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Lack of host specificity in gut colonization ability of human and chicken *Escherichia coli* isolates.

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HOJA DE APROBACIÓN DE TESIS

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RESUMEN

El objetivo de este estudio fue investigar la posible existencia de ecotipos de *E. coli* específicamente adaptados a los intestinos de mamíferos y aves. Los aislados de *E. coli* de pollos y humanos fueron sometidos a crecimiento en medio mínimo lactosado y se les permitió colonizar los intestinos de pollos. Los aislados humanos crecieron significativamente más rápido en el medio mínimo lactosado ($p=0.0188^*$); sin embargo, no se observaron diferencias cuando un aislado humano y un aislado de pollo colonizaron el intestino de los pollos. Los resultados de esta investigación muestran que los aislados de pollo crecen menos que los aislados humanos en un medio lactosado presumiblemente debido a que los primeros no han sido expuestos a leche como los segundos. Adicionalmente, parecería que las *E. coli* de pollos y humanos pueden colonizar indistintamente el intestino de uno u otro hospedero lo cual resalta la adaptabilidad de esta bacteria a diferentes ambientes.

ABSTRACT

The aim of this study was to investigate the possible existence of *E. coli* ecotypes specifically adapted to mammalian and avian intestines. *E. coli* isolates from chickens and humans were subjected to growth in lactose minimal media and allowed to colonize chicken's intestines. Human isolates grew significantly faster ($p=0.0188^*$) in lactose minimal medium; however, no differences were observed when one human and one chicken isolate colonized the chicken intestines. Results of this research showed that chicken isolates grew slowly comparing with human isolates, likely because the first were not exposed to milk as the second one. In addition, apparently chicken and human *E. coli* can equally colonize different host intestines which sign out the adaptability of this bacteria to different environments.

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Part I. GENERAL INTRODUCTION

Antimicrobial resistance in poultry

Increase of antibiotic resistance is a global public health concern. In most cases, it is related to improper use and abuse of antimicrobial drugs in livestock production (McEwen and Fedorka-Cray, 2002; Mellon et al., 2001; Sherwood and Gorbach, 2001). However, it has also been related with antibiotic use as growth promoter in poultry. Antibiotic use constitutes a selective pressure favoring antibiotic-resistant bacteria growth. Eventually, those resistant microorganisms could colonize other hosts such as human while carrying their genetic traits. Moreover, those genetic traits of resistances could be transferred by horizontal gene transmission to pathogenic bacteria into new host (Scott, 2002; Moubareck et al., 2003; Niederhäusern et al., 2011). For instance, after two years of using streptothricin as food additive in pigs, *Escherichia coli* resistant to this antibiotic were found in their intestinal microbiota. Those isolates had a transposon coding for streptothricin acetyltransferase. Even though the use of this antibiotic was stopped, the resistance disseminated to pig farmers, their families and other members of the community. It was found in urinary tract infections and in other pathogens such as *Salmonella* and *Shigella* sp. (Witte, 2000).

Another example was avoparcin, a glucopeptide structurally similar to vancomycin, which was used as growth promoter in poultry. Vancomycin resistant *Enterococcus faecium* was found in intestinal flora of food animals from farms that used avoparcin and it was transmitted to farmers and later to community members. When avoparcin was banned, a reduction of resistant *Enterococcus* was observed in Europe (Witte, 2000; Torres and Zarazaga, 2002; Sorum et al., 2004; Johnsen et al., 2005). However,

the vancomycin resistance gene *vanA* was located on transposon *Tn*1546 and integrated in a conjugative plasmid which prompted the transmission of this gene to other bacterial species such as the human pathogen *Staphylococcus aureus*. For instance, Niederhäusern and collaborators (2011) demonstrated that vancomycin-resistance was transferred in two conjugations out of 25 matings performed. The transfer was confirmed by PCR and it occurred from *E. faecalis* EMM09 to *S. aureus* STM359 and from *E. faecalis* EMB04 to *S. aureus* STM17.

As described previously, the transference of antibiotic resistance genes to humans from other animals may depend on microbial cross colonization, which may be hampered by microbiota's adaptation to specific hosts. Therefore, host specificity is the evolution of bacterial ecotypes adapted to different gut environments. It means an ecologically distinctive bacterial group with their own evolutionary lineage, its own evolutionary tendencies, and historical fate (Cohan 2002). Some examples of *E. coli* ecotypes are: commensal *E. coli*, extra-intestinal *E. coli*, entero-hemorrhagic *E. coli*, etc. (Chattopadhyay et al., 2009).

E. coli ecotypes in human and avian intestinal tracts.

There are anatomical, physiological, nutritional, and microbial differences between the gastrointestinal tract of mammals and birds. Besides, mammals (such as humans) and birds (such as chickens) have large anatomical differences in their gastrointestinal tracts: for example, the human digestive system consists of a mouth, esophagus, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (caecum, ascending colon, transverse colon, descending colon, sigmoid colon), and anus. Accessory digestive organs include salivary glands, pancreas, liver, and gallbladder. In contrast, avian digestive tract consists of a mouth (with a beak), esophagus, crop,

proventriculus, gizzard, small intestine (duodenum, jejunum, and ileum), ceca, large intestine (colon), and cloaca (Pescatore and Austin, 2011; Johnson, 2012).

In addition to their physical differences, they also differ physiologically. In human mouth, food is processed both mechanically (chewed or masticated) and enzymatically (ptyalin), while in chickens, food is collected by the beak and swallowed to the crop to be accumulated rapidly. Second, in humans, the stomach acts as a reservoir for food and also contributes to mechanical and enzymatic digestion. Internal conditions inside the stomach are strongly acidic. In contrast, chickens need a number of different organs to accomplish the same function. For instance, food is accumulated in the crop, later proventriculus act as enzymatic digestive organ while the gizzard is in charge of mechanical digestion. Finally, the urinary system in humans is separated from the digestive system; to the contrary chickens have both systems connected in the final section of the large intestine. The chicken cloaca receives a mixing of digestive wastes together with urinary system wastes (urates-uric acid and ammonia). Fecal material is usually white covered with uric acid crystals on the outer surface (Riddle, 1999).

Moreover, there are nutritional differences between human and chickens. It is very important to note that humans' diet in their first months of life is restricted to milk, which contains lactose as a primary sugar source. Later in their life, many humans continue drinking milk because it is a good source of fat, proteins, vitamins, minerals, water, and carbohydrates such as lactose; however, milk is not an indispensable nutritional requirement for human adults. Birds, on the other hand, do not drink milk in any stage of life. This means that they never receive lactose as a sugar type.

In addition, those nutritional differences could be related with the particular microbiota of human and chickens. The most abundant microbial phylum in human and chicken

intestine is Firmicutes (80% and 70% respectively). Within Firmicutes, 95% of sequences are members of the Clostridia class (Eckburg et al., 2006). The second phylum in abundance is Bacteroidetes (10%) for human and Proteobacteria (21.5%) for chicken. Moreover, the less represented phyla in humans are Actinobacteria (1.5%), Verrucomicrobia, Fusobacteria, and Proteobacteria (<1%). On the contrary, the less represented phyla in chicken are Bacteroidetes (1.9%), Actinobacteria (4.9%), and Tenericutes (<0.1%) (Jalanka-Tuovinen et al., 2011, Zhu et al 2002, Kohl 2012). The low abundance of Proteobacteria sequences (including *E. coli*) in human is known given that facultative species represent less than 0,1% in strict anaerobic environment such as the colon (Eckburg et al., 2006). In contrast, Proteobacteria is a well represented phylum in chicken with 21.5% (Kohl 2012).

On this way, *E. coli* have a number of genes enabling it to use lactose, and therefore have the ability to colonize both mammalian and avian intestines. Arguably, it is thought that some *E. coli* strains from chicken gut (lacking lactose) or human gut (constant lactose) could have evolved to increase their fitness and prosper either in chicken or mammalian intestines.

Given the differences listen above, we might expect differential adaptations. Adaptive diversification is the process that generates two derived groups from an ancestral lineage because of frequency dependent ecological interactions. An ancestral lineage could undergone a number of genetic changes until create diversified ecotypes by disruptive selection process. There is some evidence that *E. coli* have diversified in this way. For instance, Spencer et al. (2007) ran an experiment exposing *E. coli* to a medium supplemented with glucose and acetate. After 1000 generations, the ancestral lineage split on two ecotypes: fast and slow switchers. Fast switchers (FS) were large colonies that showed high growth rate on glucose, slow growth rate on acetate and a

short lag between growth from glucose to acetate in comparison with slow switcher (small colonies).

Ecological specialization in bacteria could occur by antagonistic pleiotropy when microorganisms that are adapting genetically to one environment simultaneously lose adaptations to another environment. This was suggested for a number of populations of *E. coli* that were sub-cultured in minimal media supplemented with glucose for 20.000 generations by Cooper and Lenski (2000). These populations consistently increase adaptations to glucose during the first 2000 generations but at the same time decrease fitness to other metabolic functions. In another study, Cooper et al. (2001) determined that the mutation related with decrease in ribose catabolic functions was beneficial in glucose medium selection.

Therefore, *E. coli* has the ability to adapt to their environment and change their genes expression. If changes in natural habitats are predictable, then microorganisms could prepare in advance to the following change. This phenomenon is known as adaptive prediction. For example: an evolution experiment with *E. coli* that was cultured with lactose and then maltose showed a fitness advantage compared with the ones that receive first maltose and then lactose. The promoter activity of four maltose operons was higher in strains pretreated with lactose rather than wild type (without pretreatment). This means that the bacteria turns on genes to metabolize both substrates as if they could predict that maltose will come after metabolize lactose (Mitchel et al., 2009).

Crosscolonization / horizontal gene transference – resistance

According to the adaptive-prediction ability of *E. coli,* it could be argued that commensal *E. coli* from human or chicken intestine should have different adaptations in

relation to lactose consumption. We argue that inside of some humans, *E. coli* receive a constant input of lactose because of the human habit of drinking milk, and therefore need to be adapted to this environment. On the contrary, *E. coli* chicken strains should not have this adaptation.

In order to test these hypotheses, first we will compare the growth curves of human and chicken *E. coli* isolates in a minimal media supplemented with lactose as the only carbon source. Later, we will determine the number of chicken or human *E. coli* isolates after an in vitro competition experiment in lactose minimal media as well as the colonization ability of both isolates in the chicken gut. Determining the colonization ability of these isolates will be important as they could be a resource of antimicrobial resistant determinants that could be transmitted by horizontal gene transmission or by cross-colonization to new host.

STUDY OBJECTIVES:

General objective:

- To identify ecotypes of *Escherichia coli* specialized to preferentially use lactose as the only carbon source.

Specific objectives:

- To compare growth ability of *E. coli* isolates from human and chicken using lactose as the only carbon source.
- To determine the outcome after in vitro co-culture of human *E. coli* isolates and chicken *E. coli* isolate using lactose as the only carbon source.

- To determine the colonization ability of *E. coli* isolates from human and chicken in chicken host.

HYPOTHESIS:

- There are different *E. coli* ecotypes, one adapted to chicken and other to human host.
- Human *E. coli* isolates grow better that chicken *E. coli* isolates when they use lactose as the only carbon source.
- When competing in vitro in a lactose minimal media, the human *E. coli* isolate grow faster than the chicken isolate.
- When colonizing chicken intestine, there are not differences in colonization by the chicken and human isolates regardless of their capacity to use lactose.

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Part II. PAPER.

Lack of host specificity in gut colonization ability of human and chicken *Escherichia coli* isolates.

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INTRODUCTION

The use of antibiotics in the animal industry has been associated with antibiotic resistance in human bacterial commensals and pathogens (Witte, 2000; Van den Bogaard et al., 2001; Torres and Zarazaga, 2002;Sorum et al., 2004;Johnsen et al., 2005). Antibiotic resistance could be transferred from animal to human bacterial commensals by cross colonization of antibiotic-resistant commensals and/or by lateral gene transference. One intestinal bacterial species that has been implicated in this process is *Escherichia coli* (Angulo et al., 2004; Moodley and Guardabassi, 2009).

Escherichia coli mainly colonize intestines of warm-blooded animals acting as commensal. However, some extraintestinal pathogenic ecotypes (ExPEC) are adapted to access other animal tissues and cause diseases such as urinary tract infection, newborn meningitis and sepsis. On the same way, some intestinal pathogenic ecotypes(IPEC) cause enteric and diarrheal diseases (Chattopadhyay et al., 2009; Dobrindt et al., 2003). Nevertheless, even within intestinal commensal ecotypes there may be differences between strains colonizing digestive systems of mammals (exposed to lactose) and birds (exposed to the ammonia-rich cloacae) (Kohl, 2012). Recently, it was shown that *E. coli* has a fine-tuned program to switch on the lactose and the maltose operon in one step which coincides with greater concentrations of lactose in the small intestine and greater concentrations of maltose in the mammalian large intestine (Mitchel et al., 2009). If these adaptations are important, could be a potential colonization barrier between humans and chickens, and consequently the transference of antibiotic resistance between *E. coli* strains of mammalian and avian origin may be limited. Additionally, we recently found different patterns of antibiotic resistance associated with chicken and human isolates in remote Ecuadorian communities. For

instance, 19% of chicken isolates were ciprofloxacin-resistant and 14% of isolates were gentamycin-resistant. In contrast, 3% of human isolates were ciprofloxacin-resistant and 1.3% of isolates were gentamicin-resistant. Disparity of resistance patterns would indicate that there is not dispersion of isolates between chickens and humans (Armas 2012). This study tests the ability of human and avian strains of *E. coli* to use lactose as the sole source of nutrients, and their ability to colonize chicken intestines.

MATERIAL AND METHODS

Escherichia coli isolation

From January to July 2009, a total of 2006 strains of *Escherichia coli* were isolated from human and chicken fecal samples from San Agustin a remote community in northwestern Ecuador, Latitude: 1.04304, Longitude: -78.92245 (decimal degrees). The study was part of a larger ongoing research project in this region (Eisenberg et al., 2006). All protocols were approved by the University of Michigan institutional review board and Universidad San Francisco de Quito's bioethics committees. Fecal samples were obtained from humans and chickens and inoculated onto MacConkey agar followed by overnight incubation at 37ºC. Five to seven lactose-positive colonies were selected and sub cultured onto Chromocult agar (Merk, Alemania). In addition, five glucoronidase positive colonies able to degrade the substrate 4-metilumberiferil-β-D-glucoronic (MUG+) were selected and sub cultured on nutritive agar. Each isolate was cryopreserved in brain heart infusion plus glycerol 20% at -20ºC.

McConkey and Chromocult agar are selective and differential culture media. McConkey allow differentiation of lactose positive and negative colonies. For example,

lactose positive colonies appear as pink colonies while lactose negative colonies growth transparent. In addition, chromocult allow differentiation between *E. coli* and other coliforms because of their differential components. Coliforms colonies grow red because of their ability to degrade β-galactoside and *E. coli* grow blue to purple because they degrade β-galactoside and are MUG+ (Merck, 2000).

Mutant selection

Thirty five chicken´s *E. coli* isolates ciprofloxacin and streptomycin susceptible and fifty seven human´s *E. coli* isolates streptomycin and nalidixic acid susceptible were randomly selected from the total 2006 pool of isolates from chicken and human. Streptomycin-resistant mutants were selected from chicken isolates while nalidixic acid resistant mutants were selected from human isolates as described previously (Miller, 1972). In brief, each isolate was cultured in 10ml of brain hearth infusion broth (BHI) and cultivated overnight to 37ºC 150rpm in a shaker water bath. Next day, 10ml of new BHI broth with nalidixic acid (NA) to a final concentration of 25μg/ml were added for human isolates while 10ml of BHI with streptomycin (SM) to a final concentration of 100μg/ml were added for chicken isolates followed by incubation for 48h to 37ºC, 150rpm. Nalidixic acid-resistant isolates or streptomycin resistant isolates were selected in nutrient agar supplemented with each antibiotic respectively (25μg/ml NA or 100μg/ml S). Growth curves of mutants and parental strains were compared and only mutants with no apparent replication defects (similar growth rates) were saved for co-culture experiments.

Growth curves in lactose minimal medium

Growth curves were obtained from 10 chicken *E. coli* and 10 human *E. coli* wild type isolates. On hundred microliters of each isolate (McFarland No.1) were inoculated in 10 ml M9 minimal medium supplemented with 0.4% lactose and incubated in a shaker water bath to 37º C, 150 rpm. The absorbance to 600 nm was measured every hour for 12 hours. Experiments were done in triplicate.

Lactose competition assay

To test the strains' ability to use lactose as the only carbon source, growth curves of 20 *E. coli* isolates from humans and chickens (in M9 minimal media containing lactose 0.4%) were compared. The curves were obtained using optical density (OD=600nm). Additionally, streptomycin mutant *E. coli* from chicken (14Cmut) and nalidixic acid mutant *E. coli* from human (16Hmut) were co-cultured in M9 minimal media with lactose 0.4%. Each strain was cultured in nutrient agar for 18h to 37º C and resuspended in saline solution 0.9%. For initial inoculums, both stains were counted in a Petroff-Hausser chamber and adjusted to 2 x 10^6 CFU/ml. Then both strains were mixed in a final volume of 5 ml of M9 plus lactose 0.4% and incubated 24h to 37ºC. Bacteria were plated in nutrient agar containing either streptomycin (100μg/ml) or nalidixic acid (25μg/ml) at the beginning of the experiment (0 hours) and at the final of the incubation period (24 hours). Colonies were counted after 18h incubation. Essays were done in quintuplicate.

Chicken colonization

To test the ability of human and chicken strains to colonize chicken intestines, a mixture of two strains of streptomycin-resistant mutant 14Cmut (chicken origin) and a nalidixic

acid resistant mutant 16Hmut (human origin) were inoculated in the drinking water of 17 two-day-old chicks at a concentration of $1.5x10^7$ CFU/ml (of each strain) for three days. The control group contained 11 two-day-old chicks without bacterial inoculation. All chicks received clean water for 3 additional days. After that, fecal samples from every chick were inoculated on MKL agar plus streptomycin or nalidixic acid and quantified by the colony count method described below.

Serial 10-fold dilutions of the fecal sample were carried out in a 0.9% saline solution. From each dilution, three drops of 10 μl each were inoculated in nutritive agar supplemented with nalidixic acid 0.25μg/ml or streptomycin 100μg/ml. Colonies were counted after an incubation period of 18h to 37ºC in a colony counter.

Statistical analysis

Growth curves were analyzed by a Paired t-test. For the in-vivo experiment an ANOVA test was used. Mann-Whitney test was run to analyze in vitro experiments. A p value ≤ 0.05 was considered statistically significant.

RESULTS

Ability to use lactose as the sole carbon source

Significant differences were observed between growth curves from human and chicken *E. coli* isolates when grown on lactose-only diets (Paired T-test P=0.0188*) (Fig 1).The chicken strain (14Cwt) showed a slower growth rate in lactose minimal medium. When this strain grew in tryptic soy broth, a rich broth media, no difference was observed

between 14Cwt and the other chicken and human isolates (data not shown). When the human strain 16Hmut (nalidixic acid resistant) and the chicken strain 14Cmut (streptomycin resistant) were co-cultured in M9 minimal media supplement with 0.4% lactose for 18h at 37ºC both isolates were quantified in nutrient agar supplemented with nalidixic acid 0,25µg/ml or streptomycin 100µg/ml. Under competition conditions *E. coli* human isolate grew a mean of $6.01x10^{10}$CFU/ml while *E. coli* chicken isolate grew to $1.1x10^{9}$ CFU/ml. There was no statistical difference between these rates; Mann-Whitney test $P = 0.690^{NS}$ (see Fig. 2).

Ability to colonize chicken intestines

No statistical differences were found in colony counts between *E. coli* from human and chicken origin (ANOVA $P= 0,113^{NS}$) when grown in chicken intestines (n=17). A mean of $8.01x10^{7}$ CFU/ml of 16Hmut and $1.14x10^{7}$ CFU/ml of 14Cmut were recovered from a rectal swab after the competition experiment. Controls did not have statistical differences (ANOVA $P=0,080^{NS}$) between counts of nalidixic acid resistant *E. coli* ($2.41x10^{6}$ CFU/ml) and *E. coli* streptomycin resistant ($1.16x10^{6}$ CFU/ml) (n=11) (see Fig 3).

DISCUSSION

Even though lactose utilization efficiency should not be equated with the ability to colonize mammalian intestines, it may indicate the existence of two different populations of *E. coli*. We reasoned that if there are avian adapted lineages, these bacteria lack environmental pressure to maintain intact lactose utilization genes and therefore they may have developed some mutations which may reduce the efficiency to

use lactose overtime. The present study found statistical differences in the utilization of lactose; however, no statistical differences were found in the competitive ability using lactose or in the ability to colonize chickens' intestines between human or avian *E. coli* isolates (Figures 3-4). These results demonstrate the ability of *E. coli* isolates to adapt to distinct intestinal environments.

These findings indicate that some *E. coli* strains may be able to cross-colonize both mammalian and avian intestines regardless of their ability to use lactose as their sole carbon source. Human colonization by bacteria from food animals may provide the opportunity for transference of resistant determinants to other *E. coli* lineages *in vivo* (Angulo et al., 2004;Moodley and Guardabassi, 2009). In fact, Armas (2012) found identical sequences of quinolone-resistant gene *qnrB* in 23 *E. coli* isolated from chicken and human (same pool of bacteria analyzed in this study). This is evidence that human and chicken are sharing *E. coli* resistant isolates or that resistant determinant (*qnrB*) is being horizontally transmitted. Present results support the notion that antibiotic resistant *E. coli* isolates could be transmitted between species due to cross-colonization.

In this study, chicken and human *E. coli* isolates were selected based on their ability to use lactose as the sole carbon source and later to analyze their ability to colonize chicken intestines. Although the ability to use a carbohydrate (lactose) do not have implication in their colonization abilities, we argued that their metabolic differences in conjunction with their physiological, anatomical, nutritional and microbial differences could have and strong impact to select specific *E. coli* ecotypes for each host. However, the results showed the great ability of *E. coli* to adapt to distinct host environments to survive.

Considering *E. coli* lactose consumption, *E. coli* tightly regulates the lactose operon in vivo Mitchel et al. (2009), probably because it is metabolically expensive. Additionally, it has been thought that the loss of lactose-utilization genes in enteroinvasive *E. coli* and *E. coli* Shigellae is due to the adaptation to lactose-free intracellular milieu (Lan and Reeves, 2002). However, *Escherichia coli* propagated on glucose minimum media for 20,000 generations did not lose the ability to ferment lactose (Cooper and Lenski, 2000), which may indicate that losing the lactose operon does not improve the fitness in a lactose-free environment.

Limitations of the study

One limitation of this study is related with the animal model used because chicks were not germ free. Although chicks received previous antibiotic treatment, they intrinsically contained some lactose positive bacteria resistant to nalidixic acid and streptomycin. It created a previous background for experiments. Another limitation was the type of culture media that were use for colony count. We use McConkey lactose supplemented with nalidixic acid or streptomycin. This media no not allow differentiation between *E. coli* and other coliforms.

Recommendations

- The colonization experiment should be repeating using germ free mice to compare avian with mammal colonization using the same isolates.
- For future studies, I suggest to use germ free chicks and germ free mice to avoid background of intrinsically resistant bacteria.
- Every experimental animal should be keep individually and treatment should be applied orally.

- Another type of marks could be used to differentiate isolates such as fluorescence (Ex: green fluorescent protein GFP and red fluorescent protein RFP).

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FIGURES

Figure 1. Growth curves of human and chicken *E. coli* isolates.

Experiment was done in minimal media M9+lactose 0.4%. Each value is an average of 10 isolates (three replicates of each one). Error bars are 95% confidence intervals based on the replicate populations.

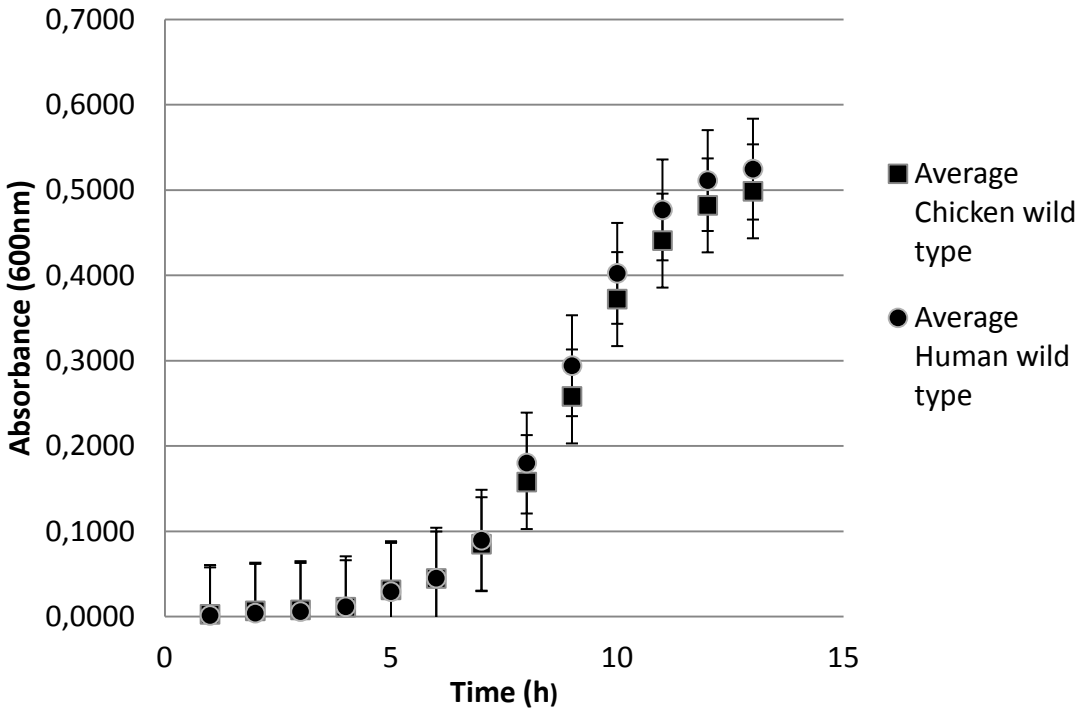

Figure 2. Ability to use lactose as the only carbon source.

Human nalidixic acid resistant isolates (Na-R, grey bar) showed an average recuperation rate $6.01x10^{10}$ CFU/ml and chicken streptomycin resistant isolate (SM-R, black bar)$1.10x10^{9}$ CFU/ml(5 replicates were done). NA-R= CFU counted in nutrient agar plus nalidixic acid; SM-R=CFU counted in nutrient agar plus streptomycin.

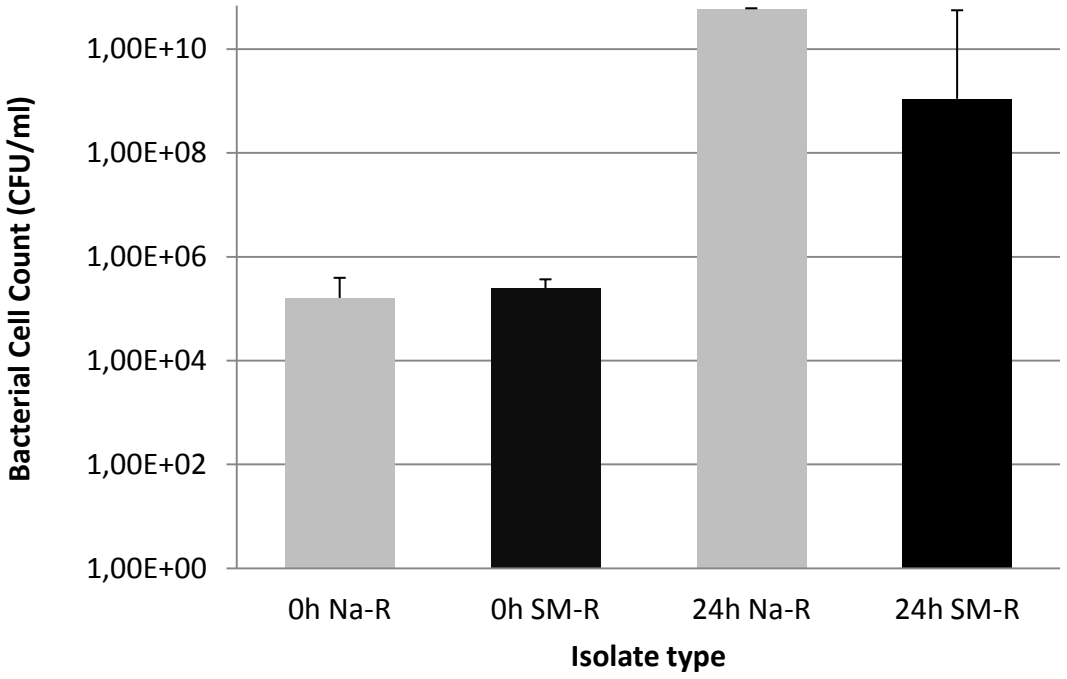

Figure 3. Colonization of chicken intestines.

Colonized chickens received water with both human (grey bar) and chicken *E. coli* (black bar) at the same concentration ($1,5x10^7$CFU/ml). Control chickens did not receive bacteria. NA=CFU in nutrient agar plus nalidixic acid; SM=CFU in nutrient agar plus nalidixic acid.