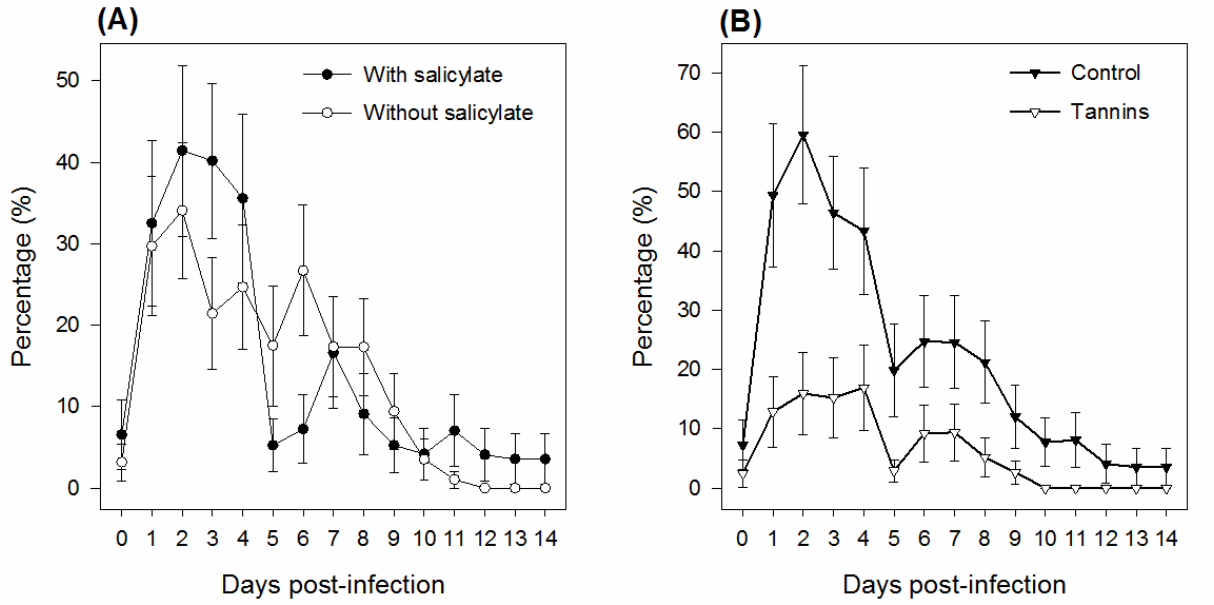

Figure 18: Effect of salicylate supply (A) and diet (B) on the percentage of piglets with diarrhoea from day 0 to 14 post-infection.

When faecal score was ≥ 3 it was termed diarrhoea. Pigs (N = 36) of the SA group were offered from days 0 to 4 a daily dose of sodium salicylate (35 mg/kg BW per day, sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Pigs (N = 36) of the CO group had *ad libitum* access to a control standard starter diet, which was formulated according to the Swiss feeding recommendations of pigs (Agroscope 2017). Pigs (N= 36) of the TA group had *ad libitum* access to the tannin supplemented diet, where wheat straw in the CO diet was substituted with 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy). P-values for the main effects: salicylate: P = 0.72; Feed: P < 0.001; Time: P < 0.001; Salicylate x Time: P = 0.003

The percentage of piglets with diarrhoea was day-dependent (P < 0.001) with the maximum percentage occurring at D2 and D3 post-infection (Figure 18 A and B). The effect of SA supply depended on the time (SA x Time interaction: P = 0.003) as in the NSA group, the percentage of piglets with diarrhoea increased until D2, on which 35% of the piglets had diarrhoea, and then decreased progressively over days to reach 0% from day 11 onwards. In contrast, in the SA group, the percentage of piglets with diarrhoea increased to reach 40% at D2 and then decreased drastically from 35% to 5% between D4 and D5 when piglets stopped receiving SA. However, compared to the NSA group, in the SA group, from D5 to D7, there was an increase in the percentage of piglets with diarrhoea, which decreased again over the following days to stabilize at 4% at the end of the second post-infection week. The addition of tannins reduced (P < 0.001) the percentage of piglets with diarrhoea. For instance, at D2 post-infection, 60% of CO piglets had diarrhoea, but only 16% of the TA piglets.

4.5.3. Days in diarrhoea

The SA diet had no effect on the number of days in diarrhoea in the first and second weeks post-infection (Table 19). In the first week post-infection, only the NSA-TA pigs tended to have fewer days in diarrhoea than the NSA-CO ones, whereas no difference was observed between the SA-TA and the SA-

CO groups (SA x Diet interaction; P = 0.06). in the second week post-infection, piglets fed the TA diet had fewer (P < 0.001) days in diarrhoea than those on the CO diet. Finally, over the two experimental weeks post-infection, TA piglets had, on average, diarrhoea (i.e., faecal score ≥ 3) for 1.4 days (P < 0.001), whereas CO pigs had 4.2 days in diarrhoea.

Table 19: Number of days in diarrhoea (i.e., Faecal Score ≥ 3) in the two weeks after infection in piglets.

Salicylate[1]	NSA		SA		SEM	P-values[2]		
Diet[3]	CO	TA	CO	TA		S	D	S x D
D 0-7	3.3^{y}	0.7^{x}	3.0^{y}	1.8^{xy}	0.61	0.41	< 0.001	0.06
D 8-14	0.9	0.1	1.3	0.2	0.35	0.44	< 0.001	0.96
D 0-14	4.1	0.8	4.3	2.0	0.88	0.35	< 0.001	0.17

[xy] row carrying no common superscript tend to differ at P < 0.10

[1] SA: Pigs (N = 18) were offered from D0 to D4 a daily dose of sodium salicylate (35 mg/kg BW × d-1; sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). NSA: Pigs (N = 18) were offered from D0 to D4 a daily dose of tap water.

[2] P-Values for the main effect of salicylate supply (S), Diet (D), S × D interaction

[3] CO: Pigs (N = 18) had ad libitum access to the control standard starter diet, which was formulated according to the Swiss feed recommendations for pigs (Agroscope 2017). TA: Pigs (N = 18) had ad libitum to the tannin supplemented diet where wheat straw in the CO diet was substituted by 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine; Italy).

4.5.4. Faecal ETEC shedding as determined using microbial culturing and using qPCR

ETEC shedding in the faeces was determined using both culturing and qPCR. Using either of the two methods, the extent of ETEC shedding was not affected by the SA supply whereas it was lower (P < 0.01) in pigs fed with TA compared with the CO diet (Figure 19 and 20). There was a time effect (P ≤ 0.05) as ETEC shedding increased at D1 post-infection. The results obtained using qPCR also revealed a diet x time interaction (P = 0.02) due to a constant shedding around a value of 0.25 overtime for the TA piglets while the excretion profile increased to a maximum of 0.90 at D2 post-infection then decreased from D2 to D7 for CO piglets (Figure 20).

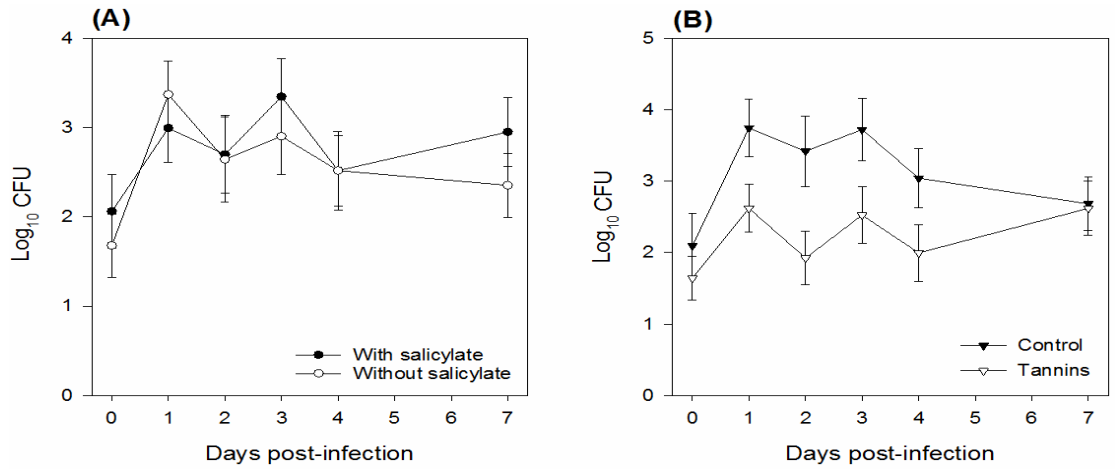

Figure 19: Effects of salicylate supply (A) and diet (B) on ETEC F4 shedding determined by plate counting in faeces samples collected from all pigs at D0, 1, 2, 3, 4 and 7 post-infection.

Pigs (N = 36) of the SA group were offered from days 0 to 4 a daily dose of sodium salicylate (35 mg/kg BW per day, sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Pigs (N = 36) of the CO group had *ad libitum* access to a control standard starter diet, which was formulated according to the Swiss feeding recommendations of pigs (Agroscope 2017). Pigs (N= 36) of the TA group had *ad libitum* access to the tannin supplemented diet, where wheat straw in the CO diet was substituted with 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy). Data are expressed as Log_{10} (Colony format units). P-values for the main effects: Salicylate: P = 0.51; Diet: P = 0.01; Time: P = 0.02

Results obtained using the plate-count technique revealed that independent of days post-infection, ETEC shedding was lower in the NSA-TA piglets than in the NSA-CO piglets, with intermediate values for the SA-TA and the SA-CO piglets (SA x Diet interaction: P = 0.03; Figure 21, black bars). Using the qPCR technique show that NSA-TA excreted less (P < 0.05) ETEC in their faeces than did piglets in the three other groups (SA x Diet interaction: P = 0.03; Figure 21, white bars).

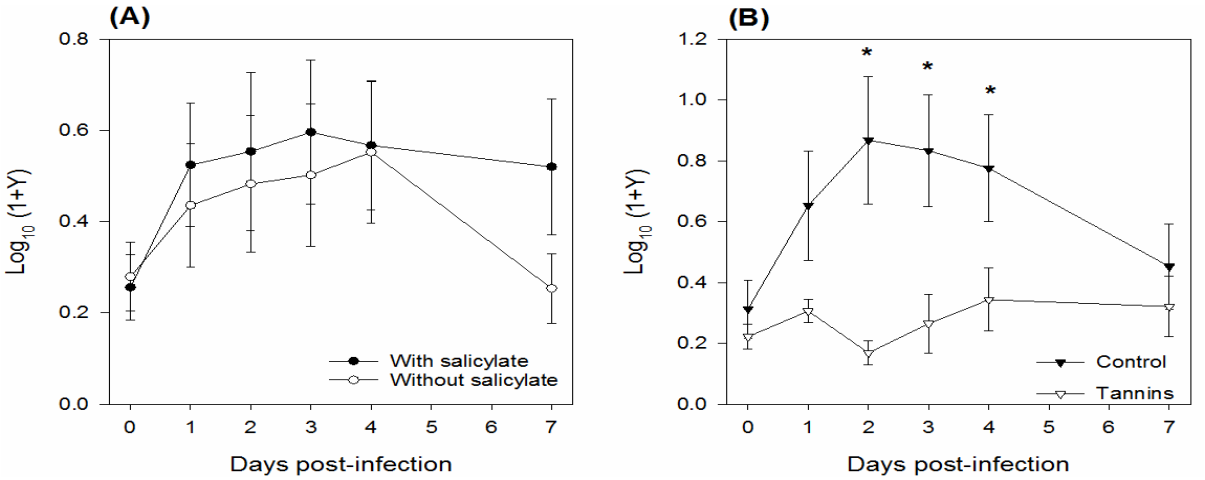

Figure 20: Effect of salicylate (A) and diet (B) on LT gene abundance determined by qPCR in the faeces collected at D0, 1, 2, 3, 4 and 7 post-infection.

Data are expressed as $Log_{10}(1+Y)$, where Y represents the ng LT-DNA per g of faeces dry matter. Pigs (N = 36) of the SA group were offered from days 0 to 4 a daily dose of sodium salicylate (35 mg/kg BW per day, sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Pigs (N = 36) of the CO group had *ad libitum* access to a control standard starter diet, which was formulated according to the Swiss feeding recommendations of pigs (Agroscope 2017). Pigs (N= 36) of the TA group had *ad libitum* access to the tannin supplemented diet, where wheat straw in the CO diet was substituted with 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy). P-values for the main effects: Salicylate: P = 0.15; Diet: P = 0.001; Time: P = 0.05; Diet x Time: P = 0.02. * marks differences between CO and TA at P < 0.05.

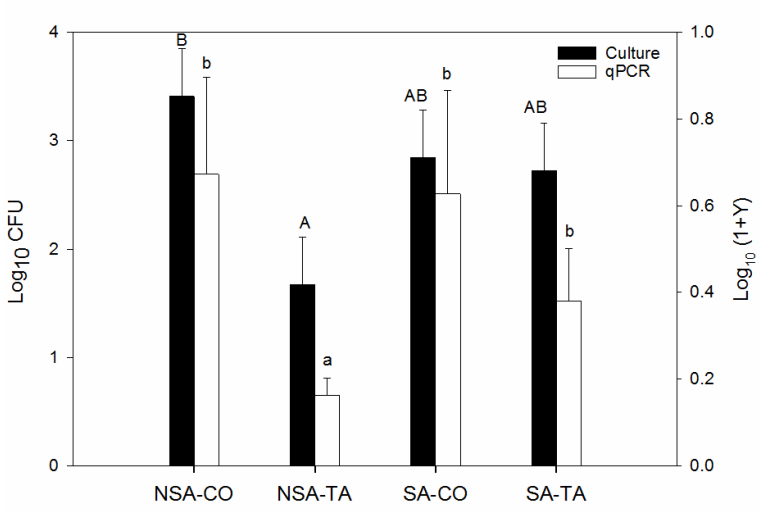

Figure 21: ETEC shedding determined by culture (black bars) and by qPCR (white bars) in faeces samples from piglets fed the CO without salicylate (NSA-CO; N = 18), piglets fed the TA diet without salicylate (NSA-TA; N = 18), piglets fed the CO diet with salicylate (SA-CO; N = 18) and piglets fed the TA diet with salicylate (SA-TA; N = 18).

Pigs (N = 36) of the SA group were offered from days 0 to 4 a daily dose of sodium salicylate (35 mg/kg BW per day, sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Pigs (N = 36) of the CO group had *ad libitum* access to a control standard starter diet, which was formulated according to the Swiss feeding recommendations of pigs (Agroscope 2017). Pigs (N= 36) of the TA group had *ad libitum* access to the tannin supplemented diet, where wheat straw in the CO diet was substituted with 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy). Data are expressed as Log_{10} (Colony Formant Units) for culture or as $Log_{10}(1+Y)$ where Y represents the ng LT-DNA per g of faeces dry matter. P-values for Salicylate x Diet: P = 0.03 (culture and qPCR). ^{A,B} bars carrying no common superscript differ significantly at P < 0.05 for the culture method. $^{a, b}$ bars carrying no common superscript differ at P < 0.05 for the qPCR method.

The ETEC shedding results obtained using the plate culture and qPCR method correlated (r = 0.67; P < 0.001; Figure 22). The faecal score also positively correlated with ETEC shedding determined using plate culturing (r = 0.43; P < 0.001; Figure 23A) and using qPCR (r = 0.42; P < 0.001; Figure 23B). Throughout the duration of the experiment, piglets in the NSA-TA group never developed watery diarrhoea (faecal score is 4).

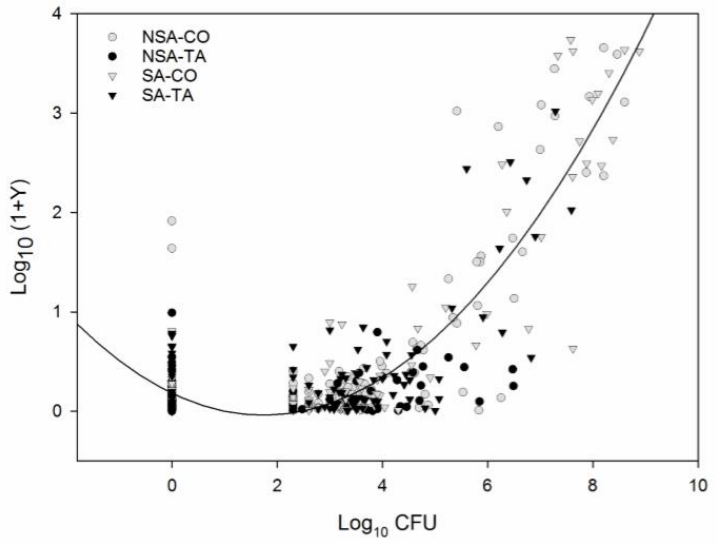

Figure 22: Relationship between ETEC F4 shedding in faeces determined by plate counting and by qPCR ($P < 0.001$; $R = 0.67$) in piglets fed the CO without salicylate (NSA-CO; N = 18), piglets fed the TA diet without salicylate (NSA-TA; N = 18), piglets fed the CO diet with salicylate (SA-CO; N = 18) and piglets fed the TA diet with salicylate (SA-TA; N = 18).

Pigs (N = 36) of the SA group were offered from days 0 to 4 a daily dose of sodium salicylate (35 mg/kg BW per day, sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Pigs (N = 36) of the CO group had *ad libitum* access to a control standard starter diet, which was formulated according to the Swiss feeding recommendations of pigs (Agroscope 2017). Pigs (N= 36) of the TA group had *ad libitum* access to the tannin supplemented diet, where wheat straw in the CO diet was substituted with 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy). Data are expressed as Log_{10} (Colony Formant Units) for culture or as $Log_{10}(1+Y)$ where Y represents the ng LT-DNA per g of faeces dry matter.

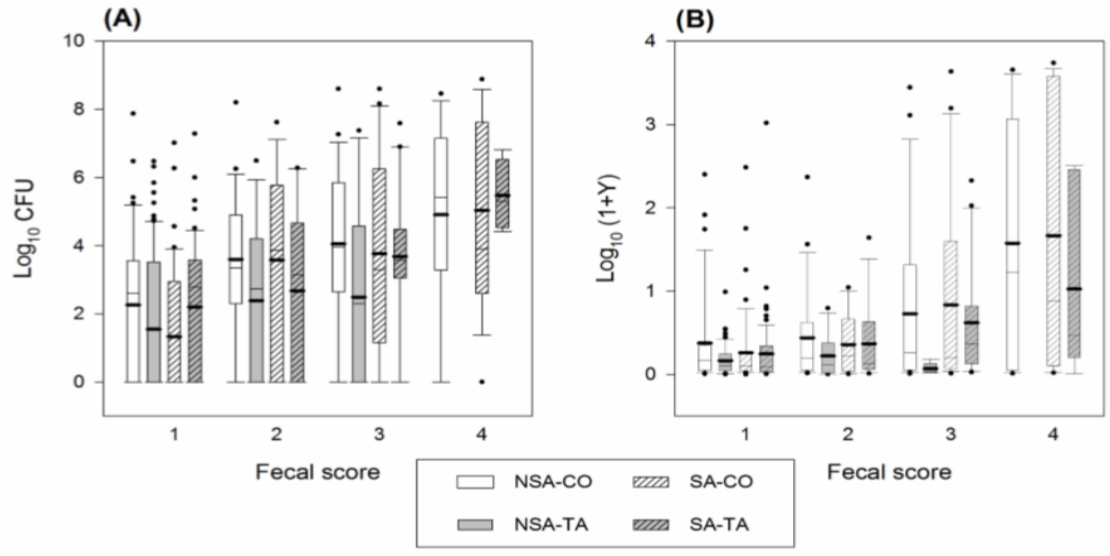

Figure 23: Relationship between ETEC shedding determined by plate counting (A) or by qPCR (B) and faecal score (1 & 2: no diarrhoea; 3 & 4: diarrhoea) in piglets fed the control diet without salicylate (NSA-CO), piglets fed the tannin supplemented diet without salicylate (NSA-TA), piglets fed the control diet with salicylate (SA-CO) and piglets fed the tannin supplemented diet with salicylate (SA-TA).

Pigs (N = 36) of the SA group were offered from days 0 to 4 a daily dose of sodium salicylate (35 mg/kg BW per day, sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Pigs (N = 36) of the CO group had *ad libitum* access to a control standard starter diet, which was formulated according to the Swiss feeding recommendations of pigs (Agroscope 2017). Pigs (N= 36) of the TA group had *ad libitum* access to the tannin supplemented diet, where wheat straw in the CO diet was substituted with 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy). Data are expressed as Log_{10} (Colony Formant Units) for culture or as $Log_{10}(1+Y)$ where Y represents the ng LT-DNA per g of faeces dry matter. ($P < 0.001$; $R = 0.43$ for plate counting and $r = 0.42$ for qPCR)

4.6. Discussion

The present experiment aimed to elucidate whether the addition of 2% extract of hydrolysable chestnut tannins with or without SA salicylate reduced the severity of diarrhoea in piglets artificially infected with ETEC F4ac. In a previous experiment, induction of diarrhoea using the same ETEC F4ac infection model (Thanner & Gutzwiller 2018). In the present study, a secondary goal was to compare the extent of ETEC shedding as determined using either culturing or qPCR. The results showed a moderate positive correlation between the two methods. Lindsay *et al.* (2014) reported a comparable Spearman's coefficient ($r = 0.61$) between the two methods in human volunteers infected with ETEC. The qPCR method amplified the DNA of both dead and living ETEC, while only the living ETEC grew on the culture plates, which could partially explain the discrepancy between the two methods.

4.6.1. Effects of salicylate

In plants, salicylate belongs to a diverse group of plant phenolics and is a major plant hormone (Raskin 1992). Some authors showed a greater average daily gain in piglets, infected with ETEC F4 or not infected, receiving aspirin, an analogue of salicylate (Xu *et al.* 1990; Kim *et al.* 2016). Van der Klis (2012) showed that, owing to its anti-inflammatory properties, SA supplementation via drinking water eliminated the negative impact of *Clostridium* infection on the BW gain of broiler. However, in the present experiment, the same dose of SA offered via electrolyte solution did not improve growth performance of artificially infected piglets. SA has also been reported that sodium salicylate possesses antibacterial activities by affecting bacterial cell growth (Cederlund & Mårdh 1993). However, in the present study, the use of SA did not reduce ETEC F4 shedding in the faeces. In addition, SA supplementation reduced neither the overall faecal score nor the percentage of piglets with diarrhoea. Furthermore, the significant effect of SA on faecal score and percentage of piglets with diarrhoea over time indicates that piglets receiving SA for 5 days post-infection did not recover faster after an episode of diarrhoea and had a second episode of diarrhoea in the second week post-infection. This result contradicts those showing a supposedly anti-secretory property of SA. Wise *et al.* (1983) observed that infusion of SA decreased fluid accumulation in intestinal loops incubated with *E. coli* ST enterotoxin in calves.

4.6.2. Effects of Tannin supplementation

Unlike with SA, piglets supplemented with a 2% extract of HT coped better with ETEC infection than piglets who received a standard weaning diet. First, piglets developed less diarrhoea with tannin supplementation, which is reflected in the reduction in faecal score, percentage of piglets with diarrhoea and days in diarrhoea during the two weeks post-infection in comparison to piglets with the CO diet. The development of coliform diarrhoea is depending on the ability of ETEC to adhere on and to colonize the intestinal epithelium. An indirect way to check for the presence of ETEC is to measure ETEC shedding in the faeces. Thus, an increase in ETEC shedding in the faeces might be the consequence of greater bacterial colonization due to the peristaltic clearance of ETEC (Rodea *et al.* 2017). In the present experiment, a moderately positive correlation was observed between diarrhoea occurrence assessed by faecal score and ETEC shedding determined by culture or qPCR. The moderate correlation is due to the high variability among piglets in the same group. Some piglets presented no clinical signs of diarrhoea but excreted a high amount of ETEC, which might translate into an improved immunity against the infection. In contrast, other piglets had diarrhoea without shedding ETEC, which might have been due to the development of nutritional or viral induced types of diarrhoea. Nevertheless, none of the 18 NSA-TA piglets developed watery diarrhoea (faecal score = 4) and in general, NSA-TA and SA-TA piglets shed less ETEC in their faeces. This result agrees with previous experiments in which infected or non-infected piglets fed different types of tannin extract excreted less ETEC or F18 verotoxigenic *E. coli* (Brus *et al.* 2013; Verhelst *et al.* 2014; Coddens *et al.* 2017).

This bacterial shedding reduction might be the result of a bactericidal and/or bacteriostatic effect of tannins on ETEC F4ac as previously observed *in vitro* (Min *et al.* 2007; Min *et al.* 2008) and/or a decrease in bacterial adhesion to the intestinal epithelium (Coddens *et al.* 2017). A previous study on uropathogenic *E. coli* reported that cranberry tannins were able to decrease *in vitro* the adhesion forces between the bacteria and a probe surface and to alter the conformation of the surface macromolecules on *E. coli* (Liu et al. 2006). Nevertheless, besides a positive effect of tannins to reduce diarrhoea, previous experiments failed to show a positive tannin effect on ETEC shedding (Verhelst *et al.* 2014; Thanner & Gutzwiller 2018). The lack of effects of tannins observed in previous studies is probably related to differences in bioactivity of tannins according to the dose (1% tannin extract, as previously administered, not sufficiently high compared to 2% in the present study) as well as to the chemical structure of tannins (Ropiak *et al.* 2017). However, caution should be taken in experiments with HT because up to now, it is unclear whether the effects of tannins are due to HT themselves and/or their metabolites (ellagic acid and urolithins), as HT can be hydrolysed in the stomach and the gut (Espín *et al.* 2007).

In the present study, supplementation with 2% tannin extract for 19 days directly at weaning improved feed intake and average daily gain. Even though tannins are often considered as an anti-nutritional factor,

the present results confirmed those of previous studies in which they did not impact negatively animal performance (Myrie *et al.* 2008; Biagi *et al.* 2010). The improved performances of the TA piglets is probably the consequence of a reduction in the occurrence of diarrhoea in piglets fed tannins, because the tannins did not improve feed efficiency.

In the present study, the substitution of 2% wheat straw with tannin extract minimized the differences in terms of ingredients and chemical compositions between the two diets. However, a slight difference in terms of fibre contents can be noticed between the two diets. The TA diet contained slightly less crude fibre and more NDF, ADF, and ADL than the CO diet. The source and chemical characteristics of fibre components affect the digesta retention time and the fermentation profile in the gut microbiota. Increasing the content of less-fermentable fibre is associated with a reduction in *E. coli* count in the faeces (Molist *et al.* 2011). In a recent study, Nepomuceno *et al.* (2016) observed a linear decrease in the occurrence of diarrhoea when NDF content was increased from 8.5 to 16.5%. Hence, in addition to the HT, the 2% unit greater NDF content in the TA diet could have partially helped in reducing both ETEC shedding and the occurrence of diarrhoea.

4.6.3. Effects of the combination salicylate and tannins

The present experiment showed a slight effect of the combination of tannins and SA on the number of day in diarrhoea (tendency) and on ETEC shedding. Interestingly, it seems that SA erased the positive effect of tannins as tannins reduced (or tended to reduce) ETEC shedding and days in diarrhoea only when they were fed alone and not combined with SA. An increase in the level of intrinsic antibiotic resistance was sometimes observed when bacteria grew in the presence of salicylate (Price *et al.* 2000). In *E. coli*, salicylate increased the transcription of the multiple antibiotic resistance operon *marRAB (Cohen et al. 1993)*. The activation of the operon decreases the accumulation of antibiotics by reducing the synthesis of porins and increases the production of multidrug efflux pump (Cohen *et al.* 1993; Ma *et al.* 1995). Such a mechanism could also be considered if tannins or their metabolites enter the bacteria. However, further investigations need to be performed to elucidate the effect of salicylate on tannins.

4.6. Conclusion

The present study demonstrated that unlike SA, 2% HT chestnut extract could be beneficial directly at weaning to reduce PWD and thus enhance piglets growth. Nevertheless, the effects of salicylate on tannins deserves further investigation. The results obtained in the present study were more promising than in a previosu study wehre the same HT chestnut extact was supplemented at a dose of 1%. Moreover, owing to the ability of HT to affect different pathogens, it would be now interesting to investigate whether this HT extract is able to affect other pathogens in vivo.

4.7. Acknowledgement

The authors gratefully acknowledge and thank the skilled technical assistance at the experimental piggery (Mr. Guy Maikoff and his team) and the analytic chemistry (Mr. Sébastien Dubois and his team) and microbiology (Dr. Nicolas Pradervand and his team) departments of Agroscope in Posieux. We also thank Dr. Werner Luginbühl of ChemStat (Chemometrik und Statistik, Berne) for his assistance with statistical analysis and Silvateam (Italy) for providing the chestnut extract.

Appendix II

1. ETEC strains culturing

A farm-specific ETEC F4ac (strain *35HI*) producing the heat-labile toxin gene (LT+), the heat-stable toxin gene types b (STb+), not type a (STa-) and resistant to sulfamethoxazole (smx^R) and rifampicin (rifR) was isolated from a case of acute PWD at the pig stable at Agroscope, Posieux (Switzerland). To obtain a convenient selection marker to retrieve the ETEC strain from faeces at the output of the infection model, a spontaneous mutant resistant to RifR was searched and isolated. To do so, the strain was cultured overnight in Luria-Bertani broth (Becton Dickinson, UK) at 37°C with 180 revolutions per minute (rpm) in a shaker incubator, and 100 µl was transferred on several Eosin-Methylene Blue (EMB) agar plates (Oxoid CM0069, UK) supplemented with 50 µg/ml rifampicin (rif50) (Figure 24). Observed the colony growth by eyes and by spectrophotometer with OD600 = 2.5 ~ 2.7. Inocula were prepared by growing overnight the strain in sterile Luria-Bertani broth. The culture was centrifuged for 10 min at 8000 rpm to move the toxin-laden supernatant. The bacterial pellet was then resuspended in 1X phosphate buffered saline (PBS) and adjusted to a final concentration corresponding to 10^8 CFU/ml (using the optical density at 600 nm absorbance, Biowave II WPA, LABGENE Scientific SA, Châtel-Saint-Denis, Switzerland) (Figure 24).

In addition, those colonies were restricted on a nutrient agar rif50 plate (Becton Dickinson, UK) and cultivated overnight at 37°C to test the presence of K88ac, STb and LT toxin gene by PCR (Table 11) to ensure that the growing strains in the faeces were the same as the infection strain from the inoculum. A Wizard® Genomic DNA Purification Kit (Promega, USA) was used to extract DNA from ETEC colonies according to themanufacturer's instructions adapted for gram-negative bacteria. GoTaq® DNA polymerase (M3001, Promega, USA) was used for PCR. The thermal cycling conditions for all these primers in Table 11 were 95˚C for 15 min followed by 40 cycles at 94˚C for 30 s, YY˚C for 30 s, and 72˚C for 45 s, followed by 72˚C for 7 min and 4˚C until termination. YY indicates the annealing temperature (K88ac: 65˚C; K88ab: 54˚C; K88ad: 50˚C; LT: 55˚C; STa: 50.2˚C; STb: 56˚C).

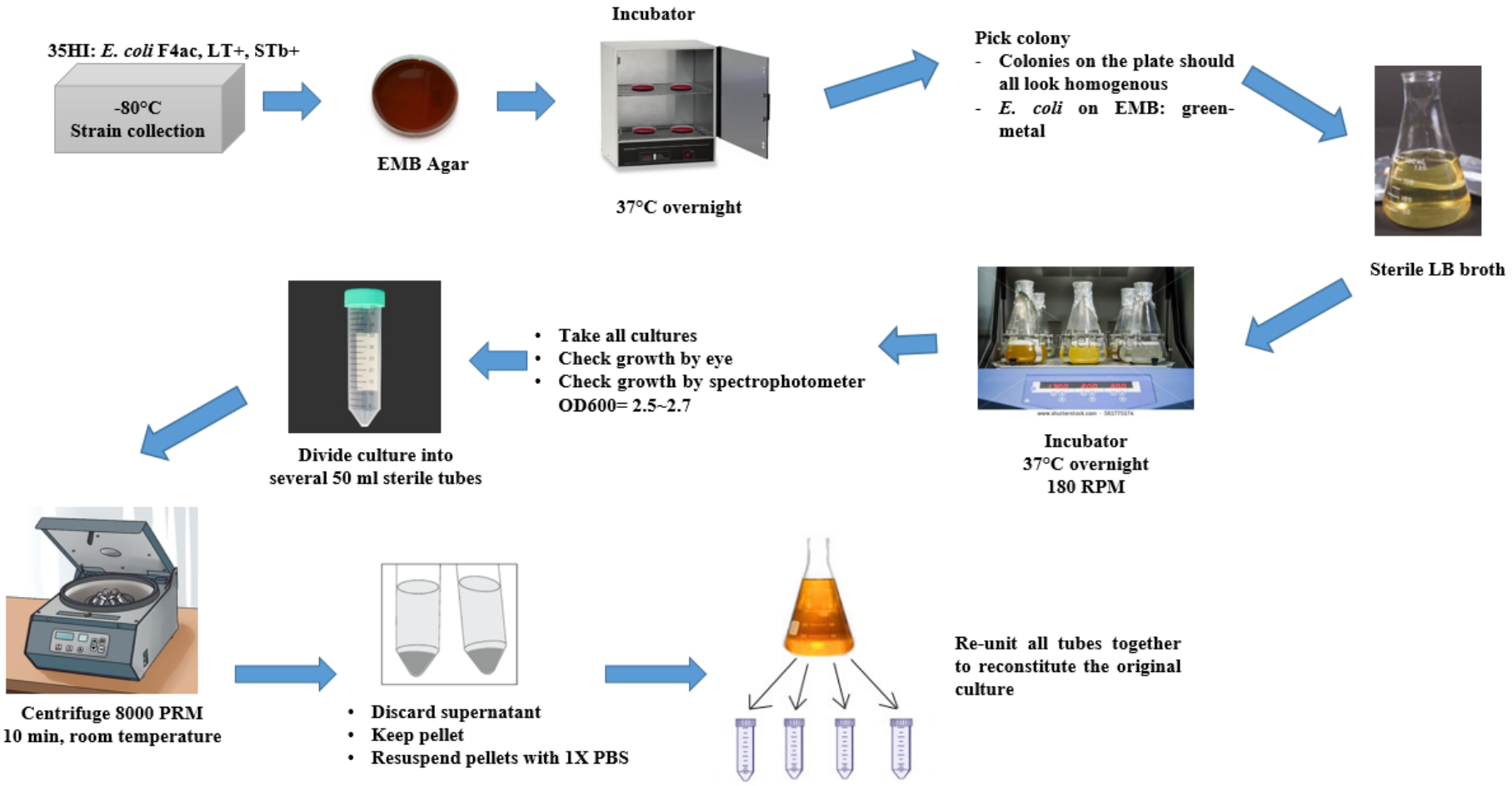

Figure 24: The procedures of culturing ETEC F4 strain.

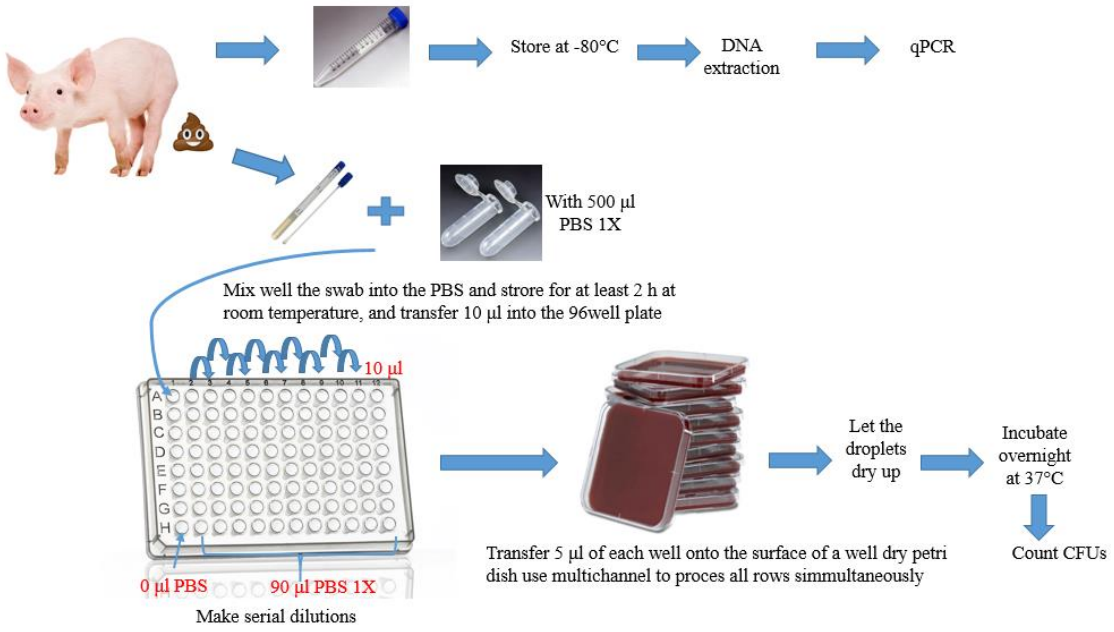

Figure 25: Procedures of counting ETEC F4 colony by plate and qPCR.

2. ETEC colony counting by plate and qPCR

Swabs were utilized to collect faeces from the rectum after infection. After collection, faecal swab samples were homogenized in 500 µl sterile PBS 1X for 2 h at room temperature before serial dilutions in sterile PBS 1X from 10^0 to 10^{-7} (Figure 25). For each dilution, 5 µl was deposited as a droplet on an EMB rif50 agar plate. Plates were incubated overnight at 37°C and colonies were counted on each droplet the next morning using binoculars. The mean of the count from dilutions was used. The details about counting were described using the example of strain ATCC as follows and in Table 20.

A total of 100 µl of ATCC strain was plated on each plate. The numbers of colonies were counted on plates 10^{-6}, 10^{-7}, 10^{-8} because the colonies were sufficiently separated and counted easily. The numbers of colonies are shown in green. On plate 10^{-6} (=0.000001): we counted 138 colonies out of 0.1 ml plated.

CFU/ml = (138/0.1)/0.000001 =$1.38E^9$ = 1380000000 colonies/ml

Then the same procedure for plates 10^{-7} and 10^{-8} and counting the average CFU/ml between the three plates. In this example, the final AVG CFU/ml of the ATCC strain is $1.36E^9$.

Table 20: The example of how CFU counting by plate.

		Plate colony count (CFU)					**CFU/ml**					**AVG CFU/ml**
		0.00001	0.000001	0.0000001	0.00000001	0.000000001	CFU/ml = number of colonies per ml plated/total dilution factor					
Culture	**Strain**	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
100%	ATCC		138	17	1			1380000000	1700000000	1000000000		1360000000

Partially faeces samples were stored in sterile tubes and stored at -80°C for further faeces DNA extraction and qPCR analysis (Figure 25). The presence of the ETEC strain was determined by quantitative real-time polymerase chain reaction (qPCR). All faeces samples were freezing dried. The DNA of the dried faeces sample was extracted using a QIAamp® Fast DNA stool Mini Kit (Qiagen GmbH) according to the manufacturer's instructions. The qPCR test was performed using primers targeting the LT toxin gene (Table 11, LT qPCR primer).

A Bio-Rad CFX96 Touch PCR machine and a KAPA SYBR® FAST qPCR universal kit were used (Kapabiosystem, USA). KAPA SYBR® FAST qPCR Master Mix (2X) is designed for high-performance real-time PCR. The kit contains a novel DNA polymerase-engineered via a process of molecular evolution- resulting in a unique enzyme specifically designed for real-time quantitative PCR (qPCR) using SYBR Green I dye chemistry. KAPA SYBR FAST DNA Polymerase has been engineered to perform optimally in stringent qPCR conditions, exhibiting dramatic improvements in signal-to-noise ratio (fluorescence), quantification cycle (Cq), linearity, and sensitivity. The KAPA SYBR FAST DNA Polymerase and proprietary buffer system improves the amplification efficiency of difficult targets, including both GC- and AT-rich templates. KAPA SYBR FAST qPCR Master Mix (2X) Kits are a ready-to-use cocktail containing all components (except primers and template) for the amplification and detection of DNA in qPCR. Quantitative PCRs were generally performed in a 20 µl reaction volume containing up to 20 ng of DNA, 10 µl 2X KAPA SYBR® Fast qPCR master mix universal, 0.4 µl 10 µM forward primer and reverse primer and PCR-grade water (Table 21).

Table 21: Required volume of each component for Bio-Rad CFX96 Touch.

Component	Volume	Final concentration
PCR-grade water	Up to 20 µl	N/A
KAPA SYBR FAST qPCR Master Mix (2X)[1]	10 µl	1X
10 µM forward primer	0.4 µl	200 nM
10 µM reverse primer	0.4 µl	200 nM
Template DNA[2]	As required	< 20 ng

[1] KAPA SYBR FAST qPCR Master Mix (2X) contains $MgCl_2$ at a final concentration of 2.5 mM.
[2] Do not exceed 20 ng per µl reaction.

The DNA of the infective ETEC F4ac strain (*35HI*) was used as a standard curve. The DNA concentration of standard 1 was 3.1 ng/µl and serial 1:10 dilutions were performed for standards 2 to 7. A total of 15 ng of DNA from each faeces sample was used for qPCR. Thermal cycling conditions were 95℃ for 3 min followed by 40 cycles at 95℃ for 10 s, 58℃ for 30 s and 72℃ for 30 s. Melting curve

analysis was followed: 95℃ for 10 s and increment 0.5℃ per 5 s from 65 to 95℃. To avoid contamination, DNA extraction and qPCR plating procedures were performed in a separate laboratory room. In a 96-well plate, the same standards 1 to 7 and no-template-control (NTC) were fixed included in every plate to test unknown samples, and the result is shown as in Figure 26. CFX manager software was used to analyse qPCR results.

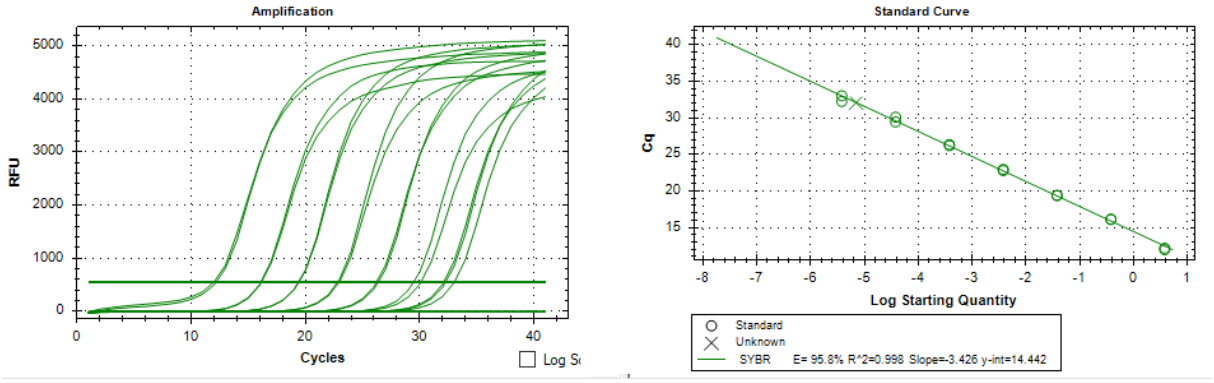

Figure 26: A real-time PCR result was performed on serial dilutions of the standard.

The left figure shows the serial dilutions of 10^0 to 10^6 which had the same number of cycles between dilution. The bottom flat line is a no-template-control (NTC). The round dot in the right figure presents the Cq number of standards and cross presents the testing sample.

Melting curve analysis was performed after PCR in the presence of the dsDNA binding fluorescent dye SYBR Green. The fluorescent dye is bound to the double-stranded DNA and fluoresces only in this bound state. During melting curve analysis, the temperature slowly increased, and the dsDNA started to dissociate into single strands (melting), releasing the dye. Therefore, the fluorescent signal decreased during melting, giving a melting curve when the fluorescent signal was plotted against temperature (Figure 27).

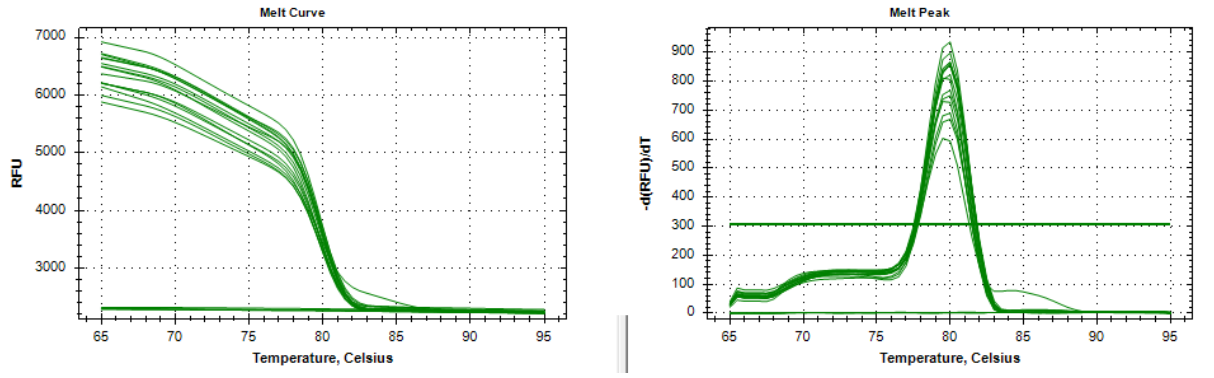

Figure 27: Melt curve and melt peak of qPCR result.

The melting point and shape of the melting curve depended on the fragment length and GC content. Therefore, a melting curve or melting peak was specific for a certain DNA fragment. Melting curve analysis could be used as a quality control for qPCR.

3. List of codes used for the statistical models

Systat Codes used for the statistical model:

Model for feed intake per pen, weight of the piglets, average daily gain and ETEC shedding determined by plate counting:

```
USE Av_daily_gain.syz
SORT RUN WEEK
MIXED
RESET
MODEL ADWG = INTERCEPT + RUN + WEEK + SALICYLATE$ + FEED$ +
SALICYLATE$*FEED$ + SALICYLATE$*WEEK + FEED$*WEEK + SEX$
CATEGORY RUN WEEK SALICYLATE$ FEED$ SEX$ LITTER$ PEN$PIGLET_ID
RANDOM PIGLET_ID / STRUCTURE = VC
RANDOM PEN$/STRUCTURE = VC
RANDOM PIGLET_ID(LITTER$)/STRUCTURE = VC
REPEATED / STRUCTURE = AR(1)
SAVE RESID / DATA
ESTIMATE / METHOD = REML TYPE = PARAMETERS CONVERGENCE = 1e-008 NNR =
20 NEM = 5 HALF = 50 TOLERANCE = 1e-012 CONFI = 0.95 CRITERION = RELATIVE
HYPOTHESIS
PAIRWISE RUN / TUKEY
TEST / CONFI = 0.95
PAIRWISE WEEK / TUKEY
TEST / CONFI = 0.95
PAIRWISE SALICYLATE$ / TUKEY
TEST / CONFI = 0.95
PAIRWISE FEED$ / TUKEY
TEST / CONFI = 0.95
PAIRWISE SALICYLATE$*FEED$ / TUKEY
TEST / CONFI = 0.95
PAIRWISE SALICYLATE$*WEEK / TUKEY
TEST / CONFI = 0.95
PAIRWISE FEED$*WEEK / TUKEY
TEST / CONFI = 0.95
PAIRWISE SEX$ / TUKEY
TEST / CONFI = 0.95
ACTIVE RESID.syz
PPLOT CRESIDUAL / NORMAL SMOOTH = LINEAR SHORT FILL = 1
PLOT CRESIDUAL*CESTIMATE CSTATISTICS CRESIDUAL / N SWTEST ADTEST
NPAR
KS CRESIDUAL / LILLIEFORS
```

R codes used for the statistical model:

Model for days in diarrhoea:

```
> dat1 <- Diarrh_tot[WEEK == 1,]
> GLM.1 <- glm(DIARRH ~ RUN + SALICYLATE.*FEED. + SEX., family=quasipoisson(log),
data=dat1)
> summary(GLM.1)
```

Model for faecal score:

```
> model7.1 <- repolr(FS ~ RUN + DAY + SALICYLATE. + FEED. + SALICYLATE.:DAY, subjects="PIGLET_ID",data=dat,times=as.numeric(levels(factor(DAY))), categories=length(levels(factor(FS))), corr.mod = "ar1",alpha = 0.5, fixed = FALSE, diffmeth = "analytic", fit.opt = c(cmaxit = 20, omaxit = 15,ctol = 0.001, otol = 0.00001, h = 0.01))
```

Model for percentage of diarrhoea:

```
> dat$DAY <- factor(dat$DAY)
> model.7 <- glmmPQL(DIARRH ~ RUN*DAY%in%RUN + SALICYLATE.*FEED. + SALICYLATE.:DAY + FEED.:DAY + SEX., data=dat, random = ~ 1 | PIGLET_ID, family = binomial)
> summary(model.7)
```

SAS code used for the statistical model:

Model for ETEC shedding determined by qPCR:

Data was ranked in ascending order and then ranks were analyzed by the MIXED procedure of SAS.

```
proc mixed data=a;
class Run_ Salicylate Feed Days ;
model &VAR = Salicylate Feed Days Salicylate*Feed Feed*Days Salicylate*Days Run_ / outp=res;
REPEATED Days / SUB = Piglet_ID TYPE = CS R RCORR;
LSMEANS Salicylate Feed Days Salicylate*Feed Feed*Days Salicylate*Days / PDIFF ADJUST = TUKEY;
```

Correlation between fecal score, ETEC shedding determined by plate counting and by qPCR:

```
proc corr data= a pearson spearman;
var Fecal_Score Log10_ETEC_bact log_1_ETEC__qPCR;
run;
```

All data were tested by Anderson-Darling Statistic method. P value < 0.05 was treated as a significant different level.

Appendix III

1. Effects of genotype and infection in piglets with the control diet

One hundred seventy Swiss Large White piglets susceptible/resistant to ETEC F4ac were then selected for the experiment. Selected piglets (average 7.2 ± 1.3 kg (mean ± SD)) were weaned at 26 ± 1 days (mean ± SD) of age. A 2.6 m^2 pen with a concrete floor and a galvanized steel floor were prepared for housing a pair of piglets. Each pen was equipped with a wooden box with a straw bed beneath infrared lamps, a nipple drinker, a drinking through and a single feeder. Selected piglets were reared at ambient temperature (> 25°C) and had free access to the feed, to clean water and to a daily prepared solution of electrolytes (NaCl hypertonic) in the drinking troughs. All piglets were fed with a standard starter control diet (CO, Table 17). Piglets were orally administered 5 ml of the ETEC F4 suspension containing 10^8 CFU/ml treated as the infected group (INF), while piglets were orally administered 5 ml PBS treated as the non-infected group (NINF). To determine the effects of genotype and infection in piglets with the control diet, four experiment groups were designed as follows: RR-INF (N = 57): ETEC F4 resistant and infected; RR-NINF (N = 41): ETEC F4 resistant and non-infected; SR-INF (N = 61): ETEC F4 susceptible and infected; and SR-NINF (N = 11): ETEC F4 susceptible and non-infected.

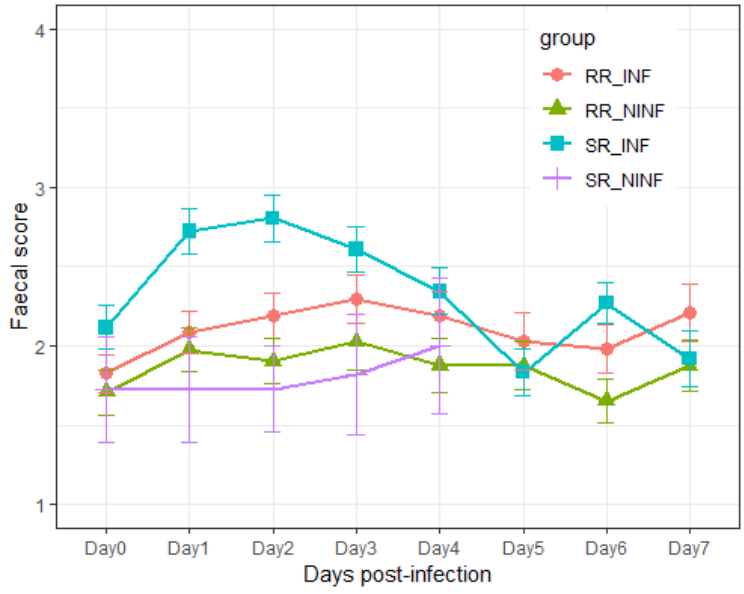

Figure 28: Average faecal score (± standard error) of infected and non-infected susceptible/resistant piglets monitored during 7 days post-infection.

The individual faecal score was visually evaluated based on the consistency of the faeces using the following scores: 1 = dry, moulded or pelleted faeces; 2 = creamy, sloppy, cow-dung appearance; 3 = liquid diarrhoea; 4 = watery diarrhoea. Piglets of RR (N = 57) and SR (N = 61) were orally administered 5 ml of the ETEC F4 suspension containing 10^8 CFU/ml at four days after weaning (day 0); piglets of RR (N = 41) and SR (N = 11) were orally administered 5 ml 1x PBS. *P*-values for main factors: genotypes: $P < 0.05$; infection: $P < 0.001$.

The faecal score was greater ($P < 0.001$) in INF than in NINF piglets for the entire duration of the study (Figure 28) regardless of genotype. In the SR-INF groups, the impact of ETEC F4 infection was evident on the first day after infection (Day 0), and the faecal score was markedly higher than in the other three groups. To determine the effect of genotypes, the faecal scores in the SR-INF and RR-INF groups were compared. Susceptible piglets had obviously higher faecal scores than resistant piglets, especially at day 1 and day 2 after infection ($P < 0.05$) (Figure 28). SR-NINF and RR-NINF piglets had faecal scores that were always lower than the other two infected groups, which presented no diarrhoea. There was no significant difference between RR-INF and RR-NINF.

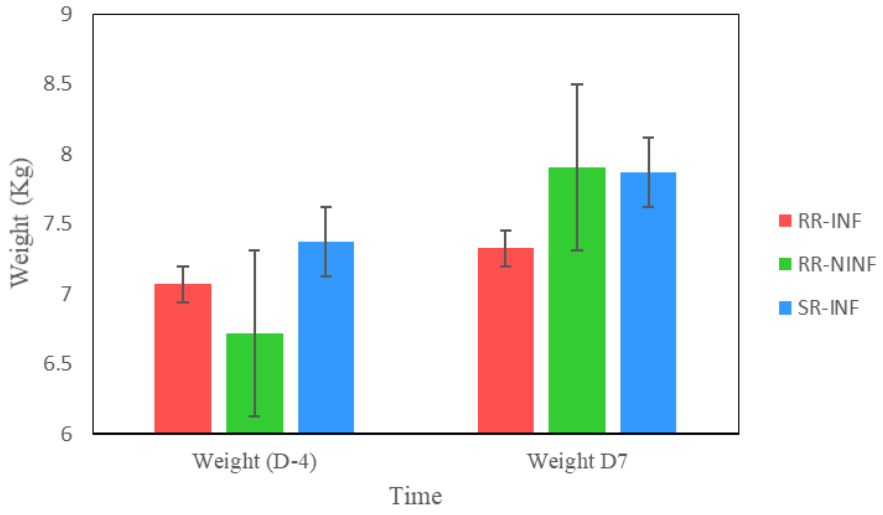

Figure 29: Average weaning body weight (kg) (± standard error) of resistant infected, susceptible-infected and resistant non-infected piglets at D-4 (four days before infection) and D7 (7 days after infection).

Piglets of RR (N = 57) and SR (N = 61) were orally administered 5 ml of the ETEC F4 suspension containing 10^8 CFU/ml at four days after weaning (day 0); piglets of RR (N = 41) were orally administered 5 ml 1x PBS. *P*-values for infection: $P = 0.71$; genotype: $P = 0.78$.

Four days before infection (D-4), RR-NINF piglets had lower weights than RR-INF and SR-INF piglets (Figure 29). After oral infection, RR-NINF piglets were heavier than infected piglets of the RR-INF and SR-INF groups. Comparing the weight difference between D-4 and D7 between RR-INF and RR-NINF, the obvious difference determined the negative effect of infection, which decreased the body weight. There was a slightly smaller increase in body weight in RR-INF than in SR-INF between D-4 and D7. However, there is no significant difference between genotypes and infection ($P > 0.05$).

Fresh faecal samples of the three groups (RR-INF [N = 21], RR-NINF [N = 22] and SR-INF [N = 18]) were collected directly in the rectum from each piglet. ETEC colony counting by plate (D0, D1, D2, D3, D4 and D7) and LT DNA/g dry faeces matter measurement by qPCR (D0, D1, D2, D3, D4, D6, D7 and D10) were performed. Average CFU values were converted into $\log_{10}$ (CFU+1) for analysis. The ETEC F4 bacteria shedding of the infected group markedly increased at day 1 after infection (Figure 30). The amount of bacteria was raised untill day 3 in SR-INF piglets whereas in RR-INF piglets, it was increased

at day 1 and dropped back to normal at day 2 after infection. There was a significant difference at day 2 post-infection ($P < 0.001$). Resistant piglets had smaller ETEC colony counting numbers on any record day than susceptible piglets. Resistant piglets recovered faster than susceptible piglets after infection. RR-NINF piglets maintained a levelled flat growing colony, which was regarded as a control group (Figure 30).

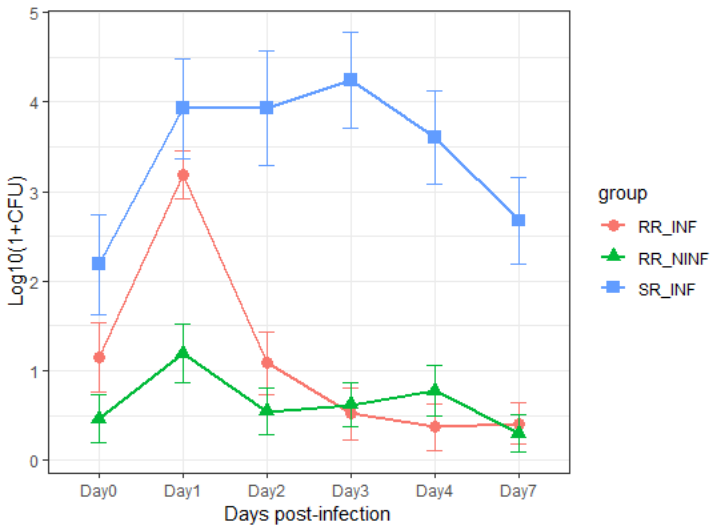

Figure 30: ETEC shedding (average log10[CFU+1] (± standard error)) in the faeces by plate counting at day 0, 1, 2, 3, 4, 7 post-infection of resistant infected, susceptible infected and resistant non-infected piglets.

Piglets of RR (N = 21) and SR (N = 18) were orally administered 5 ml of the ETEC F4 suspension containing 10^8 CFU/ml at four days after weaning (day 0); piglets of RR (N = 22) were orally administered 5 ml 1x PBS. *P*-values for genotype: $P < 0.001$.

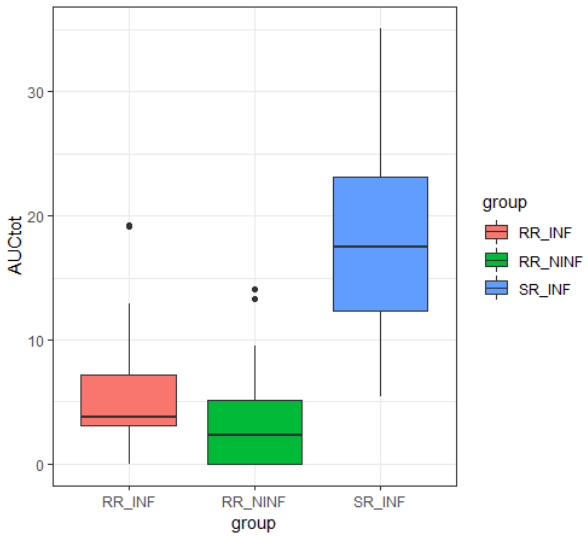

Figure 31: Total value of area under the curve (AUCtot) of day 0, 1, 2, 3, 4,7 post-infection of RR-INF, SR-INF and RR-NINF piglets were compared by boxplot in R.

Piglets of RR (N = 21) and SR (N = 18) were orally administered 5 ml of the ETEC F4 suspension containing 10^8 CFU/ml at four days after weaning (day 0); piglets of RR (N = 22) were orally 5 ml 1x PBS at day 0.

The AUC (area under the curve) of $\log_{10}$(1+CFU) on the experimental days of each piglet was calculated and summed up to a final AUCtot (AUC in total) as an output. Sixty-one piglets were analysed by boxplot to determine the relationship of effects on genotypes and infection among the three groups. Susceptible piglets showed overall higher AUC values among the whole infected period than resistant piglets, while there was no significant difference among resistant piglets with or without infection (Figure 31).

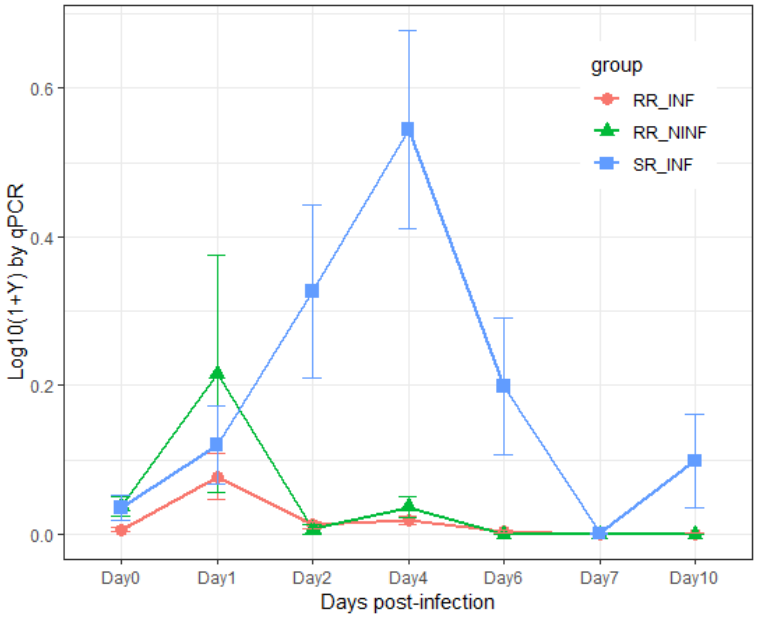

Figure 32: Effect of infection and genotypes on LT gene abundance determined by qPCR in the faeces collected at D0, 1, 2, 4, 6, 7 and 10 post-infection.

Data are expressed as Log_{10}(1+Y), where Y represents the ng LT-DNA per g of faeces dry matter. Piglets of RR (N = 21) and SR (N = 18) were orally administered 5 ml of the ETEC F4 suspension containing 10^8 CFU/ml at four days after weaning (day 0); piglets of RR (N = 22) were orally administered 5 ml 1x PBS.

The results obtained by qPCR also revealed that susceptible piglets contained a higher excretion profile after infection than resistant piglets (Figure 32). There was an increase in RR-INF at D1 after infection and back to normal from D2. RR-NINF increased at D1 without infection, which might be affected by the cross-infected bacteria in the pens. D3 data were excluded from the analysis due to missing data and difficulty in data collection.

2. Effect of 2% tannin extract and salicylate in susceptible infected pigs

Experiments designed in Chapter 4 were set up according to a completely randomized 2 x 2 factorial design. The main factors were the experimental diets (standard [CO] vs. tannins [TA]), salicylate supplementation (none [NSA] vs. sodium salicylate [SA]) and the one-way interaction. When

determining the effect of salicylate, the salicylate group (SA-CO & SA-TA) and no salicylate group (NSA-CO & NSA-TA) were used regardless of the effect of feed difference. The tannin group (NSA-TA & SA-TA) and control diet group (SA-CO & NSA-CO) were set up to determine the effect of diets. In this appendix, the two-way interaction was set up. SA-CO and NSA-CO were used to determine the effect of salicylate with a control diet. NSA-CO and NSA-TA were used to determine the effect of tannin diet without salicylate supplementation. SA-TA was used to determining the effect of the combination of salicylate and tannins. NSA-CO was used as a control to determine the effect of 2% tannin extract in the diet and salicylate supplement. The data were the same as those mentioned in the Chapter 4 and re-analysed in the new experimental design.

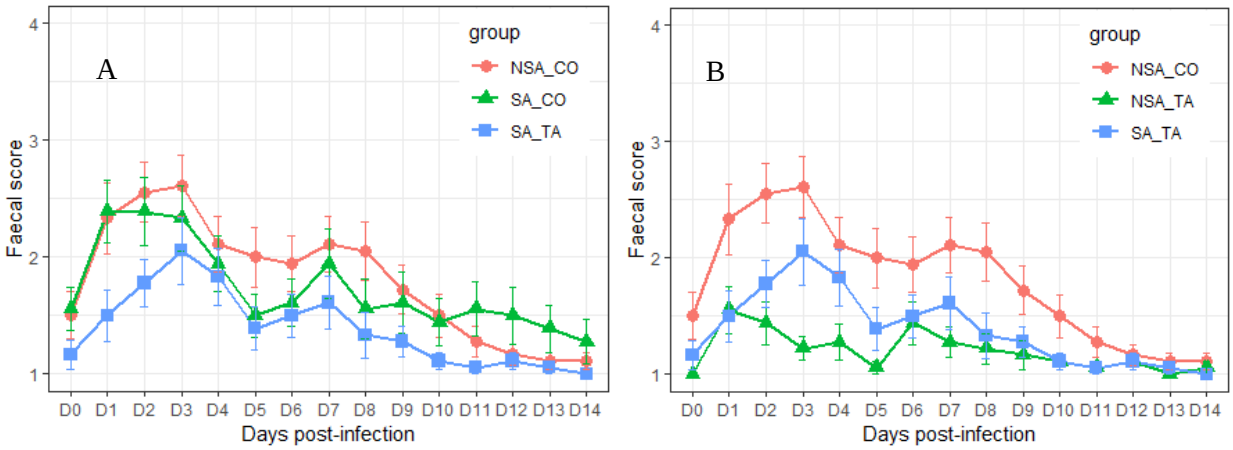

Figure 33: Average faecal score (± standard error) of infected susceptible piglets supplied with or without salicylate (A) and with/without tannins (B) monitored during 14 days post-infection.

The faecal score was assessed according to the following score scale: 1 = dry pelleted faeces, 2 = cow-dung appearance, 3 = liquid diarrhoea and 4 = watery diarrhoea during 14 days post-infection. Pigs of the SA-CO (N = 18) group were offered from d 0 to d 4 a daily dose of sodium salicylate (35 mg/kg BW × d^{-1}; sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and a control standard starter diet. Pigs of the NSA-CO (N = 18) group were offered from D0 to D4 a daily dose of tap water with a control standard starter diet. Pigs of the NSA-TA (N = 18) group had ad libitum to the tannin supplemented diet where wheat straw in the CO diet was substituted by 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy) with d 0 to d 4 a daily dose of tap water. Pigs of the SA-TA (N = 18) group had ad libitum to the tannin-supplemented diet where wheat straw in the CO diet was substituted by 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy) with d 0 to d 4 a daily dose of sodium salicylate.

The consistency of the faeces given by the faecal score varied over days with a maximal score on the second or third day post-infection (Figure 33 A&B). Supplementing piglets with salicylate for 5 days post-infection had no improvement on the faecal score (Figure 33A), whereas the addition of 2% of tannins reduced ($P < 0.001$) faecal score (Figure 33B). Except for D10 to D14, NSA-CO had higher faecal scores than SA-CO (Figure 33A). However, there was no significant difference between salicylate and no salicylate groups ($P = 0.07$). In contrast, piglets fed the TA diet had lower faecal scores in both the first and second weeks post-infection than those fed the CO diet (Figure 33B, $P < 0.001$). Comparing

the faecal score of SA-TA in Figure 33A and Figure 33B, the TA diet was observed to have a positive effect on reducing the severity of diarrhoea and SA supplementation might slightly inhibit the effect of TA.

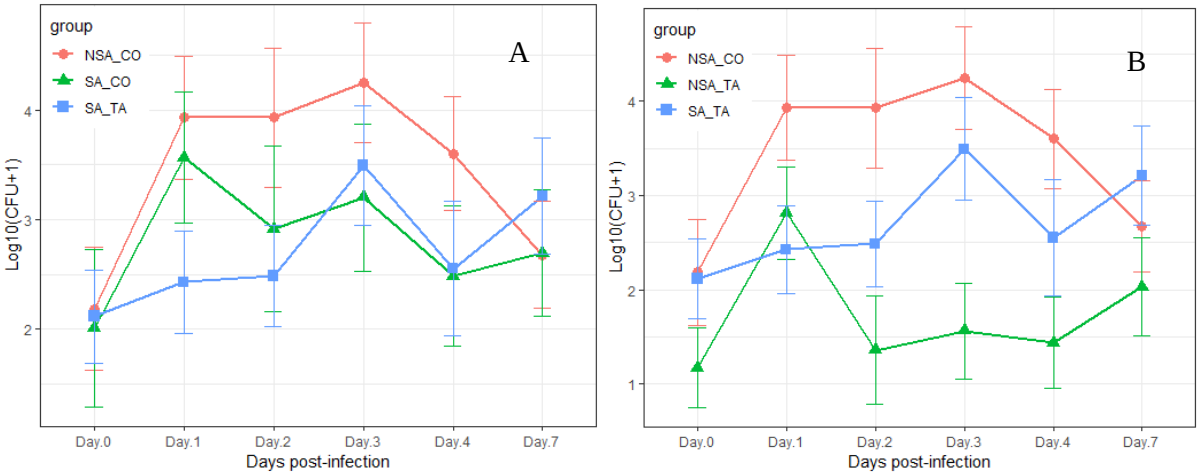

Figure 34: Effects of salicylate supply (A) and diet (B) on ETEC F4 shedding determined by plate counting in faeces samples collected from all pigs at D0, 1, 2, 3, 4 and 7 post-infection.

Pigs of the SA-CO (N = 18) group were offered from d 0 to d 4 a daily dose of sodium salicylate (35 mg/kg BW × d^{-1}; sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and a control standard starter diet. Pigs of the NSA-CO (N = 18) group were offered from D0 to D4 a daily dose of tap water with a control standard starter diet. Pigs of the NSA-TA (N = 18) group had ad libitum to the tannin supplemented diet where wheat straw in the CO diet was substituted by 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy) with d 0 to d 4 a daily dose of tap water. Pigs of the SA-TA (N = 18) group had ad libitum to the tannin supplemented diet where wheat straw in the CO diet was substituted by 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy) with d 0 to d 4 a daily dose of sodium salicylate.

ETEC shedding in the faeces were determined by both culture (Figure 34) and qPCR (Figure 35). Data were expressed as $Log_{10}(1+CFU)$, where CFU represented the average CFU obtained with the plate count technique. The extent of ETEC shedding in SA-CO is smaller than in NSA-CO, without a statistically significant difference (Figure 34A). Piglets fed TA obtained significantly lower ETEC shedding than those fed CO diet (Figure 34B, $P < 0.001$). SA supplementation inhibited the TA diet effect on ETEC F4 diarrhoea.

A B

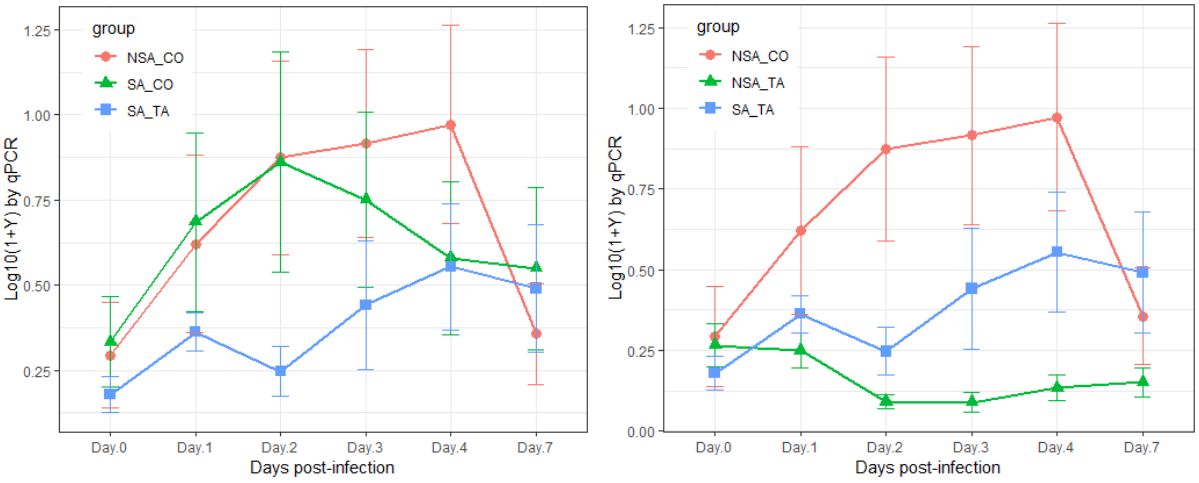

Figure 35: Effect of salicylate (A) and diet (B) on LT gene abundance determined by qPCR in the faeces collected at D0, 1, 2, 3, 4 and 7 post-infection.

Data are expressed as $Log_{10}(1+X)$, where X represents the ng LT-DNA per g of faeces dry matter. Pigs (N = 36) of the SA group were offered from days 0 to 4 a daily dose of sodium salicylate (35 mg/kg BW per day, sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Pigs of the SA-CO (N = 18) group were offered from d 0 to d 4 a daily dose of sodium salicylate (35 mg/kg BW × d-1; sodium salicylate, ReagentPlus®, S3007, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and a control standard starter diet. Pigs of the NSA-CO (N = 18) group were offered from D0 to D4 a daily dose of tap water with a control standard starter diet. Pigs of the NSA-TA (N = 18) group had ad libitum to the tannin supplemented diet where wheat straw in the CO diet was substituted by 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy) with d 0 to d 4 a daily dose of tap water. Pigs of the SA-TA (N = 18) group had ad libitum to the tannin supplemented diet where wheat straw in the CO diet was substituted by 2% of a hydrolysable chestnut tannin extract (Silvafeed Nutri P/ENC for Swine, Italy) with d 0 to d 4 a daily dose of sodium salicylate.

The results obtained by qPCR revealed a diet x time interaction (P = 0.06). The qPCR results show that NSA-CO excreted similar ETEC in the faeces as SA-CO especially at day 1 and day 2 post-infection (Figure 35A). ETEC shedding analyzed by qPCR was not significantly affected by the salicylate supply when comparing NSA-CO and SA-CO (P = 0.14). SA-TA had lower ETEC shedding in the faeces than SA-CO, which demonstrated that the TA diet could reduce diarrhoea severity (Figure 35A). NSA-TA excreted less ETEC in the faeces than NSA-CO and SA-TA (Figure 35B). Meanwhile, SA-TA had comparable higher ETEC shedding than NSA-TA after infection. SA supplementation had a negative effect on the tannin diet.

Comprehensively, contrary to sodium salicylate, 2% HT chestnut extract could be beneficial directly at weaning to reduce PWD. Nevertheless, the effects of salicylate on tannins deserve further investigation.

Chapter 5: Susceptible offspring of resistant sows show the same survival rate despite of lower antibody titres: a vaccination study and a retrospective litter analysis

This chapter is in preparation for publication.

All procedures for this study were conducted in accordance with the Swiss guidelines for animal welfare and approved by the Swiss cantonal veterinary office of Fribourg (Approval No. 26734).

*The figure is cited from http://livefromrhodeisland.com/la-vaccination-des-enfants-aux-us/.

5.1. Abstract

Pigs without specific receptors have an innate resistance against colonization by enterotoxigenic *Escherichia coli* (ETEC) expressing F4 fimbria. Resistance to colonization is a monogenetic recessive trait (RR) and RS and SS pigs are susceptible. The immunization of pregnant sows with vaccines containing F4 fimbriae (F4ab, F4ac) and *E. coli* heat-labile (LT) enterotoxin increases their colostral antibody titres and protects the suckling piglets against ETEC F4 diarrhoea. Since the immune system of ETEC F4 resistant sows is not naturally confronted with F4 fimbriae attached to their mucosa, their immune response to vaccination may be reduced. Twelve Swiss Large White (SLW) ETEC F4 resistant and 12 susceptible gilts were vaccinated twice Porcilis Porcoli, 48 ± 4 days before farrowing and 28 days thereafter. The F4 antibody titres ($-\log_2$ value of the highest dilution with a detectable antibody titres) were lower in the serum collected 7 to 14 days after the second vaccination and in the colostrum of the F4 resistant gilts (F4ab serum: 11.19 ± 1.44 vs. 12.18 ± 1.33; $P < 0.10$; F4ac serum: 10.03 ± 1.58 vs. 11.59 ± 1.43, $P < 0.05$; F4ab colostrum: 12.20 ± 2.41 vs. 14-02 ± 1.31, $P < 0.05$; F4ac colostrum: 10.93 ± 2.46 vs. 13.03 ± 5.21, $P < 0.05$). The LT antibody titres did not differ between F4 resistant and susceptible gilts (LT serum: 9.43 ± 1.45 vs. 10.18 ± 1.33, $P > 0.10$; LT colostrum: 9.83 ± 2.85 vs. 11.24 ± 1.69, $P > 0.10$). Although the resistant gilts showed a significantly reduced immune response to F4 fimbrial antigens, the small differences in colostrum antibody concentration are probably not of practical relevance. In the research herd of Agroscope all sows are routinely vaccinated against *E. coli* infection. The analysis of 5027 birth weight records of piglets born between 2010 and 2017 with known genotypes of the dam and the sire did not reveal obvious differences in birth weight among different genotypes mating. RR sows showed the lower weaning weight than RS or SS sows although no significant difference was found for average daily gain from birth to weaning. However, the highest survival rate from birth to weaning (SurBW) was found in litters of SS boars with RR sow ($P = 0.042$). Namely the vaccination is effective and protective enough even for a “risk mating”. This is contrary to average figures in the Swiss SLW herdbook population, where only approximately 40% of the sows are vaccinated against *E. coli* F4.

Key words: ETEC F4, receptors, immunization, colostrum, resistant/susceptible genotype pigs

5.2. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a significant cause of diarrhoea and mortality in neonatal piglets (Sojka *et al.* 1960). ETEC strains bear fimbriae, which adhere to specific receptors expressed on brush borders of enterocytes in the small intestinal epithelia cells and secrete heat-labile (LT) and heat-stable (STa, STb) enterotoxins, thereby causing diarrhoea (Osek 1999; Nagy & Fekete 2005).

Sows that have been infected by ETEC F4 secrete antibodies against F4 fimbriae and LT in the colostrum and milk, which offers the suckling piglets some protection against that pathogen (Rutter & Jones 1973; Deprez *et al.* 1986). Vaccines containing F4 fimbrial and LT antigens have been developed to increase the colostral antibody titres and thus efficiently protect suckling piglets against ETEC F4 diarrhoea. However, the pigs are deprived of this passive protection and becoming susceptible to ETEC infections (Van den Broeck *et al.* 1999).

The absence or presence of F4 receptors is based on genetic inheritance and can be determined *in vitro*. Breeding for ETEC F4 resistant pigs is a promising strategy for the prevention of both pre- and post-weaning ETEC F4 diarrhoea (Vögeli *et al.* 2014). Sellwood (1979) found evidence that F4 adherence is a dominant trait that is inherited in a Mendelian manner with two alleles. The loci responsible for susceptibility were assigned the candidate region to SSC13: *ALGA0072075-CHCF3*; 135'265'806-135'403'999 (Sscrofa 11.1) which is in high linkage with the *F4acR* phenotype (Hu *et al.* 2019).

The breeding goal of selecting F4ac-resistant pigs is achieved more quickly if both the sire and dam lines are selected at the same time for F4ac resistance. However, when ETEC F4 heterozygous or homozygous susceptible (SR or SS) boars are mated to ETEC F4 resistant (RR) sows, 50% or 100% of the offspring will carry F4 receptors and will therefore be susceptible to ETEC F4 infections. Therefore, the breeding program cannot be accomplished in one move.

There is a passive protection to newborn piglets by colostrum of maternal antibodies from ETEC F4 susceptible vaccinated sows against neonatal ETEC F4 diarrhoea and the protection can last about one week (Söderlind *et al.* 1982). However, ETEC F4 resistant sows without ETEC F4 receptors do not possess post-infectious antibodies against F4 fimbriae (Sellwood 1982, 1984) and must be vaccinated to protect their suckling piglets. Edfors-Lilja *et al.* (1995) have shown that F4 resistant pigs have lower antibody titres after a single injection of an F4 antigen than F4 susceptible pigs, presumably because the latter were exposed to ETEC F4 before immunization. The question therefore arises if F4 resistant sows produce protective levels of antibodies after the standard vaccination protocol developed for a commercial vaccine. In the present experiment the serum, colostrum and milk antibody titres of ETEC F4 resistant and susceptible vaccinated gilts were compared.

An analysis of the fertility traits in the SLW herdbook population revealed that offspring of genetically RR sows mated to genetically homozygous susceptible (SS) boars (all piglets are susceptible to ETEC F4 infection) had a lower survival rate from birth to weaning compared to other genotype combinations (A. Hofer 2017, Suisag internal review). No detail about the reason for the piglet's loss or the vaccination status of the sow were available. The question arises whether vaccination of the dam would correct for this drawback.

5.3. Materials and methods

5.3.1. Animals

The vaccination study was approved by the veterinary office of the canton Fribourg, Switzerland (Approval No. 26734), and was carried out in the healthy Swiss Large White pig herd (the Institute of Livestock Sciences, Posieux, Switzerland). Ear biopsy samples of all pigs intended for breeding were genotyped for ETEC F4 susceptibility/resistance by a DNA-based method. A KASP assay (LGC, Teddington, Middlesex, UK) with markers *CHCF1* and *ALGA0106330* was established to genotype (Hu *et al.* 2019). The animals were individually fed dry feed according to the Swiss feeding recommendations for pigs (Agroscope 2017).

Twelve ETEC F4 susceptible (S-, homozygous or heterozygous susceptible) and twelve ETEC F4 resistant (RR) pregnant Swiss Large White gilts were selected for the experiment to compare antibody titres among serum, colostrum and milk between different genotypes. The pregnant gilts were grouped housed in pens with straw bedding and were transferred to individual pens one week before farrowing.

5.3.2. Vaccination, sampling protocol and performance traits

All sows in Agroscope, Posieux, Switzerland were vaccinated against parvovirus and Erysipelas 1a, 1b and 2 (Parvoruvax®) and circovirus type 2 (CircoFLEX®). Then sows received the vaccine Porcilis Porcoli DF® (MSD Animal Health GmbH, Switzerland), which is administered in a 2 ml dose containing F4ab (K88ab), F4ac (K88ac), F5 (K99), the F6 (987P) fimbrial adhesins and LT toxoid.

The 24 selected gilts received the first dose of the vaccine Porcilis Porcoli DF® by injection into the neck muscle 48 ± 4 days before farrowing and were vaccinated again 28 days later. Blood samples were collected from the jugular vein when the gilts received their first and second doses of vaccine. The blood was placed into tubes without anticoagulant and centrifuged for 15 min at 3000 rpm after clotting. The colostrum and milk samples were collected within a few hours and 8 days after birth respectively. Colostrum and milk were obtained by milking several teats after milk let-down was induced by intramuscular injection of 40 IU oxytocin (Oxytocin-20, Graeub, Bern, Switzerland) All samples were stored frozen until analysis.

5.3.3. Serology

Selected 24 gilts were tested for the presence of antibodies against F4ab, F4ac and LT using an enzyme-linked immunosorbent assay (ELISA) in the laboratory of Intervet (Boxmmer, the Netherlands). The reactions were read with an ELISA reader at 450 nm and expressed as $-\log_2$ values ($-\log_2$ value of the highest dilution with a detectable signal). Animals with a titre ≥ 5.6 (signal at a dilution of 1:48 or smaller) were considered negative, $5.6 \leq$ titres ≤ 8 were doubtful and titres > 8 were considered positive.

5.3.4. Data analysis

Titres were compared with the one-factorial ANOVA. If a prerequisite for the ANOVA was not fulfilled, the Kruskal-Wallis test was used. Because the titres of most pre-vaccination serum samples and of most milk samples were below the detection limit, Fisher's exact test was used to compare the number of seropositive and seronegative first blood samples (serum 1) and milk samples.

5.3.5. Retrospective data analysis

A total of 5027 piglets from the experimental farm Posieux with birth weights out of 383 litters were used. All sows were of the Swiss Large White dam line breed. The sire breed of 4749 piglets was Swiss Large White dam line and 278 piglets of PREMO (Swiss Large White Sire Line)

Data for five performance traits were analysed with statistical packages in R (R Core Team 2014). Birth weight, average daily gain from birth to weaning and weaning weight were analysed by a linear model using the lm package. The model included the fixed effects of genotype of sire x genotype of dam, year of birth, parity of dam and sex of the piglets. The contrasts between different genotype matings were tested using a t-test. Survival rate at brith (SurB: 0 = born dead, 1 = born alive) and survival rate from birth to weaning (SurBW: 0 = not weaned, 1 = weaned) were analysed by a logistic regression model with a logit link function using the glm package and fitting the same effects as in the linear model. Odds ratios were used to compare different genotype matings.

5.4. Results

5.4.1. Antibody titres

There was no significant difference between the two groups of RR gilts and S- gilts concerning age, duration of gestation, litter size and litter weight. Before vaccination, more S- gilts tended to have serum antibodies against ETEC F4ab ($P < 0.10$), but not against F4ac ($P>0.10$, Table 22). The S- gilts responded with higher titres against F4ab ($P < 0.01$) and against F4 ac ($P < 0.001$) after the first administration of the vaccine (Table 22). That difference persisted after in the fully vaccinated gilts, although with a higher probability of error P value. The S- gilts also secreted colostrum containing more antibodies to F4ab and to F4ac ($P<0.05$, Table 22), and more S- gilts had detectable milk titres against

F4ab (P<0.10, Table 22) and against F4ac (P<0.05, Table 22). In contrast, more RR gilts had LT titres before vaccination (P < 0.05, Table 22), and the immune response to LT immunization did not differ between the two groups (P >0.10, Table 22).

Table 22: Antibody titre (-$\log_2$ mean ± standard deviation) in serum, colostrum and milk from vaccinated ETEC F4 susceptible (S) and resistant (R) gilts.

	R[a]	S[a]	P value [b]
F4ab Serum 1	0 (n=0)	6.23±0.48 (n=4)	< 0.10
F4ab Serum2	8.64±0 .88	9.99±0.93	< 0.01
F4ab Serum3	11.19±1.44	12.18±1.33	< 0.10
F4ab Colostrum	12.2±2.41	14.02±1.31	< 0.05
F4ab Milk	6.07±0.25 (n=3)	6.83±0.71 (n= 8)	< 0.10
F4ac Serum1	5.85±0.21 (n=2)	6.3±0.48 (n=3)	> 0.10
F4ac Serum2	8.01±1.16	10.19±1.04	< 0.001
F4ac Serum3	10.03±1.58	11.59±1.43	< 0.05
F4ac Colostrum	10.93±2.46	13.03±5.21	< 0.05
F4ac Milk	0 (n=0)	6.84±0.98 (n=5)	< 0.05
LT Serum1	6.66±0.80 (n=9)	7.35±0.07 (n=2)	< 0.05
LT Serum2	8.00±1.75	8.73±0.95	> 0.1
LT Serum3	9.43±1.45	10.18±1.33	> 0.1
LT Colostrum	9.83±2.85	11.24±1.69	> 0.1
LT Milk	0 (n=0)	6.2 (n= 1)	> 0.1

Serum 1: before the first vaccination; Serum 2: after the first vaccination; Serum 3: 1-2 weeks after the second vaccination. To calculate the mean, values ≥ 5.6 were used.
n: number of gilts containing antibody titre ≥ 5.6 (12 piglets per treatment)
[a] : if the $\log_2$ antibody titre < 5.6, the value was treated as 0.
[b] : When many titres were < 5.6, Fisher's exact test was used; otherwise, Kruskal-Wallis was used.

There were much more pronounced differences in antibody titres in the serum against the fimbrial antigens F4ab and F4ac after the first vaccination than after the second vaccination between RR and S- gilts (Table 22). Comparing the differences between RR and S- gilts among the three serum results, S- gilts had an immune response to ETEC F4 infection prior to vaccination. In most milk samples, the antibodies were below the detection limit. The titres of RR gilts were high in both serum and colostrum. Antibody titres of RR gilts in serum was raised after two vaccinations. The antibody titres were similar in RR gilts' colostrum and serum after two vaccinations, while S- gilts had higher antibody titres in colostrum than serum after two vaccinations.

5.4.2. Performance traits results

Mean of raw records of the five performance traits of litters of RR boar x SS/SR sow and litters of SS/SR boar x RR sow were extracted and shown in Table 23. Litters of RR boar x SR sow had the highest BW,

while SR boar x RR sow had the highest birth WW and ADGw (Table 23). Litter of SS boar x RR sow showed the highest SurB and SurBW.

Table 23: Mean of raw records of birth weight (BW, kg), weaning weight (WW, kg), average daily gain from birth to weaning (ADGw, g/day), survival rate at birth (SurB) and survival rate from birth to weaning (SurBW) by genotypes of boars and sows.

Boar	Sow	No. at birth	BW	WW	ADGw	SurB	SurBW
RR	SS	257	1.474	6.995	0.215	0.856	0.777
RR	SR	793	**1.477**	7.150	0.225	0.890	0.850
SS	RR	200	1.471	6.789	0.214	**0.935**	0.**898**
SR	RR	1181	1.472	**7.341**	**0.229**	0.855	0.813
RR	S-	1050	1.476	7.116	0.222	0.882	0.833
S-	RR	1381	1.427	7.247	0.226	0.867	0.826

Contrasts of RR boar x SS sow compared to SS boar x RR sow litters on five performance traits are shown in Table 24. There was no difference in BW and ADGw. The estimated difference for WW is 245 g but not significantly different from zero (P = 0.24, Table 24). Litters of SS boar x RR sow had estimated higher survival rate to birth, but this difference was not significant (P = 0.163, Table 24). Litters of SS boar x RR sow had significantly higher SurBW than RR boar x SS sow (P = 0.042).

Table 24: Estimated contrasts of SS sow x RR boar and RR sow x SS boar, standard errors and result of t-test for birth weight (BW, kg), weaning weight (WW, kg) and average daily gain from birth to weaning (ADGw, g/day), estimated effects of logistic regression model and odds ratios of survival rate at birth (SurB) and survival rate from birth to weaning (SurBW).

	Estimate	SE	P value[a]	Z value	Odds ratio
BW	0.038	0.038	0.317		
WW	0.245	0.208	0.240		
ADGw	0.001	0.007	0.841		
SurB	-0.486	0.348	0.163	-1.395	0.615
SurBW	-0.635	0.312	**0.042**	-2.033	0.530

[a]:P value of BW, WW and ADGw was the result of the t-test. P value of SurB and SurBW was the result of the z-test.

5.5. Discussion

Neonatal diarrhoea caused by ETEC F4 infection makes a huge economic loss in pig production worldwide, as it harboures adhesion F4ac, F4ab and LT toxins which cause diarrhoea (Fairbrother *et al.* 2005). ETEC diarrhoea impairs animal welfare and is responsible for the widespread use of antibiotics, in particular of colistin, which should be reserved for selected cases in human medicine (Liu *et al.* 2016).

Risk factors for the use of antibiotic use in suckling and in weaner pigs are recognised for a long time and are the basics of good farming practice (Kohler 1974; Hirsiger *et al.* 2015; Rhouma *et al.* 2017)

Breeding for disease resistant animals is a promising strategy to decrease antibiotics usage. In the US, as well as in Switzerland, fimbriae F18 and F4 are detected in nearly all ETEC isolates (Zhang *et al.* 2007; Schneeberger *et al.* 2017). The DNA-maker test to distinguish ETEC F4 susceptible/resistant genotype is determined to be practical (Hu *et al.* 2019).

Protection of piglets against ETEC infections through maternally derived antibodies is well-known and the present study describes the difference of immune response in ETEC F4 resistant and susceptible gilts. ETEC F4 antibodies are effective to prevent diarrhoea or moves peak prevalence of disease from the first to the second or 3rd week after birth when mortality is much lower (Moon & Bunn 1993). However, in the herd where the vaccination study was done the descendants of F4 resistant sows weighed less than those of F4 susceptible but the difference was not significant. It is prudent to take precautionary measures to protect suckling piglets of F4 resistant sows against ETEC F4 diarrhoea.

Vaccinating these resistnat sows against ETEC F4 antigens in case crossing with ETEC F4 susceptible boars during the breeding program would make sense. F4 resistant gilts had significantly lower serum antibody titres to F4 fimbrial antigen after a single intramuscular injection of a commercial vaccine (Edfors-Lilja *et al.* 1995). The present study was based on the hypothesis that a vaccine infection acted as a booster to the F4 susceptible pigs, which had been immunised by ETEC F4 colonising their intestine, and that the injection of a second dose would boost the immune system of the F4 resistant gilts sufficiently to eliminate the difference in titres seen after the first dose. The results confirm the finding of s Edfors-Lilja *et al.* (1995) that F4 resistant pigs react less to F4 fimbrial antigens than F4 susceptible pigs; although the pre-vaccinal titres did not indicate that the F4 susceptible gilts had an immune response to F4 fimbriae already. Although that difference diminished after the second dose, as we had expected, the serum and the colostrum of the fully vaccinated F4 resistant gilts had lower anti-fimbrial F4 antibody titres, and fewer F4 resistant gilts continued to secrete detectable amounts of these antibodies in the milk. On the other hand, the F4 resistant gilts did not produce fewer antibodies against LT, which indicates that the reduced response to the vaccine was restricted to the F4 fimbrial adhesions. The virulence of ETEC depends on their ability to attach to the small intestinal epithelium and to secrete LT and ST enterotoxins. Sufficient amounts of antibodies against fimbrial adhesions and against LT are probably required to protect newborn piglets from diarrhoea caused by ETEC. All fully vaccinated F4 resistant and susceptible gilts had detective amounts of serum and colostrum of F4ab titres and F4ac titres.

Significant lower antibody titres (-15%) were measured against F4ab and F4ac in the colostrum of ETEC F4 resistant sows, while there is no difference in LT when compared to susceptible gilts. It indicated that F4 susceptible offspring from vaccinated F4 resistant mother maybe more susceptible to ETEC F4

infections than those from vaccinated F4 susceptible mother. The difference of collecting time might have influence on antibody titres. Usually, the sufficient time period to transfer protective antibodies form the colostrum to the piglets was approximately 12 hours after farrowing. Immunoglobulins levels in the colostrum decreased from the highest concentration (colostrum sample 0 h after birth) to comparative lower concentration at longer time of lactation (Klobasa *et al.* 1987). However, some sows were not monitored during night and gave birth over the weekend which influence the collecting time of colostrum.

Based on performance traits analysis, effects on weights and average daily gains were rather small among different ETEC F4 genotypes. Survival rate from birth to weaning when RR sow x SS boar was significantly higher than litters of SS sow x RR boar. It seems that even RR sows had lower antibody titres in colostrum, it was still protective enough for their offspring. However, the number of the pigs in these two mating groups were limited which might influence the results. The vaccination of sows might mask the effect of ETEC F4 genotypes before weaning. Two different vaccines were administered to all gilts before Porcilis was administered and certainly not interfere with the immune response to Porcilis.

Besides, the initial vaccine was treated as a booster for all gilts since there existed pronounced differences in the antibody titres against fimbrial antigens F4ab and F4ac after the first vaccination than after the second vaccination. Offspring of SS boars X RR sows had high risk of developing ETEC F4 diarrhoea when those dam lacking ETEC F4 receptor. These offspring will then express the receptor and be susceptible to ETEC F4 infection and not be protected by the maternal antibodies. Antibody titres against fimbrial antigens of F4 resistant gilts in colostrum and serum after the second vaccination was nearly the same, while F4 susceptible gilts had higher antibody titres in colostrum than serum. F4 susceptible gilts with antigen-specific receptor can induce an immune response to ETEC F4 infection following vaccination while F4 resistant cannot. A different vaccination protocol with repeated immunizations may thus be needed for F4 resistant sows.

When compared the antibody titre against LT toxoid, there was no significant difference in serums, colostrum and milk among F4 susceptible and resistant gilts. Nine F4 resistant gilts (75%) had antibody titres against LT toxins in serum before the first vaccination above detective limit. There is probably another germ could produce LT toxoid to the same immune response in F4 resistant gilts when vaccinated. Nearly in most milk samples, the antibody titres were below the detection limit. Therefore, piglets get protection of antibody titres from their mother via colostrum. In swine, there is not an efficient materno-fetal transfer of immunoglobulins via placenta, and foetuses predominantly receive passive immunity postnatally through lactation (Matías *et al.* 2017). Milk composition differs among the moment of lactation. Milk harbours mother's antibodies against numerous pathogens with the highest concentration in the first days of lactation (colostrum) and decreasing throughout lactation (Matías *et al.* 2017). IgG is the predominant immunoglobulin in sow colostrum and IgA dominated in the mature milk.

IgG, IgA and IgM concentrations decrease from colostrum to one week later of lactation (Markowska-Daniel & Pomorska-Mol 2010; Markowska-Daniel *et al.* 2010).

Compared to unvaccinated resistant gilts, the antibody titres against fimbrial antigens and LT toxoid in vaccinated resistant gilts were much higher in both serum and colostrum (Riising *et al.* 2005). The method (EP-0692) approved by the European Pharmacopoeia Commission was used to test the efficacy of the vaccine. Piglets from vaccinated sow and non-vaccinated sows were challenged with different enteropathogenic *E. coli* strains. The efficacy of the vaccine used in this study was acceptable.

Based on the reproduction data of SLW herdbook (SUISAG, Sempach, Switzerland), litters of susceptible piglets nursed by resistant sows that have a significantly lower survival rate to weaning, especially if the sire is homozygous susceptible. As the data does not include information on vaccination or ETEC pressure in these herds, it is difficult to compare these findings to the results of this study where all sows were routinely vaccinated. Resistant sows give birth to slightly lighter piglets than susceptible sows which is probably not a big concern. Piglets born from resistant sows show a lower weaning weight than those born from susceptible sows with nearly the same average daily gain. It seems like resistant sows should be mated to resistant boars will be beneficial for selecting F4 genotypes on piglet performance. The vaccination of sows might mask the effect of F4 susceptible/resistant genotypes in a "risk mating".

Chapter 6: General discussion

The present study concentrates on efforts to eliminate post-weaning diarrhoea (PWD) in pigs. The selection of pigs genetically resistant to enterotoxigenic *Escherichia coli* (ETEC) F4 and/or F18 is considered an economically meaningful action. The vaccination of sows, food additives and rearing management can be used as alternatives to improve the health status of pigs, which are supplements for breeding programmes.

6.1. Breeding for ETEC F4 resistant pigs

An absolute or relative disease resistant pig could diminish the severity of illness, decrease pathogen proliferation and excretion, and in consequencely decrease the infection pressure and incidence of disease in healthy pigs (Figure 36). If the majority of the pigs in the population are disease resistant, the farmers could profit from lower animal loss, higher performance and better product quality compared to susceptible pigs. The usage of antibiotics will be reduced during rearing. Less zoonosis will occur due to the selection of disease resistant pigs (Figure 36).

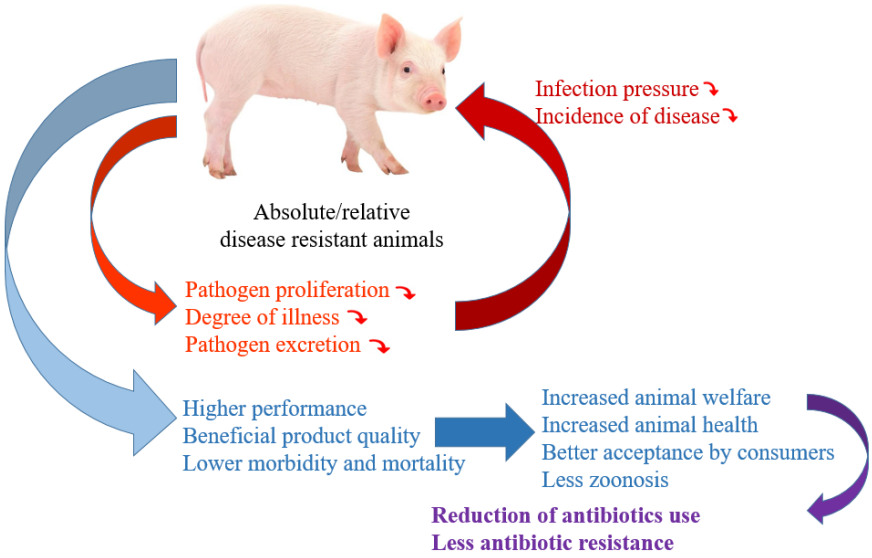

Figure 36: The advantages of breeding disease resistant pigs.

Red arrows show the positive effects on animals; blue arrows show the positive influences on economic and ethical parts; purple arrow shows that breeding disease resistant animals can reduce antibiotic usage and decrease the spread of antibiotic-resistant bacteria in human and animal productions (modified from G. Reiner, JLU Giessen personal communication).

A *FUT1* c.307 A > G transition has been identified as the mutation underlying the difference in ETEC F18 susceptibility (Meijerink *et al.* 1997; Meijerink *et al.* 2000). Since 2006, the breeding programme to select for ETEC F18 resistant (*FUT1* c.307 *A* allele as a resistant genotype) pigs have been conducted and are extremely effective in Switzerland (Luther *et al.* 2009). In Switzerland, the Large White dam line is resistant to the *E. coli* F18 strain, while the selection is still on-going in the sire line.

The feasibility of breeding for ETEC F4 resistant pigs has been discussed in the present study. Among 2034 Swiss Large White (SLW) pigs, only one recombinant pig, 5.8250.JR2 (0.04%), occurred where

CHCF1 and *ALGA0106630* showed different genotypes. Haplotypes of seven loci were tested for this recombinant pig. *MUC*-8227, *ALGA0072075*, *CHCF1* and *CHCF3* indicate the resistant genotype while *MUC13*g.15376, *ALGA0106330* and *MUC13*-226 indicate the heterozygous susceptible genotype. The phenotype of 5.8250.JR2 was unknown because the pig died before genotyping. However, 5.8250.JR2 has the same genotyping results with these seven markers as the other nine recombinant boars in Swiss Duroc, Landrace and Piétrain which showed recombination only between the markers *CHCF3* and *MUC13g.15376* (Chapter 2, Figure 8 & Table 5). Therefore, we speculated that the phenotype of 5.8250.JR2 indicated resistance. After submission of Chapter 2, another recombinant pig, 3100.CF5, was found through the SUISAG breeding system. The genotyping results of these seven markers were the same as 5.8250.JR2. The phenotype of 3100.CF5 was resistant, which verified our speculation of the phenotype of 5.8250.JR2. *CHCF1* was a reliable marker to predict the ETEC F4ac genotype even for the recombinant pig. The frequency of the resistance allele in the SLW population was 63%, which is advantageous for implementing a breeding programme to select for ETEC F4ac resistant pigs. If the frequency of the resistant allele in the population is low, the breeding programme will be time consuming and expensive which farm breeders will avoid.

The DNA marker-based method to genotype the ETEC F4 resistance/susceptibility using these two markers (*CHCF1* and *ALGA0106630*) is practicable. However, breeding for ETEC F4 resistant pigs is not feasible if there are negative effects of the resistance allele (*R*) on economic production and reproduction traits. The effects of the resistant allele on 17 important economic production traits (Table 7) were determined. The *R* allele on intramuscular fat content (IMF) of the *longissimus dorsi* muscle seems to be dominant over the *S* allele. There is a significant difference in IMF between different genotypes; however, the difference is relatively small. The heritability value for IMF is 0.58, which enables keeping the IMF% constant through conventional breeding. No negative effect of the resistant allele on the other 16 important economic production traits was determined. This diagnostic DNA marker-based method allows the pig breeders to select against susceptible animals, thereby minimizing the incidences of *E. coli* F4 diarrhoea outbreaks without considerable negative effects on production traits.

Häfliger (2016) investigated the effects of the *R* allele on reproduction traits, the number of piglets born alive, the proportion of underweighted piglets, the survival rate, and the interval weaning to oestrus. There is no other negative effect on reproduction traits by breeding for the *R* allele. Only when resistant sows (*RR*) mate with susceptible boars (*SR* or *SS*), do their susceptible suckling piglets have a significantly lower survival rate from birth to weaning (Häfliger 2016). In addition, resistant sows give birth to slightly lighter piglets than susceptible sows. Piglets born from susceptible sows are protected by ETEC F4 antibodies, which present in the colostrum if the mother had previous contact with this pathogen. Resistant sows without ETEC F4 receptors are not passively immune due to low or missing antibody titres in the colostrum. Therefore, if breeding for resistance to ETEC F4ac becomes applicable,

the immunity component should be considered regarding the breeding strategy. The breeding programme should improve the level of resistance to ETEC F4 in the sire line first. Based on the investigation mentioned above, it is feasible to breed for ETEC F4 resistant pigs without considerable negative effects on important production traits.

6.2. Vaccination and immune protection

Except for selecting disease resistant pigs, other methods can be used to implement the breeding programme. PWD is a result of multi-factorial stressors including environmental, nutritional and psychological stressors. Stress factors associated with weaning, such as removal from the sow, dietary changes, adaptation to a new environment and mixing of pigs from different farms, may negatively affect the response of the immune system and lead to intestinal gut dysfunction in pigs (McCracken *et al.* 1999; Lallès *et al.* 2004; Lallès *et al.* 2007; Rhouma *et al.* 2017). Furthermore, the breeding program cannot be accomplished in one move. The susceptible piglets born from resistant sows might be suffering from the ETEC infections during neonatal period. Sow vaccination can be an activator and a booster for immune response in sows and transfer antibody via colostrum to protect their offspring from diarrhoea.

Vaccination on sows is a practical method to decrease the morbidity of pathogenic diarrhoea in pigs. The vaccination of pregnant sows with *E. coli* antigens can stimulate the production of serum antibodies in the colostrum; however, it does not stimulate local immunity in the gut and does therefore not increase IgA titres in milk. Subsequently, commercial vaccines in pregnant sows protect suckling piglets from neonatal *E. coli* diarrhoea and have been commonly used for many years (Haesebrouck *et al.* 2004; Kosorok & Kastelic 2008). However, antibody titres in the colostrum last and function for only a few days after feeding with colostrum; therefore, they have no effective protection against PWD. To activate the immunization of piglets against PWD, a vaccine is needed to be used during the suckling period. In this study, we focus on the sow vaccination.

Piglets require the immunoglobulins from colostrum to obtain sufficient immunity during early postnatal development (Schnulle & Hurley 2003). The sow placenta is not permeable to maternal immunoglobulins (IgG, IgA, IgM) transport and therefore suckling piglets acquire maternal immunoglobulin from colostrum during the first 24-48 h of life (Nagy & Fekete 2005; Lallès *et al.* 2007). Weaning age and pre-weaning health are reported to affect the onset of PWD (Madec *et al.* 1998; Heo *et al.* 2013). Based on the investigation of profitability of weaning pigs, pigs no earlier than 26 days have less occurrence of PWD than weaning pigs before 21 days (Main *et al.* 2004). In the European Union, many pig producers wean piglets at 21 days of age. However welfare legislation encourages weaning no earlier than 28 days of age to ensure that health pigs are transferred into nursery accommodation after house cleaning (Baxter *et al.* 2013). Increasing weaning age reduces stress associated with weaning period and allows pigs to have a more mature gastrointestinal tract and become

increasingly familiar with solid feed during lactation with an improvement in immune response (McLamb *et al.* 2013; Rhouma *et al.* 2017). In this study, piglets were weaned at day 24 ±1.

Unlike vaccines for pet animals, there are very few polyvalent pig vaccines available on the EU market. Intervet International B.V. company has developed Porcilis Porcoli DF, which is an inactivated vaccine recommended for the passive immunization of sow/piglets to reduce mortality and diarrhoea due to neonatal enterotoxicosis during the first days of, caused by *E. coli* strains that express the fimbrial adhesions F4ab (K88ab), F4ac (K88ac), F5 (K99) or F6 (987P). Commercially available vaccines are given parenterally two times at approximately 6 and 3 weeks prior to farrow (Ivanov 2007). Porcilis Porcoli DF was used in the present study. The colostrum samples were collected within a few hours after gestation. However, some sows were not monitored during night and gave birth over the weekend which influence the collecting time of colostrum. IgG levels decreased from 95.6 mg/mL in first colostrum (0 h) to 64.8 mg/mL at 6 h and 32.1 mg/mL at 12 h of lactation. The concentration of IgA decreased from 21.2 mg/mL (0 h) to 15.6 mg/mL (6 h) and 10.1 mg/mL (12 h). IgM concentration decreased from 9.1 mg/mL (0 h) to 6.9 mg/mL and 4.2mg/mL (12 h) (Klobasa *et al.* 1987). The difference of colostrum samples' collecting time might influence the antibody tires results. In this study, 62.5% colostrum samples were collected within 6 hours after farrowing, 25% were between 6 hours to 12 hours and 12.5% were between 12 hours to 24 hours after farrowing. The proportion of the samples collected late was nearly the same in both F4 susceptible and resistant groups. Difference on the sample collection time might be accepted.

Significantly lower antibody titres (-15%) were measured against ETEC fimbriae F4ab and F4ac in the colostrum of ETEC F4 resistant sows, while there was no difference in antibody titres in LT when compared to susceptible gilts. Vaccination of sows and immunoglobulin uptake of pigs might mask the effect of the ETEC F4 susceptible/resistant genotype. The breeding programme of disease resistant piglets might start from the father line. Vaccination for pregnant gilts was a wise method to protect piglets from neonatal diarrhoea. However, it might confuse farm breeders that breeding for disease resistant pigs and vaccinating resistant pregnant sows were both recommended.

6.3. Food additives

To keep the swine industry profitable, it is imperative to find alternatives to in-feed antibiotics and minerals that are effective in reducing the incidence and severity of diarrhoea. The dietary polyphenol is a potential alternative to control PWD. Polyphenols containing plant extracts are widely used additives in various feed (and food) products, which have recently been suggested to be a potent inhibitor for *Vibrio cholera* (CT) *in vitro* (Morinaga *et al.* 2005). CT is similar regarding physiologic, structural and antigenic capabilities to the toxin produced by LT, with approximately 77% identity at the nucleotide level, 83% amino acid sequence homology and has a comparable mode of action (Fairbrother *et al.* 2005; Verhelst *et al.* 2014). Polyphenols might be a potent inhibitor of LT. However, whether polyphenols can

inhibit the toxicity of LT produced by ETEC *in vitro* is unknown yet (Verhelst *et al.* 2010). Tannins are natural polyphenols that are found in many vegetable feedstuffs (Kumar & Vaithiyanathan 1990). Although tannins are often reported as anti-nutritional products due to the negative effect on protein digestion (Butler 1992), some authors observed that feeding pigs with feedstuffs high in tannins such as field beans and carob powder did not affect animal growth (Flis *et al.* 1999; Lizardo *et al.* 2002). Tannic acids also seem to have a negative impact on the growth performance of weaned piglets (Lee *et al.* 2010), and the negative effects of tannins remain controversial.

An ETEC challenging model was established in this study. Susceptible piglets were identified by a diagnostic DNA marker-based method and were infected orally with a native ETEC strain, which was resistant to sulfamethoxazole and harboured the F4 fimbriae gene, LT gene and STb gene but not the STa gene. One percent and two percent hydrolysable chestnut-tannin extracts (HTE) were used as food additives in the diet comparison with weaned piglets to prevent the prevalence of diarrhoea (Chapters 3 & 4). Diet with 1% HTE did not show a significant difference in decreasing the severity of diarrhoea and improving performance compared to the control diet. However, diet with 2% HTE at weaning improved feed intake and average daily gain. Although 2% HTE showed a positive effect on growth performance and prevention of ETEC F4 diarrhoea, the perfect concentration of tannins in the diet can be analysed later if necessary.

Pathogen infections provoke inflammatory responses which manifest in loss of appetite and fever. Previous studies reported an improvement of performance and a reduction of diarrhoea in weaned pigs receiving acetylsalicylic acid also known as aspirin (Xu *et al.* 1990; Kim *et al.* 2016). Salicylate is an analogue of acetylsalicylic acid and a daily dose (D0 to D4, 35 mg/kg) of salicylate was offered via electrolyte solution (Chapter 4). Kim *et al.* (2016) hypotheses that salicylate supplementation may improve the performance of weaned pigs by reducing inflammation-associated amino acid waste through modulation of the immunosuppressive molecule prostaglandin E2 (PGE2) biosynthesis. However, based on the results, sodium salicylate did not improve the growth performance of artificially infected piglets with ETEC F4 strains. There was no clear connection between acute inflammatory responses and growth performance. The dose of salicylate acid might influence the performance results. The function of salicylate acid in improving performance and reducing diarrhoea in weaned pigs is not yet clear.

6.4. Methodological constraints

Difficulty in measuring the severity of ETEC F4 diarrhoea was met. Faecal score, ETEC colony culturing and ETEC counting by qPCR were used as measurements to represent the severity of diarrhoea caused by ETEC F4. Faecal scores were collected to compare with ETEC F4 infection and non-infection groups, susceptible and resistant genotypes, tannin diet and salicylate supplements. However, the faecal score was scaled by subjective judgement. Sometimes, it was ambiguous to distinguish the diarrhoea

severity when the faeces were similar. Furthermore, it was confusion when piglets were watery diarrhoea compared to less severe diarrhoeic piglets after peeing. Faecal scores can only subjectively judge the piglets' diarrhoea severity. If the piglets have diarrhoea, it is impossible to distinguish whether the diarrhoea is caused by ETEC F4 using only faecal scores.

Eosin-Methylene Blue (EMB) agar plates with 50 µg/ml rifampicin were used in ETEC colony culturing. Faecal samples were collected with swab, immersed in 1X PBS solution and transferred to the same amount of mixed solution in the agar plate and incubated overnight. However, it is impossible to control the same amount of faeces per sample with swab that are transferred into PBS solution. Although the ETEC colony culturing by plate cannot quantitatively measure the amount of ETEC F4 in the faeces, we were able to verify that diarrhoea in the infected piglets was caused by infective *E. coli* strains. However, apart from infective strains, fungi or other bacteria (not *E. coli*) might grow on the plate, affecting results. Therefore, before we count the colony growing on the plates, we need to distinguish the colonies of *E. coli* F4ab/ac strains. In addition, the CFU counting number is very large, and the differences between the two tested piglets might be less than the number indicated.

The real-time PCR protocol for the LT fragment was designed to quantitatively measure ETEC F4 in the faeces. Sample collection was difficult as well. The collected faecal samples were freeze dried before DNA extraction. It is difficult and complex to measure the volume and weight of faeces before drying. However, the volumes and weights of watery diarrhoea faeces and cream diarrhoea faeces changed differently after drying as well. We measured and calculated the LT PCR fragment concentration per g dry faeces matter in this study. It is sensitive for qPCR measurement, and aseptic operation is recommended to reduce cross contamination in the laboratory. ETECs adhere to small intestinal epithelial cells and thereby secrete heat-stable (STa and STb) and/or heat-labile (LT) enterotoxins. We assume that PCR results with the LT primer indicated the existence of ETEC F4 diarrhoea. However, this method still has disadvantages. When the faeces were watery diarrhoeic, there was no or less content remaining after drying which had no or less LT PCR fragment, leading to the opposite result that there was no or less ETEC F4 in this piglet. Meanwhile, faeces sample collection was a stimulate for piglets which might cause diarrhoea due to nerves. Other bacteria in the station could cross-infect piglets causing diarrhoea as well. Compared among three measurements, faecal score, colony counting and qPCR, ETEC F4 determination by qPCR is accurate to measure the level of diarrhoea caused by ETEC F4 but time consuming and expensive. Although these three methods have obvious disadvantages, there were no contradictory conclusions based on these three measurements, and we used all of them to parallel compare results that were similar and lead to the same conclusion. Meanwhile, attention to data omitted when there was no DNA extracted from faeces, which might be severe watery diarrhoea or no faeces when sample collection. No faeces collected might because there was no defecation without food intake before or just defecated before collection. No faeces or no DNA can be caused by controversial reasons, and therefore, this sample would be omitted from analysis.

6.5. Farm management

In addition to the factors mentioned above, other contributing factors, such as housing conditions, population density and the feeding regimen after weaning, play a significant role in the development of PWD (Laine *et al.* 2008). It is beyond the scope of this study to discuss in detail all the ideal conditions for pig housing during the post-weaning period.

Farm management is essential for PWD control as well. The AIAO (All-In-All-Out) system is commonly used in swine production. AIAO swine production is a system that keeps animals together in groups, which are closely matched by age, weight, production stage and condition. Animals from different groups are not mixed during their stay on the farm. In an AIAO system, sows are bred as groups to farrow during a 5- to 10-day period; while in a continuous flow system, sows are bred individually continuously and farrow continuously. Switching from a continuous flow system to AIAO can improve production and reduce costs. In addition, disease transmission and infection from other pigs or the environment is reduced or eliminated in an AIAO system because once a group is established, no pigs are added to it, and the facility can be completely emptied and sanitized between groups. In an AIAO system, pigs within each group have similar nutritional and environmental requirements; the producers can enhance better management to meet pigs' requirements and can be easier to keep records. Furthermore, AIAO has great potential for improving pig performance by increasing feed efficiency (lower feed costs) and daily gain (decrease days to market). Other risk factors of management need to be considered as well, such as to mix pigs from different suppliers in the same pen; no respect to work sequence/order; to handle sick pigs after handling healthy one; not sufficient farmer practices; no biosecurity to enter the pens and no visitor information recorded.

PWD caused by ETEC F4 is a multifactor disease. The selection of disease resistant pigs is a practical method to reduce the morbidity of PWD in pigs. Vaccination, food additives and farm management could be used as supplements to implement the breeding programme for ETEC F4 resistant pigs.

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