Research internship

Effect of in-feed administered coccidiostatics on ESBL-forming *E. coli*-bacteria, isolated from Dutch broilers

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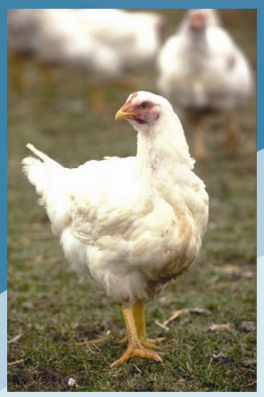

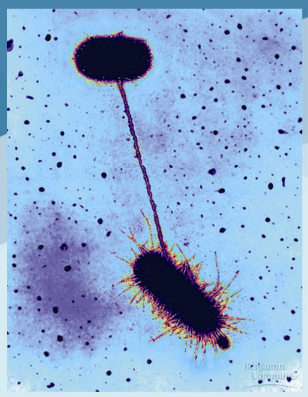

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Contents

Summary

In broiler chickens, an extensive spread of ESBL-forming *E.coli* was observed in the first week on fattening farms. This rapid spread was not observed in grandparent animals. A difference between broilers and grandparent animals is that the broilers are given coccidiostatics as feed additives, while the grandparents are vaccinated against coccidiosis. This study was performed to investigate if coccidiostatics can influence the growth of ESBL-forming *E.coli,* isolated from Dutch broilers. The effects of three ionophore coccidiostatics (used as feed additives in broiler feed) on the growth of ESBL-forming *E.coli* were studied *in vitro*. Furthermore a growth experiment with whole broiler feed was performed to detect other substances in the feed that might affect the growth of ESBL-forming *E.coli*.

Minimal Inhibitory Concentrations (MICs) were determined for salinomycin (concentration range 0,125 - 256 µg/ml), narasin (0,3125 - 64 µg/ml) and monensin (0,25 - 512 µg/ml). Six ESBL-positive strains and six ESBL-negative strains were tested. A growth experiment with a Bioscreen apparatus was performed with narasin and salinomycin for the same strains. Growth curves were made for each strain with concentrations of 1.25, 12.5 and 125 µg/ml salinomycin and 0.5, 5 and 50 µg/ml narasin (concentrations were chosen considering the concentrations of the substances in broiler feed). A growth experiment with 3 ESBL-positive and 3 ESBL-negative strains was performed with whole sterilised broiler feed suspended in the test medium. The results of determining the MICs of the tested strains for the ionophores showed that the growth of the tested ESBL-positive strains as well as the growth of ESBL-negative strains was unaffected by the presence of the coccidiostatics. For salinomycin and monensin, the MICs of all tested strains were >256 µg/ml and for narasin >64 µg/ml. The growth curves of the tested strains made with the Bioscreen showed no indication that salinomycin or narasin had a consistent inhibitory or stimulatory effect on the growth of the tested *E.coli*-strains. The inhibitory effects of salinomycin and narasin compared to the effects of the solvent ethanol were calculated, no trend in the effects on the growth of the tested strains was observed and there was no difference between the group ESBL-positive and ESBL-negative strains. The growth experiment with broiler feed showed no apparent effect of the broiler feed on the growth of the tested strains.

It can be concluded from this study that the coccidiostatics salinomycin, narasin and monensin have no consistent effect on the growth of ESBL-forming *E.coli in vitro*. The tests with broiler feed in the test medium did not indicate that other compounds of the broiler feed consistently affect the growth of ESBL-forming *E.coli*. However, the test with the broiler feed should be repeated to give more certainty about these observations.

Preface

The subject of antimicrobial resistance and the veterinary use of antibiotics is very important and a big issue in the veterinary working field these days. As a veterinary student, I intend to obtain background knowledge on antimicrobial resistance and to contribute to research in this field of experience.

The spread of ESBL-producing bacteria in poultry meat can affect human health and might be a consequence of the administration of 3rd generation cephalosporins and other antibiotics (ESBL-producing bacteria are often multi-resistant) by veterinarians. There is commotion in the Dutch society about the position of the veterinarian in livestock production. According to many, veterinarians should be able to provide their services to farmers in an independent way. The independent position of the veterinarian is under scrutiny, because veterinarians are accused of making money by selling antibiotics to farmers. The government considers depriving veterinarians of the license to have their own pharmacies. It is hard to say whether these accusations are justified, but there are still many veterinarians not well informed about the spread of ESBLS and the risks of administration of extended spectrum antibiotics (like 3rd generation cephalosporins) in livestock.

A research internship of three months is a compulsory element of my internships at the Faculty of Veterinary Medicine of Utrecht University. By contacting Dik Mevius, Head of the National Reference Laboratory on Antimicrobial Resistance in Animals at the Central Veterinary Institute, I got the opportunity to do research on the effect of coccidiostatics on ESBL-forming *E.coli*, contributing to the PhD project of Cindy Dierikx. This has led to a very pleasant and instructive internship at the CVI. The report you are about to read describes the research I've been doing during this project.

Ayla Hesp

1. Introduction

The spread of antimicrobial resistance by extended spectrum beta-lactamases (ESBLs) in the Dutch poultry industry is an unsettling matter. This topic stirred the political discussion in the Netherlands about the use of antibiotics in veterinary medicine in general (Anonymous, 2007). In livestock antimicrobial agents are widely used as growth promoters (Butaye et al., 2003). The veterinary use of antibiotics (including antimicrobial growth promoters) per kg live weight in the Netherlands was in 2007 nearly twice as high as in 1999. Van Geijlswijk et al. performed a study in which the daily dosages of antibiotics an average animal is treated with per year were calculated in 9 European countries. This study showed that the overall use of antibiotics in the Netherlands far exceeded that of the United Kingdom, Denmark, France, Italy, Sweden, Finland, Norway and Germany in 2006 and has been rising steadily until 2007 (van Geijlswijk et al., 2009).

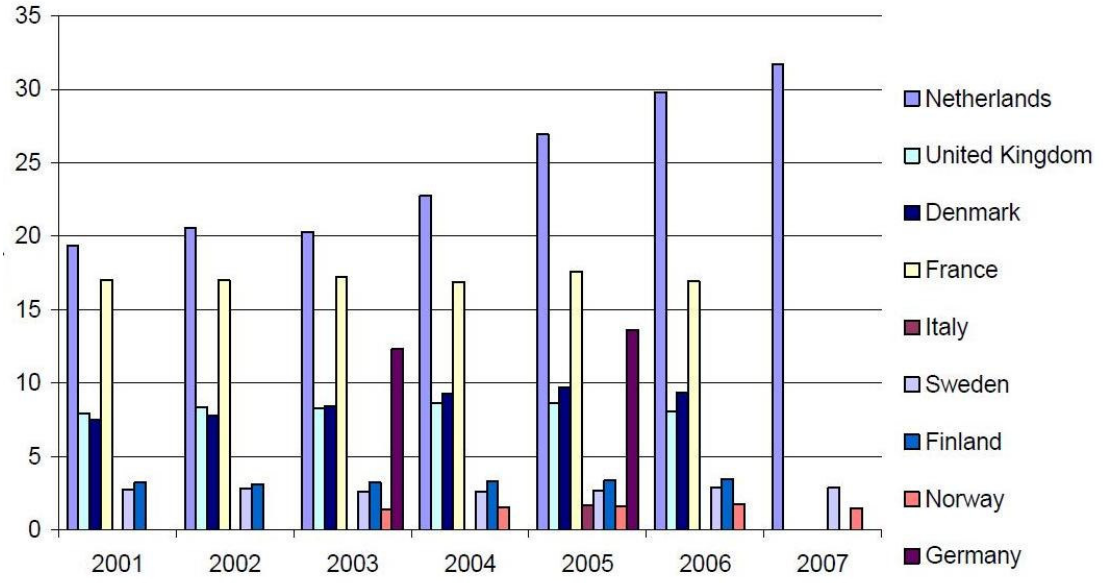


Figure 1. Daily dosages of antibiotics (calculated from the sold/delivered kg of active ingredient) per average animal per year in the various countries (Van Geijlswijk et al., 2009)

Addition of antibiotics to feed was partly forbidden in 1997 and completely prohibited in 2006 in the Netherlands, which might be an explanation for the increased therapeutic use of antibiotics (Anonymous, 2007; Mevius, 2008). The pharmacy of the Faculty of Veterinary Medicine of Utrecht University and the Agricultural Economic Institute (part of the Dutch ministry of Economics, Agriculture and Innovation) developed a quantitative measure to express the use of antibiotics in the Netherlands. Using this measure, the amount of veterinary antibiotic usage could be compared to that in human health care in hospitals in the Netherlands. It was concluded that the use of antibiotics in human health care was only a very small part of the overall use of antibiotics in the Netherlands, the vast part of it was used in livestock.

The increased use of antibiotics in veterinary medicine, specifically beta-lactam antibiotics, seems to be causing a quick spread of the resistance mechanism by the enzymes beta-

lactamases. Especially the resistance against 3rd and 4th generation cephalosporins in the Dutch poultry industry is now being scrutinized. These cephalosporins are prohibited for administration in poultry, yet resistance against 3rd generation cephalosporins was observed in Dutch broiler chickens. From 2003 until 2007 an increase in resistance to the 3rd generation cephalosporin cefotaxime was observed in *E. coli* isolates from broiler chicken intestines. This suggests that these bacteria produce ESBLs (Anonymous, 2007). This suspicion was confirmed when some of the genes encoding ESBLs were found in *E. coli* isolates from broiler intestines, namely $bla_{\text{TEM-52}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-20}}$, $bla_{\text{SHV-2}}$, $bla_{\text{CMY-2}}$, $bla_{\text{ACC-1}}$ en $bla_{\text{CTX-M-2}}$ (Dierikx et al., 2010). It was feared that bacteria with antimicrobial resistance would end up in humans by the food chain or by spreading through the environment (Witte, 1998). This fear was not unrealistic, since the ESBL-genes are located on a transmissible piece of DNA called a plasmid. These plasmids are exchanged by bacteria through a mechanism called conjugation; with a sex pilus, a needle-like tool, the plasmid is injected in the receiving micro-organism.

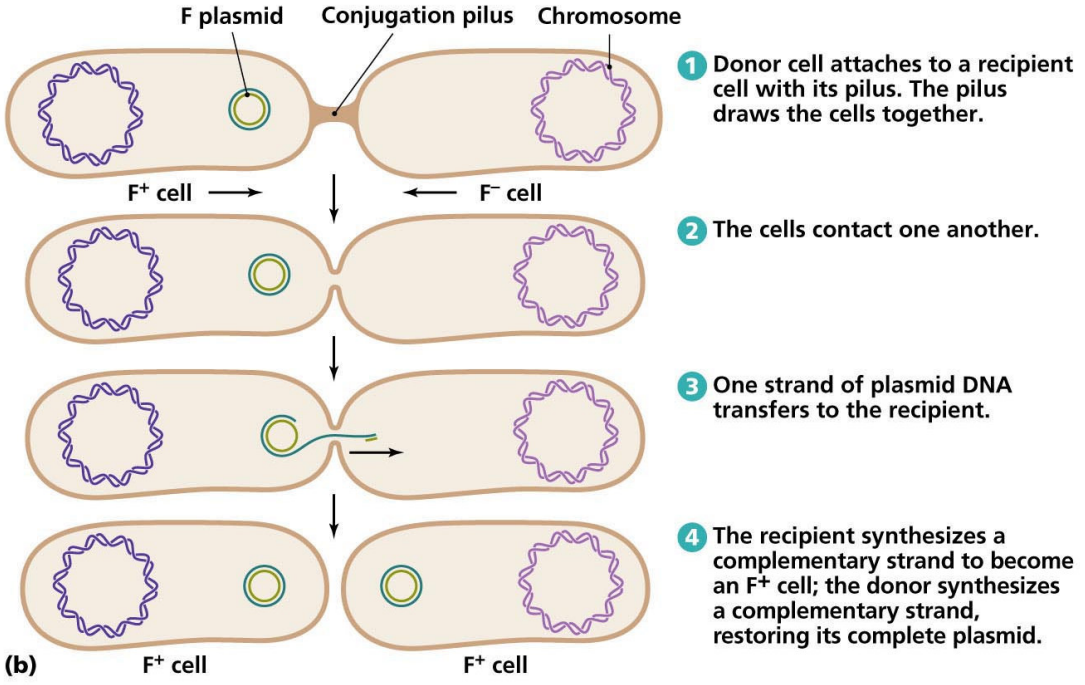


Figure 2. Bacterial conjugation of plasmids (http://academic.pgcc.edu/~kroberts/Lecture/)

This way a pathogenic micro-organism is able to receive genes for the production of ESBLs from another micro-organism and become resistant to most beta-lactam antibiotics used for both humans and animals. The observed resistance of *E. coli* to cefotaxime in broiler chickens was mainly caused by ESBL-genes $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ on plasmids of the type IncI1, these are more successful in spreading than other plasmids (Dierikx et al., 2010). The anxiety for untreatable bacterial infections in humans has stimulated research to determine the spread of antimicrobial resistance in animal reservoirs (Coque et al., 2008). A direct connection between ESBLs in humans and broiler chickens has been described in the Netherlands by a study performed by the Central Veterinary Institute in Lelystad in cooperation with the University Medical Centre of Utrecht. In this study, clinical *E. coli*-

isolates from humans were compared with isolates from broilers as well as retail chicken meat. The isolates were analysed using an ESBL-specific microarray, sequencing of ESBL genes and PCR-based replicon typing of plasmids. From the human isolates, 35% contained poultry-associated ESBL-genes and 19% contained poultry-associated ESBL-genes on plasmids that were indistinguishable from those in poultry and poultry meat. It was pointed out that 86% of the poultry associated genes in humans were $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$, which are the predominant genes in poultry (78%) and poultry meat (75%) as well (Leverstein-van Hall et al., 2011). This provides strong evidence that ESBLs are transmitted from poultry to humans by the food chain. Accordingly broiler chickens are an important animal reservoir for ESBLs in the Netherlands.

The prevalence of ESBL-producing *E. coli* in broilers is very high. A study of 26 broiler farms showed that on all farms animals were found ESBL-positive and on 85% of the investigated farms, more than 80% of the animals (n=41) carried ESBL-producing *E. coli* (Dierikx et al., 2010). Another study by the same researchers of the Central Veterinary Institute followed, which consisted of a longitudinal study on three broiler farms. Every week cloaca swabs were taken from 25 animals during 5 weeks. This survey showed that in the first week the percentage of ESBL-positive animals changed from 0-4% on day 1 to 96-100% on day 7, even on a farm where no antibiotics were administered during the first week. The same study was conducted earlier at a farm with the grandparents of the animals. A remarkable difference between both studies was that in the grandparents lower percentages of ESBL-positive animals were found on 18 and 31 weeks of age. This was not the case when antibiotics had been administered, then all animals were found positive. This suggests that on the fattening farms one or more factors are responsible for the fast spread of ESBLs through the shed, apart from administration of antibiotics. The sheds were investigated on hygiene and the researchers found it unlikely that insufficient cleaning after the production round would be the only cause of the fast spreading of ESBLs in the broiler chickens, as most samples taken from the environment after cleaning and disinfection were negative for ESBL-producing isolates. This encouraged the researchers to look into the differences in conditions between fattening farms and farms with grandparent animals. One difference that might be of interest is the administration of coccidiostatics in the feed of broiler chickens. Grandparent animals are not given coccidiostatics, instead these animals are vaccinated against coccidiosis.

Most coccidiostatics have antimicrobial activity only against Gram-positive bacteria. However Diarra et al. (2007) studied the *in vivo* effect of supplementation of bambermycin, penicillin, salinomycin, and bacitracin or a combination of salinomycin plus bacitracin to the feed of broiler chickens on the incidence and distribution of antimicrobial resistance in *E. coli* isolated from these broilers. This study describes that levels of resistance to ceftiofur, spectinomycin, and gentamicin were significantly higher in *E.coli* from broilers fed with supplementation of salinomycin. The study proved that phenotype and distribution of resistance determinants in *E. coli* can be modulated by feed supplementation. Yet the mechanism that causes these alterations in the *E. coli* in broiler intestines is still unknown. It is known that *Enterobacteriaceae* are able to acquire antimicrobial resistance (Threlfall, 2000), probably by exchange of plasmids in the intestinal lumen. It is possible that use of

coccidiostatics leads to selective pressure on ESBL-genes by co-selection. When a selective pressure for plasmids with ESBL-genes is present, the plasmids can easily be exchanged by *Enterobacteriaceae* in the broiler's intestines and spread rapidly. This might explain why a strong selective pressure for ESBL-producing *E. coli* was present on the broiler farms, even without the use of beta-lactams.

Ionophore coccidiostatics are thought to disrupt cationic cross-membrane gradients of coccidia by interfering with the ion transport of K^+, Na^+, Mg^{2+} and Ca^{2+}. The development of coccidia is inhibited early in the life cycle, before the host-cell-damaging stages have started (Scalzo et al., 2004). Because of an increase in cation concentration inside protozoan cells, the parasites are lysed by increasing osmotic pressure (Bolder et al., 1999). Coccidiostatics are active in the intestinal lumen, systemic concentrations in broiler chickens remain low (Chapman et al., 2010). The antibacterial activity is probably based on the same principle: ions are transported across the bacterial membrane and the Na^+/ K^+ gradient is disrupted. This is lethal to Gram-positive bacteria (Lowicki et al., 2010). Ionophore antibiotics are thought not to act against Gram-negative bacteria (Watanabe et al., 1981), because the cell walls of most Gram-negative bacteria cannot be penetrated by hydrophobic molecules with molecular weights of 600 or above (Westley, 1983).

The aim of this study is to test the i*n vitro* effect of ionophore anticoccidial agents on the growth of ESBL-producing and non ESBL-producing *E. coli* strains, isolated from broilers at slaughter houses. Three ionophore coccidiostatics were selected which are used as feed additives for broiler chickens: salinomycin, narasin and monensin. Salinomycin and narasin are both administered in-feed during the first week on fattening farms, monensin is not administered until after two weeks. Possibly there are other agents in the diet of the broiler chickens that contribute to the selective pressure of ESBL-forming *E. coli.* Therefore feed experts of Wageningen UR Livestock Research were consulted to find out which compounds might be responsible for a selective pressure on ESBLs.

2. Materials and methods

2.1 Broiler chicken diet

The feed of the broiler chickens described above was examined in this study. Compounds and feed additives were discussed with feed experts *Prof.dr.ir. Nico Lenis*, specialist in additives for animal feed, and *Dr.ir. Alfons Jansman,* expert in broiler feed, of Livestock Research, Wageningen UR. The objective was to identify compounds of the broiler feed that might contribute to selection of ESBL-forming *E. coli*. Since antibiotics as feed additives were prohibited in 2006, the poultry industry had to look for alternatives. According to him, antibiotics enhanced feed conversion with 1-3%. Organic acids, plant extracts, fatty acids and pre- and probiotics are suggested as alternatives. Organic acids are widely used in pigs but their effects in broilers are probably less because they may be absorbed by the gullet and not pass the stomach. Most manufacturers of broiler feed in the Netherlands have

abandoned antibiotics as feed additive five years ago. The substances of the studied broiler feed are presented in table 1.

Table 1. Substances of studied broiler feed (Select Minislag Ross of De Hoop Mengvoeders B.V.)	
Ingredients	wheat, toasted and peeled soybean meal, (produced from genetically modified soy), corn, fatty acids (produced from genetically modified soy beans), fatty acids, rapeseed flakes, dicalcium phosphate, feed lime, beetroot, salt
Analytic compounds	raw protein 212.3 g – raw fat 74 g – raw cellulose 25 g - raw ash 59 g – calcium 10 g – phosphor 6.8 g – lysine 14 g – methionine 6 g – sodium 1 g
Additives per kilogram:	
Anti-oxidants	butylhydroxyanisol (BHA) E320 1.0 mg – butylhydroxytolueen E321 (BHT) 1.0 mg – propylgallate 2.0 mg
Vitamins	vitamin A (E672) 17172 IU – vitamin D3 (E671) 5051 IU – vitamin E (DL-alpha-tocoferyl acetate) 121 mg -
Spore elements	copper 15 mg – iodine 3 mg – selenium 0.34 – zink 61 mg – iron 69 mg – manganese 79 mg
Enzymes	endo-1,4-betaxylanase EC 3.2.1.8 (E1606) 13 IU
Coccidiostatics	nicarbazin (E 772) 50 mg – narasin (E 772) 50 mg

Among the additives are enzymes, these are added to break down carbohydrates, to prevent them from becoming substrate for bacteria. Other enzymes are used to prevent the inhibition of micelle forming.

An important factor in the broiler feed is the protein percentage, especially the part of proteins that can be digested enzymatically, like proteins out of soy and fishmeal. Broiler chickens have a more protein rich diet than grandparent animals, because the grandparents have to grow up more gradually. With these animals, reproduction is the most important objective, while for broiler chickens this is growth. Therefore breeding animals get less protein rich feed, with more cellulose, so it leads to quicker saturation. Broiler chickens have more risk to suffer from dysbacteriosis, because of the big amount of substrate for bacteria in the gut.

The only antimicrobial agents present in the diet of the chickens are coccidiostatics. The concentrations of the feed additives selected for this study are presented in table 1.

Table 2. Concentrations of examined coccidiostatics in broiler feed	
Salinomycin-sodium	125 mg/kg (feed manufacturer: Agrifirm)

Narasin	50 mg/kg (feed manufacturer: De Hoop mengvoeders B.V.)
Monensin	138 mg/kg (feed manufacturer: De Hoop mengvoeders B.V.)

Apart from the three anticoccidial agents described, another one is present in the broiler diet: nicarbazin. Nicarbazin is a synthetic drug, the others are naturally occurring substances. This feed additive was not examined in this study for practical reasons, because it is not solvable in water, ethanol or dimethyl sulfoxide (DMSO).

2.2 Strains

All *E.coli* strains used were isolated from broiler chickens at slaughterhouses, as part of the Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in The Netherlands (MARAN). Of these strains, 6 ESBL-forming strains (numbered 38.16, 38.27, 38.52, 38.53, 38.34 and 39.76) and 9 non ESBL-forming strains (numbered 38.08, 38.17, 38.18, 38.19, 38.20, 38.21, 38.22, 38.63 and 38.66) were selected. The susceptibility patterns of these strains are presented in table 3. Of the selected ESBL-forming *E.coli*-strains, 4 carried ESBL-gene CTX-M-1 and 2 carried ESBL-gene TEM-52. The strains were stored in glycerol at a temperature of -80 °C. The strains were grown overnight at 37 °C on plates with heart infusion sheep blood (HIS) agar, together with 4 control strains. For the control strains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603*, S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were selected. For each test fresh cultures of all strains were used.

Table 3. Selected strains isolated from broilers and their MICs (in µg/ml) for several antibiotics

	AMP	FOT	TAZ	GEN	NEO	TET	SMX	TMP	CIP	NAL	FFN	CHL	Antibiotics strains are resistant for
38.17	4	≤ 0.12	0.25	0.5	≤ 1	2	≤ 8	≤ 0.5	0.5	**> 128**	8	8	NAL
38.18	**> 64**	≤ 0.12	≤ 0.12	0.5	≤ 1	2	**> 1024**	**> 64**	0.25	**64**	4	8	AMP-SMX-TMP-NAL
38.19	**> 64**	≤ 0.12	≤ 0.12	0.5	≤ 1	> **64**	**> 1024**	**> 64**	0.25	**128**	8	8	AMP-TET-SMX-TMP-NAL
38.20	**> 64**	≤ 0.12	≤ 0.12	0.5	**64**	4	**> 1024**	**> 64**	0.25	**64**	8	8	AMP-NEO-SMX-TMP-NAL
38.21	1	≤ 0.12	≤ 0.12	0.5	2	**> 64**	≤ 8	≤ 0.5	≤ 0.06	≤ 2	4	8	TET
38.22	2	≤ 0.12	≤ 0.12	0.5	≤ 1	**64**	≤ 8	≤ 0.5	≤ 0.06	≤ 2	8	8	TET
38.16 ESBL	**> 64**	**> 16**	**1**	0.5	≤ 1	**> 64**	**> 1024**	**> 64**	≤ 0.06	≤ 2	4	8	AMP-FOT-TAZ-TET-SMX-TMP
38.27 ESBL	**> 64**	**> 16**	**2**	0.5	2	**> 64**	**> 1024**	**> 64**	≤ 0.06	≤ 2	8	**> 128**	AMP-FOT-TAZ-TET-SMX-TMP-CHL
38.34 ESBL	**> 64**	**16**	**8**	0.5	≤ 1	**> 64**	**> 1024**	**> 64**	0.25	**128**	8	**> 128**	AMP-FOT-TAZ-TET-SMX-TMP-NAL-CHL
38.52 ESBL	**> 64**	**> 16**	**1**	2	≤ 1	4	**> 1024**	**> 64**	0.25	**128**	4	8	AMP-FOT-TAZ-SMX-TMP-NAL
38.53 ESBL	**> 64**	**> 16**	**1**	1	≤ 1	64	**> 1024**	**> 64**	≤ 0.06	**64**	8	8	AMP-FOT-TAZ-TET-SMX-TMP-NAL
39.76 ESBL	**> 64**	**16**	**16**	8	2	1	**> 1024**	**> 64**	0.25	**> 128**	8	**> 128**	AMP-FOT-TAZ-SMX-TMP-NAL-CHL
38.08	2	≤ 0.12	≤ 0.12	0.5	≤ 1	1	≤ 8	≤ 0.5	≤ 0.06	≤ 2	4	8	
38.63	2	≤ 0.12	≤ 0.12	0.5	≤ 1	1	≤ 8	≤ 0.5	≤ 0.06	≤ 2	4	8	
38.66	4	≤ 0.12	≤ 0.12	1	≤ 1	2	≤ 8	≤ 0.5	≤ 0.06	≤ 2	8	8	

AMP = ampicillin, FOT = cefotaxime, TAZ = ceftazidime, GEN = gentamicin, NEO = neomycin, TET= tetracyclin, SMX = sulphametathoxazole, TMP = trimethroprim, CIP = ciprofloxacin, NAL = naladixic acid, FFN = florfenicol, CHL = chloramphenicol

In bold: values considered resistant using EUCAST epidemiological Cut-off values (www.eucast.org).

2.3 Ionophores

The three ionophore coccidiostatics selected for this study were bought online from Sigma Aldrich®: salinomycin monosodium salt hydrate, narasin from *Streptomyces auriofaciens* and monensin sodium salt. Stock solutions were made of each of the ionophores in 99% ethanol because ionophores are not soluble in water. Concentrations of the stock solutions were calculated in consideration of the purity of each compound (table 4). Information about the potency of the compounds was not available, therefore potency was not taken into account in this experiment.

Table 4. Purity of ionophore compounds according to Certificate of Analysis manufacturer	
Salinomycin monosodium salt hydrate	80.3 %
Narasin from *Streptomyces auriofaciens*	97 %
Monensin sodium salt	91 %

2.4 MICs

2.4.1 Broth microdilution test

The inhibitory activity of the ionophore compounds was measured by a broth microdilution assay in a 96 well sterile microtitre tray with a U-shaped bottom. First a test measurement of salinomycin was made, to test the potential inhibitory effect of ethanol. For this test only *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were used. Twelve dilutions of salinomycin were made in Mueller Hinton broth (concentration range 0,125 – 256 µg/ml). Furthermore dilutions of ethanol were made in MH-broth with a concentration range of 0,001389 - 5,69% (corresponding with the concentration of ethanol in the dilutions of salinomycin). Inocula were prepared by making solutions of cultures in 0.9% NaCl-solution to a density of 0.5 on the McFarland turbidity scale and diluting these 200-fold in MH-broth. The 96 wells microplate with 50 µl of the dilutions in each well was inoculated with 50 µl of the culture medium in each well. Then the microplate was incubated aerobically overnight at 35 °C.

After the first test with two control strains, twelve dilutions of salinomycin were made in the same way (concentration range 0,125 - 256 µg/ml), now all strains were tested in duplo.

In the same way, twelve dilutions of monensin (concentration range 0,25 - 512 µg/ml) and narasin (concentration range 0,3125 - 64 µg/ml) were made. These dilutions were based on the different concentrations of the compounds in the broiler diets. All tests were performed in twofold. The concentration ranges of ethanol for the other tests were 0,001418 - 5.81% for the monensin test and 0,000977 - 4% for the narasin test.

The highest concentration of monensin was not fully dissolved when diluted in Mueller Hinton broth. This could affect the results, because the active concentration would be lower than 512 µg/ml when the compound was not fully dissolved. The test was repeated with

256 µg/ml as highest concentration. This time the compound was more dissolved, but still a little suspended because the solution was not clear.

The Central Veterinary Institute had Sensititre NMMCS4B5 plates in stock from a former project. The plates were far older then the expire date, but were used for a control measurement of the salinomycin MICs determined by the described microdilution test. The plates contained eight antimicrobial substances: nitrofurantoin, avilamycin, bacitracin, Flavomycin®, salinomycin, synercide, tilmicosin, and virginiamycin. Solutions of 0,5 McFarland were prepared and diluted 200 times, by adding 55 µl of the solution to 11 ml Mueller Hinton broth. Then 50 µl of the bacterial suspension was added to each well with a multistep pipette. Sixteen Sensititre plates were inoculated this way to test all strains. The plates were incubated overnight at 35 °C.

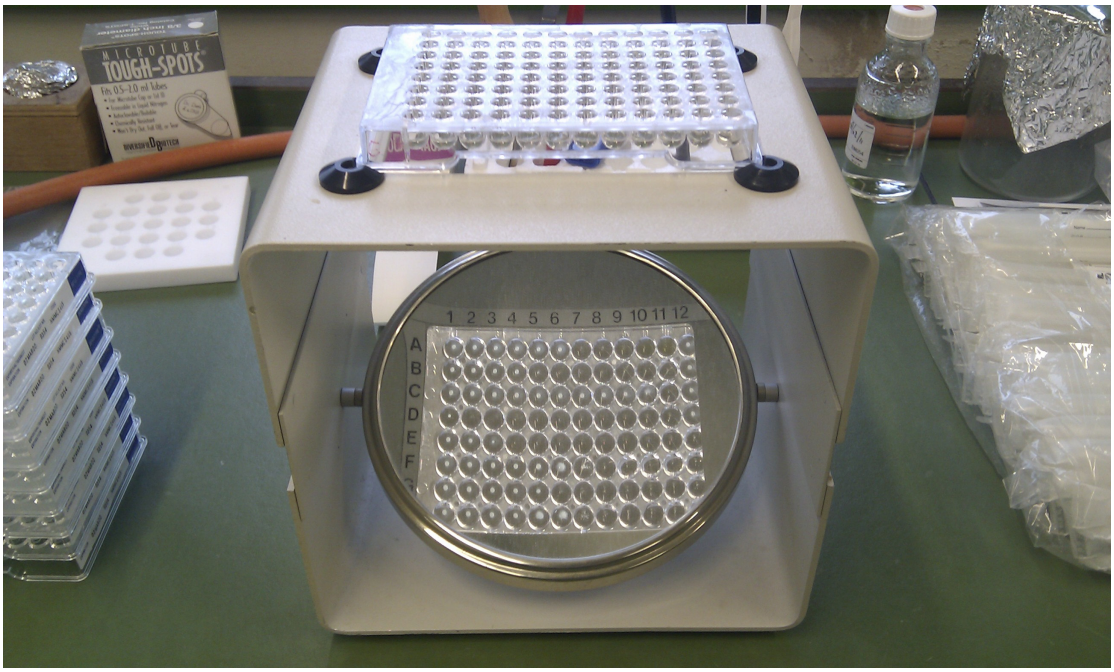


Figure 3. Determination of MICs of strains for salinomycin with Sensititre plates.

2.4.2 Agar dilution test

Because the results of determining MICs with the broth microdilution test were not satisfying, another method was used: the agar dilution test. In this test 12 dilutions of coccidiostatics were tested in Mueller Hinton agar. This method is different from the microtitre plates because agar is coagulating quickly, so there is no time for the dissolved or suspended compound to sink. Secondly, the solution in ethanol is first diluted in water and not directly in broth or agar as in the first method.

Stock solutions of all compounds were made 40 times higher than the highest concentration. Highest concentrations were 256 µg/ml for monensin and salinomycin and 64 µg/ml for narasin. The necessary stock solutions were calculated, after correcting the necessary concentrations with the purity of each compound. The concentration of 64 µg/ml for narasin was chosen for practical reasons, because there was not enough of the substance in stock for a concentration of 256 µg/ml. A concentration this high should not be necessary for

narasin, since it is often tested with 50 µg/ml as highest concentration and the concentration in the broiler diet is 50 mg/kg.

For two agar plates, a bottle of 45 ml agar was used. This was a practical way to make doubling dilutions in duplo. The agar was melted and cooled down until it was used in a bath of 50°C. First the stock solution was diluted 4 times in water, then 12 doubling dilutions were made in water. Thereafter the tenfold dilution step in Mueller Hinton agar was made by adding 5 ml of the solutions in the agar bottles of 45 ml. Each bottle was poured out in two empty plates and put away to coagulate. Furthermore doubling dilutions of ethanol were made with a concentration range of 0,078125 – 2,5%. Four plates with nothing except agar were made for control measurements. The concentrations of monensin of 128 µg/ml and 256 µg/ml were not fully dissolved, but spread reasonably well through the plates when these were coagulated. The clotted plates were turned upside down, stacked and stored overnight in the refrigerator at 4°C.

The next day half of the plates were incubated with all 16 strains. For this a solution of 0,5 McFarland was diluted tenfold in physiologic saline water, then the agar plates were seeded with spots of 5µl (approximately 10^7 CFU) of each strain. The Gram-positive control strains were double tested at different spots on the plate, to test if the ionophores were spread evenly through the agar. A control plate with just agar was inoculated before and after inoculating the test plates to verify whether the testing conditions were even for all plates. The plates were incubated aerobically overnight at 35°C. The next day the plates were viewed to determine inhibitory concentrations. The remaining plates were incubated in the same way.

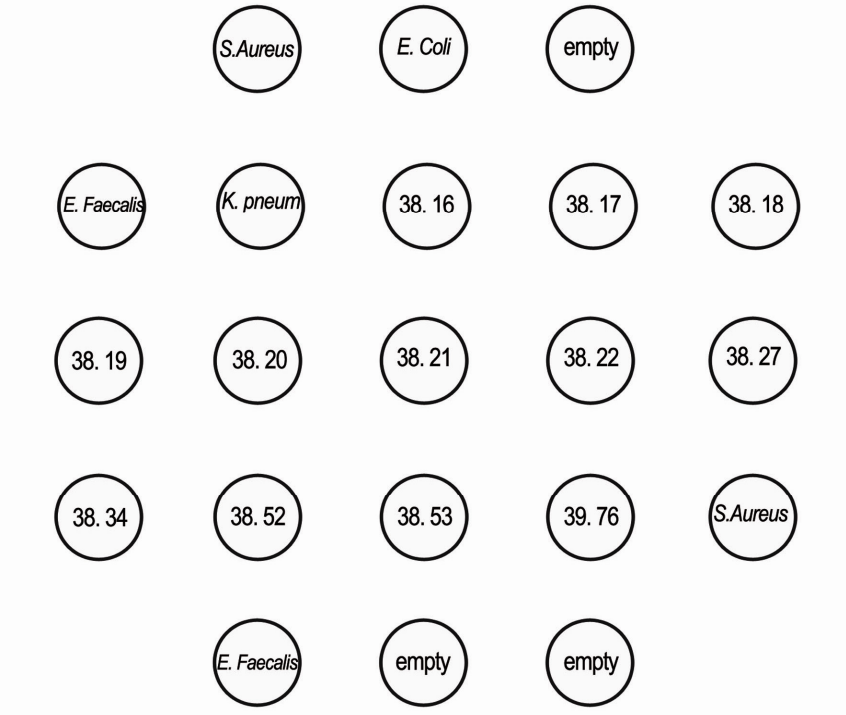


Figure 4. Overview of location of different strains on agar plates

2.5 Growth experiments

2.5.1 Bioscreen experiment

To investigate the effect of the ionophores on the *E. coli*-strains, growth experiments were performed with a Bioscreen C MBR apparatus. This is a culture growth monitoring instrument, which is computer controlled and is an incubator, as well as shaker and optical density reader. The optical density (OD) is kinetically measured and the forthcoming data can be interpreted as bacterial counts (Lambert et al., 1998). This provides detailed data on the growth of the incubated strains. In this study, the Bioscreen apparatus was used to monitor the growth of the *E. coli*-strains when different concentrations of narasin and salinomycin were dissolved in the growth medium.

With the Bioscreen system, 200 wells can be tested at once. To test the effect of ethanol on the growth of the strains, a growth experiment with different concentrations of ethanol was performed first. Two sterile honeycomb micro plates with 100 wells were filled with different dilutions of ethanol in Mueller Hinton broth. After adding the strains, the tested concentrations in the wells were 0, 0.05, 0.5 and 5%. All 16 strains were triple tested. The filled micro plates were put in the incubator and the computer program was instructed to incubate the plates for 7 hours and measure the OD every 12 minutes. The wells were shaken before every measurement. These testing conditions were used for all tests with the Bioscreen apparatus.

After interpreting the ethanol test measurement, it was decided to use 0.5% ethanol as highest concentration for testing salinomycin and narasin. Because of that, high stock solutions of the ionophore compounds had to be made. To make a tenfold dilution of three different concentrations, a stock solution of 200 times the tested concentrations were made. To get useful results, it was important that the solutions of the ionophore compounds in ethanol would stay clear after dissolving them in Mueller Hinton broth. If the coccidiostatics would be suspended instead of dissolved, the OD measurements would not be accurate and could not be used as bacterial counts. When preparing the dilutions in Mueller Hinton agar, no precipitation of the ionophore compounds could be observed. Because the wells were shaken before every measurement by the Bioscreen apparatus, it was assumed that the ionophore compounds stayed dissolved.

2.5.2 Bioscreen experiment narasin

Narasin was tested in three stages, to have enough control measurements of ethanol. Because not more than 200 wells could be tested at once, two non ESBL-forming strains, two ESBL-forming strains and two control strains were tested each day. *E. faecalis* and *E. coli* were used as control strains in every experiment to have good comparison. For narasin, three concentrations were tested: 0.5 µg/ml, 5 µg/ml and 50 µg/ml. The purity of narasin is 97%, so it was presumed that no correction for the purity would be necessary when preparing the stock solutions. In each microplate, two rows of ten wells were used to test one strain. In one row were three wells with each concentration of narasin. In the second row were three wells with the three corresponding concentrations of ethanol: 0.5%, 0.05%

and 0.005%. The last well of each row was only filled with Mueller Hinton broth to have a blank control measurement. Two microplates were filled identically. The first day of testing, strains 38.16, 38.17, 38.18 and 38.27 were tested. For these four strains control measurements of the used ethanol concentrations were made. The control strains *E. faecalis* and *E. coli* were also tested, but for these no control measurements of ethanol concentrations were made, because there were not enough wells on the microplates.

The second day, strains 38.19, 38.20, 38.34 and 38. 52 were tested and again *E. faecalis* and *E. coli* as control strains. This time, the control strains were also tested in different concentrations of ethanol by using a more efficient way to fill the microplates. Now one microplate was only filled with concentrations of narasin, in the same way as described earlier. Two rows were used to test one strain. The second microplate was used to test ethanol concentrations, the strains were put in the wells corresponding to the wells of the first microplate.

At the third day of testing narasin, strains 38.21, 38.22, 38.53 and 39.76 were tested, together with the control strains *E. faecalis* and *E. coli.* The microplates were filled in exactly the same way as described above.

2.5.3 Bioscreen experiment salinomycin

For the growth experiment with salinomycin, concentrations were based on the concentrations in the broiler feed. The concentration of salinomycin in the studied broiler feed was 125 mg/kg. Therefore concentrations of 1.25 µg/ml, 12.5 µg/ml and 125 µg/ml were chosen. Because the purity of the purchased salinomycin was 80.3%, this time a correction was made when preparing the stock solutions. It was calculated that dilutions of 1.56, 15.6 and 156 µg/ml would be necessary. A stock solution was again prepared 200 times higher than the highest concentration. The test was again performed in three stages. The strains used for each stage were exactly the same as in the narasin experiment. The Bioscreen microplates were filled in the same way as described for narasin on each day of testing.

2.5.4 Feed experiment

After testing the effect of coccidiostatics on the growth of ESBL-forming and non ESBL-forming strains, another test was performed to discover if substances in the feed of the broilers might have influence the growth of ESBL forming strains. Therefore a growth experiment with whole broiler feed was performed.

For this experiment, pellets of broiler feed were suspended in culture medium. The broiler feed was first sterilised by γ-radiation. The medium used was Luria Bertani (LB) medium. One ESBL-producing strain and one non ESBL-producing strain were tested together. To determine if the broiler feed had a significant effect on the strains in vitro, a control group with both strains tested in LB-medium was used. A control growth experiment of just the feed suspended in LB medium was used to determine whether the feed had been contaminated after sterilisation. In total six strains were tested on three different days. The ESBL-producing strains used were 38.16, 38.27 and 38.34. For the non ESBL-producing

strains, this time completely sensitive *E. coli*-strains, isolated from broiler chickens, were used: 38.08, 38.63 and 38.66. The susceptibility patterns of the strains can be read from table 3.

Five test-tubes were filled with LB medium by the following method:

1. 10 ml LB medium + 100µl 0,5 McFarland sensitive strain
2. 10 ml LB medium + 100µl 0,5 McFarland sensitive strain + 1 g feed
3. 10 ml LB medium + 100µl 0,5 McFarland ESBL-strain
4. 10 ml LB medium + 100µl 0,5 McFarland ESBL-strain + 1 g feed
5. 10 ml LB medium + 1 g feed

The tubes with feed added were mixed as well as possible, to suspend the feed through the test medium. The tubes were placed in an incubator and shaker at 37°C. After 2, 4, 6 and 24 hours tenfold dilutions were made in physiologic saline water. The dilutions 10^{-3},10^{-4},10^{-5}, 10^{-6}, 10^{-7} were inoculated on McConkey agar plates and incubated at 37°C overnight. The following day, colonies were counted with a colony counter. The bacterial counts were made using the agar plates of the three dilutions that were best countable.

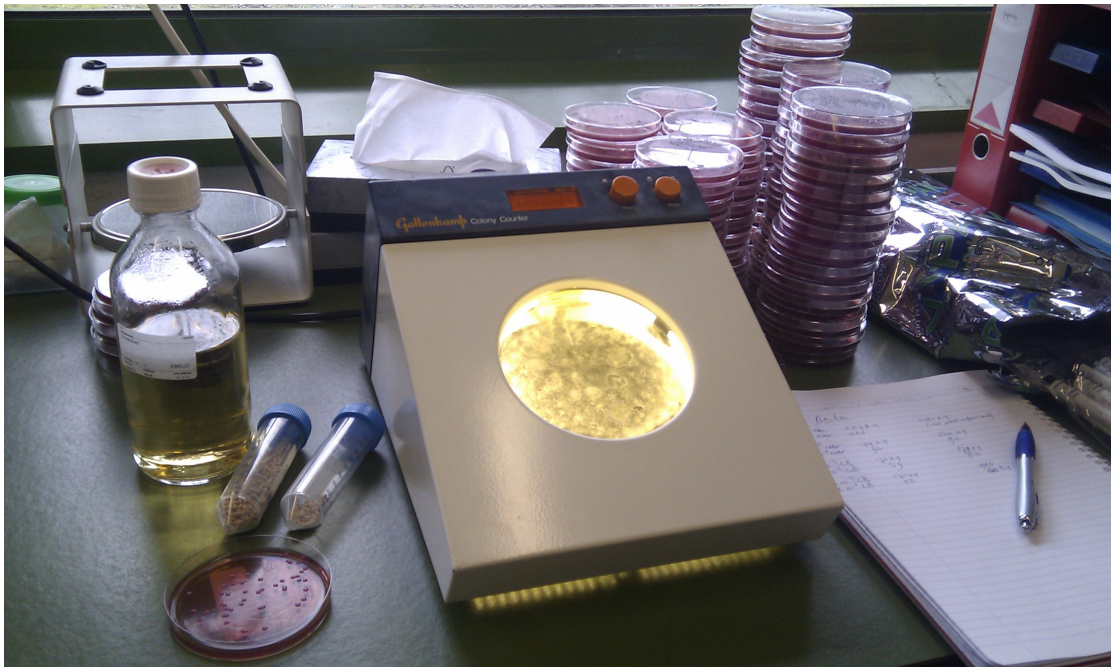


Figure 5. LB medium, test tubes with broiler feed, McConkey agar plates with colonies and Colony Counter.

3. Results

3.1 MICs

3.1.1 Broth microdilution test

The first small test with salinomycin and two control strains showed that a concentration of 5,69% ethanol did not visibly inhibit the growth of both strains and that *S. aureus* ATCC 29213 was inhibited at a concentration of 8 µg/ml salinomycin. *E. coli* ATCC 25922 was not inhibited in growth. The results of the broth microdilution tests of all three ionophore compounds are visible in table 5.

The selected ESBL-forming and non ESBL-forming *E. coli*-strains were not inhibited at all by the tested concentrations of salinomycin, neither were the control strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603. The MIC was therefore determined to be >256 µg/ml for all strains and the two Gram-negative control strains. The Gram-positive control strains *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were both inhibited in growth.

Table 5. MICs determined by microdilution assay in Mueller Hinton broth

	Strain	Microplate 1	Microplate 2
Salinomycin	All tested *E. coli* –strains	>256 µg/ml	>256 µg/ml
	E. coli ATCC 25922	>256 µg/ml	>256 µg/ml
	K. pneumoniae ATCC 700603	>256 µg/ml	>256 µg/ml
	S. aureus ATCC 29213	2 µg/ml	1 µg/ml
	E. faecalis ATCC 29212	1 µg/ml	0,5 µg/ml
Narasin	All tested *E. coli* –strains	>64 µg/ml	>64 µg/ml
	E. coli ATCC 25922	>64 µg/ml	>64 µg/ml
	K. pneumoniae ATCC 700603	>64 µg/ml	>64 µg/ml
	S. aureus ATCC 29213	64 µg/ml	64 µg/ml
	E. faecalis ATCC 29212	64 µg/ml	64 µg/ml
Monensin	All tested *E. coli* –strains	>512 µg/ml	>512 µg/ml
	E. coli ATCC 25922	>512 µg/ml	>512 µg/ml
	K. pneumoniae ATCC 700603	>512 µg/ml	>512 µg/ml
	S. aureus ATCC 29213	128 µg/ml	128 µg/ml
	E. faecalis ATCC 29212	128 µg/ml	8 µg/ml

With the broth microdilution assay using Sensititre plates, for both *S. aureus* and *E. faecalis* an MIC of 1 µg/ml was found for salinomycin. *E. coli* and *K. pneumoniae* were not inhibited by a concentration of salinomycin up to 128 µg/ml. The ESBL-strains and non ESBL-strains were both not inhibited by salinomycin. Only strain 38.21 seemed slightly inhibited at a concentration of 128 µg/ml. All tested strains were resistant to avilamycin, bacitracin, and virginiamycin, but most strains appeared sensitive to nitrofurantoin, Flavomycin®, tilmicosin and synercide. Because the Sensititre plates were expired and there was no control measurement for compounds other than salinomycin, the MICs of the strains for these substances are not described here.

3.1.2 Agar dilution test

As expected, all Gram-negative strains tested in agar showed MICs above the highest tested concentrations of ionophores. The results are presented in table 6. On the second day, the test results were a little different. Some MIC's turned out to be one dilution step lower than the day before. For the tested *E. coli*-strains as well as *E. coli* ATCC 25922, a MIC of >256 µg/ml was determined for salinomycin and monensin and of >64 µg/ml for narasin. For the Gram-positive control strains, a MIC of 4 µg/ml was determined for monensin on both days. For salinomycin, the MIC of *S. aureus* was determined to be 1 µg/ml on day 1 and 0,5 µg/ml on day 2. For *E. faecalis*, MICs were 0,5 µg/ml on day 1 and ≤0,125 µg/ml on day 2. The MICs of *S. aureus* for narasin were 0,5 µg/ml on day 1 and 0,25 µg/ml on day 2. The other Gram-positive strain, *E. faecalis,* was inhibited by narasin at a concentrations of 0,25 µg/ml on day 1 and 0,125 µg/ml on day 2.

The control measurements with only agar were very reliable, the plates before and after the inoculation were exactly the same. This provided good comparison for determining whether any inhibiting effect had taken place. The inoculated plates with six dilutions of ethanol in agar (concentration range 0,078125 - 2,5%) were not visibly different, all 16 strains seemed uninhibited by ethanol in this concentration range.

Strain 38.17 (a non ESBL-forming strain) seemed to be inhibited by narasin at a concentration of 64 µg/ml and by monensin at a concentration of 256 µg/ml. This effect was not observed at day 2 of testing in agar. All strains showed differences in plaque diameter between the highest and lowest concentrations of ionophores tested in agar. This effect was observed on both days of testing.

Table 6. MICs determined by doubling dilutions in Mueller Hinton agar

Salinomycin	Strain	Day 1	Day 2
	Tested *E. coli* –strains	>256 µg/ml	>256 µg/ml
	E. coli ATCC 25922	>256 µg/ml	>256 µg/ml
	K. pneumoniae ATCC 700603	>256 µg/ml	>256 µg/ml
	S. aureus ATCC 29213	1 µg/ml	0,5 µg/ml

	E. faecalis ATCC 29212	0,5 µg/ml	≤0,125 µg/ml
Narasin	Tested *E. coli* –strains	>64 µg/ml	>64 µg/ml
	E. coli ATCC 25922	>64 µg/ml	>64 µg/ml
	K. pneumoniae ATCC 700603	>64 µg/ml	>64 µg/ml
	S. aureus ATCC 29213	0,5 µg/ml	0,25 µg/ml
	E. faecalis ATCC 29212	0,25 µg/ml	0,125 µg/ml
Monensin	Tested *E. coli* –strains	>256 µg/ml	>256 µg/ml
	E. coli ATCC 25922	>256 µg/ml	>256 µg/ml
	K. pneumoniae ATCC 700603	>256 µg/ml	>256 µg/ml
	S. aureus ATCC 29213	4 µg/ml	4 µg/ml
	E. faecalis ATCC 29212	4 µg/ml	4 µg/ml

Because of the difference in growth between the highest and lowest concentrations, the diameters of all growth plaques were measured to decide whether there had been a significant effect of the ionophore compounds on the tested strains. The difference in plaque diameter between the highest and the lowest concentrations were calculated for both days, the results are presented in table 7. As can be seen in table 7, all Gram-negative strains are inhibited slightly in growth. When looking at the mean difference in plaque size, it can be concluded that there is a difference between ESBL-producing and non ESBL-producing strains on the first day of testing. On the second day of testing, no apparent difference between the two groups was observed. The mean difference in diameter of the group ESBL-positive strains (for all twelve values) and the group ESBL-negative strains and the corresponding standard deviation were calculated. A T-Test with a 95% confidence interval was performed to compare the difference between two means. The outcome of the test was that no significant difference between the two groups existed.

Table 7. Difference in plaque diameter of strains on agar plates (in mm) based on results obtained from experiments performed on 2 consecutive days.

Strain	Monensin				Salinomycine				Narasin			
	Δ in diameter Day 1	Δ in diameter Day 2	MEAN	STDEV	Δ in diameter Day 1	Δ in diameter Day 2	MEAN	STDEV	Δ in diameter Day 1	Δ in diameter Day 2	MEAN	STDEV
***S. aureus* ATCC 29213**	4	4	4	0.000	4	2	3	1.414	5	4	4.5	0.707
***E. coli* ATCC 25922**	2	2	2	0.000	4	1	2.5	2.121	4	2	3	1.414
***E. faecalis* ATCC 29212**	3.5	3	3.25	0.354	3	0	1.5	2.121	3	3	3	0.000
***K. pneumoniae* ATCC 700603**	1	1	1	0.000	0.5	0	0.25	0.354	1	1	1	0.000
ESBL *E. coli* 38.16	2	3	2.5	0.707	2	1	1.5	0.707	2	2	2	0.000
ESBL *E. coli* 38.27	1	0	0.5	0.707	1.5	1	1.25	0.354	3	2	2.5	0.707
ESBL *E. coli* 38.34	1	1	1	0.000	2	1	1.5	0.707	1	2	1.5	0.707
ESBL *E. coli* 38.52	1	1	1	0.000	2	2	2	0.000	2	2	2	0.000
ESBL *E. coli* 38.53	2	2	2	0.000	1	1.5	1.25	0.354	2	1.5	1.75	0.354
ESBL *E. coli* 39.76	1	1	1	0.000	1.5	2	1.75	0.354	2	1.5	1.75	0.354
		MEAN group		1.333		MEAN group		1.542		MEAN group		1.917
		STDEV group		0.779		STDEV group		0.452		STDEV group		0.469
Non ESBL *E. coli* 38.17	4	1	2.5	2.121	5	1	3	2.828	4	2	3	1.414
Non ESBL *E. coli* 38.18	2	2	2	0.000	3	1.5	2.25	1.061	4	3	3.5	0.707
Non ESBL *E. coli* 38.22	1	0	0.5	0.707	1	0.5	0.75	0.354	1	2	1.5	0.707
Non ESBL *E. coli* 38.21	1	0	0.5	0.707	2.5	3	2.75	0.354	2	3	2.5	0.707
Non ESBL *E. coli* 38.2	2	1	1.5	0.707	3	2	2.5	0.707	4	2	3	1.414
Non ESBL *E. coli* 38.19	1	0	0.5	0.707	1	0	0.5	0.707	1	0	0.5	0.707
		MEAN group		1.250		MEAN group		2.333		MEAN group		1.958
		STDEV group		1.138		STDEV group		1.572		STDEV group		1.405

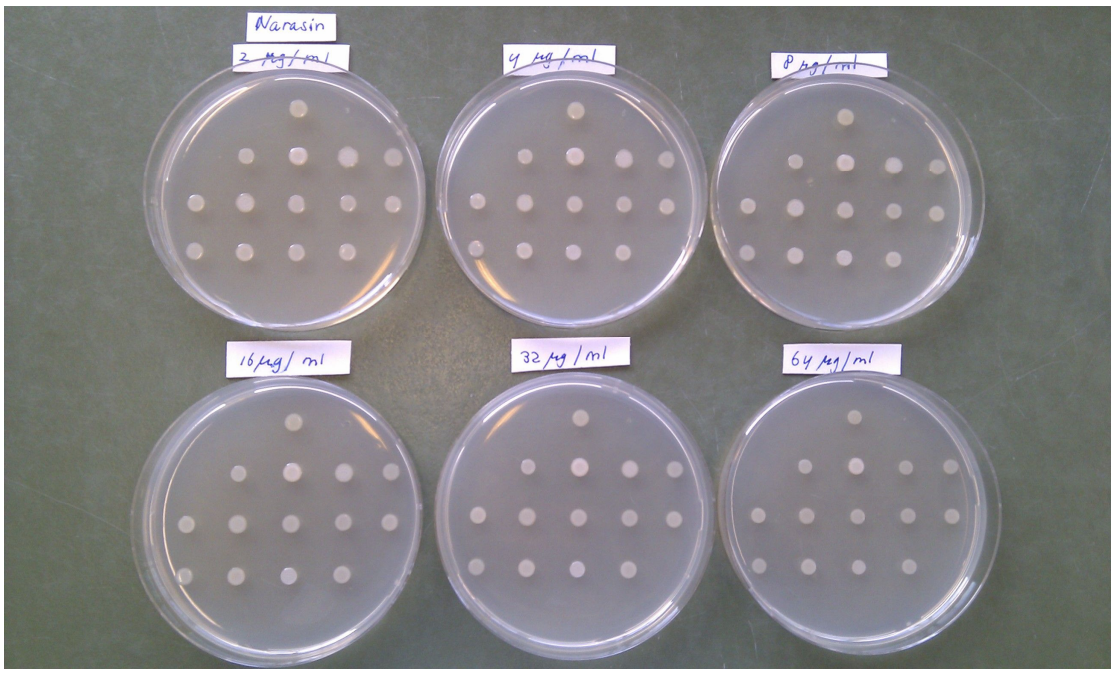


Figure 6. Agar plates with six different concentrations of narasin, inoculated with the tested strains

3.2 Growth experiments

Of the OD measurements that were made by the Bioscreen every 12 minutes, growth curves were made to compare the growth of the different strains with or without the addition of the ionophores. The OD values were used to calculate the area under the curve (AUC) by the formula below. The formulas were retrieved from a growth inhibition test for alga from the Organisation for Economic Co-operation and Development in Paris. In this test spectrophotometry was used to interpret the inhibitory effect of chemicals on an unicellular algal species (OECD, 1984). The area below the growth curve can be calculated by:

$$A = (N_1-N_0)/2 * t_1 + (N_1 + N_2 - 2N_0)/2 * (t_2-t_1) + (N_{n-1}+N_n-2N_0)/2 * (t_n-t_{n-1})$$

A = area
N_0 = nominal number of cells/ml at time t_0
N_1 = measured number of cells/ml at time t_1
N_n = measured number of cells/ml at time t_n
t_1 = time of first measurement after beginning of test
t_n = time of n^{th} measurement after beginning of test

The OD values were used to denote the number of cells/ml. The area under the curve could be used to compare the inhibiting effects of the ionophore compounds to the ethanol control measurements. The percentage inhibition of the cell growth at each test substance concentration was calculated by the formula:

$$I_A = (A_c - A_t)/ A_c * 100$$

This is the difference between the area under the control growth curve A_c and the area under the growth curve at each test substance concentration (A_t). The I_A values describe the inhibiting effect of the added coccidiostatics. Of every tested strain, six growth curves in

total could be made for each concentration of coccidiostatic and six growth curves could be made for each concentration of ethanol. Of the blank control (without test substance or ethanol), four growth curves were made.

5.2.1 Narasin Bioscreen experiment

From the Bioscreen experiment with narasin, the mean of the areas under the curve ($\bar{x}$) of each six measurements of one strain are presented in table 8, together with the standard deviation (STDEV). From the areas under the curve, the percentage inhibition was calculated with the described formula. The mean calculated I_A values ($\bar{x}$ I_A) are presented in table 9 and in figure 6 to 8. The standard deviation (STDEV) of the six measurements are presented by the black lines in the diagrams and graphs.

Table 8. Mean areas under the curve (AUC) in Bioscreen experiment with different concentrations of narasin or different concentrations of ethanol, and control in MH-broth

	Concentrations narasin	**50 µg/ml**		**5 µg/ml**		**0.5 µg/ml**		**Control (blank)**	
ESBL-strains									
		$\bar{x}$	STDEV	$\bar{x}$	STDEV	$\bar{x}$	STDEV	$\bar{x}$	STDEV
38.16	AUC test	60.7	2.0	60.3	0.4	62.4	1.2	64.8	0.5
	AUC ethanol	59.4	3.0	56.6	3.0	59.8	1.3		
38.27	AUC test	55.3	4.0	46.5	1.6	47.6	1.7	52.1	1.3
	AUC ethanol	50.2	2.0	48.1	0.7	48.4	1.3		
38.34	AUC test	48.4	1.6	47.5	1.1	46.6	3.8	53.4	3.1
	AUC ethanol	48.2	1.1	46.1	2.0	47.0	1.5		
38.52	AUC test	67.0	1.7	70.3	1.0	52.0	1.8	76.2	0.9
	AUC ethanol	70.9	3.3	68.6	3.8	7.1	1.0		
38.53	AUC test	48.6	2.2	56.5	2.4	55.9	1.9	62.5	1.4
	AUC ethanol	58.7	2.1	55.4	4.8	51.4	5.2		
39.76	AUC test	55.9	2.3	54.4	2.7	58.3	1.7	62.5	2.0
	AUC ethanol	55.5	1.2	52.9	2.4	54.9	2.2		
Non ESBL-strains									
38.17	AUC test	55.0	2.3	46.3	3.2	44.8	2.7	52.9	3.0
	AUC ethanol	49.9	3.4	45.2	3.2	44.9	2.4		
38.18	AUC test	46.7	5.2	46.0	2.9	44.3	5.2	50.5	3.0
	AUC ethanol	46.2	4.2	44.6	2.2	46.2	3.0		

38.19	AUC test	58.2	1.7	62.0	2.0	63.0	1.7	66.7	0.7
	AUC ethanol	61.3	2.1	62.5	1.0	61.2	0.8		
38.2	AUC test	62.4	1.6	63.9	1.5	63.1	1.6	71.5	1.7
	AUC ethanol	69.5	1.8	68.3	1.9	67.3	0.9		
38.21	AUC test	66.4	1.8	68.5	1.1	67.9	1.4	73.3	1.2
	AUC ethanol	71.6	11.6	67.9	1.7	66.8	2.1		
38.22	AUC test	56.7	1.1	62.7	1.2	62.4	0.8	61.7	0.8
	AUC ethanol	58.3	3.3	59.7	4.2	54.8	1.6		
Control strains									
***E. faecalis* ATCC 29212**	AUC test	0.0	0.3	0.0	0.3	6.6	1.6	38.6	3.9
	AUC ethanol	35.9	1.0	35.1	0.9	35.2	1.5		
***E. coli* ATCC 25922**	AUC test	59.6	1.4	62.1	4.9	63.1	1.2	66.0	2.6
	AUC ethanol	60.3	1.3	61.7	2.4	62.2	0.7		

Table 9. Mean percentage of growth inhibition ($\bar{x}$ I_A) in Bioscreen experiment by different concentrations of narasin

	Concentrations	**50 µg/ml**		**5 µg/ml**		**0.5 µg/ml**	
Strains		$\bar{x}$ I_A	STDEV	$\bar{x}$ I_A	STDEV	$\bar{x}$ I_A	STDEV
ESBL-strains	38.16	-2.3	3.8	-6.8	6.1	-4.4	2.8
	38.27	-10.3	11.5	3.5	3.5	1.6	3.7
	38.34	-0.5	3.8	-3.0	4.9	0.9	6.4
	38.52	5.3	5.0	-2.6	4.8	-2.5	4.5
	38.53	17.2	1.2	-2.8	12.2	-9.3	8.1
	39.76	-0.8	4.5	-3.1	8.2	-6.2	3.5
Non ESBL-strains	38.17	-10.7	8.3	-2.5	7.0	0.1	3.4
	38.18	-1.1	7.0	-3.1	3.5	3.9	11.0
	38.19	5.1	3.6	0.9	3.0	-2.9	2.6
	38.20	10.2	2.2	6.4	2.7	6.2	3.5
	38.21	5.7	11.9	-1.0	1.7	-1.7	2.4
	38.22	2.4	5.4	-5.4	5.8	-13.9	3.9

Control strains	*E. faecalis* ATCC 29212	100.0	1.0	100.1	1.0	81.1	5.1
	E. coli ATCC 25922	1.1	1.3	1.1	9.6	-0.8	3.3

No consistent effect of narasin on the tested *E. coli*-strains was observed, neither a stimulating nor an inhibiting effect. Some strains seemed either inhibited or stimulated in growth, but this was independent of carriage of ESBL-genes. As can be seen from the data, the Gram-positive control strain *E. faecalis* was about 80% inhibited by a concentration of 0.5 µg/ml and completely inhibited by 5 µg/ml and 50 µg/ml.

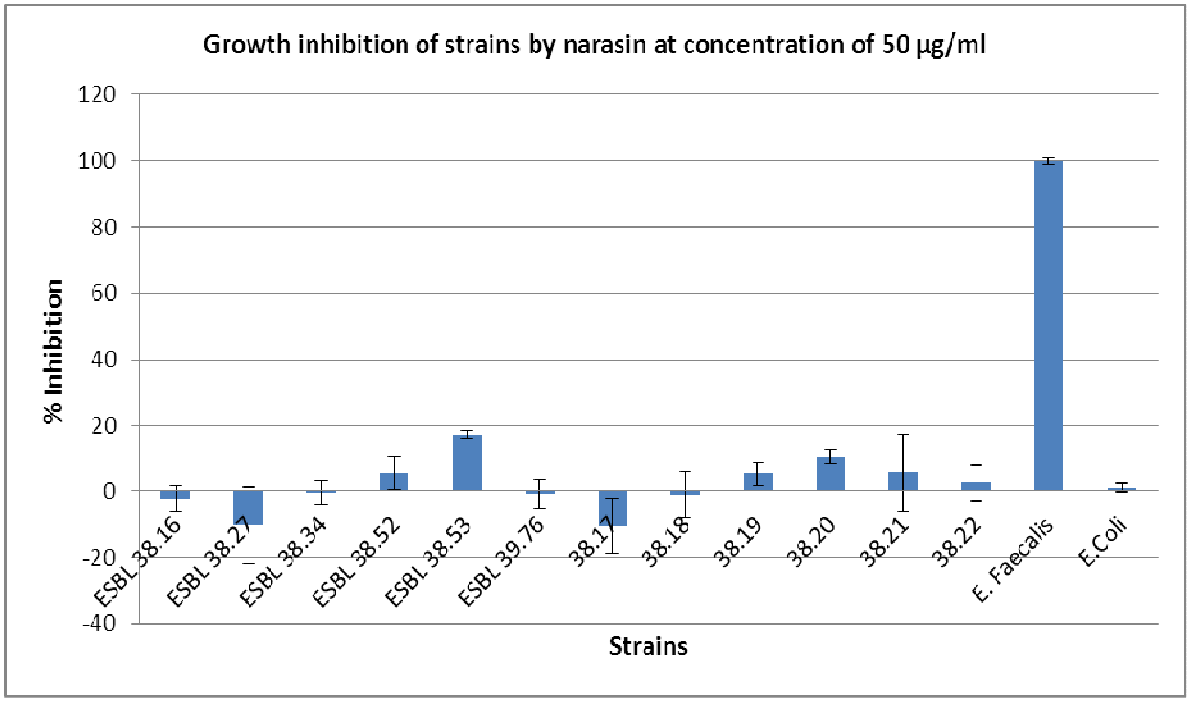
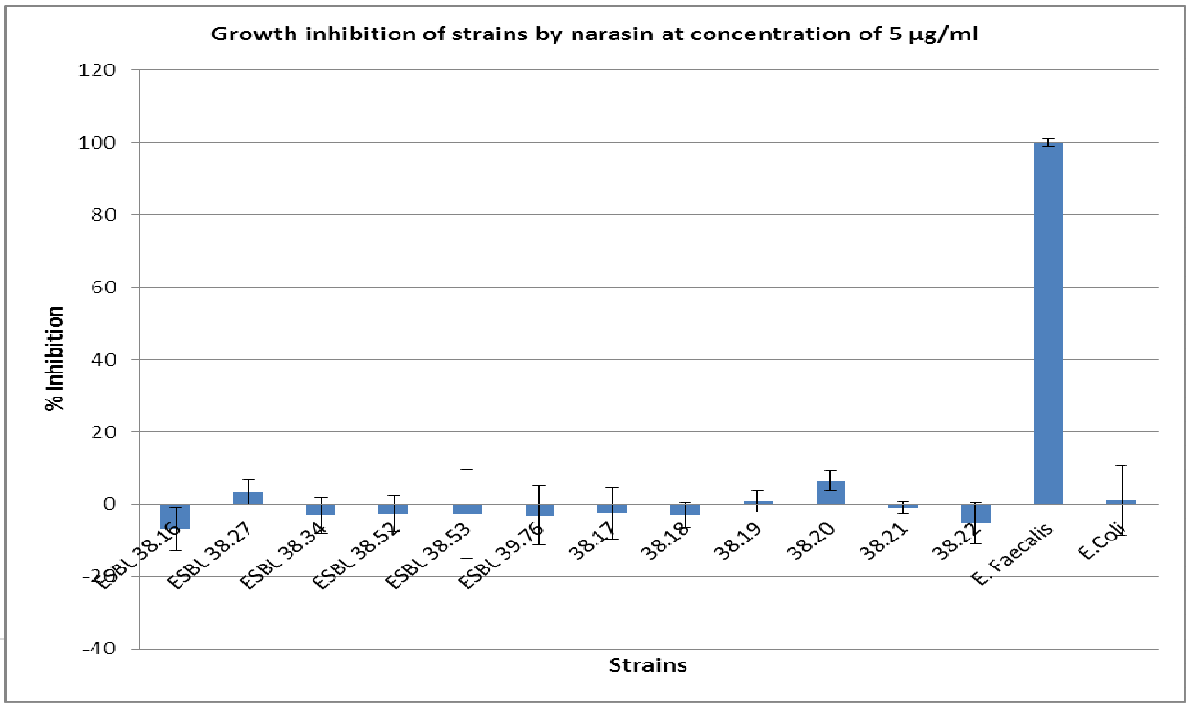


Figure 7. Percentage growth inhibition of strains by narasin at concentration of 50 µg/ml

Figure 8. (Below) Percentage growth inhibition of strains by narasin at concentration of 5 µg/ml



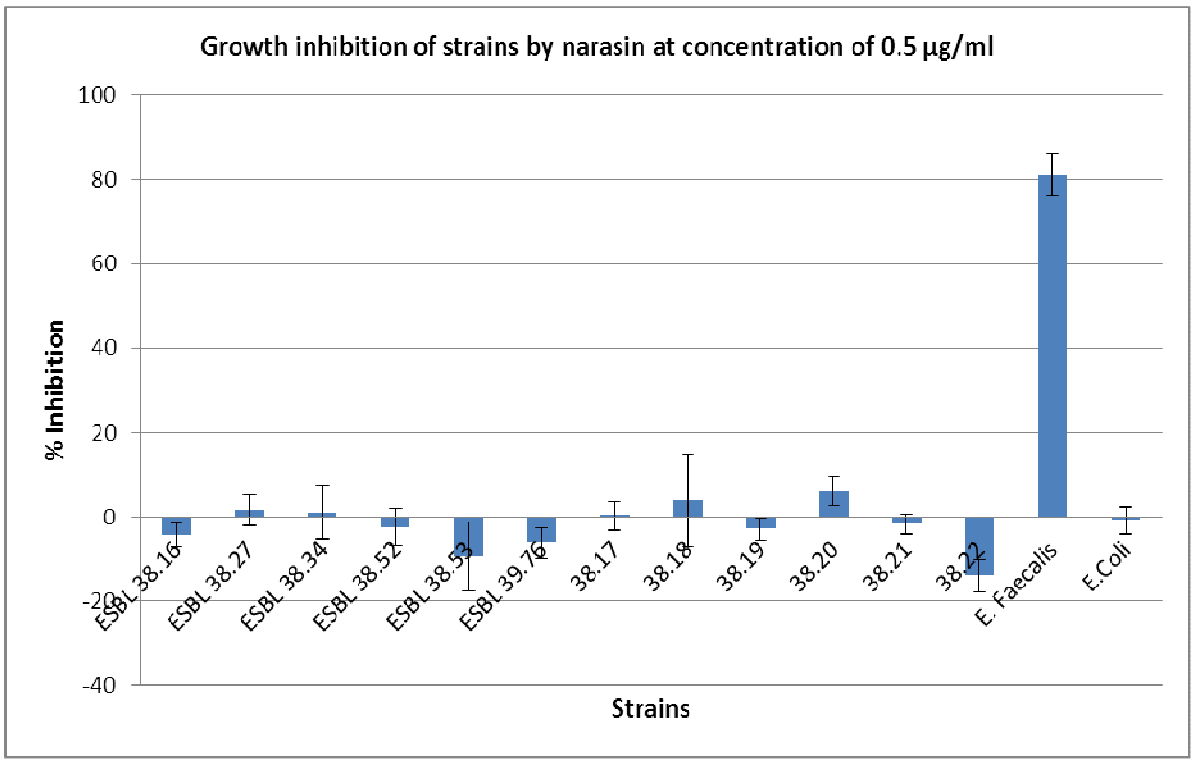


Figure 9. Percentage growth inhibition of strains by narasin at concentration of 0.5 µg/ml

Growth curves of strain 38.17 and 38.53 were made for each concentration to illustrate the growth of a ESBL-negative and ESBL-positive strain, presented in figure 9 to 14.

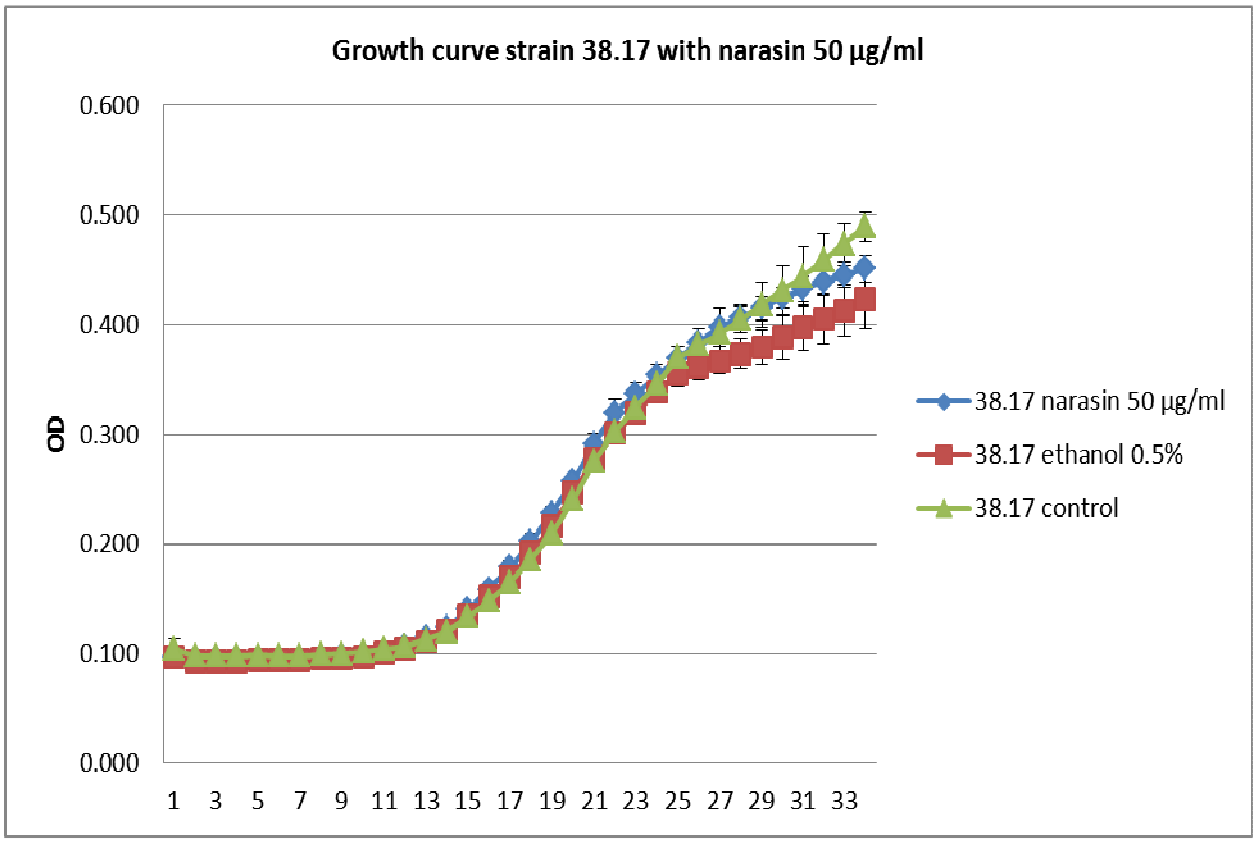


Figure 10. Growth curve of ESBL-negative strain 38.17 with narasin 50 µg/ml

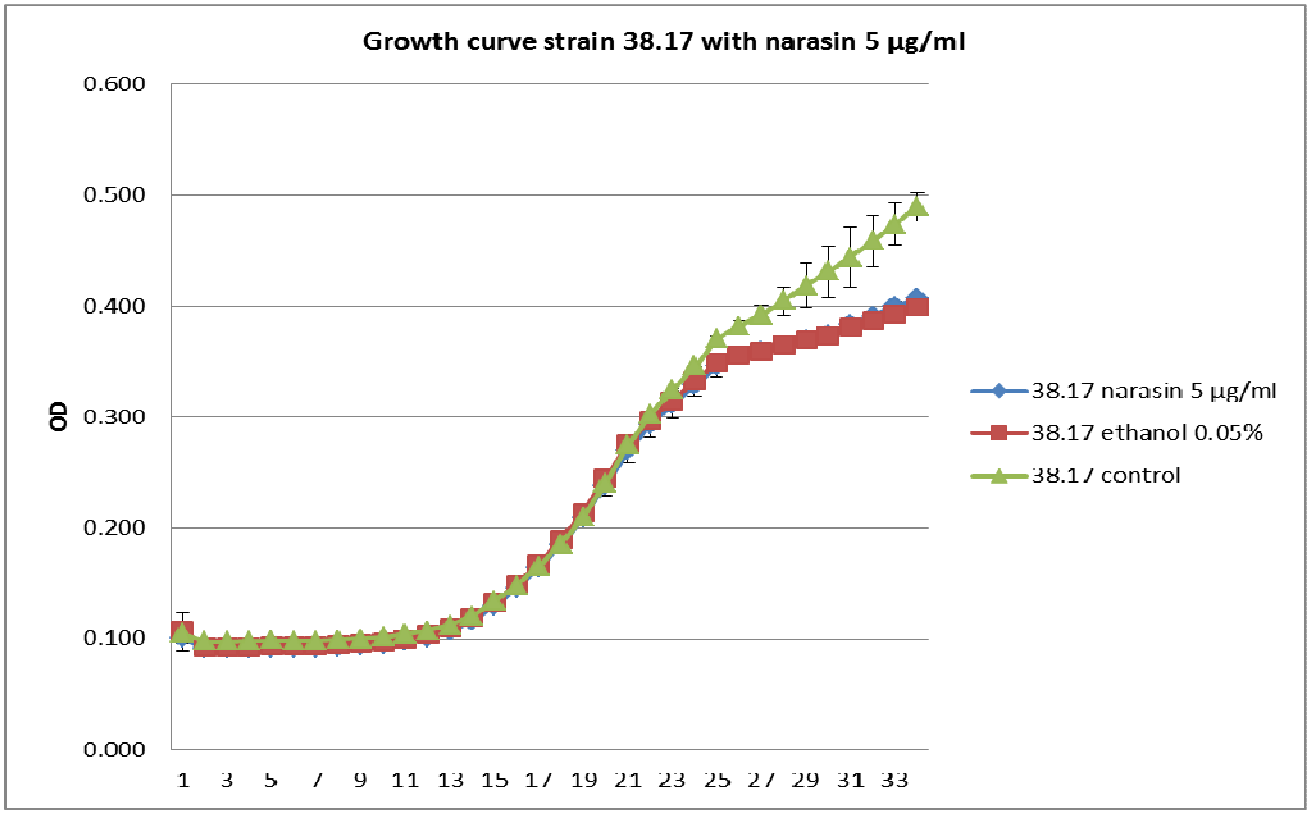
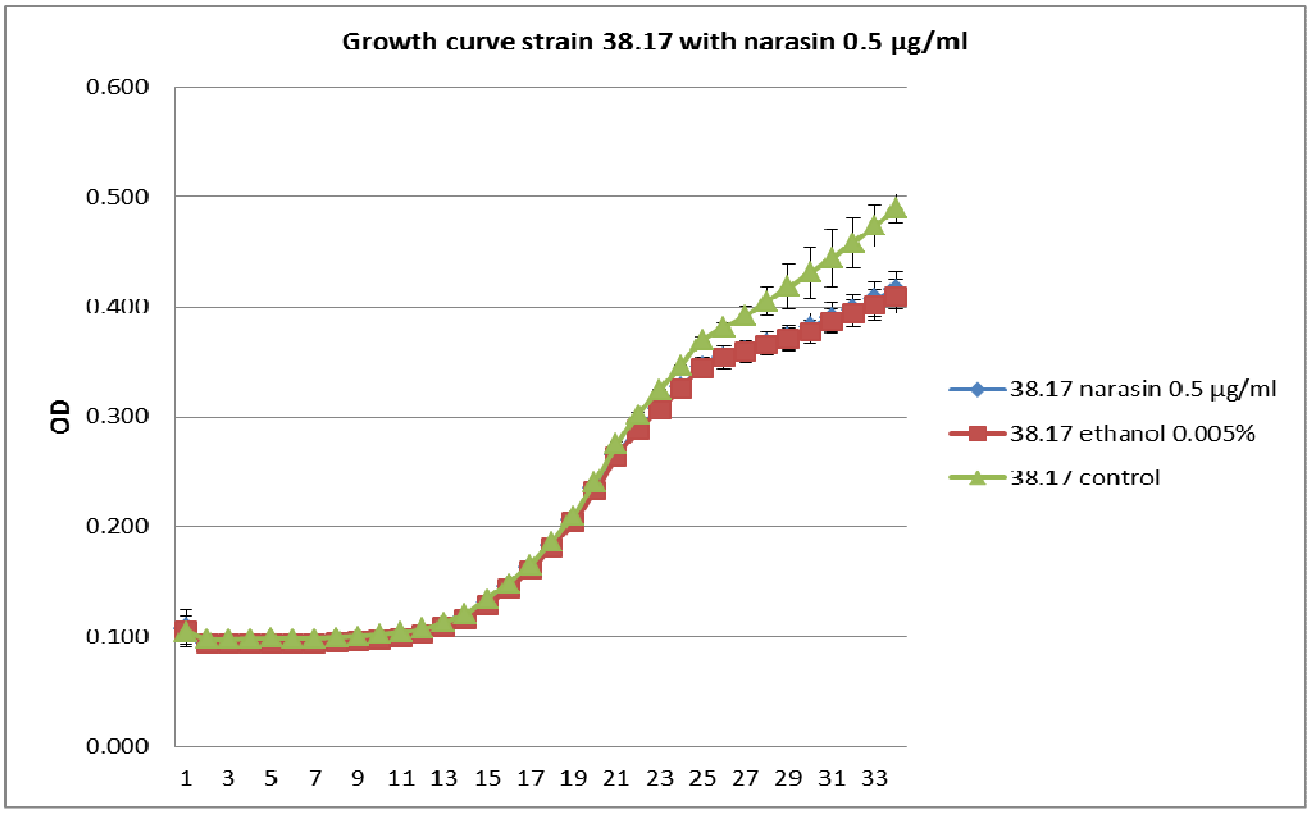


Figure 11. Growth curve of ESBL-negative strain 38.17 with narasin 5 µg/ml

Figure 12. Growth curve of ESBL-negative strain 38.17 with narasin 0.5 µg/ml



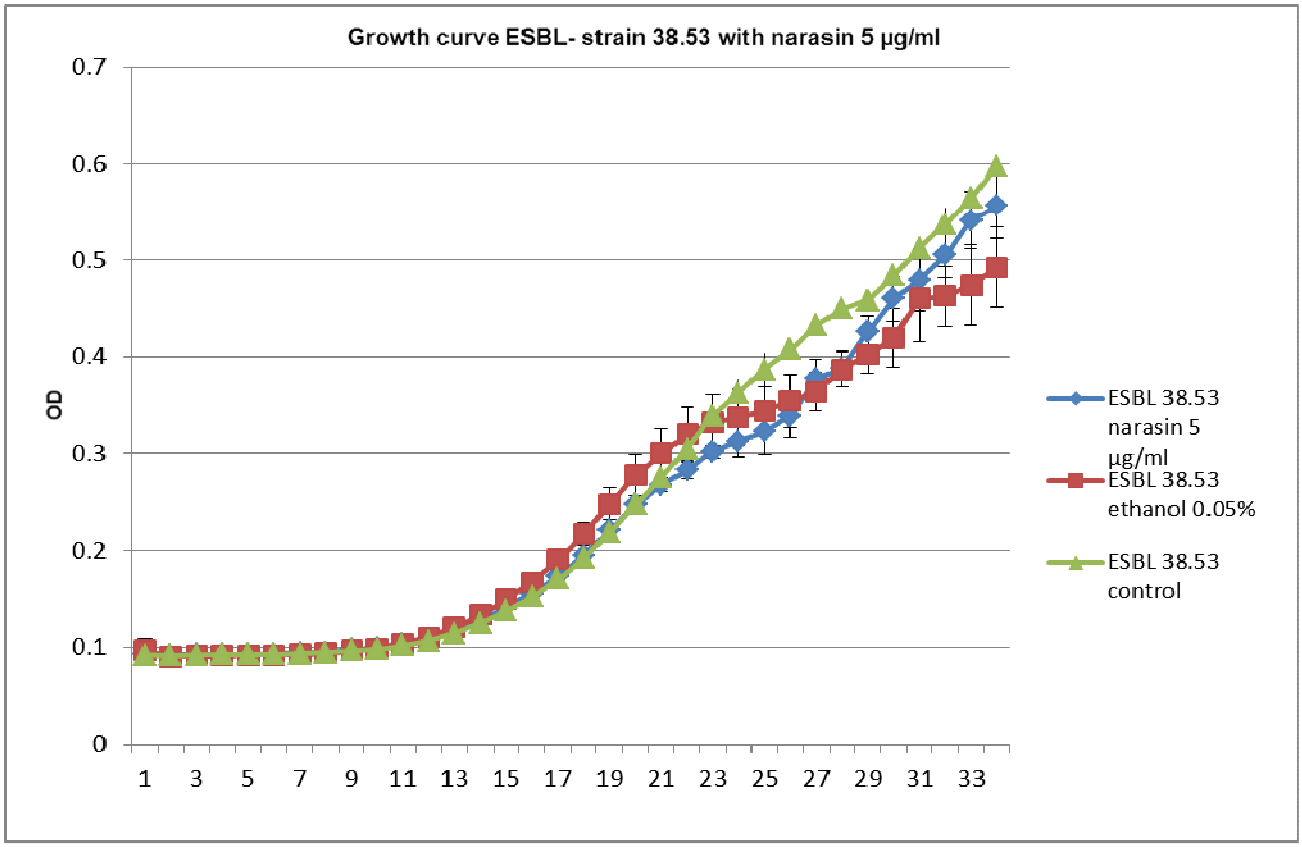


Figure 13. Growth curve of ESBL-positive strain 38.53 with narasin 50 µg/ml

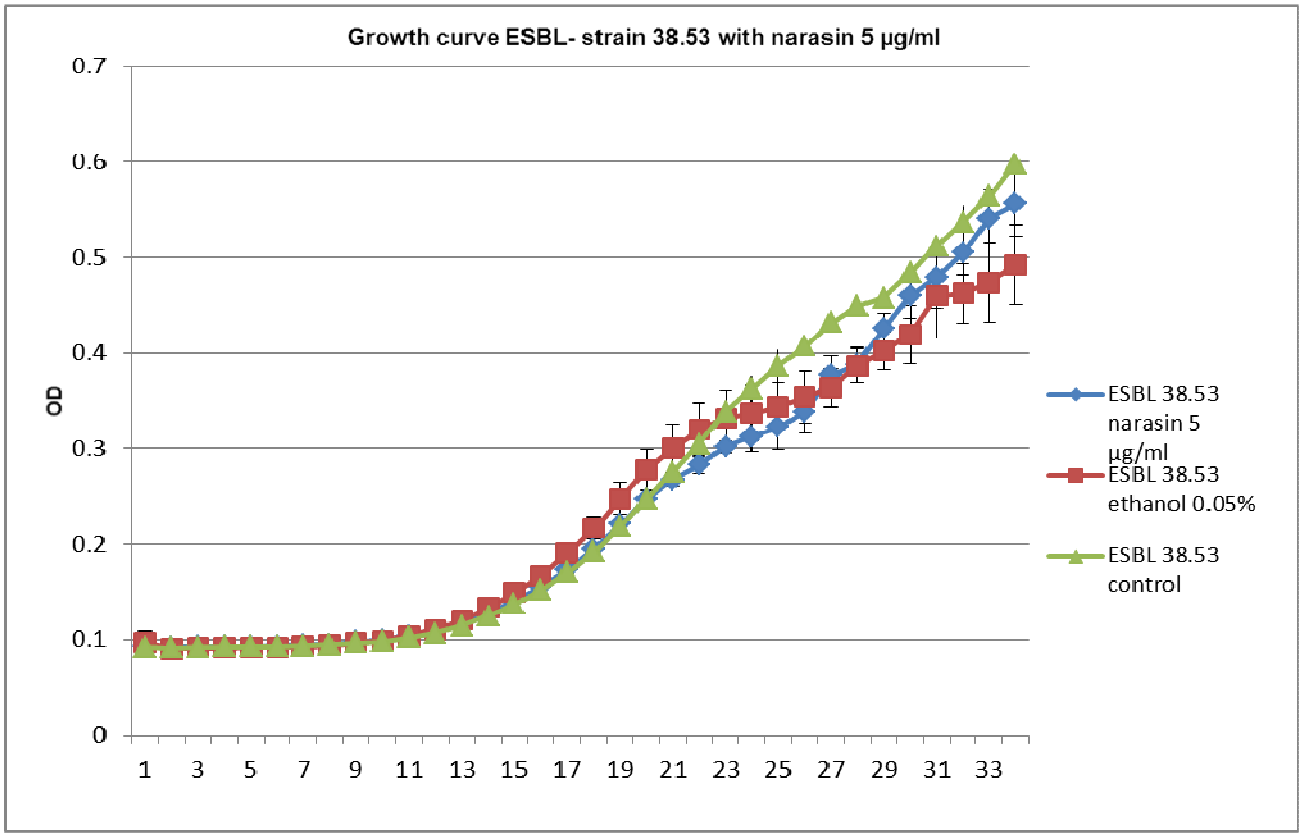


Figure 14. Growth curve of ESBL-positive strain 38.53 with narasin 5 µg/ml

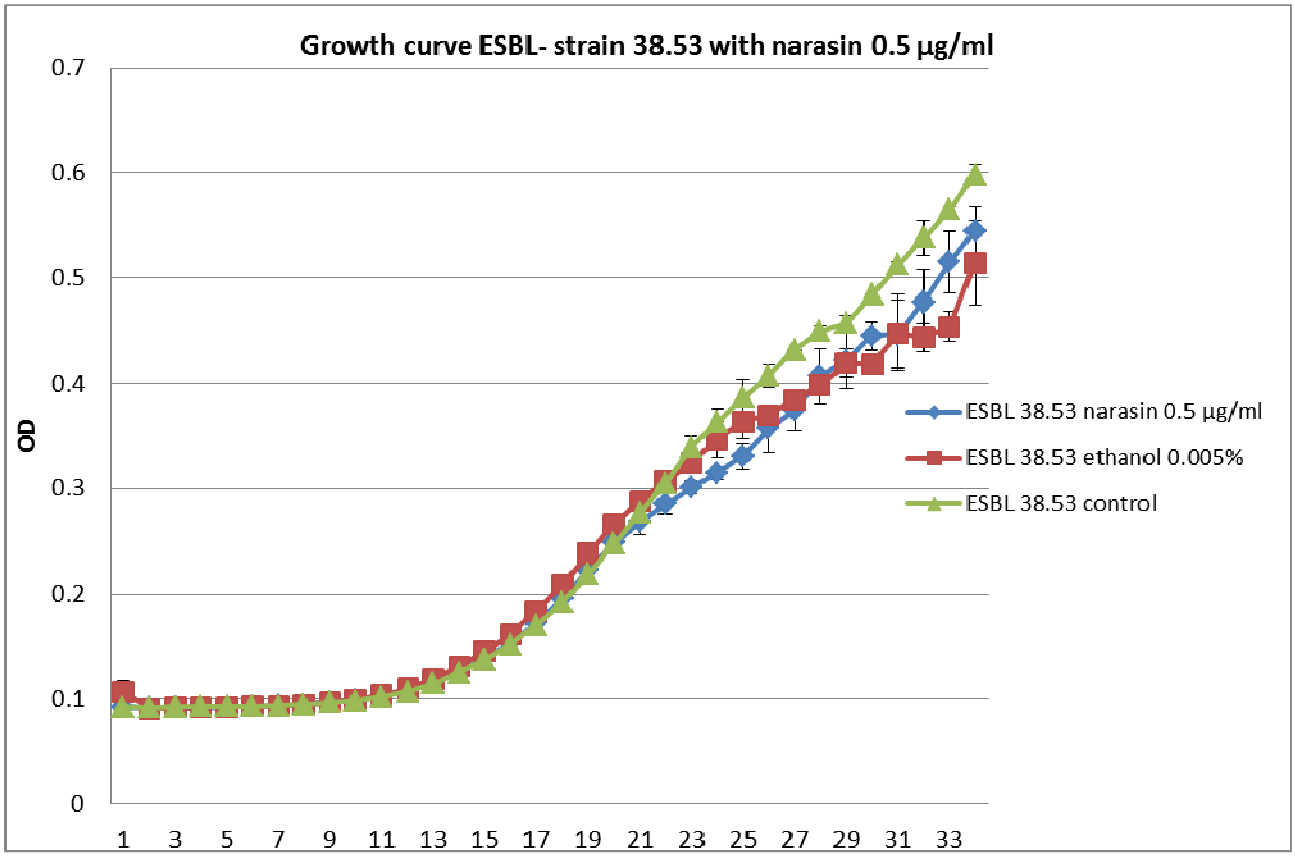


Figure 15. Growth curve of ESBL-positive strain 38.53 with narasin 0.5 µg/ml

3.2.2 Salinomycin Bioscreen experiment

The mean of the areas under the curve (from six growth curves) for all tested strains from the Bioscreen experiment with salinomycin can be seen in table 10. The mean calculated I_A values of each strain for each concentration are presented in table 11 and in figure 15 to 17.

Table 10. Mean areas under the curve (AUC) in Bioscreen experiment with different concentrations of salinomycin or different concentrations of ethanol, and control in MH-broth

	Concentrations salinomycin	125 µg/ml		12.5 µg/ml		1.25 µg/ml		Control (blank)	
ESBL-strains									
		$\bar{x}$	STDEV	$\bar{x}$	STDEV	$\bar{x}$	STDEV	$\bar{x}$	STDEV
38.16	AUC test	67.6	0.4	69.5	1.3	71.0	1.3	74.2	0.5
	AUC ethanol	69.5	2.9	66.5	1.3	67.9	1.9		
38.27	AUC test	58.9	2.1	50.7	1.4	54.1	2.8	26.1	35.5
	AUC ethanol	53.3	0.9	55.4	5.4	52.5	1.5		
38.34	AUC test	41.8	6.4	32.7	2.6	34.2	2.4	44.1	8.8
	AUC ethanol	47.8	0.4	48.0	0.9	48.3	0.4		
38.52	AUC test	61.8	3.1	66.1	5.1	65.7	8.4	70.6	6.5
	AUC ethanol	69.9	1.7	69.8	0.9	71.3	0.6		
38.53	AUC test	53.7	1.5	56.3	1.5	48.7	2.3	54.3	0.7
	AUC ethanol	51.7	2.1	51.1	2.4	50.1	2.1		

39.76	AUC test	43.7	4.0	43.4	0.4	43.8	3.3	47.5	0.7
	AUC ethanol	46.0	1.3	45.9	1.2	45.1	0.4		
Non ESBL-strains									
38.17	AUC test	67.9	16.2	49.8	24.8	58.3	0.6	63.8	1.1
	AUC ethanol	58.9	2.3	60.0	4.8	56.4	2.0		
38.18	AUC test	53.1	4.3	49.1	0.8	51.3	1.7	28.3	29.6
	AUC ethanol	52.0	1.5	49.5	1.0	51.2	0.8		
38.19	AUC test	50.0	6.9	52.7	10.5	59.5	3.1	62.6	1.3
	AUC ethanol	59.1	1.9	59.2	0.9	58.9	1.2		
38.2	AUC test	41.0	8.0	39.3	9.0	41.6	4.3	49.6	7.2
	AUC ethanol	51.7	0.7	53.3	1.8	55.8	9.1		
38.21	AUC test	58.6	0.7	64.9	2.7	60.2	5.8	66.6	0.3
	AUC ethanol	60.0	1.2	64.8	1.3	60.1	1.0		
38.22	AUC test	57.3	1.4	55.4	3.0	53.3	3.3	58.2	1.2
	AUC ethanol	58.0	1.2	62.2	1.4	59.4	1.7		
Control strains									
***E. faecalis* ATCC 29212**	AUC test	-2.1	4.4	-1.2	3.0	0.3	0.2	34.1	2.2
	AUC ethanol	33.3	2.0	33.1	1.1	33.0	1.4		
***E. coli* ATCC 25922**	AUC test	50.3	7.4	54.7	55.0	54.7	54.8	51.3	14.3
	AUC ethanol	53.0	6.1	9.5	5.7	5.9	6.2		

Table 11. Mean percentage of growth inhibition ($\bar{x}I_A$) in Bioscreen experiment by different concentrations of salinomycin

	Concentrations	**125 µg/ml**		**12.5 µg/ml**		**1.25 µg/ml**	
Strains		$\bar{x}I_A$	STDEV	$\bar{x}I_A$	STDEV	$\bar{x}I_A$	STDEV
ESBL-strains	38.16	2.6	3.8	-4.5	0.6	-4.5	1.3
	38.27	-10.4	2.8	7.9	8.4	-3.2	5.8
	38.34	12.5	12.8	31.9	5.2	29.2	5.1
	38.52	11.7	4.2	5.3	6.5	7.8	11.4
	38.53	-4.0	6.2	-10.4	6.4	2.7	5.6
	39.76	5.0	8.0	5.4	2.7	3.0	7.9
Non ESBL-strains	38.17	-15.2	27.1	17.1	41.7	-3.4	3.0
	38.18	-2.1	8.5	0.7	2.2	-0.2	2.4
	38.19	15.6	10.1	10.9	18.1	-1.0	6.7
	38.20	20.7	15.0	26.3	16.0	23.6	14.7

	38.21	2.4	1.0	-0.2	6.3	-0.1	9.8
	38.22	1.3	3.5	11.0	5.8	10.2	5.3
Control strains	*E. faecalis* ATCC 29212	106.6	13.6	103.7	9.2	99.1	0.5
	E. coli ATCC 25922	5.4	6.0	1.0	10.5	0.2	1.6

Some strains, like ESBL-forming strains 38.34, 38.52 and non ESBL-forming strains 38.19 and 38.20 were inhibited by salinomycin. This effect is stronger at concentrations of 1.25 and 12.5 µg/ml than at a concentration of 125 µg/ml and was not consistent. No difference between the group ESBL-positive and ESBL-negative strains was observed. The control strain *E. faecalis* is inhibited at al concentrations, while the control *E. coli*-strain is not inhibited significantly.

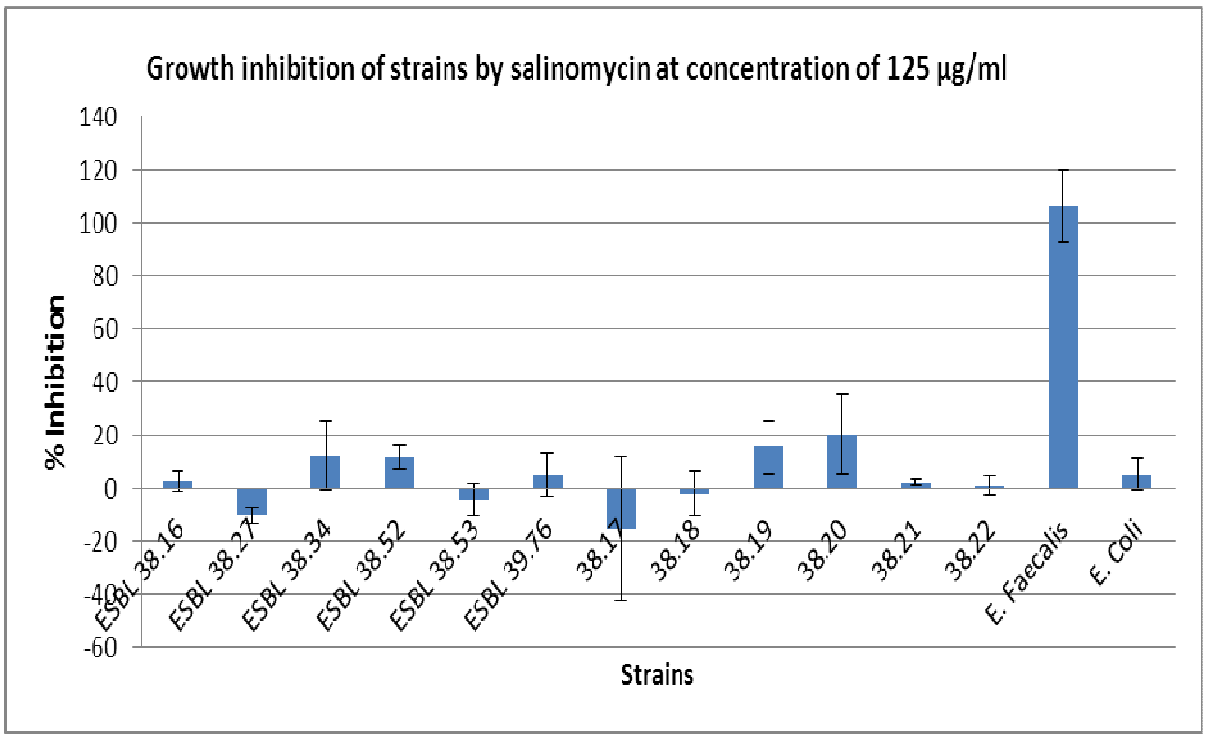


Figure 16. Percentage growth inhibition of strains by salinomycin at concentration of 125 µg/ml

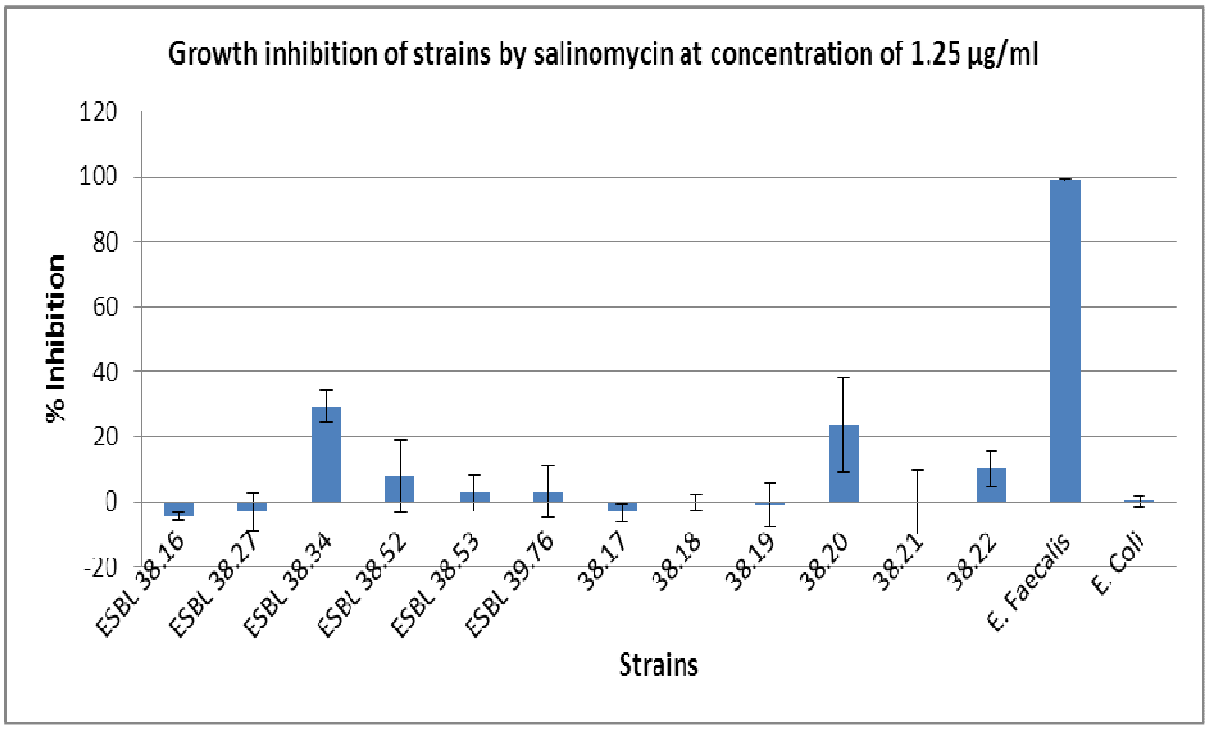


Figure 17. Percentage growth inhibition of strains by salinomycin at concentration of 12.5 µg/ml.

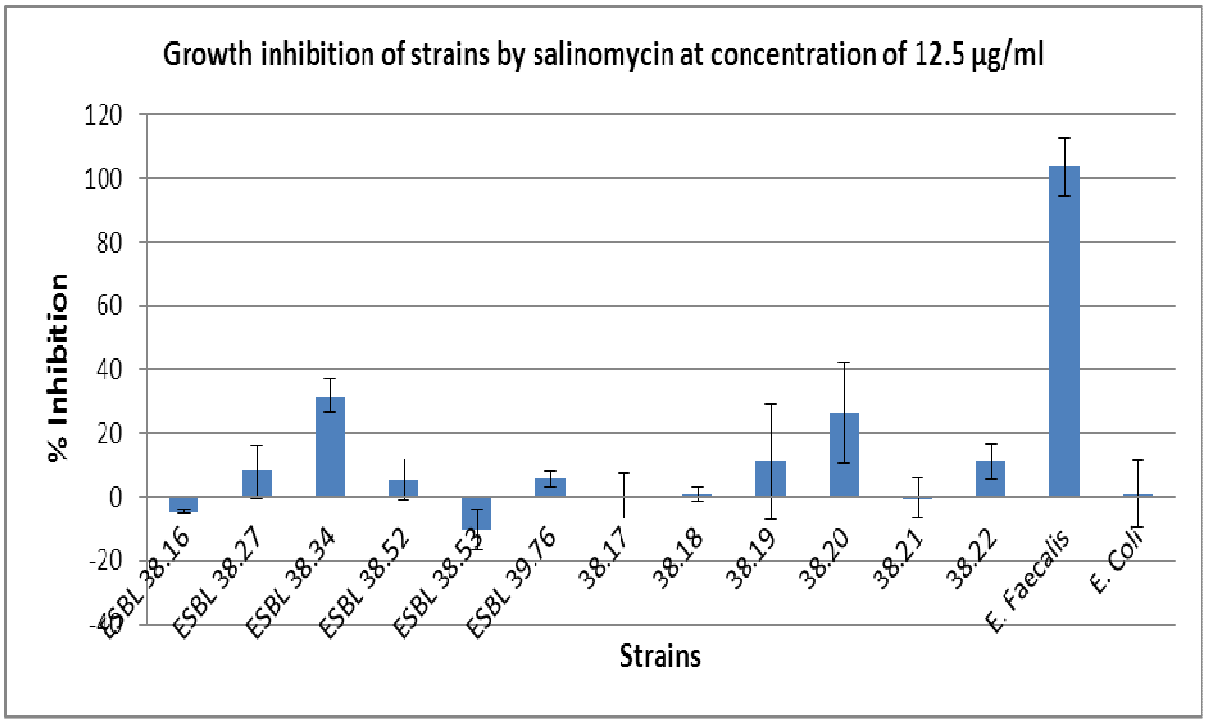


Figure 18. Percentage growth inhibition of strains by salinomycin at concentration of 1.25 µg/ml.

Growth curves of strain 38.34 were made at different concentrations of salinomycin to illustrate the growth of an ESBL-forming strain (figure 18 to 20).

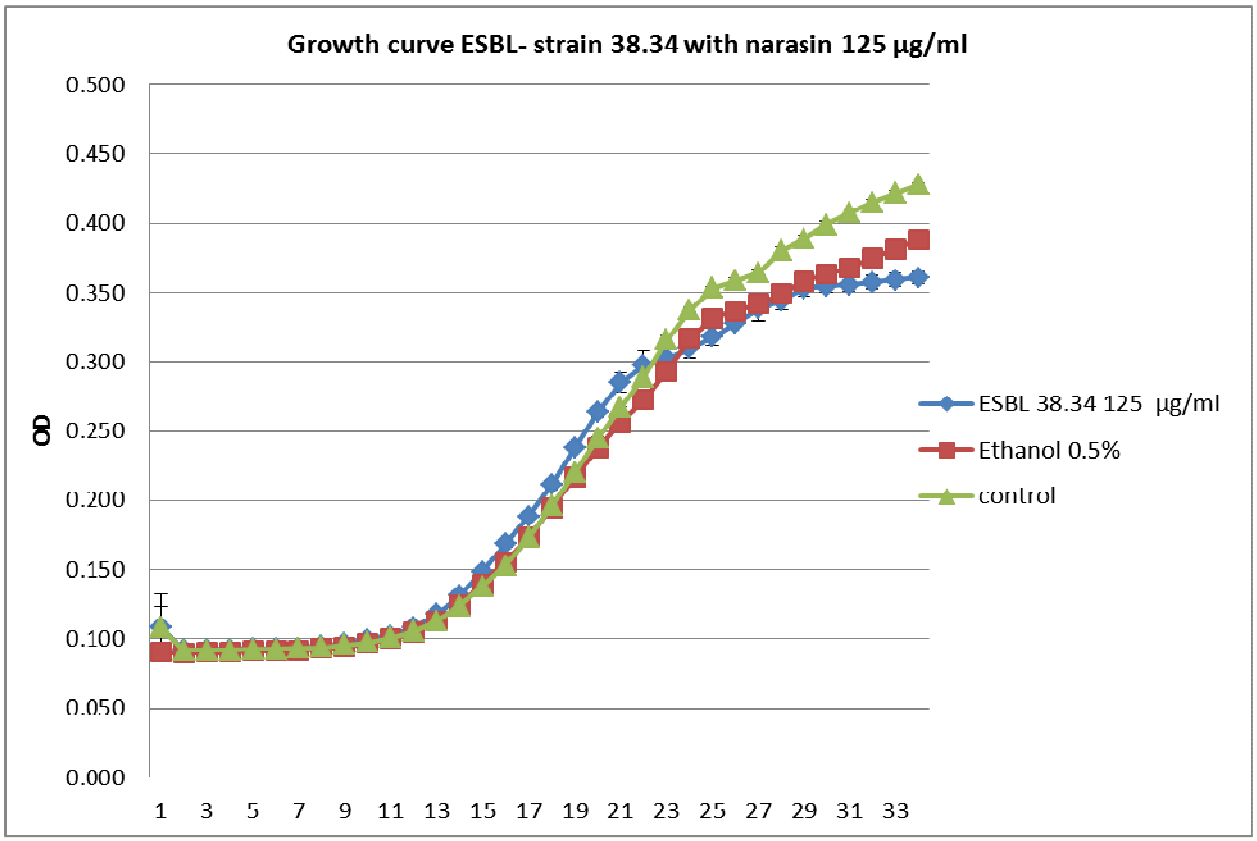


Figure 19. Growth curve of ESBL-positive strain 38.34 with salinomycin 125 µg/ml

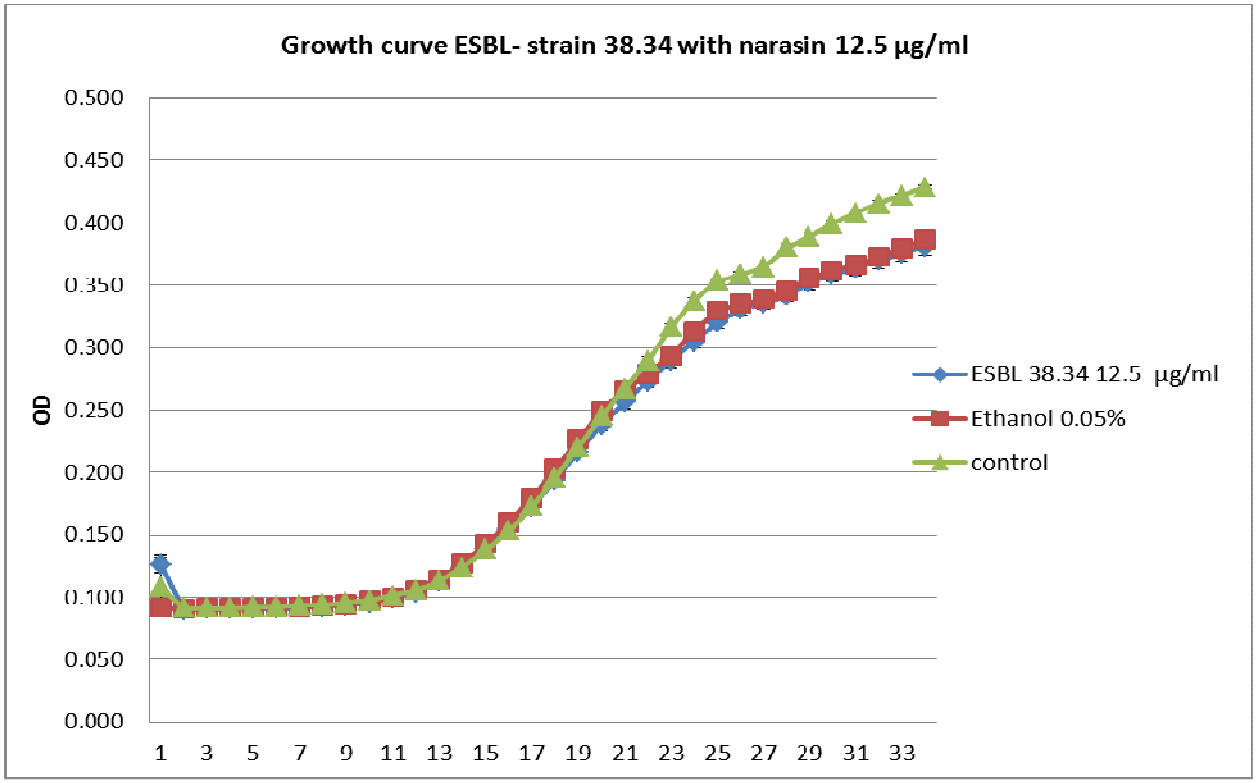


Figure 20. Growth curve of ESBL-positive strain 38.34 with salinomycin 12.5 µg/ml

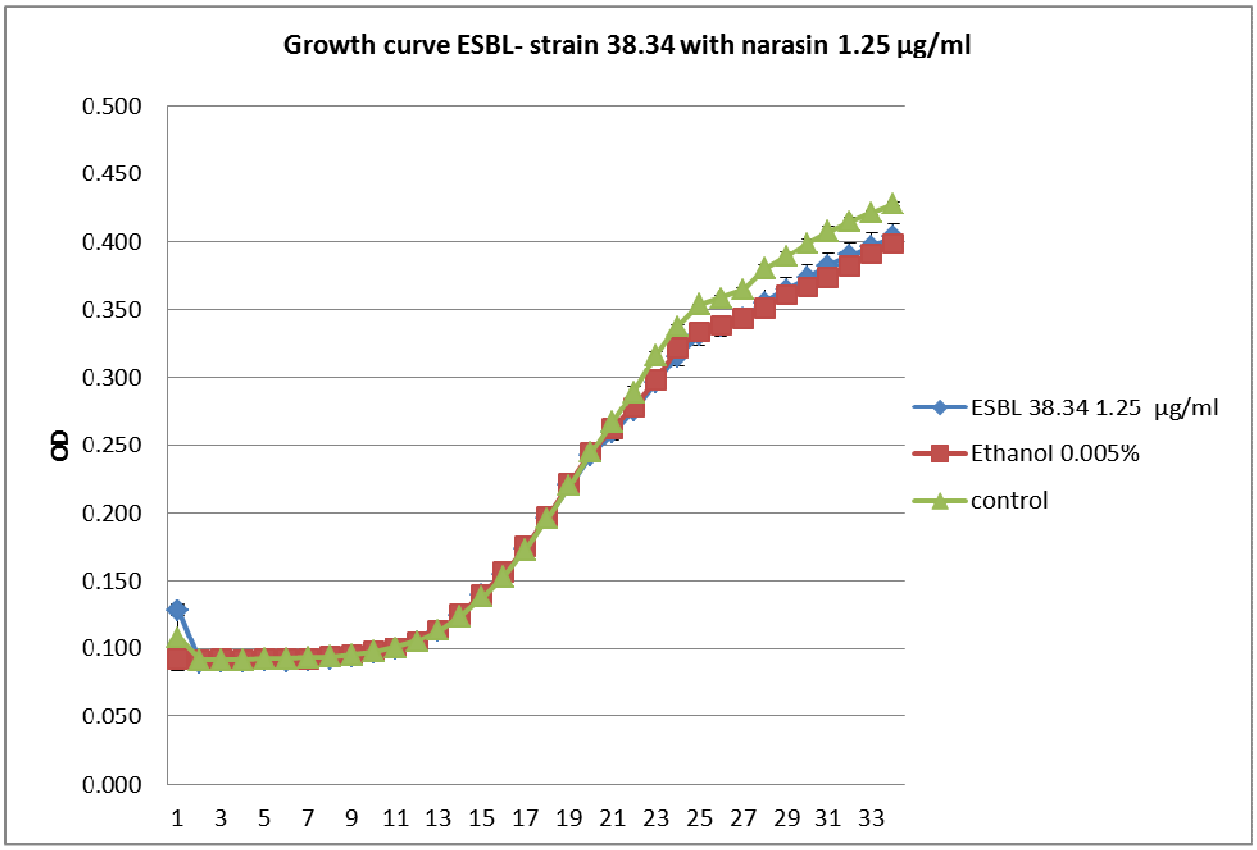


Figure 21. Growth curve of ESBL-positive strain 38.34 with salinomycin 1.25 µg/ml

As can be seen in figure 18 to 20, the growth curves of strain 38.34 is similar for all concentrations of salinomycin. The growth curves of the wells with ethanol and the wells with narasin are almost the same and are a little below the blank control growth curve.

3.2.3 Growth experiment feed

From the bacterial counts that were made by testing strains with the addition of broiler feed, growth curves were made to compare the growth of the strains. The results are visible in figure 21 to 26. No apparent effect of the broiler feed on the growth of the tested strains was observed.

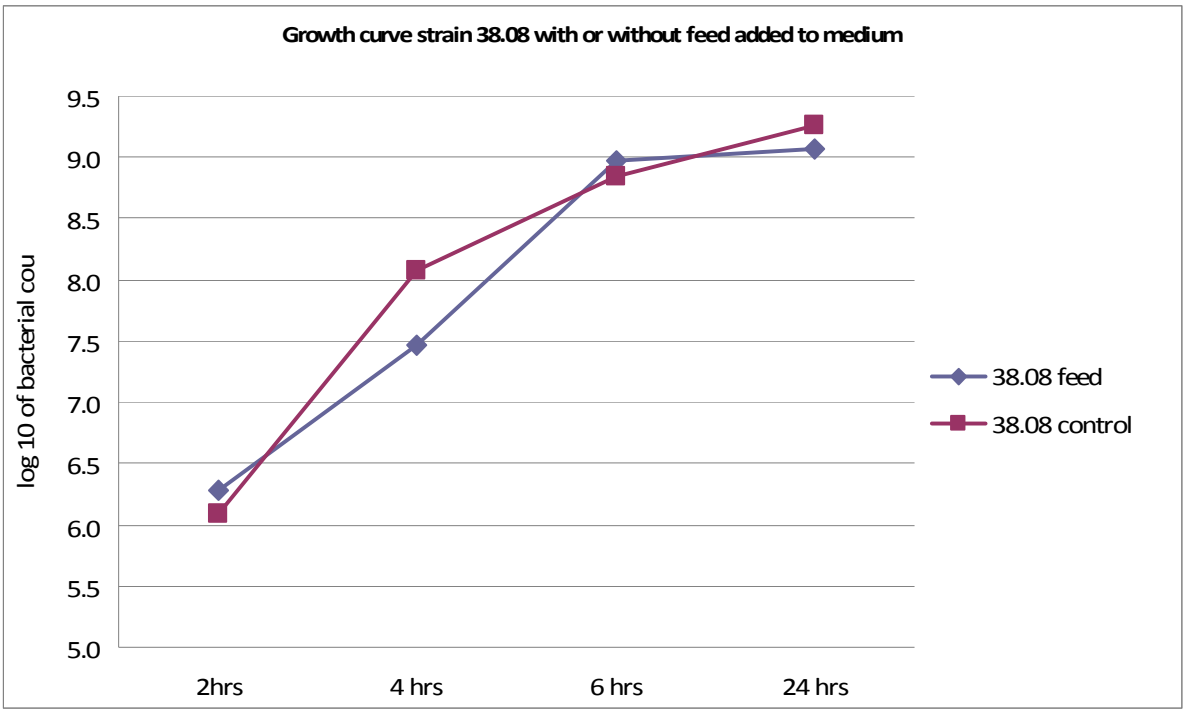


Figure 22. Growth curve of ESBL-negative strain 38.08 with and without feed added to the LB-broth

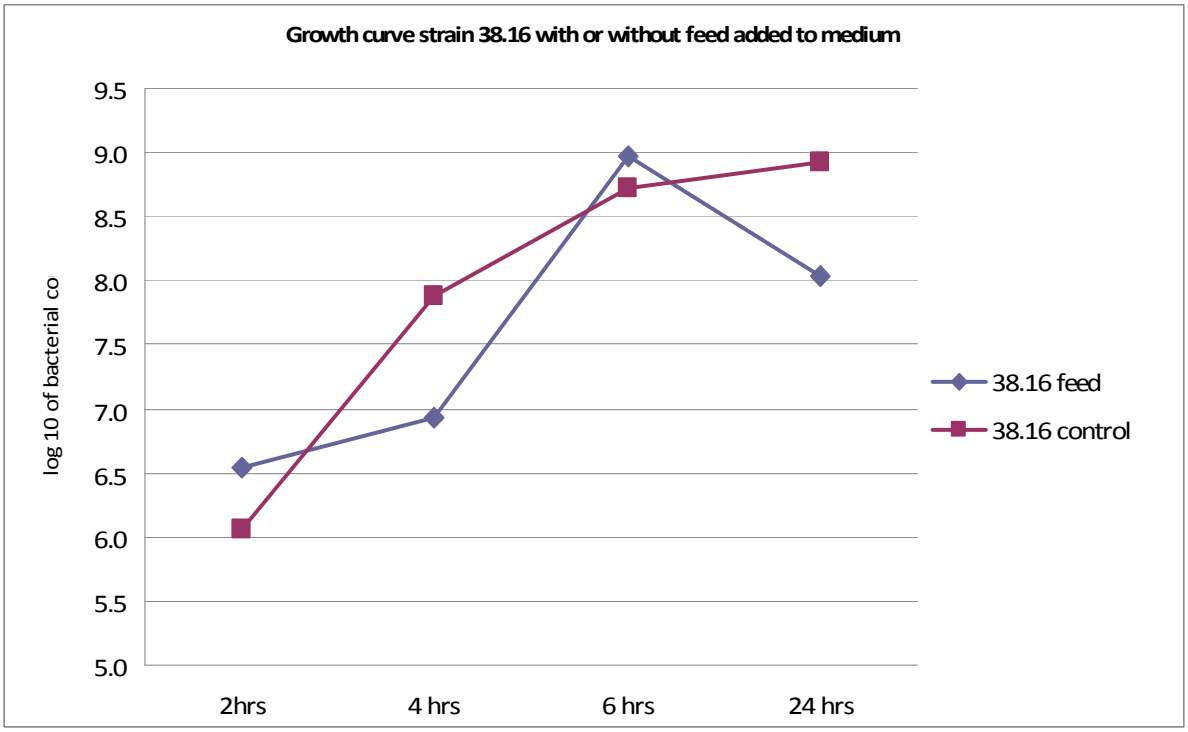


Figure 22. Growth curve of ESBL-positive strain 38.16 with and without feed added to the LB broth

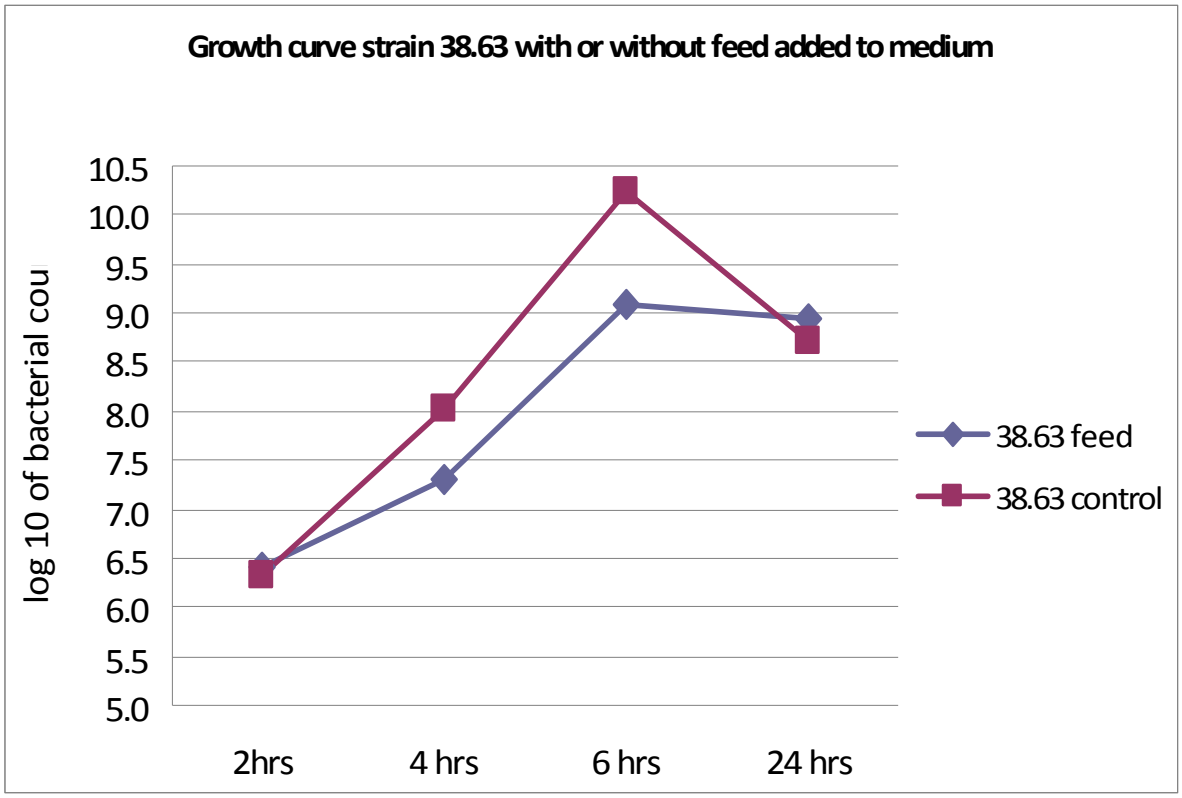


Figure 24. Growth curve of ESBL-negative strain 38.63 with and without feed added to the LB broth

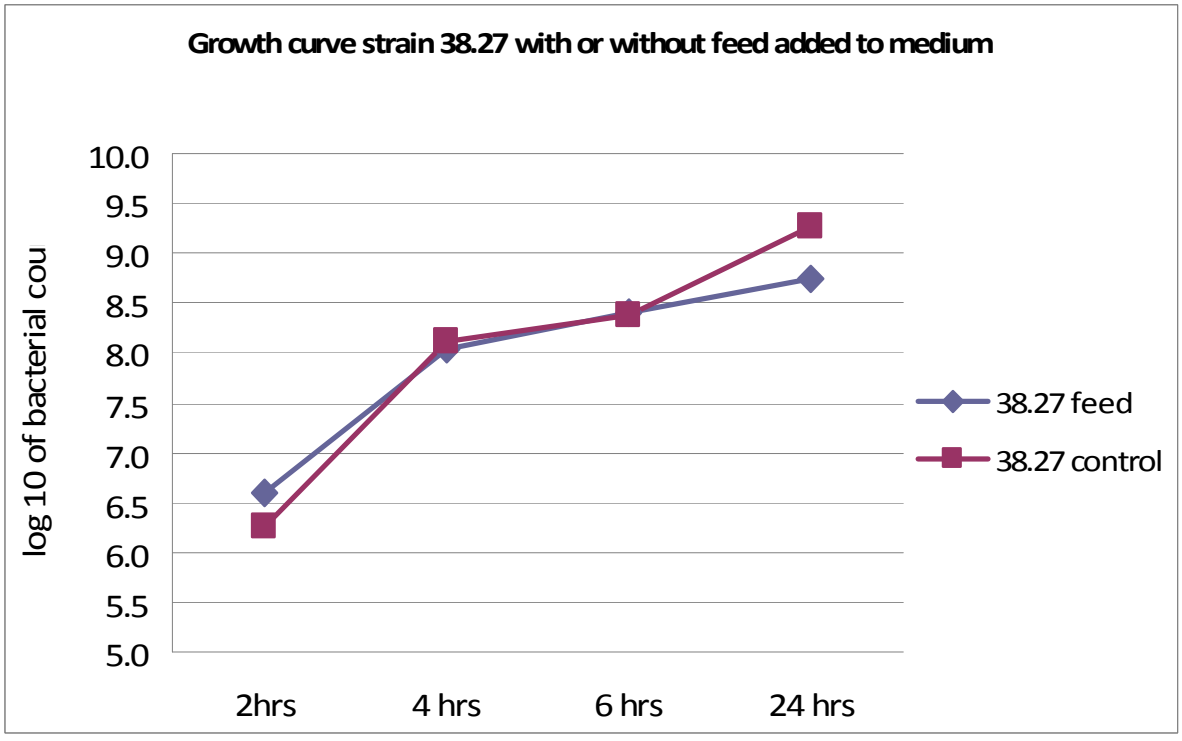


Figure 25. Growth curve of ESBL-positive strain 38.27 with and without feed added to the LB broth

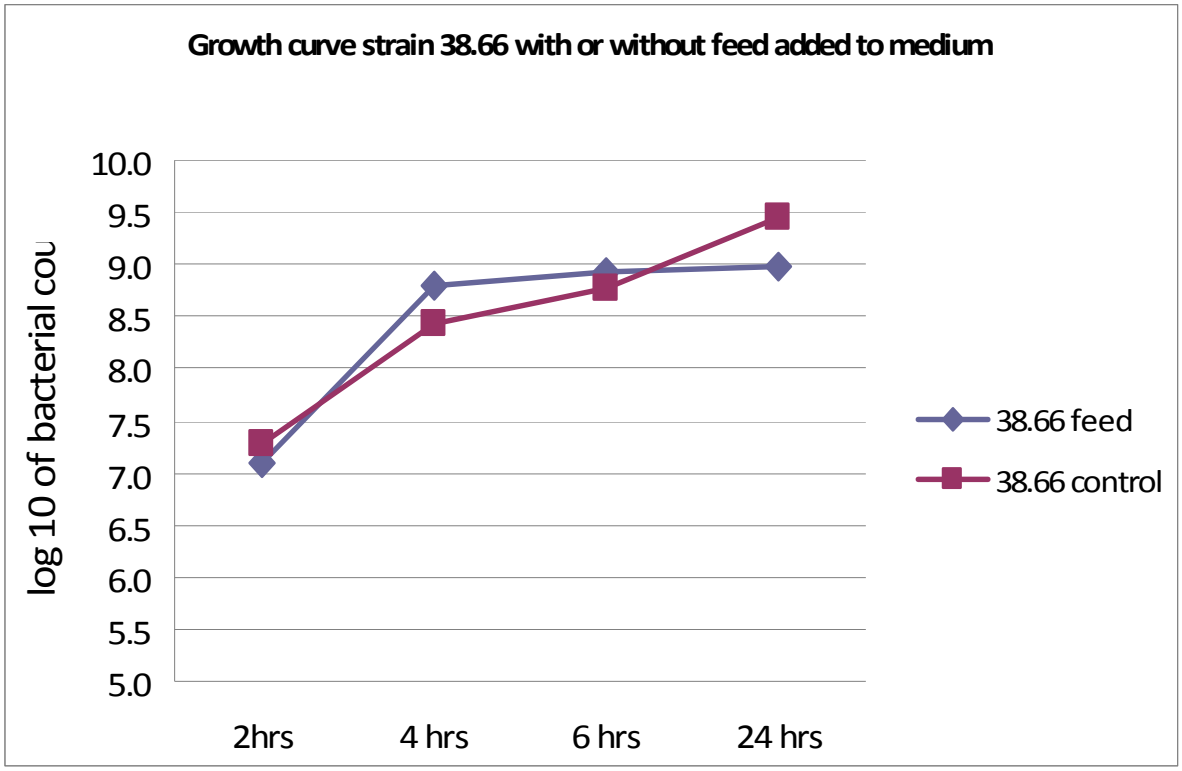


Figure 26. Growth curve of ESBL-negative strain 38.66 with and without feed added to the LB broth

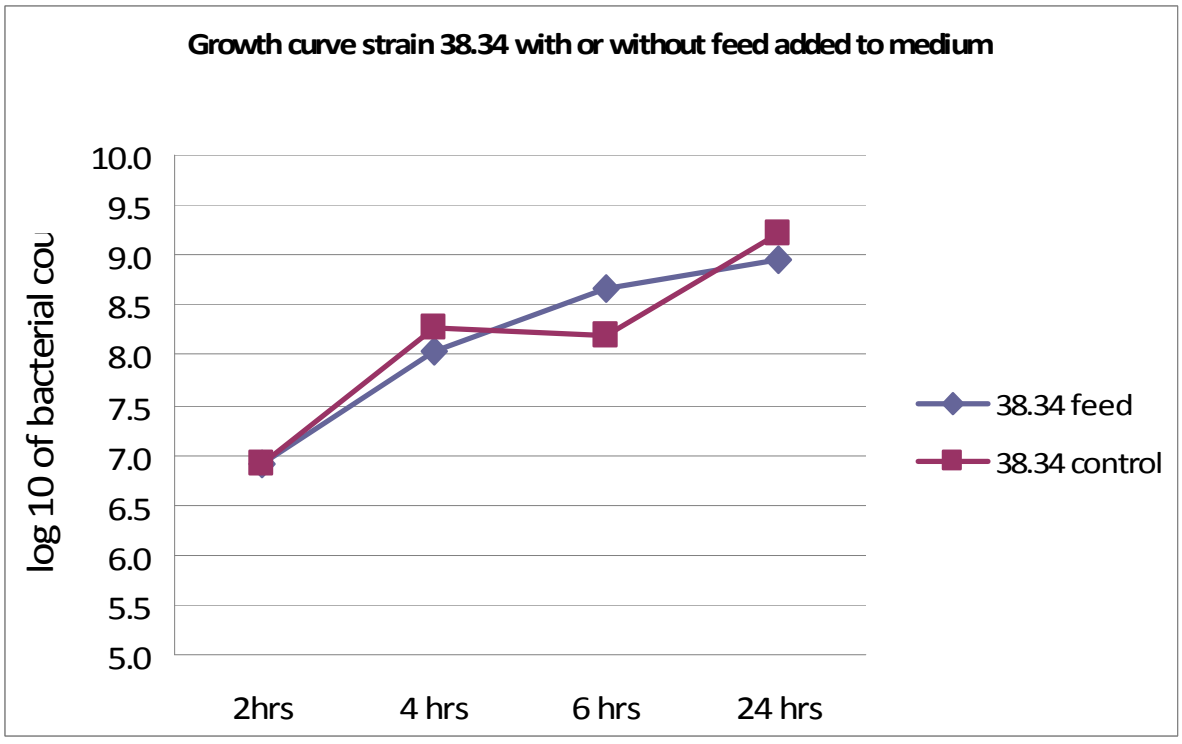


Figure 27. Growth curve of ESBL-positive strain 38.34 with and without feed added to the LB broth

4. Conclusion & discussion

Looking at the results of this study, the following conclusions about the acquired data can be made.

MICs determined with the broth microdilution test. Looking at the results of the broth microdilution test with salinomycin, narasin and monensin, it becomes clear that the selected ESBL-forming and non ESBL-forming *E. coli*-strains were not inhibited by the tested concentrations of ionophores, neither were the control strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603. The Gram-positive control strains *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were both inhibited in growth, but the results between the test performed in duplo were often more than one dilution step different for both strains. It was expected that the Gram-negatives would have intrinsic resistance to the ionophores and would be uninhibited. However, because of the differences in MICs found in the Gram-positive control strains, the broth microdilution test results might not be reliable. For example, the MICs for monensin found in the control strains *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were much too high and did not correspond with MICs found in literature. Carson and Statham found MICs for monensin of 12 µg/ml and 24 µg/ml for *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 respectively, while in this study 128 µg/ml was found in duplo in *S. aureus* and 128 µg/ml and 8 µg/ml were found in *E. faecalis*. Lowicky et al. found a MIC for monensin of 12 µg/ml in *E. faecalis*. The MICs found for narasin in the control strains were considered too high as well. A MIC of 64 µg/ml was found in the Gram-positive control strains, while Butaye et al. found 0,5 µg/ml in both Gram-positive strains and Carson and Statham found 1,5 µg/ml in both strains. An explanation for this could be that the ionophore compounds were not fully dissolved and therefore the active concentrations were lower than the concentrations aimed for. Most studies of ionophores were done in agar instead of Mueller Hinton broth. The MICs obtained in the above mentioned studies from literature are all obtained by methods using agar dilution method, except for the experiment of Carson and Statham. To be sure that the testing conditions would be comparable to those in literature, it was decided to test the MICs again with agar dilution test.

MICs determined with agar dilution test. With the agar dilution test, all Gram-negative strains showed MICs above the highest tested concentrations of ionophores. This was as expected and these test results were more reliable, because the MICs found in the Gram-positive strains were the same in twofold and were in the same range as MICs found in literature. The MIC's of the control strains for salinomycin corresponded with the MIC's found with the broth microdilutions using Sensititre plates. MICs of 1 µg/ml and 0,5 µg/ml were found for *S. aureus*, corresponding with the MIC found with Sensititre plates of 1 µg/ml. For *E. faecalis*, MICs of 0,5 µg/ml and ≤0,125 µg/ml were found and a MIC of 1 µg/ml with Sensititre plates. On the second day of testing with the agar dilution method, the test results were a little different. Some MIC's turned out to be one dilution step different (mostly smaller) than the day before. However, the measurements in duplo were the same and therefore the results were thought to be accurate. When a difference between two following days of tests was observed, one would have expected the MICs to be higher

instead of lower. It was not considered feasible that the ionophores would become more potent after one day in agar, so a likely explanation for this was not available. Remarkable was that for most strains, the highest and lowest concentrations showed a difference in the size of the growth pellets. There seemed to be some inhibiting effect of the ionophores on the tested *E. coli*-strains. From measuring the diameters followed by statistical analysis, it could be concluded that there was an inhibitory effect of the ionophore compounds dissolved in ethanol, but there was no difference between ESBL-forming and non ESBL-forming strains ($p = 0{,}308$, 0.339, 0.836 for narasin, salinomycin and monensin respectively). It is most probable that the observed effect was caused by the ethanol instead of the coccidiostatics, because after performing the Bioscreen experiment, it was concluded that a concentration of 5% of ethanol had strong effects on the growth of the strains. This effect was not visible when the MICs were determined.

Growth experiment with Bioscreen. The results of the Bioscreen test showed no consistent positive or negative effect on the growth of the tested strains and there was no difference between the group of ESBL-positive and group of ESBL-negative strains. The growth curves of the tested strains pointed out that most of the effect on the growth of the tested strains was caused by ethanol. It was expected that the ionophore compounds would not inhibit the *E. coli*-strains, since the MIC determination had shown that the strains were resistant to the ionophores at the tested concentrations. The observation that there was no stimulating effect on the growth of the ESBL-forming strains was a bit disappointing, because that would have provided an explanation for the quick spread of ESBL-forming strains on the broiler farms. Because the inhibiting effect of the tested substances were compared to an ethanol control growth curve, the results are considered as a proper indication of the effects of coccidiostatics on the growth of the tested strains. From the performed ethanol control test with the tested strains, it was concluded that ethanol at a concentration of 5% had strong effects on the growth of the strains. These effects were much less at a concentration of 0.5%, but still visible. For the OD measuring of the Bioscreen, the test medium with dissolved test substances had to be a clear solution and the compound would have to be fully dissolved, so it was not possible to work with a concentration lower than 0.5% ethanol. The objective of this study was to determine if there was a general difference between ESBL-forming and non ESBL-forming strains in effect of the ionophore compounds on the growth. The Bioscreen is considered as a good way to study these effects.

Growth experiment with feed. No obvious effect of the broiler feed on the growth of the tested strains was observed. The dilutions of the LB broth with the feed suspended in it might have led to less accurate dilutions of the bacteria, because the feed parts were not evenly spread through the suspension. Differences between the growth curves are most likely due to the inaccuracy of the dilutions. To get more accurate results, the test would have to be repeated. Furthermore, after performing these growth experiments, it was discovered that broiler feed might still contain large bacterial counts because of the way of sterilisation. The feed is heated by the manufacturer, but this leads to a reduction of log 1 or 2 of the bacterial count. It is possible that bacteria in the broiler feed affect the growth of other strains or might influence the spread of ESBLs. However this has not been tested.

More research will have to be done to detect which factors are important in the spread of ESBLs. However coccidiostatics as feed supplementation remain an interesting subject of study in this matter, because they have antibacterial activity and might influence antimicrobial resistance of *Enterobacteriaceae.* Theoretically, ionophores might cause a more favourable position of Gram-negatives among the competitive micro flora of the broiler's intestines, because of their antibacterial effect on Gram-positives. But an *in vivo* experiment with *Salmonella enterica* subsp. *enterica* serotype Typhimurium showed that changes in antimicrobial sensitivity of *Salmonella* can be observed independently of ionophore anticoccidial agents in the feed of broiler chickens (Scalzo et al., 2004). Scalzo et al. found no significant decrease in *Salmonella* shedding caused by the administration of a diet containing 120 mg/kg monensin, 60 mg/kg salinomycin or semduramicin (the latter tested at a dose of 20 mg/kg and 25 mg/kg). Another *in vivo* study showed that administration of 60 mg/kg Salinomycin Sodium (Sacox®) caused a slight decrease in shedding of *Salmonella enteritidis* at 6 weeks of age (Bolder et al., 1999). Even if there is an effect of anticoccidial agents on Gram-negative bacteria in the gut, this wouldn't explain selection of ESBL-forming *E. coli* specifically. In this study, genetic differences between ESBL-forming isolates were not taken into account, because the intent of the research was to observe a difference in effect between both groups. It might be interesting to perform another Bioscreen experiment with an ESBL-forming strain and a non ESBL-forming strain which are genetically identical and compare the growth curves with or without the addition of ionophores. It is possible to produce ESBL-forming strains from sensitive strains, but it would be hard to obtain completely sensitive strains from Dutch broiler chickens. Another future project could be to put an ESBL-forming and a non ESBL-forming strain together with feed added to the medium, to see if the ESBL-forming strain has an advantage in growing under these conditions. Furthermore, coccidiostatics or other substances in the feed might influence the transmission of plasmids among the bacterial flora of the gut, which we did not investigate in this study. Coccidiostatics have a bactericidal effect on Gram-positive bacteria, because the function of the bacterial membrane is affected. Gram-negative bacteria have intrinsic resistance to ionophores because their membranes cannot be penetrated by the ionophore compounds. However, salinomycin in combination with citric acid has been described to affect Gram-negative bacteria as well, because this acid damages the bacterial membrane. The function of the bacterial membrane might affect the transmission of plasmids among bacteria. Organic acids are administered to broiler chickens in the drinking water and are used to eliminate micro-organisms in the drinking water systems. Another interesting follow-up of this study would therefore be to investigate the transmission of plasmids under different testing conditions, with or without the addition of broiler feed, or with or without adding ionophore compounds (possibly with addition of organic acids).

To conclude, the objective of this study was to test the *in vitro* effect of ionophores on growth of ESBL-producing *E. coli* and to identify substances in the broiler feed that might enhance the growth of ESBL-forming *E.coli* and cause rapid spread of ESBL-producing *E.coli*. From the performed tests, it can be concluded that there was no difference in growth observed between the groups of ESBL-forming *E.coli* and non ESBL-forming *E.coli* when salinomycin, narasin or monensin were added to the growth medium. When whole pellets of

broiler feed were added to the test medium, no difference in growth was observed either. Hence no new discoveries about the cause of the excessive spread of ESBLs on the broiler farms are made and further research is necessary.

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