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The effect of formic acid on *Escherichia coli´s* susceptibility to different antibiotics

Effektene til maursyre på *Escherichia coli´*s sensitivitet til ulike antibiotikum

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1 Abstract

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Since the ban of antibiotics as an additive in livestock feed in Europe in 2006, organic acids have emerged as an alternative and is now commonly used. However, we still see examples that absence of antibiotics does not prevent the development of resistance. It has been questioned if formic acid can affect the gut microbiota to develop and express antibiotic resistance mechanisms. The aim of this study was to study the effect of formic acid on *E. coli´s* susceptibility to antibiotics. Three different isolates of *E. coli* were adapted to formic acid (1M pH 3,9), and subsequently treated with three different antibiotics (tetracycline 12 mg/L, gentamicin 1,6 mg/L and ciprofloxacin 0,06 and 0,08 mg/L). Severe variation in percent survival after acid adaptation was observed both between the strains and between the trials. For gentamicin and ciprofloxacin there was an increased susceptibility for antibiotics in the formic acid adapted group (gentamicin 0,0003-0,0010 %, ciprofloxacin 0,09-0,62 %), compared to the control group (gentamicin 13,7-20,3 %, ciprofloxacin 11,62-12,38 %). Tetracycline showed a lower percent survival, but a minimal difference between the acid adapted (0,34-1,18 %) and the control group (2,44-3,20 %). The result suggests a synergistic effect with formic acid combined with gentamicin and ciprofloxacin, but not combined with tetracycline. Additional research is needed to further elucidate this topic.

2 Abbreviations

AR	Acid resistance
CFU	Colony forming units
GI-tract	Gastrointestinal tract
GRE	Glycopeptide resistant *E. faecium*
LPS	Lipopolysaccharide
NE	Necrotic enteritis
OD	Optical density
OMPs	Outer membrane porins
PLP	Pyridoxal-5´-phosphate
PBS	Phosphate buffered saline
QREC	Quinolone resistant *E. coli*
RPM	Rounds per minute
SCFA	Short chain fatty acid
TSB	Tryptic soy broth
TNTC	Too numerous to count
VRE	Vancomycin resistant *Enterococcus*
WHO	World Health Organization

3 Introduction

3.1 Feed additives in production animals

3.1.1 Antibiotics as feed additives

In 1946 it was reported increased growth among chickens given small concentrations of antibiotics added to the feed (Moore et al., 1946). After this observation, it became common worldwide to use antibiotics at low levels as growth promotors to increase growth among animals, but also to improve feed efficiency and lower the incidence of infections (Aarestrup, 2000; Khachatourians, 1998). However, this widespread use of antibiotics as feed additives over time has contributed to the appearance of antibiotic resistant bacteria, among them strains of *Escherichia coli*, *Salmonella*, *Campylobacter* and *Enterococcus* (Khachatourians, 1998). These findings created concerns that existing antibiotics no longer would work to combat diseases in farm animals, but also concerns that these bacteria could be transferred to humans.

Later, confirmed associations were reported between bacteria isolated from infections in humans and bacteria isolated from livestock animals. For instance, in 1988, multidrug resistant *Salmonella typhimurium* definite type 104 (DT 104) was reported in cattle in England and Wales. It was isolated from meat and meat products from several other domestic animals, along with unpasteurized milk from different locations. Humans were then reported to be infected with the bacteria either through meat products as beef, chicken and pork sausages, or through direct contact with farm animals (Threlfall et al., 1997). Another example is vancomycin resistant *Enterococcus* (VRE) which was reported in Europe in 1988 (Uttley et al., 1988) and outside the health care setting in 1993 (Bates et al., 1993). In 1994 it

was reported findings of VRE not only in livestock feces, but also from uncooked chicken, which suggested that farm animals could be reservoirs for VRE causing infections in humans (Bates et al., 1994). Results from a study in Denmark with genetical analysis on VRE in feces from pigs and poultry, also suggested a genetical similarity to resistance genes found in VRE from human isolates (Aarestrup et al., 1996). Both vancomycin used as a therapeutic agent in humans and avoparcin used as a feed additive in production animals are glycopeptide antibiotics. An association between the resistance to these two components was therefore suggested when the same gene responsible for vancomycin resistance (vanA) was detected in VRE from animals given avoparcin to the feed (Klare et al., 1995). This association was later confirmed by epidemiologic studies in several countries including Denmark (Aarestrup, 1995) and Norway (Kruse et al., 1999).

Scandinavian countries were among the first to take actions on the concerns about using antibiotics as feed additives, starting in 1986 when Sweden banned all use of antibiotics as growth promoters. Norway and Denmark followed up with the same decision in 1995 and 1998-1999, respectively (Grave et al., 2006). The actions taken by the Scandinavian countries influenced the European Union and countries internationally, which led to an increased focus on both the general use of antibiotics and the use of antibiotics as feed additives (Bengtsson & Wierup, 2006). The European Union (EU) followed up by phasing out substances from 1997, and then totally banning the use and marketing of all antibiotics as growth promoters from the 1st of January 2006 (European Commission, 2003). The termination led to a massive reduction in the usage of antibiotics in feed production. By 2004 the use had decreased by 65 % in Sweden, 47 % in Denmark and 40 % in Norway after the termination, according to the nations monitoring programs (DANMAP, 2004; NORM/NORM-VET, 2004; SVARM, 2004). Fecal samples from animals also showed a decrease in the incidence of antibiotic resistant bacteria

after antibiotics were removed from the feed. For instance, a study in Denmark showed a significant decrease ($p = 0,000000$) of glycopeptide resistant *E. faecium* (GRE) in poultry between 1995–1998. However the same study also reported the number to be unchanged in samples from pigs during the same time period (Bager et al., 1999).

Before the removal, antibiotics as feed additives were mainly used in the pig and poultry industries in the Scandinavian countries. There was also some use in feed for calves in Denmark and Sweden, but in the beginning of the 1970s there were reported doubts in Sweden about the effect on growth in calves (Johnson & Jacobsson, 1973). The concerns about resistance to antibiotics in calves were also brought up at the time (Wierup et al., 1975), which led to antibiotics as feed additives in calves and general beef production to be almost completely removed in Sweden even before the total termination in 1986 (Bengtsson & Wierup, 2006). In Norway, antibiotics in feed were mainly used for pigs and poultry at the time of termination. Because of its positive effects there were concerns that the removal would lead to increased infection rates as well as reduced animal welfare and reduced efficiency in the production.

3.1.2   Organic acids as feed additives

3.1.2.1   Pig industry

Antibiotics as feed additives in pigs were used to promote growth and increase feed efficiency, but also to decrease morbidity and mortality, especially among young piglets (Cromwell, 2002). In the pig industry, there has always been an interest of weaning pigs at a young age to maximize the production and keep it as efficient as possible. This creates problems as it exposes the piglets to several stress factors such as changes in nutrition,

environment and social life, at the same time as having a naive immune system. A sudden feed change from milk to solid food can therefore result in a syndrome most known as "post weaning diarrhea", which is characterized by malnutrition and sometimes overgrowth of pathogenic bacteria. This is usually manifested by diarrhea, dehydration, reduced growth and death, and antibiotics as feed additives used to be the general solution to overcome this problem.

The discovery of antibiotic resistant bacteria (and eventually the termination of antibiotics as feed additives) therefore led to a search of alternative additives to reduce these problems. Organic acids were brought in early as a possible replacement after several studies indicating that they may have a prophylactic effect similar to antibiotics (Mathew et al., 1991; Scipioni et al., 1978) as well as improved weight gain and feed efficiency in both pigs and poultry (Patten & Waldroup, 1988; Skinner et al., 1991). It was also reported that it might reduce the incidence of diarrhea in pigs (Kirchgessner & Roth, 1987; Kirchgessner & Roth, 1990).

3.1.2.2 *Poultry industry*

The use of antibiotics in feed in poultry was mainly introduced to reduce the rates of necrotic enteritis (NE) caused by *Clostridium perfringens*. Shortly after the removal of avoparcin and other antibiotic feed additives on 31st of May 1995, a new epidemic of clinical NE in Norwegian broilers emerged. Grave et al. reported an increase in treated broilers for NE (relatively to the broilers produced) from 1,2 % before 31st of May 1995, to 11,3 % in the rest of the year. However, they also reported a decrease to 5 % in 1996, following a further decrease to similar levels as before the ban of avoparcin (Grave et al., 2004).

The reported increase in NE made the Norwegian poultry industry look for an alternative feed additive. After the ban in Sweden in 1986, narasin was introduced as an alternative, which also showed beneficial effects on the occurrence of NE. The Norwegian poultry industry did the same after the experiences of narasin as a feed additive in Sweden was shared (Grave et al., 2004).

Narasin is a polyether monocarboxylic acid derived as a fermentation product from strains of *Streptomyces aureofasciens* and was first described in 1977 (Boeck et al., 1977). It was further characterized the following year when its antibiotic effect on gram-positive bacteria, anaerobic bacteria (among them *C. perfringens*) and fungi was described. Its protection against coccidial infections in chicken was also reported in this study (Berg & Hamill, 1978). Anticoccidial activity for other similar polyether antibiotics such as monensin had previously been claimed (Haney et al., 1970). It was first described for narasin in 1977, when its activity against *Eimeria acervulina*, *E. tenella* and *E. maxima* was reported (Weppelman et al., 1977). This was confirmed by Ruff et al. through a series of three battery trials in broilers. Their research also showed increased growth among broilers with narasin given in the feed, as well as the discovery of effect against other coccidia as *E. mitis*, *E. necatrix* and *E. brunetti* (Ruff et al., 1979). This was further confirmed by later trials which also reported significant increase in weight gain and feed efficiency when increasing the amounts of narasin given (Jeffers et al., 1988). As the effects of narasin was confirmed it was approved as a coccidiostat feed additive for chickens in Norway in November 1995. There was however no doubts in the substance antibiotic effects, which also explains the reduction of prevalence of NE in Norway after the introduction of narasin, as previously described (Grave et al., 2004).

Since narasin was approved as a coccidiostat and not an antibiotic feed additive, it was not affected by the termination of antibiotic feed additives in Scandinavia or in the EU. However, narasin was also removed from the broiler production in Norway in June 2016 after the Norwegian poultry industry announced to start phasing out the additive in December 2014 due to being heavily pressured by the media and consumers at the time. The coccidiostat was replaced by vaccinating every one-day old chicken against coccidiosis along with an increased focus on management issues (Nortura, 2014). These were however not the only measures taken by the industry when narasin was removed. Since 2016, formic acid has systematically been added to broiler feed (Sanson, 2018).

Data provided by The Norwegian Food Safety and presented in NORM-VET 2018 showed that these actions led to a massive reduction in the use of ionophore coccidiostats from 13 722 kg sold in 2014 to 1436 kg in 2016. The report also show that the termination did not result in a significant increase in use of antibiotics, as only 0,18 % of broiler flocks were treated with antibiotics in 2017, compared to 0,16 % in 2013 (NORM/NORM-VET, 2018). Measures taken were therefore considered successful as the removal of narasin did not increase the prevalence of diseases or therapeutic use of antibiotics.

3.2 Organic acids

Compounds containing carbon and acidic properties are defined as organic acids. The most common are carboxylic acids, which are weak acids whose acidity is associated with the carboxylic (-COOH) part of the molecules (Theron & Lues, 2010). Organic acids are distributed in plants and animals as well as being produced by the process of microbial fermentation of carbohydrates, predominantly in the large intestine (Partanen & Mroz, 1999).

They are known as effective feed preservatives that protect food against unwanted bacterial and fungal growth, and by that improve the feed quality (Frank, 1994). Organic acids used in food animal production are classified into two different groups: **1.** <u>Monocarboxylic acids</u>, such as formic-, acetic-, propionic- and butyric acids, and **2.** <u>Hydroxyl group bonded carboxylic acids</u>, such as lactic-, malic-, tartaric- and citric acids. (Dibner & Buttin, 2002). They can be used in their form as acids, salts or a mixture of multiple acids, and can be given either through feed or drinking water (Huyghebaert et al., 2011). Studies done in broiler chickens have shown that using multiple acids in blends can increase the beneficial effects compared to using a single acid alone (Samanta et al., 2008; Samanta et al., 2010)

3.2.1 Action mechanisms

The action mechanisms of organic acids as feed additives are not clearly understood, but several suggestions have been proposed based on research in pigs and chickens.

1. Increased proteolytic activity and digestibility of nutrients

Reduced pH in the diet results in reduced pH of the gastrointestinal tract. In the stomach, pepsinogen is converted to the active enzyme pepsin which is important in digesting proteins. However this conversion is dependent on a pH below 5,0. Pepsin is most active at a pH between 2,0 and 3,5 and have no activity at pH levels above 6,0 (Taylor, 1959). It has been documented that weaned pigs have reduced production of hydrochloric acid which results in higher pH values than the optimal pH 2,0-3,0 that is seen in older pigs (Kidder & Manners, 1978). The acid production only reaches these levels 2-3 weeks after weaning (Cranwell & Moughan, 1989). Supplements of organic acids can therefore be useful in weaned pigs as the lower pH values will increase the production and activity of pepsin. This will therefore reduce

the risk of undigested proteins reaching the intestines, and therefore reduce the risk of osmotic diarrhea.

The end product of·pepsin digestion can also stimulate the secretion of pancreatic enzymes and bicarbonate. It has been reported that short chain fatty acids (SCFA) might stimulate both endocrine and exocrine secretions from the pancreas in pigs, sheep and calves (Harada & Kato, 1983; Harada et al., 1986; Kato et al., 1989; Sano et al., 1995). This increase digestibility, absorption and retention of protein and amino acids.

Studies in chickens have shown that organic acids can increase the digestibility of nutrients by elevating the retention of protein and dry matter, as well as improving the absorption of minerals and the utilization of phosphorous (Nezhad et al., 2011; Rafacz-Livingston et al., 2005). Increased absorption and retention of minerals has also been shown in pigs. Absorption of calcium and phosphorous seems to be particularly improved by organic acids (Höhler & Pallauf, 1993; Jongbloed et al., 1995; Jongbloed & Jongbloed, 1996; Kirchgessner & Roth, 1980). It has also been reported that adding fumaric acid to the feed of weaned pigs improve the balance of different minerals such as calcium (Ca), phosphorous (P), magnesium (Mg) and zink (Zn), which indicates that organic acids can influence the retention of minerals (Kirchgessner & Roth, 1980). However, the effect of this can vary and depends on the diet and how much minerals it contains (Partanen & Mroz, 1999).

2. *Reduced number of pathogenic bacteria*

It is documented that low pH values in the gastrointestinal tract can inhibit growth of unwanted bacteria, coliforms included (Maxwell & Stewart, 1995). A study in pigs showed that organic acid supplements can reduce the number of bacteria such as *E. coli* and

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Enterococci significantly in duodenum and jejunum. The amount of *Lactobacilli* was also reduced, but to a smaller degree (Roth & Kirchgessner, 1998). It has been described that non-dissociated organic acids are lipophilic and can penetrate the bacterial cell wall and disrupt the normal physiology of the bacteria. When the non-dissociated organic acid gets exposed to the internal pH of the bacteria, it dissociates, releasing cations (H^+) and anions (A^-). This results in decreased pH inside the bacteria, and pH sensitive bacteria such as *E. coli* are not able to tolerate the big difference between internal and external pH. This results in increased H^+-ATPase pump activity to pump H^+ out of the cell, as an attempt to bring the internal pH back to normal. This can stop bacterial growth or result in death of the bacteria, as it requires a lot of energy (Lambert & Stratford, 2003). The anionic part of the acid can only diffuse through the cell wall in its non-dissociated form. This results in accumulation of anions within the bacteria which can lead to osmotic problems and toxic effects (Roe et al., 1998). I has also been suggested that the ions themselves might inhibit the protein synthesis (Lück, 1986), and by that prevent the bacteria to replicate (Roth & Kirchgessner, 1998)

Studies in chickens have shown that acidic conditions have bactericidal effects by making the environment more favorable for *Lactobacilli* (Fuller, 1977). It has been suggested that *Lactobacilli* might inhibit the colonization and proliferation of *E. coli* by blocking adhesion sites. Another suggested inhibiting mechanism is the production of lactic acid and other metabolites which decrease the pH (Partanen & Mroz, 1999).

3. *Source of energy and improved gut health*

It has been suggested that the growth promoting effect of organic acids might be caused by them being utilized as an energy source after absorption (Bosi et al., 1999). Organic acids can as part of the tricarboxylic acid cycle reduce the needs of gluconeogenesis and lipolysis

(Giesting & Easter, 1985; Partanen & Mroz, 1999). Short chain fatty acids (SCFA) are a source of energy for the growth of epithelial cells and can therefore improve gut health in chickens (Gadde et al., 2017). It is documented that SCFA produced during fermentation of carbohydrates can stimulate the proliferation of epithelial cells. (Lupton & Kurtz, 1993; Marsman & McBurney, 1996; Sakata et al., 1995). The same effect has also been seen when SCFA have been administered orally, intravenously or through gastrointestinal infusions to animals (Frankel et al., 1994; Sakata et al., 1995). A study done by Gálfi and Bokori showed that adding sodium butyrate in the diet to pigs resulted in an increase in number of cells with microvilli in the ilium, increased length of the microvilli itself, as well as increased depth of cecal crypts (Gálfi & Bokori, 1990). Since organic acids can influence the fermentation process it has been suggested that they might also indirectly influence the morphology of the intestinal tract (Partanen & Mroz, 1999), and thereby improve gut health. Similar studies on the morphology of the gut has also been done in chicken. Studies by Garcia et al. showed that adding formic acid to the diet increased the height of the intestinal villi as well as the depth of the crypts in jejunum (Garcia et al., 2007). Increased height of intestinal villi has also been reported in other studies when butyric acid, fumaric acid and lactic acid were added to the chicken feed (Adil et al., 2010).

4. *Effect on gastric emptying rate*

As mentioned, lowering of the pH in the stomach stimulates the conversion of pepsinogen to pepsin, and thereby increases the digestion of proteins. However, the end products of pepsin digestion are also a part of the regulation of emptying the gastric content (Maner et al., 1962). The rate of gastric emptying is stimulated by the pH in the pyloric region of the stomach (Kidder & Manners, 1978; Mayer, 1994). The emptying rate decreases with increased acidity of the food, which in turn gives more time for protein digestion in the stomach (Mayer, 1994)

3.2.2   Formic acid

The properties of formic acid are summarized in table 1. It is an organic acid belonging to the monocarboxylic acids and has the chemical formula HCOOH. Formic acid is a weak acid with a pKA value of 3,75, it exists as a liquid and is soluble in water (Pubchem, 2020). It is colorless, transparent and has a pungent smell. When ingested, it can easily be absorbed through mucus membranes and diffuse across cell membranes in its undissociated form (Partanen & Mroz, 1999).

Table 1. Properties of formic acid.

Chemical name	Formic acid
Formula	HCOOH
pKa	3,75
Solubility in water	Soluble in all proportions
Physical form	Liquid (in pure state), colorless, transparent, fuming
Odor/taste	Pungent odor, emission of strong odors
Production	Synthetically: from methyl formate and formamide, by-product of acetic acid production and by laboratory methods Naturally: in many fruits (apples, strawberries, raspberries), honey, nettles

It is widely used as a preservative in the production of livestock feed. Preservatives reduce the incidence of microbes in the feed, and therefore the quantity of microbes consumed by the animal (Quitmann et al., 2014). Formic acid is used to promote the fermentation of lactic acid as well as to suppress the formation of butyric acid. Other benefits include allowing the fermentation process to happen fast, at a lower temperature, as well as reducing the loss of nutritional value (Reutemann & Kieczka, 2000). Formic acid can inhibit or kill yeasts and some bacteria, while fungi and bacteria producing lactic acid seem to be more acid resistant (Lueck, 1980). It is shown to be effective against *E. coli* in low concentrations as well as

effectively remove *Salmonella* from contaminated feeds (Frank, 1994). In vitro studies have shown that the minimum inhibiting concentration (MIC) of formic acid is 0,1 for *S. typhimurium*, *C. perfringens*, *Listeria monocytogenes* and *Campylobacter jejuni*, and 0,15 for *E. coli* and *Staphylococcus aureus* (Strauss & Hayler, 2001).

The beneficial effects from adding formic acid to the feed has been documented through several studies. Supplementing 6-18 g/kg of formic acid to the diet has shown to improve weight gain, feed intake, protein accretion and utilization, as well as to reduce the incidence of diarrhea in weaning piglets (Kirchgessner et al., 1992). Partanen and Mroz reported increased daily growth when formic acid at 46–444 mequiv/kg was added to the diet of weaning piglets. The feed:gain ratio was also reported to decrease slightly with increased amounts of acid in the same study (Partanen & Mroz, 1999). Formic acid has also been reported to have anti-agalactia properties when added to the diet of lactating sows (Mroz et al., 1998). Studies done in growing pigs have shown that adding 1,2 % potassium diformate to the diet can reduce the amount of coliform bacteria in the duodenum, jejunum and rectum of these animals (Øverland et al., 2000). Excessive formic acid supplementation can disturb the acid-base balance, leading to metabolic acidosis, which results in reduced feed intake and slower growth rate (Kim et al., 2005). Acute toxicity (LD_{50}) of formic acid is 1-2 g/kg body weight after oral application (Lueck, 1980). In the European union, the maximum value approved in feed for pigs is 12 g/kg with 12 % moisture (Luise et al., 2020).

3.3 Antibiotic resistance

Antibiotics are medicines used to prevent bacterial infections in both human and animals. The discovery of antimicrobial compounds revolutionized modern medicine, and antibiotics are one of the most important tools to combat infections. Antibiotic resistance develops when a bacteria no longer responds to a drug to which it was originally sensitive. The prevalence of antibiotic resistant bacteria is rising to dangerously high levels in all parts of the world, and it is one of the greatest threats to global health, food security and development (WHO, 2014). When infections no longer can be treated by first-line antibiotics, infectious diseases will be difficult to control. A prolonged duration of illness and treatment, and often a longer hospitalization, result in increased health care costs. Medical treatments that are widely used today, such as surgery, transplantations and chemotherapy will become much more dangerous and will involve a greater risk without effective antibiotics for prevention and treatment of infections.

3.3.1 Antibiotic resistance in Norway

The Norwegian monitoring program for antimicrobial resistance in the veterinary and food production sectors, NORM-VET, monitors *E. coli* from healthy animals of different species, and the bacteria are tested for sensitivity to a range of substances. Clinical isolates of different bacteria from different animal species are also monitored. There is resistance to several groups of antibiotics in Norway. However, the conclusion of NORM-VET 2019 is that the prevalence of antibiotic resistant bacteria in animals is still low (NORM/NORM-VET, 2019). This is due to low usage of antibiotics in human and veterinary medicine, a beneficial usage pattern and effective measures against the spread of resistant bacteria. However, continuous

effort and awareness is important to maintain the favorable situation and to ensure the effectiveness of antibiotics when needed.

Quinolones

The NORM-VET program reveals that quinolone resistant *E. coli* (QREC) is present in samples from some healthy animal species. This is interesting since quinolones are not used prophylactically in Norway and the veterinary therapeutic use is also very limited. The program indicates that there is a difference in occurrence of quinolone resistance between different species, with it being found most frequently in broilers and pigs.

Quinolones are a group of broad-spectrum antibiotics which are used in human and veterinary medicine. Nearly all quinolones in use are fluoroquinolones, for example ciprofloxacin. Quinolones are bactericidal, and the mechanism of action is inhibiting the activity of DNA-gyrase and topoisomerase IV, which are two essential enzymes that modulate the chromosomal supercoiling required for nucleic acid processes, such as transcription and DNA synthesis (Correia, Poeta et al. 2017). Quinolones are classified as "Highest priority critically important antimicrobials" (WHO, 2018). Resistance to quinolones has become widespread in Europe, and the occurrence is increasing (ECDC, 2018).

Kaspersen et al. made a study where they compared QREC in various species in relation to human population density (Kaspersen et al., 2018). They analyzed data from the NORM-VET reports from 2006 and 2016. The bacteria isolates originated from broilers, layers, cattle, turkeys, dogs, wild birds, red foxes, reindeers, sheep, horses and pigs. They found that in total, 1,4 % of the isolates were quinolone resistant, which is low compared to other countries in Europe. There was interspecies variation, with the highest occurrence in broilers and wild

birds. The results also showed that human population density was not associated with occurrence of QREC. Fluroquinolones are not used prophylactically in Norway, only therapeutical in very small amounts. Therefore, the interspecies variation in the prevalence of QREC suggests that other factors than the use of fluoroquinolones may be important in the development of resistance. These factors are presently unknown, and further research is needed to examine possible explanations. It has been suggested that the difference in occurrence of QREC between different animal species may be due to different production management and environment. Broilers have the highest population density among the production animals and the highest prevalence of quinolone resistance, while cattle have the lowest population density and also the lowest prevalence of QREC (Kaspersen et al., 2018). It has also been hypothesized that there is a variation in the prevalence of QREC between ruminants and monogastric animals, with a much lower prevalence of QREC in ruminants (Bjelland Mohn, 2020). An increase in quinolone resistance in broilers from 2011 to 2018 has also been reported (NORM/NORM-VET, 2018). As mentioned in section 4.1.1.2, narasin was removed and formic acid was added to the poultry feed in 2016. It has therefore been questioned if this change might have influenced the increase in quinolone resistant bacteria in broilers.

The acquisition of quinolone resistance is recognized to be multifactorial and complex. It has been shown that bacterial stress factors can induce chromosomal mutations, which is typical for quinolone resistance (Qin et al., 2015). The main resistance mechanism is one, or a combination of target-site gene mutations, which modify the drug-binding affinity of target enzymes (Aldred et al., 2014). However, there are other mechanisms that may contribute to resistance. For example, mutations that lead to reduced intracellular drug concentrations by either decreased uptake or increased efflux, and plasmid-encoded resistance genes that

produce either target protection proteins, drug-modifying enzymes or multidrug efflux pumps (Aldred et al., 2014) (Correia et al., 2017). The cellular changes associated with each mechanism are not mutually exclusive and they can therefore accumulate and create high levels of resistance.

Tetracycline

Tetracycline is a broad-spectrum antibiotic class, with bacteriostatic effect on Gram-positive and Gram-negative aerobic and anaerobic bacteria, *Rickettsiae, Spirochetes, Chlamydiae*, *Mycoplasma* and some protozoans such as *Anaplasma*. The mechanism of action is by reversibly inhibiting bacterial protein synthesis by binding to the 30S ribosome subunit and preventing attachment of tRNA to the mRNA-ribosome complex, and thereby blocking the addition of amino acids to the growing peptide chain. Resistance mechanisms against tetracycline include efflux pumps, ribosome protection and enzymatic inactivation of the tetracycline molecule (Speer et al., 1992). Tetracycline is classified as "Highly important antimicrobials" on WHO´s list of critically important antimicrobials (WHO, 2018). The use of tetracyclines in food producing animals and horses has gradually decreased since the mid 1990´s (NORM/NORM-VET, 2019). In 2019, tetracycline represented 0,9 % of the total amount of antibiotics prescribed to cattle, 1,4 % of the total amount of antibiotics prescribed to pigs, and 1,9 % of the total amount of antibiotics prescribed to sheep. Tetracycline was one of the antibiotics where resistance was most frequently found. 2,2 % of *E. coli* isolates from caecal samples of healthy cattle less than one year of age were resistant to tetracycline. NORM-VET 2019 also reported that 9,0 % of *E. coli* isolates from dogs with clinical urinary tract infections and 14,0 % from other clinical infections were resistant to tetracycline.

Gentamicin

Gentamicin is an aminoglycoside and a broad spectrum, bactericidal antibiotic. The mechanism of action is by binding to the 30S ribosome subunit and inhibit the mRNA translation and protein synthesis, which result in the synthesis of abnormal proteins (Hsu, 2008). Aminoglycosides are effective against Gram-negative aerobic bacteria and are synergistic with β-lactams against many Gram-positive bacteria. The most widespread mechanism of resistance to aminoglycosides is by inactivation by modifying enzymes. Other resistance mechanisms are efflux pumps, decreased permeability of the bacterial cell wall, mutations and modification of the ribosomal target (Garneau-Tsodikova & Labby, 2016). Aminoglycosides are classified as "High priority critically important antimicrobial" on WHO´s list of critically important antimicrobials (WHO, 2018). The use of aminoglycosides in food producing animals and horses has markedly decreased since the mid 1990´s (NORM/NORM-VET, 2019). In 2019 it represented 3,5 % of the total amount of antibiotics prescribed to cattle, 1,9 % of the total amount of antibiotics prescribed to pigs, and 7,1 % of the total amount of antibiotics prescribed to sheep. 0,3 % of *E. coli* isolates from healthy animals were resistant to gentamicin, when analyzing caecal samples of cattle less than one year of age. It was also reported resistance in 2,3 % of *E. coli* isolates from dogs with clinical urinary tract infections and in 4,7 % of *E. coli* from other clinical infections in dogs.

3.3.2   Mechanisms of antibiotic resistance

Antimicrobial resistance is ancient and an expected result of the interaction of many organisms with their environment. Most antimicrobial molecules are natural, and bacteria have evolved mechanisms to overcome their actions in order to survive. These bacteria are intrinsically resistant to one or more antibiotics. Originally susceptible bacteria can gain acquired resistance as a result of mutations in the genome, or due to acquisition of external

genetic material obtained from organisms in the environment. Acquisition of external genetic material trough horizontal gene transfer can occur through three main strategies; transformation (incorporation of free DNA), transduction (phage mediated DNA transfer) and conjugation (plasmid is transferred from a donor cell to a receiving cell trough pilus). Resistance to one type of antibiotic can be achieved through multiple biochemical mechanisms and one bacterium can use more than one mechanism of resistance. Mutations that make the bacteria less sensitive to an antibiotic can occur in a susceptible population. This subpopulation will then survive after being exposed to that antibiotic, while the sensitive subpopulation will be eliminated. The changes are often costly to the cell´s homeostasis and is only maintained in the presence of the antibiotic. (Munita & Arias, 2016) The mechanisms of antibiotic resistance are illustrated in figure 1 and can be classified as follows:
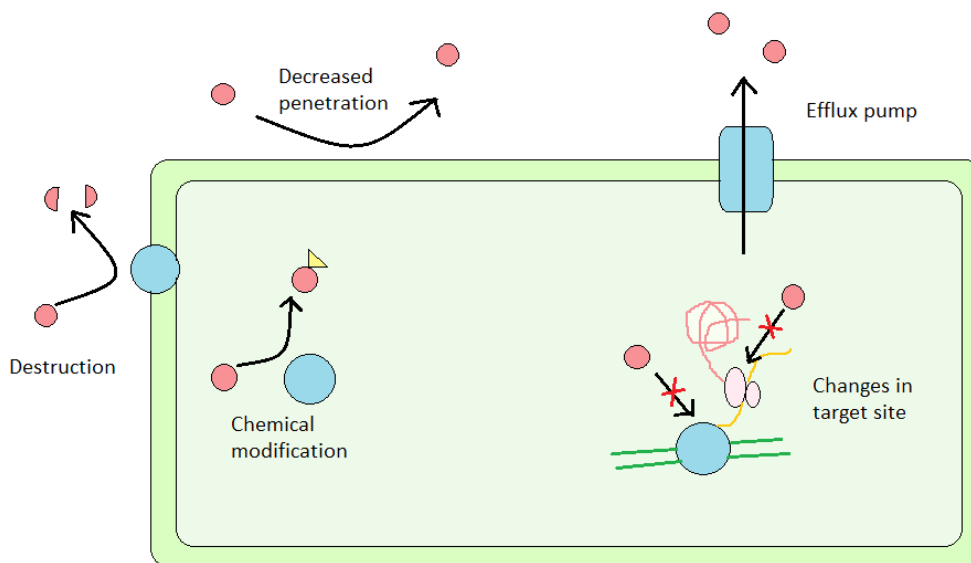

Figure 1. Mechanisms of antibiotic resistance includes modification or destruction of the antibiotic molecule, changes in target site, decreased penetration and increased efflux.

1. Modification or destruction of the antibiotic molecule

Modification of the antibiotic molecule is a successful strategy of the bacteria to defend itself against antibiotics. They can produce enzymes that inactivate the antibiotic molecule by adding specific chemical moieties to the compound or enzymes that destroy the molecule, both leaving the antibiotic unable to interact with its target. Many types of modifying enzymes have been described, and the most common reactions they catalyze are acetylation (aminoglycosides, chloramphenicol, streptogramins), phosphorylation (aminoglycosides, chloramphenicol), and adenylation (aminoglycosides, lincosamides) (Munita & Arias, 2016). The effect of the modified antibiotic molecule is often related to steric hindrance to reach its target. The most widespread mechanism of resistance to aminoglycosides is by inactivation of the antibiotic by modifying enzymes (Garneau-Tsodikova & Labby, 2016). An example of enzymes that destroy the antibiotic molecule is the beta-lactamases. These enzymes destroy the bond of the beta-lactam ring, leaving the antibiotic ineffective.

2. Changes in target sites

Antimicrobial resistance can be caused by changing target sites, either by protection of the target site or by modification of the target site. Modification of target site is a common mechanism, affecting almost all families of antimicrobial compounds. The modifications can consist of point mutations in the genes encoding the target site, enzymatic changes of the binding site (for example methylation) and replacement or bypass of the original target. The effect is decreased affinity of the antibiotic for the target site. One example of point mutations leading to quinolone resistance is mutations in one or both of the two target enzymes, usually in a localized domain of the GyrA and ParE subunits of the respective enzyme, which reduce drug binding to the enzyme-DNA complex (Hooper & Jacoby, 2015). An example of target protection is the quinolone resistance protein Qnr, encoded by a plasmid-mediated gene, and

was first described in the mid-1990s (Martínez-Martínez et al., 1998). Qnr competes for the binding site of DNA gyrase and topoisomerase IV, and thereby inhibits the effect of quinolones (Rodríguez-Martínez et al., 2011). The Qnr-encoding genes result in a low-level quinolone resistance, but has been shown to promote the development of highly resistant isolates by facilitating the selection of isolates with mutations in genes encoding the DNA gyrase and/or topoisomerase (Aldred et al., 2014).

3. *Decreased antibiotic penetration*

Many antibiotics have intracellular targets, or targets located in the cytoplasmic membrane (the inner membrane of Gram-negative bacteria). Bacteria have developed different mechanisms to prevent the antibiotic to reach its intracellular target through decreasing the uptake of the substance. This mechanism is especially important in Gram-negative bacteria. The outer membrane acts as the first defense against toxic substances, such as antimicrobial compounds. Hydrophilic molecules are particularly affected by changes in permeability of the outer membrane, since they often use porins, which are water-filled diffusion channels, to cross this barrier. Examples of hydrophilic antimicrobial agents are beta-lactams, tetracyclines and some fluoroquinolones. (Munita & Arias, 2016)

4. *Efflux pumps*

Efflux pumps are protein transporters localized in the cytoplasmic membrane of the cell (Nikaido, 2011). They are active transporters, thus functioning via an energy-dependent mechanism. Some are primary active transporters using ATP hydrolysis as a source of energy, while others are secondary active transporters (transport is coupled to an electrochemical potential gradient) such as uniporters, symporters or antiporters (Amaral et al., 2014).

Efflux pumps can remove a variety of different substrates out of the cell through active transport such as antibiotics, organic pollutants, heavy metals, and other toxins (Quinn et al., 2011). The efflux system that pumps tetracycline out of the cytoplasm of *E. coli* was one of the first to be described, in the early 1980s (McMurry et al., 1980). This tetracycline efflux pump is the best studied and most familiar mechanism of tetracycline resistance (Speer et al., 1992). Today, many classes of efflux pumps have been characterized. They can either be substrate-specific or have broad specificity and be able to pump different substrates out of the cell. There are five major families of efflux pumps; the small multidrug resistance family (SMR), the resistance-nodulation-cell-division family (RND), the ATP-binding cassette family (ABC), and the multidrug and toxic compound extrusion family (MATE). The differences between the families are in terms of structural conformation, energy source, range of substrates they can extrude and in the type of bacterial organisms they are distributed in. All microorganisms have sequences in their chromosomes that code for efflux pumps. Expression of these genes are highly regulated, and the presence of the right stimulator will induce the expression (Blanco et al., 2016), and thereby induce the production of the proteins involved. Expression of more than one type of efflux pump or an efflux pump with broad substrate specificity in a bacterium may lead to a broad-spectrum antibiotic resistance.

The main efflux pump of *E. coli* is the AcrAB-TolC efflux pump. It consists of three distinct proteins (Okusu et al., 1996) and is illustrated in figure 2. The transporter component of the efflux pump, AcrB, is attached to the plasma membrane. There are two AcrA fusion proteins that flank the transporter and are believed to assist the movement of substrate through the AcrB transporter by peristaltic action, which in turn drives water through the transporter (Nikaido, 2011). TolC is the third component of the efflux pump and is continuous with the TolB transporter and provides a channel for the extrusion of the substrate (Lorca et al., 2007).

The precise structural changes that take place and the way which the transporter recognizes its substrate is not yet completely understood.
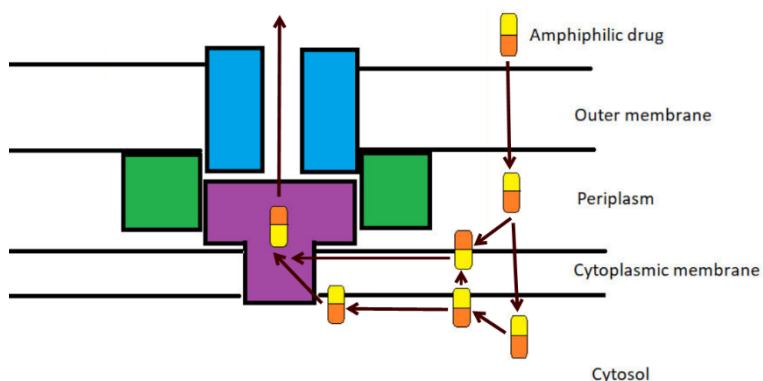

Figure 2. A schematic figure of the AcrAB-TolC efflux pump. It consists of three different proteins, the transporter component AcrB (violet), two fusion proteins, AcRA (green), and TolC (blue) which provides a channel for the extrusion of the substrate. The amphilic drugs (yellow represent hydrophobic parts and orange represent hydrophilic parts of the molecule), can be captured from the periplasm-plasma membrane interface or possibly from the cytosol. The figure is modified from (Nikaido, 2011).

Transport of drugs from the inside to the outside of the cell by this system is coupled to proton motive force from the periplasm to the cytoplasm. Studies indicate that the binding and release of the substrate are pH dependent (Su & Yu, 2007). At low pH the dissociation of the substrate is high and at pH 7 it is very slow. In a physiological environment with pH 7, one would expect that the pump would be very ineffective since the dissociation of the substrate would be very slow. However, it has been suggested that the function of the pump at environmental conditions involve lowering the pH of the internal cavity to which the substrate is bound, to enable the substrate to be released. This way, the pump can continue to function even in environments with pH values that are not ideal for the pump itself. To accomplish this, it has been postulated that the decrease in pH in the pocket takes place by generation of hydrogen ions from the metabolism, which pass from the cytoplasmic side of the plasma membrane trough the transporter (Amaral et al., 2011).

3.4   *Escherichia coli*

In this study we used *E. coli* as a model bacterium for studying the effect of formic acid adaptation on the bacteria's susceptibility to antibiotics. *E. coli* is frequently used as a model organism and is a member of the Enterobacteriaceae family. It is a Gram-negative rod-bacterium. The colony morphology of *E. coli* is round, convex, opaque and sometimes mucoid. Certain strains have hemolytic activity on blood agars. Somatic- (O), flagellar- (H), capsular- (K) and fimbria- (F) antigens are used for serotyping *E. coli*. Gram-negative bacteria, like *E. coli* are intrinsically more resistant than Gram-positive bacteria. This is due to the outer membrane of Gram-negative bacteria, which are composed of an outer leaflet of lipopolysaccharides and an inner leaflet of phospholipids, acting as a barrier that prevents antibiotics from reaching their intracellular targets. (Quinn et al., 2011)

E. coli inhabits the intestinal tract of animals and humans and can contaminate vegetation, soil and water. *E. coli* in the environment colonize the mammalian intestinal tract shortly after birth and persist as part of the normal flora throughout life. Most strains of *E. coli* are commensal organisms and of low virulence. However, they may cause opportunistic infections. Pathogenic strains of *E. coli* which produce extraintestinal disease, frequently colonize the intestinal tract of healthy animals. Strains that cause enterocolitis are not usually a part of the normal flora of healthy animals and infection is a result of contact with other infected animals, contaminated food or water. Pathogenic strains of *E. coli* have virulence factors which enable them to colonize mucosal surfaces and cause disease. (Quinn et al., 2011)

3.4.1   *E. coli´s* response to acidic environment

Bacterial cells use a combination of passive and active acid resistance (AR) systems to counteract acid stress. Passive AR systems consists of low membrane permeability and buffering capacity of the cytoplasm. The active AR systems can be divided into physiological, metabolic and proton-consuming systems. During acid stress, the systems must be regulated and coordinated to achieve a favorable acid stress response. This regulation process is poorly understood (Kanjee & Houry, 2013).

3.4.1.1   Active acid resistance systems

Physiological adaptations to acid stress

E. coli can change the composition of the cell membrane (reduce the amount of unsaturated lipids and increase the amount of cyclopropane fatty acids) to decrease membrane fluidity and the permeability to protons, thus reducing the influx of protons (Brown et al., 1997). Proton influx is also reduced by blocking outer membrane porins (OMPs) (Samartzidou et al., 2003). There are chaperones both in the periplasm and the cytoplasm that are activated by acid stress and bind to acid-denatured proteins (Kanjee & Houry, 2013). When the pH increases the chaperones release the proteins in a refolded competent conformation. Dps is a DNA-binding protein that contributes to acid tolerance in *E. coli*, by binding and protecting the bacterial DNA (Choi et al., 2000).

Metabolic adaptations to acid stress

There are several metabolic changes that provide protection against acid. One example is the increase in genes involved in transport and metabolism of secondary carbon sources, such as

sugars other than glucose, and sugar derivatives that produce fewer acids during metabolism compared to glucose (Kanjee & Houry, 2013). This is beneficial in an acid-stressed cell. During aerobic growth under mild acid stress there is an upregulation of several components of the electron transport chain. Under normal conditions this system is involved in generating the proton motor force, and protons are exported from the cell in the process. The effect of the upregulation is a higher capacity to export protons, and the cell can counteract the decrease of cytoplasmic pH (Maurer et al., 2005).

Nove et al. studied the pump activity of the *E. coli K-12 AG100* strain expressing the AcrAB-TolC pump system, at pH 7 and pH 5 in the presence of efflux pump inhibitor promethazine. The efflux pump inhibitory activity of promethazine was more effective at neutral pH. This indicates that pH 5 induces a stress response in the bacteria, leading to upregulation of involved genes, and thereby creating a more effective efflux pump. It was concluded that the genetic system that regulates the activity of the main efflux pump is pH dependent (Nove et al., 2020). Another study has shown connections between survival and growth in acidic environment and the expression of the TolC outer membrane channel, as well as the EmrB and MdtB, which all are components of multidrug resistance (MDR) efflux pumps (Deininger et al., 2011).

Proton-consuming acid resistance mechanisms

The main action of this acid resistance system is to consume intracellular protons. There are two major classes: the hydrogen-gas-producing formate hydrogen lysase (FHL) complex which is important for survival under anaerobic extreme acid stress, and the pyridoxal-5´-phosphate (PLP)-dependent amino acid decarboxylase AR systems (Kanjee & Houry, 2013).

Four amino acid-dependent AR systems are characterized. They consist of two components, a cytoplasmic PLP-dependent decarboxylase that catalyzes a proton-dependent decarboxylation of an amino acid to a product and CO_2, and an inner membrane substrate/product antiporter that enables the continued operation of the system by exchanging external substrate for internal product (Foster, 2004). The decarboxylases have optimal enzyme activity when pH is lower than neutral. The pH optima range from ~pH 4 to pH 7 for the different amino acid-dependent systems, and *E. coli* can therefore mount a robust acid stress response due to overlapping activities of the different AR systems (Kanjee et al., 2011).

3.4.1.2   *Long-term effects of formic acids on E. coli*

The long-term effects of formic acid on non-pathogenic *E. coli* strains under different acidic conditions have also been studied. Although *E. coli* possess properties to counteract acid stress, it has been shown that exposure to formic acid over time leads to increased susceptibility to acid as well as altered acid resistance response (Novoa-Garrido et al., 2009). The authors stated that the increased susceptibility to formic acid was mainly due to an alternation in the expression of O-antigen lipopolysaccharides (LPS), and that the adding of formic acid to animal feed might help to control the microbiota of the intestines and replace the use of antibiotics as growth promoters.

4 Aim of study

Several advantageous factors such as a positive effect on the microbiota in the gastrointestinal tract and increased growth rate, have been associated with the use of organic acids as feed additives. Organic acids have therefore become a widespread alternative for using antibiotics in livestock feed. However, questions have been raised about how intestinal bacteria such as *E. coli* react to the acidic stress caused by these feed additives, and a concern about potential development of synergetic acid adaptation and antibiotic resistance mechanisms has emerged. We wanted to contribute to the knowledge on this matter by studying the effects of formic acid adaptation on *E. coli´*s susceptibility to tetracycline, gentamicin and ciprofloxacin. Our sub-goals during the study were as follows:

1. To find the optimal concentration of formic acid that would initiate a stress response in *E. coli* without causing major cell death.

2. To find the sub-MIC concentration of tetracycline, gentamicin and ciprofloxacin to three different *E. coli* isolates.

3. To study the effect of tetracycline, gentamicin and ciprofloxacin on acid adapted *E. coli.*

4. To evaluate if there are any strain-specific differences in the response against formic acid and antibiotics within *E. coli* by using three different bacterial isolates.

5 Materials and methods

5.1 Bacterial strains

Three different strains of *Escherichia coli* were used in this study: a lab strain, a bovine strain and a canine strain, as shown in table 2.

Table 2. Strains used in this study.

Strain	Description
A	Lab strain, ATCC25922
B	Bovine strain, from sample delivered to the laboratory
C	Canine strain, form the sample delivered to the laboratory

Bacteria were grown overnight at 37°C on blood agar base No. 2 (Oxoid, Cambridge UK) supplemented with 5 % bovine blood. The agar diffusion method was performed to confirm sensitivity to tetracycline, gentamicin and ciprofloxacin for all strains prior to the study. To prepare for this test, cultures of each isolate were made by transferring a few colonies to 5 mL of physiological saline. Physiological saline was then used to adjust the cultures to match a 0,5 McFarland turbidity standard, before each culture was transferred to a Mueller Hinton agar plate by using a sterile swab and an aseptic technique. The following Neo Sensitabs disks were added to the plates before being incubated overnight at 37°C: tetracycline 30 µg, gentamicin 10 µg and ciprofloxacin 5 µg.

The principle of this method is that the antibiotic will diffuse into the agar and inhibit the bacteria from growing if being susceptible to the antibiotic. This results in an area around the disk called the inhibition zone where there will be no sign of bacterial growth. Depending on the diameter of this zone, the bacteria is found either susceptible, moderately susceptible or

resistant to the chosen antibiotic. These zones were measured the day after incubation and compared to the reference values provided by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) as shown in table 3.

Table 3. Reference values provided by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) for Enterobacteria and the chosen antibiotics from version 10.0, valid from 01.01.2020. S = Sensitive, I = Intermediate, R = Resistant.

Antibiotic	Disk content (µg)	Zone diameter breakpoints (mm)		
Tetracycline	30	$S \geq 15$	$I = 14\text{-}12$	$R < 11$
Gentamycin	10	$S \geq 17$	-	$R < 17$
Ciprofloxacin	5	$S \geq 25$	-	$R < 22$

5.2 Preparation of media and solutions

5.2.1 Tryptic soy broth (TSB)

30 g Tryptic soy broth granula (Merck KGaA, Darmstadt, Germany) was dissolved in 1000 mL deionized water. It was then autoclaved and stored in a refrigerator until used.

5.2.2 Phosphate-buffered saline (PBS)

8 g of NaCl, 0,2 g of KCl, 1,44 g of Na_2HPO_4 and 0,24 g of KH_2PO_4 were added to 800 mL distilled water. The pH was adjusted to 7,4 with HCl and distilled water was added to a total volume of 1 L. The solution was dispensed to aliquots and sterilized by autoclaving (20 min, 121°C, liquid cycle), and stored at room temperature.

5.2.3   Formic acid stock solution 1,0 M pH 3,9

To make a 1 M solution of formic acid, 1,925 mL of 98-100 % formic acid (25,974 M) (Merck KGaA, Darmstadt, Germany) was slowly added to 12,5 mL deionized water. The pH of the solution was adjusted to 3,9 with 6,075 mL 5 M NaOH to avoid the formation of sodium formate (HCOONa). Another 3,926 mL deionized water was added to get a final volume of 50 mL.

5.2.4   Antibiotic stock solutions

The following antibiotics were purchased from Merck KGaA (Darmstadt, Germany) to be used in this study:

- Tetracycline hydrochloride powder (T7660)

- Gentamicin 10 mg/mL, liquid solution in deionized water (G1272)

- Ciprofloxacin powder, ≥ 98,0 % HPLC (17850)

The aimed concentrations of the stock solutions were as follows: tetracycline 100 mg/L, gentamicin 10 mg/L and ciprofloxacin 1 µg/mL. These solutions were made as described below.

- *Tetracycline*: 10 mg powder of tetracycline was dissolved in 100 mL deionized water to get a final concentration of 100 mg/L, and a total volume of 100 mL.

- *Gentamicin*: 10 µl of the 10 mg/mL solution was transferred to 9,990 mL deionized water, to get a final concentration of 10 mg/L, and a total volume of 10 mL.

- *Ciprofloxacin*: 25 mg of ciprofloxacin was dissolved in 1 mL 0,1 M HCl, to get a concentration of 25 mg/mL. 100 µl of this solution was then transferred to

24,9 mL deionized water, to get a concentration of 0,1 mg/mL. 100 µl of this solution was then transferred to 9,900 mL deionized water, to get a final concentration of 1 µg/mL, and a total volume of 10 mL.

The solutions were stored following the recommendations provided by The British Society for Antimicrobial Chemotherapy as shown in table 4 (Andrews, 2001).

Table 4. Recommendations on how to store solutions as provided by the British Society for Antimicrobial Chemotherapy.

	4°C	-20°C
Tetracycline	-	Not recommended
Gentamicin	6 months	Not recommended
Ciprofloxacin	2 weeks	3 months

5.2.5 Antibiotic test solutions

Test solutions of antibiotics were made by diluting the antibiotic stock solutions in different volumes of TSB, as shown in table 5. The solutions used in the final study were made as described below.

- *Tetracycline*: 3,6 mL of the 100 mg/L stock solution was added to 26,4 mL of TSB to get a final concentration of 12 mg/L and a total volume of 30 mL.

- *Gentamicin*: 4,8 mL of the 10 mg/L stock solution was added to 25,2 mL TSB to get a final concentration of 1,6 mg/L and a total volume of 30 mL.

- *Ciprofloxacin*: For this antibiotic it was decided to use two different concentrations:

  - 1,8 mL of the 1 µg/mL stock solution was added to 28,2 mL of TSB to get a final concentration of 0,06 mg/L and a total volume of 30 mL.

- 2,4 mL of the ciprofloxacin stock solution 1 µg/mL was added to 27,6
mL of TSB to get a final concentration of 0,08 mg/L and a total volume of
30 mL.

Table 5. Description of the making of antibiotic test solutions at different concentrations.

Tetracycline	AB stock concentration mg/L	AB stock mL added	TSB mL added	Total mL	Final concentration mg/L
	100	0,15	29,85	30	0,5
	100	0,30	29,70	30	1,0
	100	0,45	29,55	30	1,5
	100	0,60	29,40	30	2,0
	100	0,75	29,25	30	2,5
	100	0,9	29,1	30	3
	100	1,2	28,8	30	4
	100	1,5	28,5	30	5
	100	2,4	27,6	30	8
	100	3,0	27,0	30	10
	100	3,6	26,4	30	12
Gentamicin	**AB stock concentration mg/L**	**AB stock mL added**	**TSB mL added**	**Total mL**	**Final concentration mg/L**
	10	0,6	29,4	30	0,2
	10	0,9	29,1	30	0,3
	10	1,2	28,8	30	0,4
	10	1,5	28,5	30	0,5
	10	1,8	28,2	30	0,6
	10	2,4	27,6	30	0,8
	10	3,9	26,1	30	1,3
Ciprofloxacin	**AB stock concentration mg/L**	**AB stock mL added**	**TSB mL added**	**Total mL**	**Final concentration mg/L**
	1	0,15	29,85	30	0,005
	1	0,30	29,70	30	0,01
	1	0,45	29,55	30	0,015
	1	0,60	29,40	30	0,02
	1	1,2	28,8	30	0,04
	1	1,8	28,2	30	0,06
	1	2,4	27,6	30	0,08

5.3   Initial cultures prior to adaptation and sensitivity assay

Cultures of *E. coli* for each isolate were made by transferring one colony from a blood agar plate to 5 mL TSB and incubated overnight at 37°C with gently shaking (120-130 rpm). The optical density (OD) between the isolates were compared by using a spectrophotometer (Genesys 20, Thermo scientific) with absorbance at 600 nm (A600). The volume transferred to test tubes containing 4 mL TSB was adjusted to get the aimed OD after approximately the same time. 10 µl was transferred from the culture with the highest density, and the volume for the other two cultures were adjusted according to their OD values. The test tubes were incubated at 37°C with gently shaking (rpm 120-130) until OD_{600} reached 0,2. To decide the **initial cell population** (CFU/mL) of these pre-adaptation cultures, 10 µl was transferred to blood agar plates for the final dilution of 10^{-2} during the establishment studies. During the final study, serial dilutions in TSB were made and 10 µl was transferred for the final dilutions of 10^{-4} and 10^{-6}. The colonies were counted after incubation overnight at 37°C.

5.4   Establishment of formic acid adaptation assay

Different concentrations of formic acid were added to the initial cultures of $OD_{600} = 0,2$ as shown in table 6. Formic acid was added to two tubes per isolate, per concentration. The test tubes were incubated at 37°C with gently shaking (rpm 120-130) for 1 hour. To decide the **cell population (CFU/mL) after exposure to formic acid**, 10 µl was transferred to blood agar plates for the final dilution of 10^{-2}. The colonies were counted after incubation overnight at 37°C

Table 6. Concentrations of formic acid used to establish the final concentration.

Concentration of formic acid (mM)	Amounts added to the test tubes (µl)
70	300
92	400
110	500
150	690
200	1000
300	1650

5.5 Establishment of antibiotic sensitivity assay

Test solutions with antibiotics of different concentrations were prepared by diluting the antibiotic stock solutions as described in section 5.2.5 and shown in table 5. 4 mL of the antibiotic test solutions were transferred to test tubes before 100 µl of the initial cultures of $OD_{600} = 0,2$ were added into separate tubes. The test tubes were incubated for 1 hour at 37°C with gently shaking (rpm 120-130). Test tubes with 4 mL pure TSB were used as negative controls. To decide the **final cell population (CFU/mL) after exposure to antibiotics**, 10 µl was transferred to blood agar plates for the final dilution of 10^{-2}. The colonies were counted after incubation overnight at 37°C.

5.6 Final protocol

Cultures of *E. coli* for each isolate were made as described in section 5.3. Three test tubes were made for each isolate: 1 for measuring, 1 for acid adaptation and 1 for negative control. A serial dilution in TSB was made for each test tube when OD_{600} reached 0,2. 10 µl was transferred to blood agar plates for final dilutions as shown in table 7. We attempted to use dilutions with CFU between 25-250, but most blood agar plates contained more CFU, therefore the dilution with the least CFU was chosen.

Table 7. Dilutions used for blood agar plates with initial cell population, after formic acid adaptation and for the antibiotic sensitivity assay. Dilutions used for gentamicin and ciprofloxacin were adjusted according to the results of the tetracycline trial.

	Initial	**1 hour acid adapted**	**1 hour negative control**	**AB acid adapted**	**AB negative control**
Tetracycline	10^{-4} and 10^{-6}	10^{-2} and 10^{-4}	10^{-4} and 10^{-6}	10^{-2} and 10^{-4}	10^{-2} and 10^{-4}
Gentamicin	10^{-6}	10^{-6}	10^{-6}	10^{-2}, 10^{-4} and 10^{-6}	10^{-2}, 10^{-4} and 10^{-6}
Ciprofloxacin	10^{-6}	10^{-6}	10^{-6}	10^{-2}, 10^{-4} and 10^{-6}	10^{-2}, 10^{-4} and 10^{-6}

5.6.1   Formic acid adaptation assay

An illustration of the acid adaptation process is shown in figure 3. To adapt the bacteria to formic acid, 690 µl of the formic acid 1 M stock solution was added to one of the test tubes for each isolate containing the initial culture of $OD_{600} = 0,2$, to a final concentration of 150 mM. The other duplicate test tube was used as a negative control by adding 690 µl PBS. All cultures were incubated at 37 °C with gently shaking (rpm 120-130) for 1 hour. To decide the **cell population (CFU/mL) after exposure to formic acid**, the cultures were serial diluted in TSB. 10 µl was transferred to blood agar plates for final dilutions as shown in table 7. Colonies were counted after incubation overnight at 37°C. Survival after acid adaptation relative to the initial bacterial population was calculated as change in percent using the following formula: (acid adapted population – initial population) / initial population * 100 %.
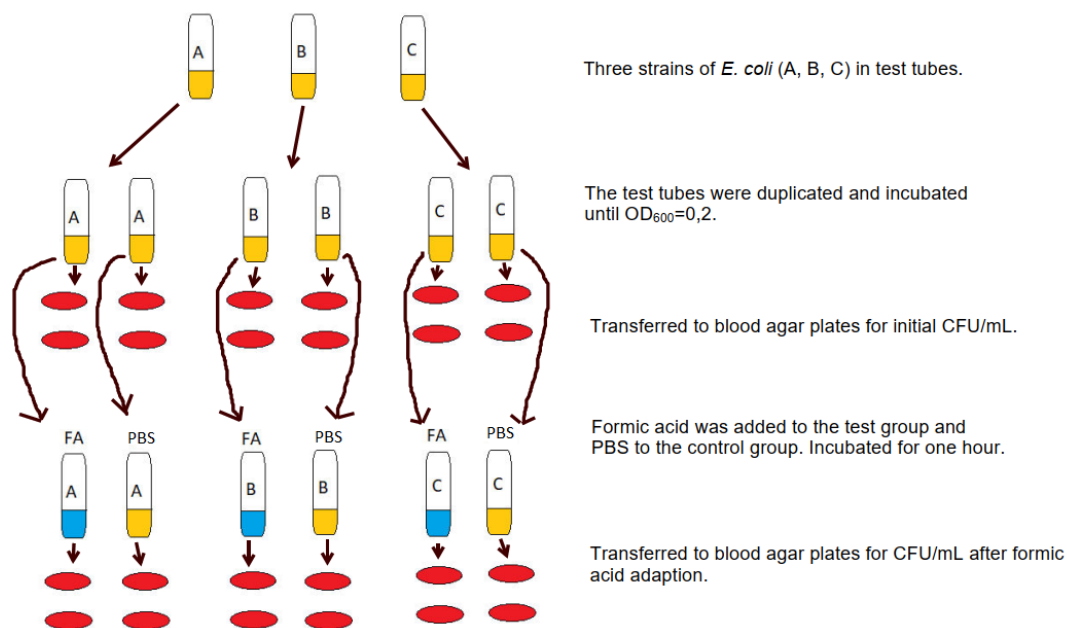
Figure 3. Illustration of the process of acid adaptation. Three strains of E. coli (A, B, C) were incubated in TSB overnight at 37° C. The test tubes were duplicated and incubated until OD$_{600}$ = 0,2, whereupon they were transferred to blood agar plates and incubated overnight at 37° C. Initial CFU/mL was calculated. Formic acid was added to one of the duplicate test tubes (test group), and PBS to the other (control group). They were both incubated for 1 hour before transferred to blood agar plates and incubated overnight. CFU/mL after formic acid adaptation was calculated, as well as change in percent relative to the initial bacterial population. FA = formic acid, PBS = phosphate buffered saline.

5.6.2   Antibiotic sensitivity assay

An illustration of the antibiotic sensitivity assay is shown in figure 4. 100 µl of each formic acid adapted culture as well as their respective negative PBS-control was transferred to two duplicate test tubes containing 4 mL of each of the following antibiotic test solutions: tetracycline 12 mg/L, gentamicin 1,6 mg/L, and ciprofloxacin 0,06 mg/L and 0,08 mg/L. They were then incubated at 37°C with gently shaking (120-130 rpm) for 1 hour. To calculate the **final cell population in CFU/mL after antibiotic exposure**, a serial dilution in TSB was made for each test tube. 10 µl was transferred to blood agar plates for final dilutions as shown in table 7. The colonies were counted after incubation overnight at 37 °C. The percent survival after antibiotic treatment relative to the bacterial population after acid adaptation was calculated using the following formula: (cell population after antibiotics / cell population after formic acid adaptation) * 100 %.
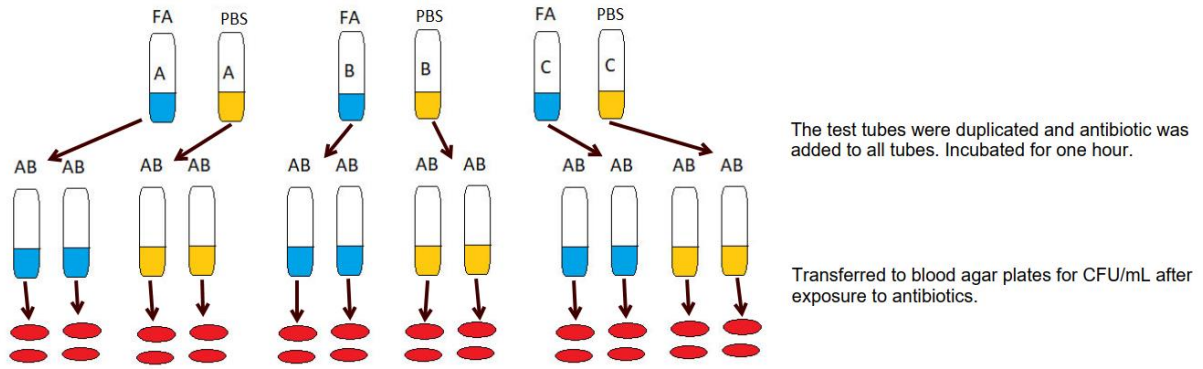
Figure 4. Illustration of the antibiotic sensitivity assay. The test tubes with formic acid and the control tubes with PBS were duplicated before antibiotics were added to all tubes. All tubes were incubated at 37 ℃ for 1 hour, whereupon they were transferred to blood agar plates and incubated overnight at 37 ℃. CFU/mL after exposure to antibiotics was calculated, as well as the percent survival relative to the bacterial population after acid adaptation. FA = formic acid, AB = antibiotics, TSB = tryptic soy broth

5.7 Statistics

We were unfortunately forced to reduce our lab work as a result of the COVID-19 epidemic. The results are therefore only based on one trial with two duplicates for each antibiotic. Other studies have performed two or more independent trials to get a reliable result (Guilfoyle & Hirshfield, 1996; Kwon & Ricke, 1998; Marcusson et al., 2009). For this study we only have provisional estimates and further studies are needed to establish the statistical significance of the results.

6 Results

6.1 Establishment of formic acid adaptation assay

To establish the formic acid adaptation, it was important to decide which concentration of formic acid that affected the bacteria without being lethal to the population (the sub-MIC concentration). It was only used one dilution (10^{-2}) as the focus was to compare the effect between the different formic acid concentrations and the negative controls, rather than knowing the exact bacterial population in CFU/mL. Initially, concentrations of formic acid of 70 mM, 92 mM and 110 mM were tested to evaluate the effect on the growth of *E. coli*. None of these concentrations resulted in a visible effect on the *E. coli* cell population. Therefore, higher concentrations of 150 mM, 200 mM and 300 mM were tested, and the results are presented in table 8.

Strain A showed zero visible colonies when concentrations of 200 mM and 300 mM formic acid was added, while 150 mM resulted in an approximately log 1 reduction in CFU/mL compared to the initial bacterial population. Strain B showed a reduction when adding formic acid at concentrations of 200 mM and 300 mM, while 150 mM resulted in too numerous colonies to count on the blood agar plate. For strain C there was a dose dependent reduction in CFU when adding formic acid at concentrations of 150 mM, 200 mM and 300 mM. It was not possible to calculate a log reduction for strain B and C, since there were too numerous colonies to count on the initial blood agar plates.

To compare, there were severe differences between the three strains. This is probably due to different initial concentrations of bacteria, as the OD_{600} values were 0,013 for strain A, 0,442 for B and 0,033 for C before adaptation to formic acid. The strains were therefore not

completely comparable. However, it was apparent that concentrations of 200 mM and 300 mM formic acid gave large reductions in CFU/mL compared to the initial bacterial populations. 150 mM formic acid resulted in countable colonies for strain A and C, but not for B. As mentioned, strain B had a higher OD_{600} value, so a countable number would be expected if the value was lower. Of the tested concentrations of formic acid (70-300 mM), 150 mM was the lowest concentration that resulted in a visible reduction in CFU/mL. Thus, this concentration was chosen for acid adaptation in the final study.

Table 8. Results from the acid adaptation assay in CFU/mL. TNTC = Too numerous to count

		Strain A	Strain B	Strain C
Initial OD_{600} value		0,013	0,442	0,033
Initial CFU/mL		$2,0*10^4$	TNTC	TNTC
CFU/mL after acid adaptation	150 mM	$1,2*10^3$	TNTC	$3,5*10^5$
	200 mM	0	$3,5*10^5$	$2,6*10^4$
	300 mM	0	$3,6*10^4$	$2,0*10^2$

6.2   Establishment of antibiotic sensitivity assay

All three strains of *E. coli* were found susceptible to tetracycline, gentamicin and ciprofloxacin during the susceptibility testing as described in section 5.1. To establish the antibiotic sensitivity assay, *E. coli* was exposed to different concentrations of the mentioned antibiotics to decide a sub-inhibitory concentration that would stress the bacteria without being lethal to most of the population. Bacterial enumeration was only performed using one dilution (10^{-2}) for the reasons mentioned in section 6.1.

The CFU/mL of bacteria exposed to all tested concentrations of tetracycline and gentamicin were too numerous to count. There was however less confluent growth on the highest concentrations (12 mg/L of tetracycline and 1,6 mg/L of gentamicin) compared to the control groups. Therefore, it was decided to use these concentrations in the final study. No differences in susceptibility to tetracycline and gentamicin were observed between the bacterial isolates. The ciprofloxacin concentrations between 0,005-0,02 mg/L resulted in too numerous colonies to count without any visible differences in CFU compared to the control groups. The results from ciprofloxacin concentration 0,04 mg/L, 0,06 mg/L and 0,08 mg/L are presented in table 9. Strain A showed greater susceptibility to ciprofloxacin compared to strain B and C by demonstrating a clear dose-dependent reduction in CFU/mL with increased antibiotic concentration. For strain B and C there was also a reduction in CFU/mL with increased antibiotic concentration, but it was not as pronounced as for strain A. Because of differences between the strains it was decided to use both 0,06 mg/L and 0,08 mg/L of ciprofloxacin in the final study.

Table 9. Results after ciprofloxacin in CFU/mL. TNTC = too numerous to count

CIPROFLOXACIN	Strain A	Strain B	Strain C
Initial OD$_{600}$ value	0,209	0,264	0,343
Initial CFU/mL	TNTC	TNTC	TNTC
Control group	TNTC	TNTC	TNTC
0,04 mg/L	$1,3*10^4$	$4,0*10^5$	$4,0*10^5$
0,06 mg/L	$7,0*10^2$	$2,6*10^5$	$1,2*10^5$
0,08 mg/L	$3,0*10^2$	$1,0*10^5$	$2,9*10^4$

6.3 Formic acid adaptation and antibiotic sensitivity assay

The goal of this study was to observe the effects of formic acid adaptation on *E. coli´s* susceptibility to tetracycline, gentamicin and ciprofloxacin. We were unfortunately forced to reduce our lab work as a result of the COVID-19 epidemic. The results are therefore only based on one trial for each antibiotic as presented in table 10 and attachment 1. Strain A was excluded from the trial with ciprofloxacin due to a mistake in the making of the initial culture.

Table 10. Results from the final study in CFU/mL. The survival after added antibiotics are presented as a mean of the two duplicates.

	Test group in CFU/mL			Control group (PBS) in CFU/mL		
Tetracycline	**Initial**	**After acid**	**After AB**	**Initial**	**After acid**	**After AB**
Strain A	$2,2*10^8$	$4,5*10^7$	$5,3*10^5$	$2,2*10^8$	$8,0*10^8$	$2,0*10^7$
Strain B	$2,1*10^8$	$4,5*10^7$	$1,6*10^5$	$2,1*10^8$	$8,0*10^8$	$2,3*10^7$
Strain C	$2,2*10^8$	$4,0*10^7$	$3,2*10^5$	$2,2*10^8$	$6,1*10^8$	$2,0*10^7$
Gentamicin	**Initial**	**After acid**	**After AB**	**Initial**	**After acid**	**After AB**
Strain A	$6,3*10^7$	$1,7*10^8$	$1,7*10^3$	$2,5*10^8$	$5,8*10^8$	$1,2*10^8$
Strain B	$2,0*10^8$	$1,5*10^8$	$4,5*10^2$	$1,5*10^8$	$7,6*10^8$	$1,0*10^8$
Strain C	$1,7*10^8$	$1,4*10^8$	$1,0*10^3$	$1,6*10^8$	$8,1*10^8$	$1,2*10^8$
Ciprofloxacin	**Initial**	**After acid**	**After AB**	**Initial**	**After acid**	**After AB**
Strain A	-	-	-	-	-	-
Strain B, 0,06 mg/L	$1,1*10^8$	$1,2*10^8$	$7,3*10^5$	$1,1*10^8$	$4,6*10^8$	$5,3*10^7$
Strain B, 0,08 mg/L	$1,1*10^8$	$1,2*10^8$	$5,8*10^5$	$1,1*10^8$	$4,6*10^8$	$5,6*10^7$
Strain C, 0,06 mg/L	$1,1*10^8$	$5,8*10^7$	$7,0*10^4$	$6,5*10^7$	$4,1*10^8$	$5,1*10^7$
Strain C, 0,08 mg/L	$1,1*10^8$	$5,8*10^7$	$5,0*10^4$	$6,5*10^7$	$4,1*10^8$	$4,9*10^7$

6.3.1   Effect of acid adaptation on *E. coli* growth

The effect of formic acid on the growth of *E. coli* is presented in table 11. The effect of the three antibiotics on acid-adapted *E. coli* was tested separately in three different trials (TET-trial, GEN-trial and CIP-trial). Thus, each trial used separate *E. coli* cultures. The growth of *E. coli* after exposure to 150 mM formic acid showed severe variation between the different trials. In the TET-trial, all three strains showed a similar reduction in growth (-78-81 %) after exposure to formic acid, compared to an increase of 178-281 % in the control group. In contrast, the effect of formic acid on *E. coli* growth varied significantly in the GEN-trial. Strain A demonstrated an increase in growth of 173 % compared to strain B and C where the growth was reduced with 25 % and 17 %, respectively. Also, the growth of the control groups varied greatly in the GEN-trial, from 133 % for strain A, to 424 % and 405 % growth for strain B and C, respectively. Thus, strain A showed an increase in growth after exposure to formic acid, as well as demonstrating greater growth than the control group. This deviates from strain B and C in the same trail as well as all the strains in the TET-trial which all showed a decrease in the bacterial population after formic acid exposure, and an increase in the population in the control group. Variation between strains was also observed in the CIP-trial where strain B demonstrated a minor increase in growth of 10 %, in contrast to strain C where the growth was reduced with 45 %. In this trial, the control groups of strain B and C increased their growth with 322 % and 534 %, respectively. In total, the effect on the growth of *E. coli* after one hour exposure to 150 mM formic acid varied between a reduction of 81 % to an increase of 173 %. The growth of the control groups varied between an increase of 133-534 %.

Table 11. Survival of E. coli after one hour exposure to 150 mM formic acid in three different trials. The results are presented as change in percent (%) relative to the initial bacterial population (100 %).

	Acid adapted group			Control group		
	TET-trial	GEN-trial	CIP-trial	TET-trial	GEN-trial	CIP-trial
Strain A	-79 %	173 %	-	269 %	133 %	-
Strain B	-78 %	-25 %	10 %	281 %	424 %	322 %
Strain C	-81 %	-17 %	-45 %	178 %	405 %	534 %

6.3.2 Effect of antibiotic treatment

Tetracycline

The survival of *E. coli* after exposure to 12 mg/L tetracycline is shown in table 12 and figure 5. Only 0,34–1,18 % of the bacterial population after acid adaptation survived in the test groups, compared to a 2,44–3,20 % survival in the control groups. The reduction of growth after exposure to tetracycline was similar for all three strains. The difference in percent survival was small between the acid adapted bacteria and the control groups, which is equivalent to a log 2 difference in CFU/mL for all three strains.

Table 12. Survival of E. coli after exposure to tetracycline. The results are presented in CFU/mL and survival in percent relative to the bacterial population after acid adaptation.

		After formic acid/PBS, CFU/mL	After exposure to tetracycline, CFU/mL	Percent survival
Strain A	Acid adapted group	$4,5*10^7$	$5,3*10^5$	1,18
	Control group	$8,0*10^8$	$2,0*10^7$	2,44
Strain B	Acid adapted group	$4,5*10^7$	$1,6*10^5$	0,34
	Control group	$8,0*10^8$	$2,3*10^7$	2,88
Strain C	Acid adapted group	$4,0*10^7$	$3,2*10^5$	0,79
	Control group	$6,1*10^8$	$2,0*10^7$	3,20

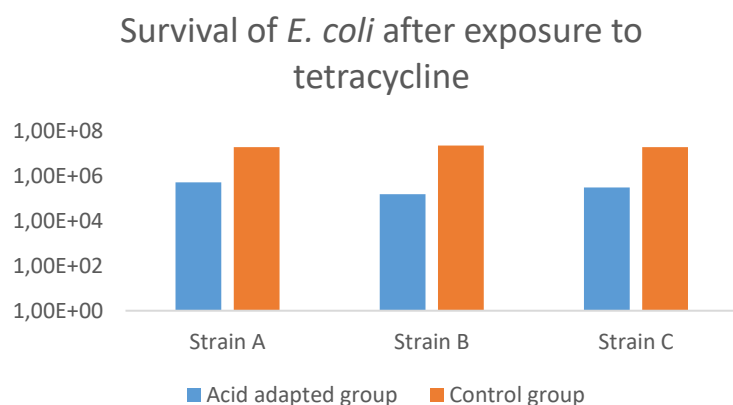*Figure 5. Survival of three strains of E. coli (A, B, C) after exposure to 12 mg/L tetracycline for one hour. Test groups were treated with 150 mM formic aid and control groups were treated with PBS for one hour prior to exposure to tetracycline. Bacterial survival is expressed as log CFU/mL. There was a log 2 difference in survival between the test groups and the control groups for all three strains.*

Gentamicin

The survival of *E. coli* after exposure to 1,6 mg/L gentamicin is shown in table 13 and figure 6. Only 0,0003–0,0010 % of the bacterial population after acid adaptation survived in the test groups compared to a 13,7–20,3 % survival in the control groups. This is equivalent to a log 5 difference in CFU/mL between the two groups for strain A and C, and log 6 difference for strain B. The survival was relatively similar among the strains with strain A deviating slightly from strain B and C with a higher percent survival in both groups.

Table 13. Survival of E. coli after exposure to gentamicin. The results are presented in CFU/mL and survival in percent relative to the bacterial population after formic acid adaptation.

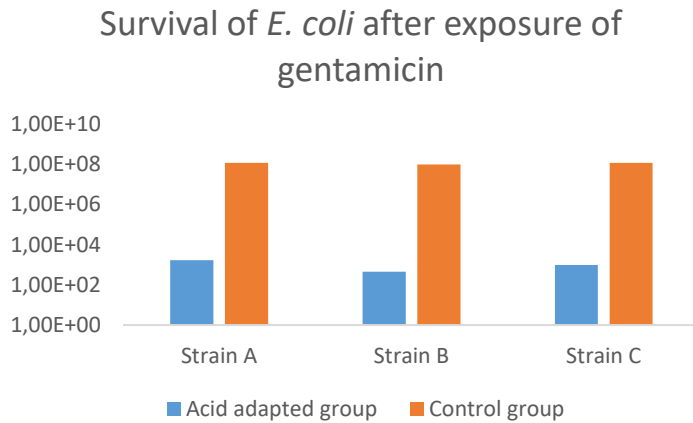|		After formic acid/PBS, CFU/mL	After exposure to gentamicin, CFU/mL	Percent survival
Strain A	**Acid adapted group**	$1,7*10^8$	$1,7*10^3$	0,0010
	Control group	$5,8*10^8$	$1,2*10^8$	20,2609
Strain B	**Acid adapted group**	$1,5*10^8$	$4,5*10^2$	0,0003
	Control group	$7,6*10^8$	$1,0*10^8$	13,6843
Strain C	**Acid adapted group**	$1,4*10^8$	$1,0*10^3$	0,0007
	Control group	$8,1*10^8$	$1,2*10^8$	14,5421

Survival of *E. coli* after exposure of gentamicin

Figure 6. Survival of three strains of E. coli (A, B, C) after exposure to 1,6 mg/L gentamicin for one hour. Test groups were treated with 150 mM formic acid and control groups were treated with PBS for one hour prior to exposure to gentamicin. Bacterial survival is expressed as log CFU/mL. The difference between the two groups was log 5 for strain A and C, and log 6 for strain B.

Ciprofloxacin

The survival of *E. coli* after exposure to ciprofloxacin is shown in table 14 and figure 7. After being exposed to 0,06 mg/L ciprofloxacin, 0,12–0,62 % of the acid adapted bacterial population survived, compared to 11,62–12,38 % of the bacteria in the control groups. Exposure of 0,08 mg/L resulted in 0,09–0,50 % survival in the acid adapted groups, compared to 11,89–12,28 % survival in the control groups. This is equivalent to a log 2 difference in CFU/mL between the two groups for both concentrations of ciprofloxacin for strain B, and a log 3 difference for both concentrations for strain C. The percent survival was relatively similar among the two strains for both concentrations, but strain C was slightly more affected with a lower percent survival compared to strain B for both concentrations.

Table 14. Survival of E. coli after exposure to ciprofloxacin. The results are presented in CFU/mL and survival in percent relative to the bacterial population after formic acid adaptation.

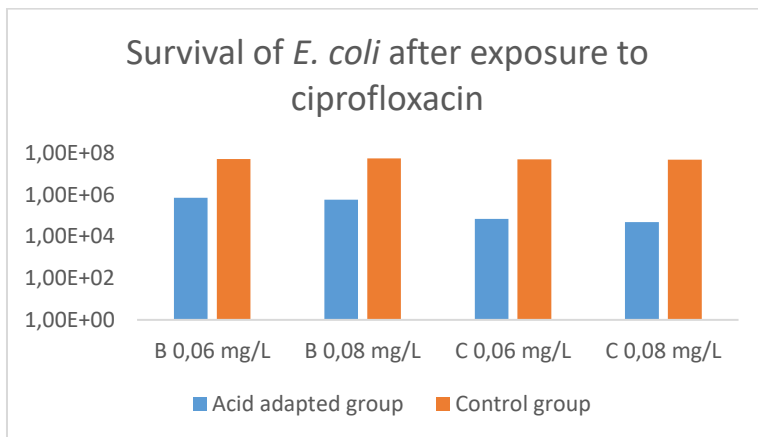|		After formic acid/PBS, CFU/mL	After exposure to ciprofloxacin, CFU/mL	Percent survival
Strain B 0,06 mg/L	**Acid adapted group**	$1,2*10^8$	$7,3*10^5$	0,62
	Control group	$4,6*10^8$	$5,3*10^7$	11,62
Strain B 0,08 mg/L	**Acid adapted group**	$1,2*10^8$	$5,8*10^5$	0,50
	Control group	$4,6*10^8$	$5,6*10^7$	12,28
Strain C 0,06 mg/L	**Acid adapted group**	$5,8*10^7$	$7,0*10^4$	0,12
	Control group	$4,1*10^8$	$5,1*10^7$	12,38
Strain C 0,08 mg/L	**Acid adapted group**	$5,8*10^7$	$5,0*10^4$	0,09
	Control group	$4,1*10^8$	$4,9*10^7$	11,89

Figure 7. Survival of two strains of E. coli (B, C) after exposure to 0,06 mg/L and 0,08 mg/L ciprofloxacin for one hour. Test groups were treated with 150 mM formic acid and control groups were treated with PBS for one hour prior to exposure to ciprofloxacin. Bacterial survival is expressed as log CFU/mL. The difference between the two groups was log 2 for strain B and log 3 for strain C for both concentrations.

7 Discussion

The main goal of this study was to observe the effects of formic acid adaptation on *E. coli´*s

susceptibility to antibiotics (tetracycline, gentamicin and ciprofloxacin). We also wanted to

observe if there were any differences in response within *E. coli* by using three different

strains: a lab strain (A), a bovine strain (B) and a canine strain (C). Our hypothesis was that

acid adaptation might reduce the susceptibility and therefore increase antibiotic resistance due

to induction of acid resistance mechanisms that the bacterium also can use to defend itself

against antibiotics. A concentration of 150 mM formic acid was chosen for acid adaptation,

while concentrations of 12 mg/L tetracycline, 1,6 mg/L gentamicin and 0,06 mg/L and 0,08

mg/L ciprofloxacin were chosen for antibiotic treatment. The results showed a reduced

percent survival after antibiotic treatment among formic acid adapted *E. coli* compared to the

control groups. There was a minimal difference between the two groups during the trial with

tetracycline, while formic acid resulted in increased susceptibility for antibiotics during the

trials with gentamicin and ciprofloxacin. Severe variation in percent survival after acid

adaptation was also observed both between the strains and between the trials.

7.1 Methodological considerations

Dilution series and bacterial enumeration

The study showed varying results in percent survival after acid adaptation and after treatment

with antibiotics, which suggests that improvements should be made in later studies. The goal

of exposing bacteria to formic acid and different antibiotics was to stress the bacteria without

killing the majority of the population. However, up to 81 % of the initial bacteria population

was killed after one hour exposure to 150 mM formic acid. To compare, a concentration of

100 mM was used when Kwon and Rickie studied the effect of SCFA on acid resistance in

Salmonella typhimurium (Kwon & Ricke, 1998). After antibiotic treatment, only 2,4-20,3 % of the bacteria in the control groups survived after treatment with the chosen concentrations of tetracycline, gentamicin and ciprofloxacin. These results suggest that the concentrations of formic acid and the different antibiotics were too high. As mentioned in section 6.1 and 6.2, these concentrations were chosen because they were the first of the tested concentrations where a reduction in CFU/mL could be visualized compared to the initial cell population. However, only one dilution of 10^{-2} was used during the establishment trials. Only severe differences in CFU between the bacterial cultures were possible to observe for this dilution, and smaller changes were therefore difficult (if not impossible) to evaluate. To be able to compare CFU for different bacterial cultures of high density, the bacterial concentration should be decreased to a number which is easier to count when plated on an agar plate. Multiple dilutions should therefore be performed in later studies to make sure that more appropriate concentrations are found, but also to increase the accuracy in the final trials. During the trial with tetracycline, several thousand colonies were present on the highest dilution (10^{-4}) after one hour exposure to formic acid, as well as in the control group after added antibiotics. However, during the trial with gentamicin there were only 0-20 colonies present on the lowest dilution (10^{-2}) for the acid adapted cultures after added antibiotics. These results are therefore not completely accurate and more optimal dilutions would probably give different results in later trials.

Exposure time to formic acid

An adaptation time of one hour to formic acid was chosen in this study based on former literature as well as practical considerations. However, this might not have been long enough to completely induce the mechanisms of acid resistance in the bacteria. Kwon and Ricke reported that the acid resistance in *S. typhimurium* was greatly increased after exposure to

SCFA, and that the resistance was further enhanced by extending the exposure time to four hours (Kwon & Ricke, 1998). It is therefore possible that a longer adaptation time along with lower concentrations of formic acid and antibiotics would have given different results and should be taken into consideration in later studies.

Technical errors by pipetting

Another source of error that needs to be considered is the possibilities for technical errors, since none of us are professional lab workers. This increases the risk of errors, for instance while making the different solutions, measuring, or during transfer of material with pipettes etc. There was observed a great variation in bacterial survival after exposure to formic acid between the trials, from a reduction of the initial population by 81 %, to an increase of the population by 173 %. However, all cultures were given the exact same treatment prior to being treated with antibiotics, and similar results were thus expected. As the formic acid stresses the bacteria it was also expected to see a reduction and not an increase in CFU/mL. Technical errors are therefore suspected. During the trial with gentamicin there was an increase of 173 % for strain A after acid adaptation, compared to a reduction of 25 % and 17 % for strain B and C, respectively. The bacterial population for the initial test and control cultures of strain A were $6,3*10^7$ CFU/mL and $2,5*10^8$ CFU/mL, respectively. However, these initials bacterial cultures were duplicates and therefore expected to be approximately the same. A technical error during pipetting is therefore suspected. If the survival of strain A was calculated relative to the initial population of the control group, the result would be similar to the results of strain B and C with a reduction of 30,4 % after acid adaptation. However, the same error cannot explain the 10 % increase in CFU/mL after acid adaptation for strain B during the trial with ciprofloxacin, as the initial test and control cultures were both $1,1*10^8$ CFU/mL. In this trial, a technical error during the adding of formic acid is instead suspected

to be the cause. For later studies it should be considered to work with two, or more replicates to get more accurate results.

Technical errors are also suspected during the treatment with different antibiotics as there was some variation between the duplicates. For instance, in the control group after treatment with gentamicin there was $2,9*10^7$ CFU/mL and $2,0*10^8$ CFU/mL present for the duplicates for strain A. The percent survival was calculated based on the mean of the two duplicates, but as there was a great variation between some duplicates these numbers are not completely accurate.

7.2 Varying effect of formic acid adaptation on *E. coli*'s susceptibility to different antibiotics

Even though there are several sources of errors and room for improvement in later studies, there are still some interesting results in this study. The results showed a reduced percent survival after antibiotic treatment among formic acid adapted *E. coli* compared to the control group. There was a minimal difference between the test and control groups during the trial with tetracycline, while formic acid adaptation resulted in increased susceptibility for antibiotics during the trials with gentamicin and ciprofloxacin. But why is *E. coli* more susceptible to gentamicin and ciprofloxacin after formic acid adaptation, but not to tetracycline?

Tetracycline is a bacteriostatic antibiotic that works by binding to the bacterial ribosomes and reduce the number of active ribosomes, which in turn inhibits bacterial protein synthesis and cell growth (Levin et al., 2017). This results in a low-rate bacterial killing. Formic acid in its

non-dissociated form can pass through the bacterial cell wall and dissociate into cations (H^+) and anions (A^-) in the cytoplasm. It has been suggested that the anions might inhibit the protein synthesis (Lück, 1986). Thus, the effect of tetracycline by reducing the number of active ribosomes could be less pronounced when the protein synthesis is already inhibited. Gentamicin and ciprofloxacin are bactericidal antibiotics. In contrast to the observed effect of tetracycline, the bactericidal effect of gentamicin and ciprofloxacin seem to work synergistic to the effect of formic acid. Gentamicin causes misreading of tRNA, leaving the bacterium unable to synthesize proteins vital to its growth (Hsu, 2008), while ciprofloxacin inhibits DNA synthesis by exerting its effect on the gyrase and topoisomerase enzymes (Correia et al., 2017). It is tempting to suggest that these differences in action mechanisms might explain the synergistic effect that was observed in our study, since the bacteria is "attacked" from several directions.

Another possible explanation to the synergistic effect observed for gentamicin and ciprofloxacin combined with formic acid, might lie in how the bacterial cell wall is affected by formic acid. Acid resistance mechanisms of *E. coli* include altered composition of the cell membrane and blocking of outer membrane proteins (OMPs), leading to lower permeability of protons. This should also make the cell membrane less permeable to antibiotics, leaving the acid adapted bacteria more resistant to antibiotics. That is, the opposite of our result. When entering the bacterial cell, tetracycline passively diffuses through the outer membrane porins OmpF (Mortimer & Piddock, 1993). Blocking of these porins could therefore explain the less potentiating effect of formic acid combined with tetracycline, compared to gentamicin and ciprofloxacin. However, ciprofloxacin also enters bacterial cells trough porins (Aldred et al., 2014), and should therefore be affected in the same way, which it was not in our study.

Gentamicin on the other hand, enters the bacterial cell through three distinct stages, the first of which increases the bacterial membrane permeability by binding to the negatively charged components of the cell membrane, such as phospholipids and lipopolysaccharide (LPS) of Gram-negative bacteria (Krause et al., 2016). Formic acid alters the composition of the bacterial cell membrane by reducing the amount of unsaturated lipids and increasing the amount of cyclopropane fatty acids, which results in decreased membrane permeability (Brown et al., 1997). These changes could possibly affect the ability of gentamicin to enter the cell. However, if this is the case, the bacteria should become less susceptible to gentamicin after formic acid adaptation, which is the opposite of our result. However, it is possible that a lower formic acid concentration and longer adaptation time would have given different results.

7.3 External validity

Only one trial per antibiotic was performed in this study. The results showed a variation within and between the trials in percent survival after acid adaptation and after treatment with antibiotics. The external validity of the results is therefore considered to be low. However, two duplicates as well as three different strains of *E. coli* were used. Even though the percent survival varied, the result of all trials showed reduced percent survival for acid adapted bacteria after antibiotic treatment. This increases the probability that the same results would be found in other trials, if the same concentrations of formic acid and antibiotics were used. Yet, it is well known that observations made in *in vitro* studies cannot be directly translated to *in vivo* responses. Organic acids are widely used feed additives with several documented beneficial effects (Kirchgessner & Roth, 1987; Kirchgessner & Roth, 1990; Mathew et al., 1991; Mroz et al., 1998; Partanen & Mroz, 1999; Patten & Waldroup, 1988; Scipioni et al.,

1978; Skinner et al., 1991). In this work we were not able to find evidence for our hypothesis that organic acids make bacteria susceptible to antibiotics, our result rather indicates the opposite. However, the environment in the gastrointestinal tract of animals, including pH, buffering capacity and oxygen availability, may not be directly comparable to the condition in a culture medium in the lab. The components of the digesta (nutrients, host cells, peptides and other molecules) can reduce the effective concentrations of organic acids in the gut. Bacteria can also adhere to mucosal surface of the gut wall where there is a higher buffer capacity and the pH is close to constant (McNeil et al., 1987). These factors contribute to the thought that the effective concentration of formic acid in the gut might not be that high. A longer exposure time with lower formic acid concentration could therefore have different effects on the bacteria, as previously discussed. Furthermore, in the GI-tract *E. coli* interact with other bacteria and may develop other defense mechanisms against antibiotics, that are difficult to predict and replicate in the lab.

7.4   Practical implications (of the results)

The result of our study, an increased susceptibility for antibiotics as a consequence of formic acid, would actually be beneficial for both the feed-producers, farmers and the animals. However, the results are most uncertain as previously discussed, and could not be the base for a statement of a potentiating effect on antibiotics caused by formic acid. On the other hand, if organic acids would make the bacteria less sensitive to antibiotics, it could have serious consequences. Organics acids are commonly used in animal feed and the normal intestinal microbiota of animals in a population receiving organic acids might consist of bacteria that are more tolerant to organic acids. These bacteria are transferred to new animals that are born in that population, as well as trough contamination of the environment. Thus, organic acids in

animal feed could affect the intestinal microbiota for generations of animals, and there might be a selection of certain populations of acid tolerant bacteria. If the acid adaptation mechanisms also can be used against antibiotics, the bacteria population are not only more tolerant for organic acids, but also less sensitive to antibiotics. The common practice of supplementing animal feed with organic acids could, if this is the case, contribute to the development of antibiotic resistant bacteria. However, further research is needed to make any reliable conclusions.

8 Conclusion

Our results showed a lower percent survival among acid adapted bacteria when exposed to tetracycline, gentamicin and ciprofloxacin, compared to the control groups. This is the opposite of what we expected when starting this study. There was a minimal difference between the two groups during the trial with tetracycline, while formic acid resulted in increased susceptibility for antibiotics during the trials with gentamicin and ciprofloxacin. However, with better tuning of parameters, such as lower concentrations and longer time of exposure, as well as using more optimal dilutions, it is possible that the results would be different. Therefore, it is not possible to draw any reliable conclusions from this study, and additional research is needed to further elucidate this topic. Nevertheless, this work could be useful for further establishment and optimization of the method in later studies.

9 Acknowledgements

10 Sammendrag

Tittel: Effektene til maursyre på *Escherichia coli*´s sensitivitet til ulike antibiotikum

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Etter at antibiotika ble forbudt som fôrtilskudd til matproduserende dyr i Europa i 2006 har organiske syrer vokst frem som et alternativ. Det finnes dog eksempler på at fjerningen av antibiotika i foret ikke forhindrer resistensutviklingen. Det har derfor blitt stilt spørsmål om maursyre kan påvirke tarmmikrobiotaen på en slik måte at det dannes resistensmekanismer mot antibiotika. Målet for denne studien var å studere effektene til maursyre på *Escherichia coli*´s sensitivitet til antibiotika. Tre ulike *E. coli* isolater ble adaptert til maursyre (1M pH 3,9) og deretter behandlet med tre ulike antibiotikum (tetrasyklin 12 mg/L, gentamicin 1,6 mg/L, i tillegg til ciprofloksacin 0,06 mg/L og 0,08 mg/L). Det ble observert stor variasjon i prosent overlevelse etter syreadaptering både mellom isolatene og mellom forsøkene. For gentamicin og ciprofloksacin var det en økt sensitivitet for antibiotika i den syreadapterte gruppen (gentamicin 0,0003-0,0010 %, ciprofloksacin 0,09-0,62 %), sammenlignet med kontrollgruppen (gentamicin 13,7-20,3 %, ciprofloksacin 11,62-12,38 %). Tetrasyklin hadde en lavere prosent overlevelse etter antibiotikabehandling, men det var en minimal differanse mellom den syreadapterte gruppen (0,34-1,18 %) og kontrollgruppen (2,44-3,20 %). Resultatene kan tyde på en synergistisk effekt for maursyre sammen med gentamicin og ciprofloksacin. Denne effekten kunne derimot ikke observeres med tetrasyklin. Mer forskning er nødvendig for videre utredning av dette temaet.

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12 Attachments

Attachment 1: Results from the final study in CFU/mL. Isolate A was removed due to a

mistake during the trial with ciprofloxacin.

	Acid adapted group			Control group		
Tetracycline	**Initial**	**After formic acid, CFU/mL**	**After antibiotic, CFU/mL**	**Initial**	**After PBS, CFU/mL**	**After antibiotic, CFU/mL**
A1	$2,2*10^8$	$4,5*10^7$	$4,8*10^5$	$2,2*10^8$	$8,0*10^8$	$2,1*10^7$
A2			$5,8*10^5$			$1,8*10^7$
B1	$2,1*10^8$	$4,5*10^7$	$2,7*10^5$	$2,1*10^8$	$8,0*10^8$	$1,8*10^7$
B2			$4,0*10^4$			$2,8*10^7$
C1	$2,2*10^8$	$4,0*10^7$	$2,9*10^5$	$2,2*10^8$	$6,1*10^8$	$1,9*10^7$
C2			$3,4*10^5$			$2,0*10^7$
Gentamicin	**Initial**	**After formic acid, CFU/mL**	**After antibiotic, CFU/mL**	**Initial**	**After PBS, CFU/mL**	**After antibiotic, CFU/mL**
A1	$6,3*10^7$	$1,7*10^8$	$1,9*10^3$	$2,5*10^8$	$5,8*10^8$	$2,9*10^7$
A2			$1,4*10^3$			$2,0*10^8$
B1	$2,0*10^8$	$1,5*10^8$	$4,0*10^2$	$1,5*10^8$	$7,6*10^8$	$1,1*10^8$
B2			$5,0*10^2$			$1,0*10^8$
C1	$1,7*10^8$	$1,4*10^8$	0	$1,6*10^8$	$8,1*10^8$	$9,0*10^7$
C2			$2,0*10^3$			$1,5*10^8$
Ciprofloxacin	**Initial**	**After formic acid, CFU/mL**	**After antibiotic, CFU/mL**	**Initial**	**After PBS, CFU/mL**	**After antibiotic, CFU/mL**
B 0,06 mg/L	$1,1*10^8$	$1,2*10^8$	$7,3*10^5$	$1,1*10^8$	$4,6*10^8$	$5,3*10^7$
B 0,08 mg/L			$5,8*10^5$			$5,6*10^7$
C 0,06 mg/L	$1,1*10^8$	$5,8*10^7$	$7,0*10^4$	$6,5*10^7$	$4,1*10^8$	$5,1*10^7$
C 0,08 mg/L			$5,0*10^4$			$4,9*10^7$