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Algae in Chicken Feed

The Colonization of Two Different Strains of Campylobacter Jejuni in Chicken Fed with Laminarin Extract

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Master's Degree Project in Infection Biology, 30 credits. Spring 2020

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Abstract

In the last two centuries, the amount of carbon dioxide in the atmosphere and nitrogen in the oceans have steadily risen due to human action. This has resulted in a changing climate with higher mean temperatures globally and more eutrophic sea waters. Lowering the amount of carbon dioxide while also lowering the amount of nitrogen, by harvesting algae from the oceans has been proposed as a way in alleviating some of the problems.

Algae and algae extracts have been shown to have a beneficial effect on the growth of animals. The aims of this study were to determine whether supplementing chicken feed with the algae extract laminarin has any effect on the colonization of *Campylobacter jejuni* in chicken intestine, while also studying strain specific differences in colonization ability between *C. jejuni* strains of different host origins.

This study showed no difference in *C. jejuni* colonization between the chickens raised on feed supplemented with laminarin or conventional feed, nor between the two different strains of *C. jejuni*. These results show that the amount of *C. jejuni* are neither lower nor higher in the chickens raised on feed supplemented with laminarin. Supplementing laminarin may therefore be a feasible way of increasing the growth of chickens without increasing the bacterial load, all while being beneficial to the environment.

Key words

Laminarin, Algae, *Campylobacter*, Chicken, Host specificity, Agriculture

Popular scientific summary

Do algal supplements lower the number of bacteria in chickens?

Algal supplements in animal feed is a hot topic, but what are the actual benefits of supplementing the diet of chickens with algal extracts? Researchers at Uppsala University and SLU have been assessing the potential health benefits of adding algae to the chicken feed.

The vast seas have many untapped and unexplored resources. From the deepest trenches to the shallowest shores, there is a plethora of different thriving organisms. These organisms all share a common trait: they are all able to use the nutrients in the water efficiently. Too much nutrients are however not a good thing either. The presence of too much nutrients in the water leads to algal bloom, which is a floating, unpleasant, and smelly sludge. When this sludge decomposes, it creates dead sea floors.

In previous studies researchers have found that by adding an algal extract called laminarin to the feed of chickens, the substance could increase the growth rate of the chickens from a very young stage. They also looked at if adding laminarin to the chicken feed would affect the bacterial flora in the chickens, but they did not find any evidence for that.

Campylobacter is one of the leading causes of bacterial gastrointestinal disease in the developed world. In Sweden, it causes about 10 000 documented cases every year but is estimated to cause ten times as many undiagnosed cases. In chickens and other birds, *Campylobacter* does not cause disease, but still causes problems when not handled properly. The prevalence of *Campylobacter* in Swedish broilers has, according to the trade organization Svensk fågel, steadily been decreasing since 1991 when a surveillance programme was introduced. In 2019 the prevalence was 4.6%. Reducing the amount of *Campylobacter* in the chickens would mean that the likelihood of someone being affected by the disease decreases.

Researchers at SLU, along with Uppsala University, have been researching the effect of adding laminarin to the diet of chickens. The researchers at SLU have been looking at aspects such as egg quality, growth rate and immune system, while researchers at Uppsala University have been looking at the occurrence of *Campylobacter* in the chickens that were given laminarin.

Researchers at Uppsala University have found that there is no difference in occurrence of *Campylobacter* in chickens that were given laminarin as compared to those that were not. The number of *Campylobacter* was similar in both test groups, meaning that adding laminarin to the chicken feed does not provide any benefits when it comes to the occurrence of *Campylobacter*. However, laminarin does not result in any pertinent downsides either, meaning that the benefits regarding growth rate still can be utilized.

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1. Introduction

1.1. Background

Climate change is a growing threat to all different species all around the world. An increasing amount of carbon dioxide in the atmosphere is one of the driving forces towards a changed climate, leading to raised sea levels, changed weather patterns and no longer habitable ecosystems. As a result of human activity, it has been estimated that the global mean temperatures have increased by 1°C, and it is likely to increase to 1.5°C in the coming decades. (IPCC, 2018) By lowering the amount of emitted carbon dioxide, it would be possible to slow this process down and lowering the impact of climate change.

The rising production of meat and other agricultural products is one of the major sources of carbon dioxide. Raising animals to produce meat is a very carbon dioxide intensive industry, in large due to the need of feed for the animals. Animals require a greater input of energy in the form of feed than they produce in form of meat. Growing feed for the animals requires large amounts of fertilizer, which is difficult to produce. The Haber-Bosch process, which is used to turn atmospheric nitrogen into ammonia in the fertilizer, requires a substantial amount of energy. About 1.2 % of the total primary energy output globally is devoted to the production of ammonia for fertilizer. (Everett, 2016) By lowering the use of fertilizer, the emissions would be lower and the whole industry could lower its carbon footprint.

Highly nutritious runoff from the agriculture often end up in the sea making it eutrophic. The Baltic sea is an example where this has happened. The eutrophic sea water is highly nutritious for different kinds of bacteria, which proliferate during summers when the water gets warmer which creates algal bloom. When the bacteria and algae die, they start to decompose at the bottom of the sea. Aerobic decomposition is the most efficient way to decompose matter but uses up the oxygen dissolved in the sea water. When the sea water is devoid of oxygen, anaerobic decomposition becomes the primary decomposition method. This process of anaerobic decomposition creates toxic hydrogen sulphide, making the sea floor even less habitable. (Rheinheimer, 1998)

Aquatic agriculture has been proposed to alleviate both problems regarding emissions of carbon dioxide and the eutrophication of the Baltic Sea. By harvesting crops such as seaweeds from the sea, the nutrients in the sea is used as fertilizer. This eliminates the need of carbon dioxide intensive manufactured fertilizers, while at the same time also lowering the nutrients available for the bacteria and algae that thrive on the nutrients and give rise to algal bloom. This lowers the emissions from agriculture, while also lowering the eutrophic state of the sea. (Amenorfenyo, 2019)

There is an interest in using micro- and macroalgae as both feedstock for animals and in different industrial processes. Microalgae are rich in lipids, and macroalgae are rich in carbohydrates. In the energy industry, the lipid rich microalgae can be turned into biodiesel and the carbohydrate rich macroalgae can be turned into biogas or ethanol. It has been estimated that the available marine biomass could be converted to the equivalent amount of 100 EJ per annum of methane, comparable to one fifth of the total primary energy consumption globally. This would reduce the need of fossil fuels, which would result in lower emissions globally. (Huesemann, 2010) (Everett, 2016)

Algae and algae extract have been shown to be beneficial to the growth of animals. One of these algae extracts is laminarin. Laminarin belongs to a class of storage β-glucans found in several different kinds of seaweeds, such as the brown algae *Saccharina latissima.* Laminarin has a low molecular weight and can be extracted from seaweeds using several different methods. (Kadam, 2014) Chicken fed with laminarin had a higher intake of feed and had a higher body weight gain compared to chicken raised with conventional feed. The chicken fed with algae extracts also had larger villus, and key genes involved in the immune response were upregulated compared to the chickens raised on the conventional feed. It is believed that the larger villi lead to a higher absorption of nutrients in the intestines, and therefor to an increased growth rate. Early in the lifetime of the chickens, the colonization of *C. jejuni* did not differ between the chickens raised on feed supplemented with algae extracts and conventional feed. However, the effect later in life on the bacterial load has not been as well studied. (Sweeney, 2017)

C. jejuni is a gram-negative pathogenic bacterium known to affect humans. The main reservoir is in birds, where it is not regarded as pathogenic. *C. jejuni* is one of the most common causes of bacterial gastrointestinal disease in humans and the disease is most often attributed to undercooked meat. (Kaakoush, 2015) Infection with *C. jejuni* in humans is notifiable under the Swedish Communicable Diseases Act. (Smittskyddslag, 2004) About 10 000 cases of gastrointestinal illness in Sweden is attributed to the bacteria, but it is estimated that there are ten times as many undiagnosed cases of campylobacteriosis each year. (Hanninen, 2009)

C. jejuni in broilers is also notifiable. Sweden has a surveillance programme put in place by the trade organisation Svensk fågel since 1991, to catch the cases of *C. jejuni* in the chicken industry. *C. jejuni* normally colonizes the intestines and inner organs of the birds. As part of the surveillance programme, the caeca of the chicken are sent for analysis to the National Veterinary Institute of Sweden where the prevalence of *C. jejuni* is assessed. The prevalence varies between seasons, with a peak during the summer months. Since 1991, the prevalence of *C. jejuni* in Swedish broilers has been declining and in 2019 the prevalence was 4.6%. (Svensk fågel, 2019)

Different *C. jejuni* strains have been shown to have strong host specificity in wild birds (Griekspoor, 2013). *C. jejuni* strains isolated from mallards colonize mallards better than strains isolated from either chicken or thrush. While the strains from chicken and thrush both were worse at colonizing the mallards than the strain isolated from mallards, the chicken strain was better at colonizing the mallards than the thrush strain. Phylogenetic analysis of these three strains showed a closer relationship between the mallard strain and the chicken strain, than between the thrush strain and either chicken or mallard strains. (Atterby, 2018)

It is possible to quantify the number of bacteria in the intestine using several different methods, each having different advantages and disadvantages towards the other ones. Sampling can be done by killing the animals and taking intestinal samples from different parts of the intestine or by taking faecal samples. (Atterby, 2018) Killing the animals prior to sampling makes it impossible to follow the same animal over time but it is possible to observe the bacterial load in specific areas in the intestine, whereas the opposite is achieve by faecal sampling. Analyzing the samples could also be done using several different methods. qPCR is a robust and sensitive quantitative method that may be used for species identification, but it is also sensitive towards inhibitors that are present in the faeces. (Smith, 2008) 16s amplicon sequencing for microbiota analysis is another possible method, which combined with mapping the data to an already available library, to capture the total population of all bacteria but is not optimal if looking for a specific bacterium. (Marguerat, 2009) Growing the faeces on agar plates also has the problem of being unspecific, but different kinds of selective agar could be used to circumvent that issue. (Bolton, 1983) To be selective for *Campylobacter spp*. the agar needs to contain a mixture of different antibiotics. One of the used formulations is mCCDA agar, which combines charcoal, cefoperazone, sodium deoxycholate and amphotericin B. This is routinely used to cultivate and select for *Campylobacter spp*. in different labs. (Bolton, 1983) (Hutchinson, 1984)

Growing bacteria from faecal samples with unknown bacterial concentrations raises some issues. Using faecal samples, the number of bacteria taken from each sample may vary. As concentration of bacteria is unknown, several dilutions and replicates are needed for more precise data, requiring many plates. A simpler method, where it is possible to combine dilutions and replicates on the same plate is the 6x6 drop plate method. (Chen, 2003) Being able to load 36 samples onto one agar plate, reduces the amount of agar plates needed drastically. (Chen, 2003)

1.2. Aim

This project had two aims. The first aim was to assess whether the algae extract had any effect on how well *Campylobacter jejuni* could colonize the chicken intestine, as compared to chickens raised on conventional feed.

The second aim was to assess whether there is any difference in strain specificity between *C. jejuni* isolated from chickens or from thrush when colonizing chicken.

2. Results

2.1. The effect of laminarin

To determine whether the addition of laminarin to the feed of chicken had any effect on *Campylobacter jejuni* colonization, the chickens were raised on either a conventional feed or a conventional feed supplemented with 300 ppm laminarin starting from the day of hatching. When the chickens were about 19 days old, faecal samples were taken from the chickens to assess if they had any prior *C. jejuni* infections, before being inoculated intra-oesophagically with *C. jejuni*. Out of the 48 chickens, 24 were inoculated with a strain of *C. jejuni* isolated from chickens (*C. jejuni* 65), and the other 24 were instead inoculated with a strain of *C. jejuni* isolated from thrush (*C. jejuni* 4:628). Both strains were isolated from previous studies (Atterby, 2018). None of the chickens had any prior *C. jejuni* infection.

After inoculation with *C. jejuni* from chickens, the faeces of the chickens were collected on 1 dpi, 2 dpi, 3 dpi, 5 dpi, 7 dpi, 14 dpi and 19 dpi. The faeces were grown on mCCDA-plates using the 6x6 drop plate method. After incubation, the colonies were counted. **Figure 1** shows the chickens inoculated with the *C. jejuni* strain 65 previously isolated from chickens and it shows that the number of *C. jejuni* shed by the chickens increased over time from a median of zero cfu/g faeces on 1 d.p.i. to a median of between 10^5-10^7 cfu/g faeces on 2-19 d.p.i. The difference in the amount of *C. jejuni* shed by the chickens in the two chicken treatment groups was however not enough to be considered statistically significant when comparing the two groups for each of the sampling dates in Graphpad Prism using a t-test.

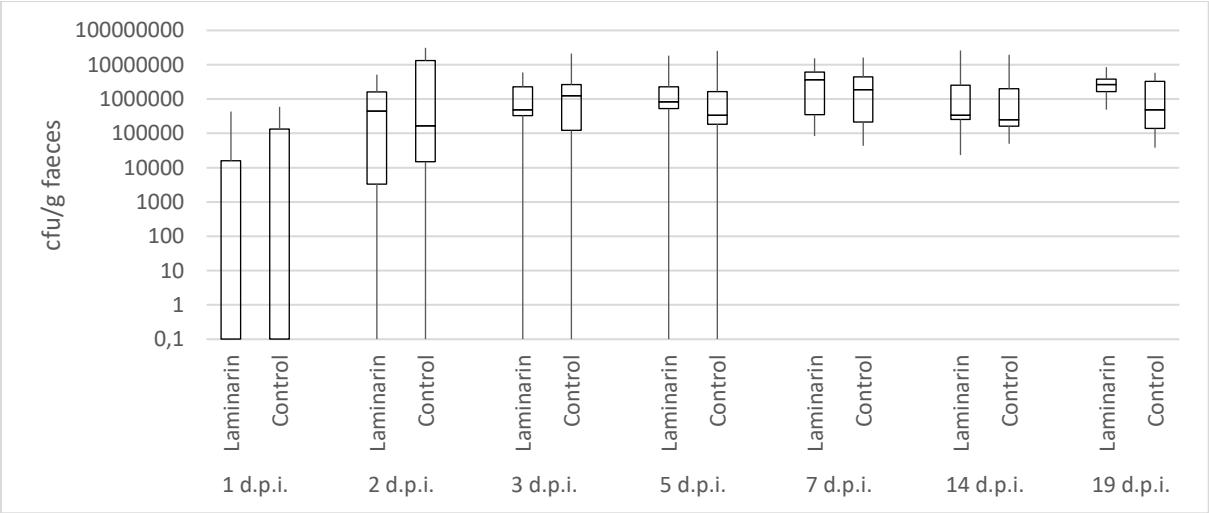

Figure 1. *Laminarin does not affect the colonization of C. jejuni isolated from chickens in chicken. The chickens were raised on either control feed without a supplement of laminarin, or one with laminarin supplemented. cfu/g of faeces in the two different chicken treatment groups where the chickens were inoculated by the strain 65 from chicken origin over the course of the trial was measured by plating the samples on mCCDA agar. Samples that showed no growth of bacteria were counted as having 0.1 cfu/g faeces.*

The chickens that were inoculated with *C. jejuni* isolated from thrush were also sampled faecally at 1, 2, 3, 5, 7, 14 and 19 d.p.i. and were plated on mCCDA-plates using the 6x6 drop plate method. **Figure 2** shows the chickens inoculated with the *C. jejuni* strain 4:628 previously isolated from thrush and it shows that the number of *C. jejuni* shed by the chickens increased over time from a median of zero cfu/g faeces on 1 d.p.i. to a median of between 10^5-10^7 cfu/g faeces on 2-19 d.p.i. No statistically significant differences were observed in the amount of *C. jejuni* between the two different chicken treatments in the chicken inoculated with the strain isolated from thrush.

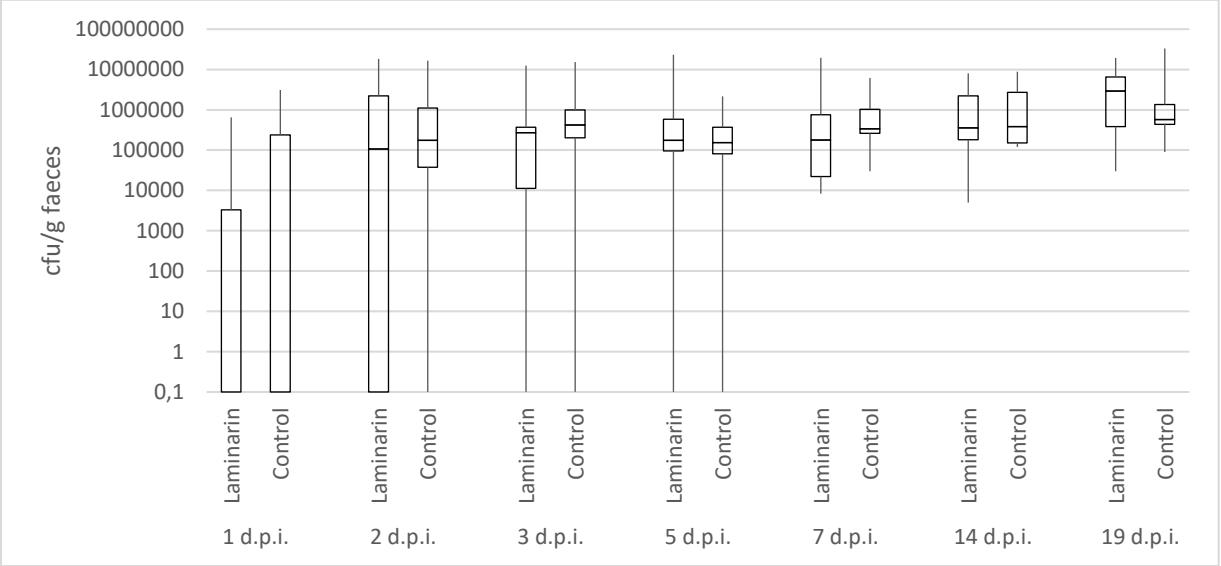

Figure 2. *Laminarin does not affect the colonization of C. jejuni isolated from thrush in chicken. The chickens were raised on either control feed without a supplement of laminarin, or one with laminarin supplemented. cfu/g of faeces in the two different chicken treatment groups where the chickens were inoculated by the thrush strain over the course of the trial. Samples that showed no growth of bacteria were counted as having 0.1 cfu/g faeces.*

To see if laminarin affects chickens, independent on what strain of *C. jejuni* has colonized the chickens, the data from both strains of *C. jejuni* was combined. In practice the exact strain of *C. jejuni* that colonizes the chickens might vary from the strains used in this study, but by combining that data a broader conclusion can be drawn. **Figure 3** shows the chicken inoculated with either of the two strains of *C. jejuni* in the two different chicken treatment groups. It shows that the number of *C. jejuni* shed by the chickens increased over time from a median of zero cfu/g faeces on 1 d.p.i. to a median of between 10^5-10^7 cfu/g faeces on 2-19 d.p.i. No statistically significant differences were observed in the amount of *C. jejuni* between the two different chicken treatments.

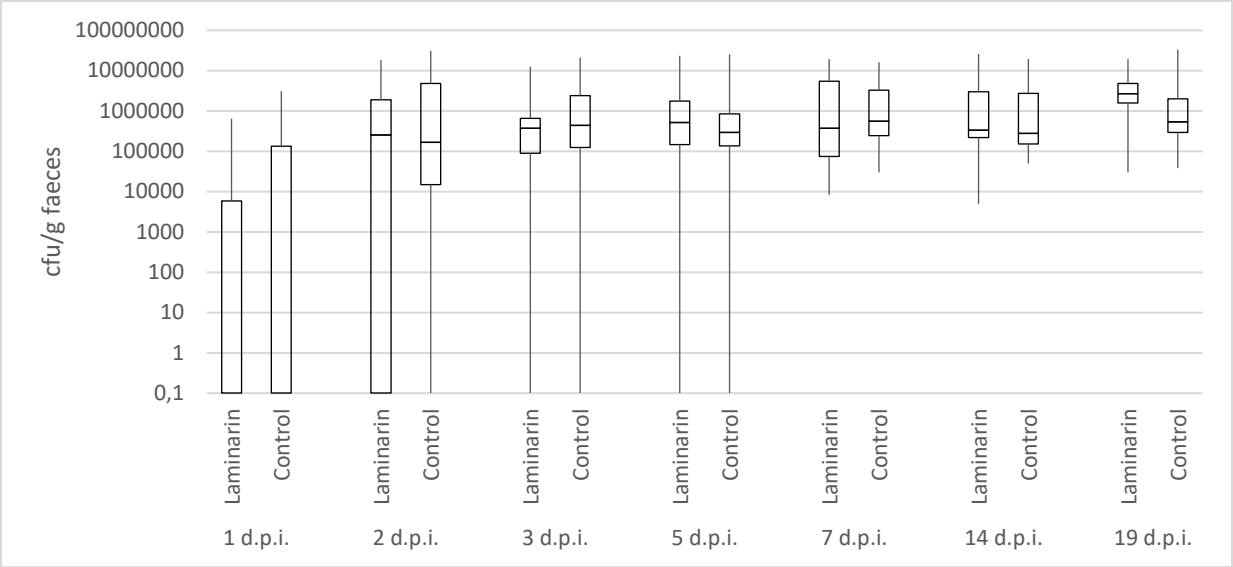

Figure 3. *Laminarin does not affect the colonization of C. jejuni isolated from chickens or thrush in chicken. The chickens were raised on either control feed without a supplement of laminarin, or one with laminarin supplemented. cfu/g of faeces in the two different chicken treatment groups where the chickens were inoculated by either strain over the course of the trial. Samples that showed no growth of bacteria were counted as having 0.1 cfu/g faeces.*

2.2. Colonization capability of different *Campylobacter jejuni* strains

Previous studies in mallards examined the colonization capability of different strains of *C. jejuni*. To examine if the colonization capability also differs between strains of *C. jejuni* in chicken, two different strains of *C. jejuni* were used as inoculates. The data from the chickens used in the feed study was also used in this study, albeit visualized differently.

One of the strains, *C. jejuni* 65, was isolated from chicken and the other strain, *C. jejuni* 4:628, was isolated from thrush. 24 of the 48 chicken were inoculated with *C. jejuni* 65 and the other 24 chickens with *C. jejuni* 4:628. To avoid cross infection between the two different strains, the chickens were kept isolated from each other.

The amount of *C. jejuni* shed by the chickens in the control group with no feed additives increased over time, regardless of which strain was inoculated, illustrated in **Figure 4**. The number of *C. jejuni* shed by the chickens increased over time from a median of zero cfu/g faeces on 1 d.p.i. to a median of between 10^5-10^7 cfu/g faeces on 2-19 d.p.i. However, no statistically significant differences were found between the chicken inoculated with *C. jejuni* 65 and *C. jejuni* 4:628 when comparing the two groups for each of the sampling dates in Graphpad Prism using a t-test.

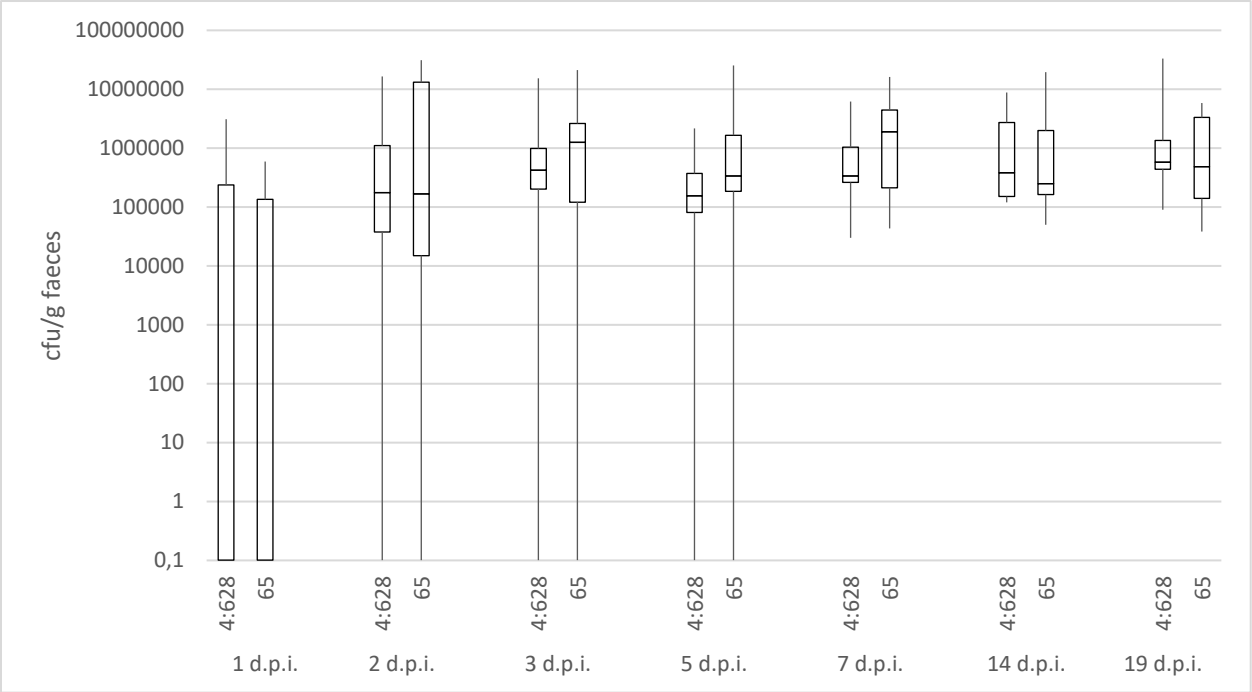

Figure 4. *No difference in host specificity between two different strains of C. jejuni in control treated chickens. The chickens were inoculated with either a strain of C. jejuni isolated from chicken (C. jejuni 65) or a strain isolated from thrush (C. jejuni 4:628) and given the control treatment. cfu/g of faeces in the chicken inoculated with either of the two different strains where the chickens were in the control group over the course of the trial. Samples that showed no growth of bacteria were counted as having 0.1 cfu/g faeces.*

In the chickens raised on feed supplemented with laminarin, no statistically significant differences were observed between the chickens inoculated with the strain of *C. jejuni* isolated from chickens or the strain isolated from thrush. The number of *C. jejuni* shed by the chickens increased over time from a median of zero cfu/g faeces on 1 d.p.i. to a median of between 10^5-10^7 cfu/g faeces on 2-19 d.p.i. This is illustrated in **Figure 5**.

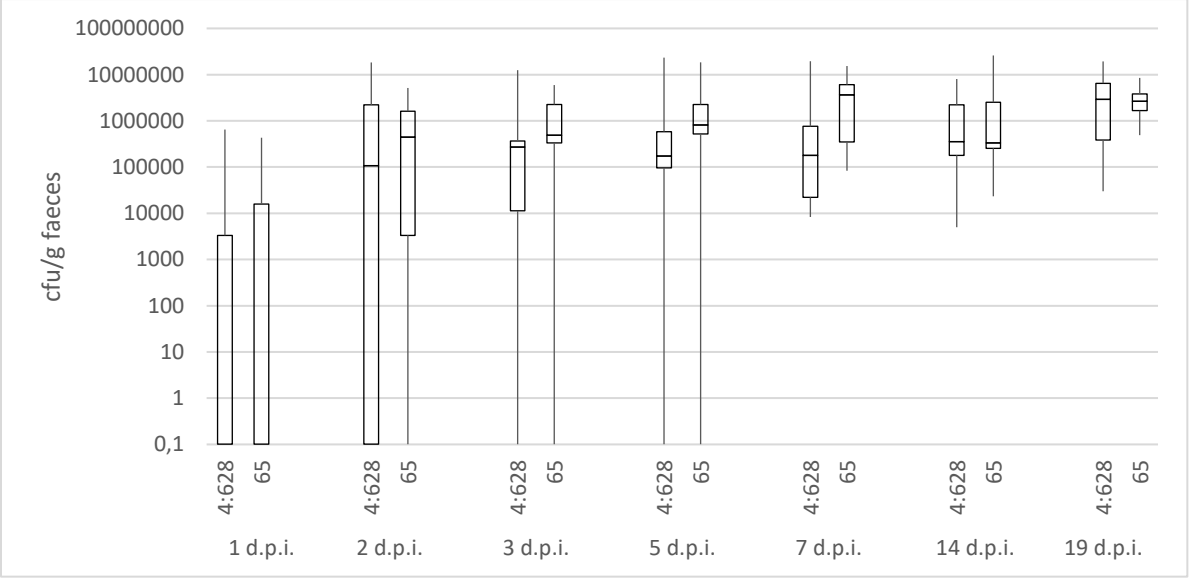

Figure 5. *No difference in host specificity between two different strains of C. jejuni in laminarin treated chickens. The chickens were inoculated with either a strain of C. jejuni isolated from chicken (C. jejuni 65) or a strain isolated from thrush (C. jejuni 4:628) and given the laminarin treatment. cfu/g of faeces in the chicken inoculated with either of the two different strains where the chickens were in the laminarin group over the course of the trial. Samples that showed no growth of bacteria were counted as having 0.1 cfu/g faeces.*

To see if there is a difference in host specificity between the two strains in chickens, independent on what treatment the chickens were subjected to, the data from both chicken treatments were combined. In practice the exact feed given to the chickens might vary from the exact feed used in this study, but by combining that data from both chicken treatments a broader conclusion can be made about the colonization capability of *C. jejuni*. When combining the results from either chicken treatment group, the results follow the same pattern as for the different treatments separately. The number of *C. jejuni* shed by the chickens increased over time from a median of zero cfu/g faeces on 1 d.p.i. to a median of between 10^5-10^7 cfu/g faeces on 2-19 d.p.i. as illustrated in **Figure 6**, however the difference between the two strains is not statistically significant.

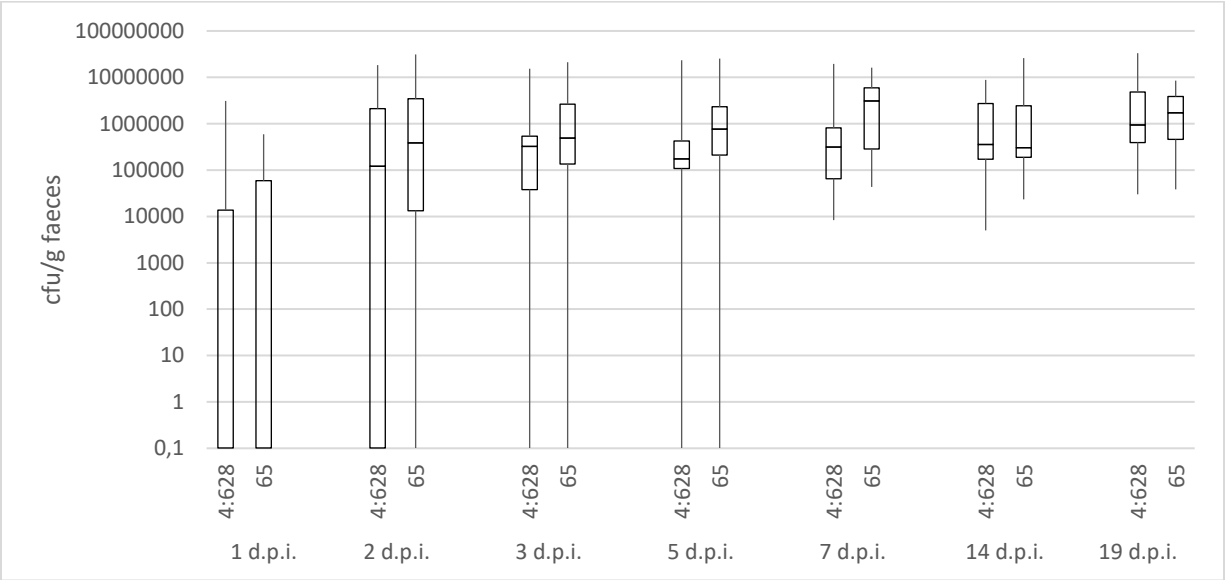

Figure 6. *No difference in host specificity between two different strains of C. jejuni in chickens. The chickens were inoculated with either a strain of C. jejuni isolated from chicken (C. jejuni 65) or a strain isolated from thrush (C. jejuni 4:628) and given either chicken treatment. cfu/g of faeces in the chicken inoculated with either the chicken strain or the thrush strain where the chickens were in either group over the course of the trial. Samples that showed no growth of bacteria were counted as having 0.1 cfu/g faeces.*

To assess whether the strain of *C. jejuni* had any effect on the treatments, the two treatments were compared regarding what strain the chickens were infected with. The results show an increase in the amount of *C. jejuni* over time regardless of treatment or strain of *C. jejuni*, and within each group the number of *C. jejuni* shed was similar for the same day. No statistically significant differences were found between any of the groups, except for between laminarin 65 and control 65 on 2 d.p.i. ($p = 0.0423$) when comparing the four groups using a 2-way ANOVA in Graphpad Prism. This is illustrated in **Figure 7.**

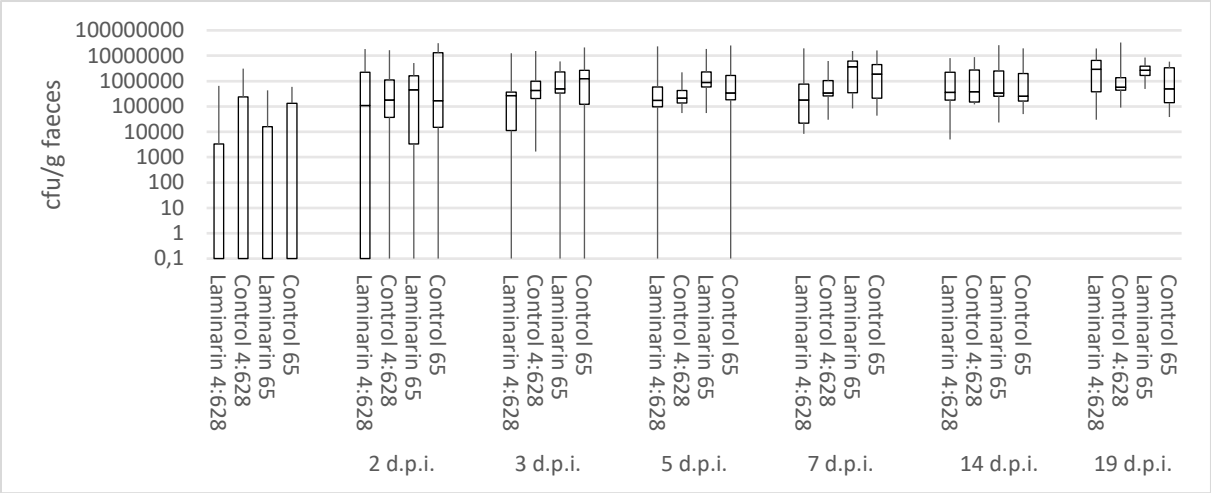

Figure 7. *Laminarin does not affect the colonization of C. jejuni in chicken. The chickens were inoculated with either a strain of C. jejuni isolated from chicken (C. jejuni 65) or a strain isolated from thrush (C. jejuni 4:628) and given either laminarin or control treatment. cfu/g of faeces in the chicken inoculated with either of the two different strains and where the chickens were in either group over the course of the trial. Samples that showed no growth of bacteria were counted as having 0.1 cfu/g faeces.*

The chickens used in this study were hatched from hens that were also subjected to different treatments. The hens were either given a control feed, a control feed with laminarin or a control feed with whole algae. This information was used to assess whether this had any effect on the colonization of *C. jejuni* in the chickens. The setup for which of the chickens got which treatment can be found in **Supplementary 1**.

As seen in **Figure 8**, the amount of *C. jejuni* shed by the chicken in the different chicken and hen treatments were similar to each other for all sampling days, with an exception on 3 d.p.i. On 3 d.p.i., the number of *C. jejuni* shed by the chickens treated with the control feed, hatched from the hens treated with a control feed and inoculated with the chicken strain *C. jejuni* was statistically higher than some of the other treatment groups. This was done by doing a 2-way ANOVA using Graphpad Prism.

Between the chickens treated with the control feed, hatched from the hens treated with a control feed and inoculated with the chicken strain *C. jejuni* and the chickens treated with the laminarin feed, hatched from the hens treated with the whole algae feed and inoculated with the thrush strain *C. jejuni*, the difference was statistically significant ($p = 0.0254$).

Between the chickens treated with the control feed, hatched from the hens treated with a control feed and inoculated with the chicken strain *C. jejuni* and the chickens treated with the laminarin feed, hatched from the hens treated with the laminarin feed and inoculated with the thrush strain *C. jejuni*, the difference was statistically significant ($p = 0.0205$).

Between the chickens treated with the control feed, hatched from the hens treated with a control feed and inoculated with the chicken strain *C. jejuni* and the chickens treated with the control feed, hatched from the hens treated with the control feed and inoculated with the thrush strain *C. jejuni*, the difference was statistically significant ($p = 0.0489$).

Between the chickens treated with the control feed, hatched from the hens treated with a control feed and inoculated with the chicken strain *C. jejuni* and the chickens treated with the laminarin feed, hatched from the hens treated with the control feed and inoculated with the chicken strain *C. jejuni*, the difference was statistically significant ($p = 0.0111$).

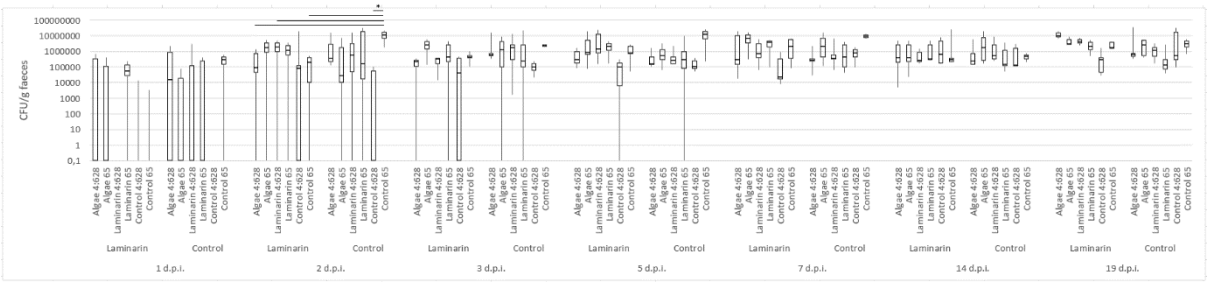

Figure 8. *Laminarin does not affect the colonization of C. jejuni in chicken hatched from hens who received different treatments. The chickens were hatched from hens that were treated with either a control feed, a control feed with laminarin, or a control feed with whole algae. The chickens were raised on either a control feed or a control feed with laminarin and later inoculated with either a strain of C. jejuni isolated from chicken (C. jejuni 65) or a strain isolated from thrush (C. jejuni 4:628). cfu/g of faeces in the chicken inoculated with either of the two different strains from either of the three hen treatment groups and where the chickens were in either group over the course of the trial. Samples that showed no growth of bacteria were counted as having 0.1 cfu/g faeces.*

2.3. Colonisation in different organs

To assess if the different chicken treatments had any effect on the *C. jejuni* load in other organs, the livers and spleens of the chickens were dissected out and homogenized by hand in PBS. The livers were homogenized in 10 ml of PBS, while the spleens were homogenized in 5 ml of PBS. 100 µl of the homogenate was plated onto separate mCCDA-plates using the glass bead spread method. After incubation at 42°C for 48 hours, there were no growth of *C. jejuni* on any of the mCCDA-plates.

2.4. Validation of plating method

Biological samples often carry some degree of uncertainty. When plating faeces onto agar plates for the enumeration of bacteria, the results are dependent on where in the faeces the sample is taken. Depending on where in the faeces the sample is taken, there might be more or less bacteria. To get a more representative result, it is possible to plate several replicates from the same faecal sample. This is what has been done during this study.

A method for plating six replicates in six tenfold dilutions each has been used. To assess how reliable the study design chosen, the variation in the 6x6 drop plate method was analyzed. To assess this, the standard deviation between the six replicates for each sample for each of the sampling days was calculated and the result can be seen in **Figure 9**.

The standard deviation between replicates within the same sample varied greatly. Some samples, such as the sample from chicken 1:2 on 1 d.p.i. had a standard deviation of 0 between the different replicates, whereas the sample from chicken 4:27 on 1 d.p.i. had a standard deviation of $7.19*10^5$ cfu/g faeces.

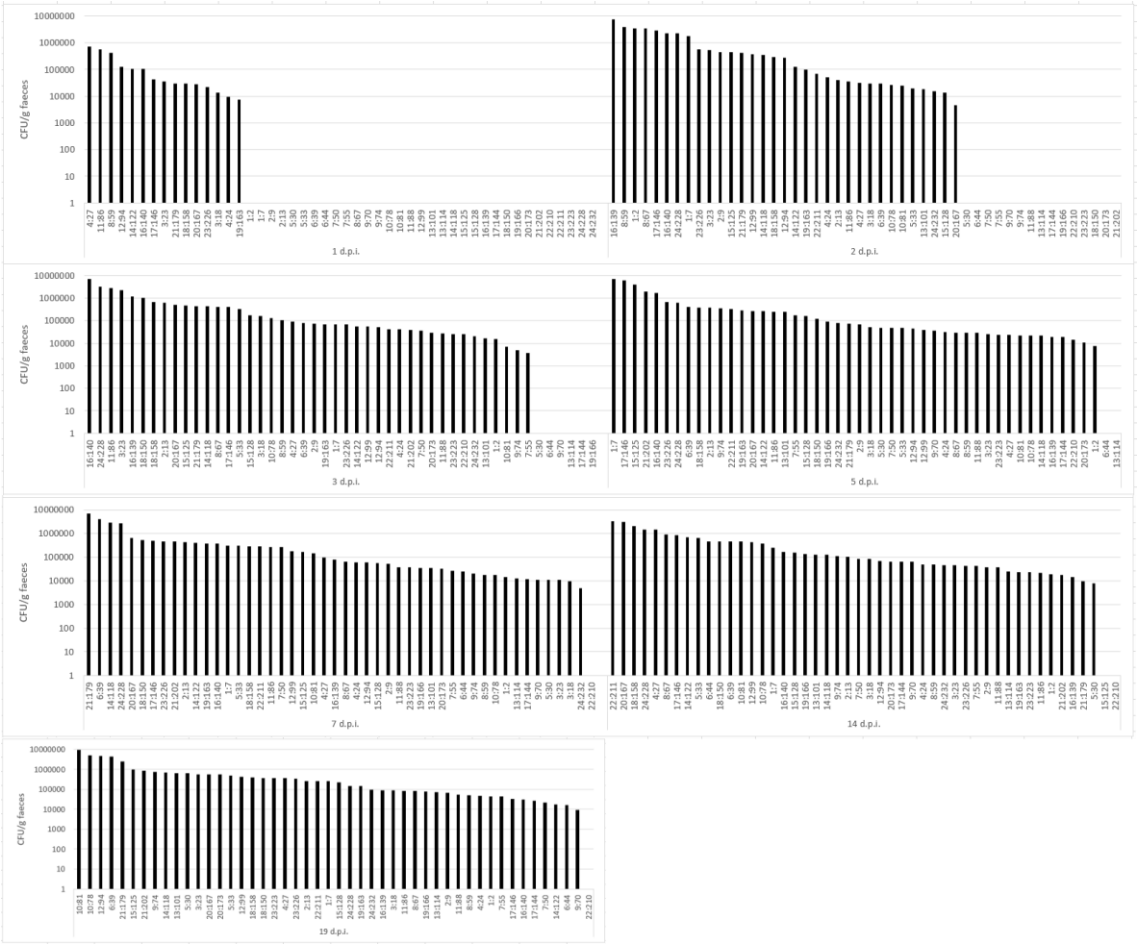

Figure 9. *Standard deviation within faecal samples was high, indicating uncertain data. When sampling the chickens for this project, six replicates of the same faecal sample was plated. The colonies were counted and based on the cfu/g faeces from each of the six replicates, the standard deviation within each sample was calculated. This was done for each of the sampling days.*

3. Discussion

The aim of this project was twofold. The first aim was to assess how supplementing the feed given to chickens with laminarin affected the levels of *C. jejuni* in the intestine, whereas the second aim was to assess whether the different strains of *C. jejuni* isolated from chicken and thrush had different colonization capabilities in chicken.

During this project, the main technique used has been bacterial plating on selective agar with enumeration. After counting, the data from each chicken could be assessed in many ways, such as by chicken treatment, hen treatment, inoculated strain of *C. jejuni* or a combination of any of the mentioned ways. This has been possible to achieve since the chickens were given different treatments, were hatched from hens who had been given other treatments and since the chicken were inoculated with two different strains of *C. jejuni*. It has been possible to assess whether there is a difference between the chicken given a feed supplemented with laminarin and chicken given conventional feed, and also whether there is a difference in how the two different strains colonize the chickens. By using this experimental approach, it has been possible to reduce the number of chickens used.

Sampling for quantitative analysis poses several challenges, compared to sampling for qualitative analysis. Analysing biological samples is highly dependent on how the samples were taken. In this study, the variance between samplings from the same faecal sample was analysed by having six replicates for each faecal sample. The higher concentration of *C. jejuni* in the faecal sample, the higher the variance and standard deviation. The difference in standard deviation between replicates from different samples varied between 0 and 9440000. This highlights the difficulty in taking samples representative for the whole of the sample. Depending on how the samples are taken, the results could vary greatly. This introduces some degree of uncertainty to the results. With enough samples and replicates, these variations are believed to cancel each other out, making the end results trustworthy.

In total, 48 chickens were used over the time this experiment carried on. This meant that for every sampling timepoint, 48 chickens had to be sampled. Handling the chickens took some time, but the most time-consuming part was plating the samples on mCCDA agar plates. The samples were plated one after the other and by the time the last one was plated, the first one had already had at least four hours in the incubator. The time it took between the first sample and the last sample varied between each day, but four hours was the minimum amount of time. Having a variation in time when the samples were plated, could have affected the results. *C. jejuni* does not grow at room temperature or in an oxygen rich environment, but there may be bacteria that have died in the faecal samples that were plated towards the end.

Previous studies done on younger chicks showed no difference in levels of *Campylobacter* between chickens raised on feed supplemented with laminarin or conventional feed. It was expected that this study would not come to any different conclusions than the study by Sweeney (Sweeney, 2017), which stated that there were no effect on the colonization of *C. jejuni* in young chickens treated with laminarin. As expected, this study did not find any differences either. There was no statistically significant difference between the chickens raised on the feed supplemented with laminarin or the conventional feed, for neither of the strains of *Campylobacter*. The chickens were hatched from hens that were given different treatments. The different hen treatments did not seem to impact the amount of *C. jejuni* in the intestines of the chickens either, except for a few comparisons on 3 d.p.i. Since these are just found on 3 d.p.i., it was assumed that these statistically significant differences were not due to a true difference, but rather that the samples taken accidentally contained enough *C. jejuni* to become statistically significant.

We speculated that supplementing laminarin to the chicken feed might have had an influence on the amounts of *C. jejuni* in other organs than the intestines as well, but no colonies could be seen on any of the plates. This would indicate that there is no difference in the amount of *C. jejuni* that translocated from the intestines into other organs between the different chicken treatments. Plating the inner organs proved very time consuming. The homogenization of the chicken livers and spleens by hand in the PBS, proved difficult. The lack of equipment that could homogenize the tissue, but at the same time preserve the viability of the *C. jejuni*, meant that the tissues had to be homogenized by hand. Homogenizing the tissues by hand did not homogenize the tissues completely, which meant that the tissue clogged the pipette used for dispersing the homogenate onto the agar plate. The tissue matter that got onto the plate made it difficult to assess any growth of *C. jejuni* on the plates. It is not possible to draw any conclusions from this organ assay due to these problems. If a proper homogenizer was used, the tissues could have been properly homogenized, and the results might have been different. It would also possible to examine the tissue samples present with molecular methods, such as qPCR. It is also possible that there has not occurred any translocation of the *C. jejuni* into the liver or spleen.

In a study done by Atterby (Atterby et al, 2018) the host specificity of three different strains of *C. jejuni* isolated from mallards, chickens, and thrush towards mallards was assessed. In that study, there was a clear difference in colonization capability between the different strains. In mallards the mallard strain was the best colonizer, while the chicken strain was the next best and the thrush strain were the third best colonizer.

This study was conducted using the strain of *C. jejuni* isolated from chicken and the strain isolated from thrush, also used in the study done by Atterby (Atterby et al, 2018). Since the chicken strain *C. jejuni* was isolated from chickens, we assumed that the chicken strain would be adapted to the chicken host in a way that the thrush strain was not. Previous genetic analysis done by Atterby (Atterby et al, 2018) showed that the two strains used in this study were genetically different, which would indicate a difference in adaptation to their respective host. This study however did not find any differences in the colonisation capability between the two different strains when inoculated into chickens. The results were therefore surprising but may be explained by the fact that the chicken is a domesticated animal, while the thrush is a wild one. Chicken might be susceptible to colonization of a variety of different strains of *C. jejuni* with a greater genetic variation than most of the wild bird species.

The amount of *C. jejuni* in the inoculates were quite low as compared to the inoculates used in the study done by Atterby (Atterby, 2018), where the inoculates contained a cfu/ml of 0.6–1.8 × 10^9 cfu/ml. The difference in concentration of the inoculates used in this study was in the range of 2.3–4.0 × 10^3 cfu/ml, which is a narrower range than in the study done by Atterby. The slight difference in concentration between the two different strains would probably not have influenced the colonization of the *C. jejuni*.

3.1. Ethics

To conduct this experiment, research animals have been used. The research animal ethical permit was given by the Uppsala regional research animal ethics board and was given the permit number 5.8.18-10572/2019.

When conducting animal studies, it is important to consider the wellbeing of the research animals. If it is possible to replace the research animals for a system where the animals are not used, this must be considered. If replacing the animals is not plausible, the number of animals used must be reduced as much as possible without sacrificing the statistical power. The animals used must also have refined living conditions. Their basic and social needs should be met if possible. These three factors are important to consider when conducting animal studies.

Chickens are social animals and they should be kept in groups. To limit the number of chickens in each cage too much could make the chickens devoid of their social interactions. Limiting the number of chickens too much would also result in insecure data. This experiment was also affected by how many chickens the bigger project run by SLU used. Reducing the number of animals was therefore not feasible.

SLU looked at parameters such as egg quality, growth rate and immunological status in chickens raised using feed supplemented with laminarin or conventional feed. They therefore needed to use chickens. This experiment was a part of this experiment and needed to use chickens. Replacing the chickens was not an option.

When it comes to refining the experiment, there were some measures put in place. The chickens were kept in 24 different cages, with four chickens in each cage. The cages were lined with wood shavings. The chickens had a constant supply of feed and water, and the ambient environment was moistened and heated.

48 out of 96 chicken were inoculated intra-oesophagically with one ml of *C. jejuni* inoculate. The inoculate was transported to the lab on ice, which made the inoculate quite cold and uncomfortable for the chickens. Inoculating the chickens with *C. jejuni* however does not affect the chickens since *C. jejuni* is part of their normal intestinal flora.

Sampling of faeces was done by placing the chicken in a cardboard box and closing the lid until the chicken had left a faecal sample at the bottom of the box. The chickens that did not leave a faecal sample were swabbed in their cloaca with a cotton swab.

3.2. Future studies

There are several different ways this study can be improved upon and continued. The issues raised in this study regarding the difficulty of reaching a representative view of the samples, as well as the issues raised regarding the homogenization of the tissues are some of the problems that needs further assessment. Both issues could be improved upon by homogenizing the samples better. If more faeces could be sampled, the sample could reflect the level of *C. jejuni* in the chickens better. For this to be possible, a better system for homogenizing the samples would be needed.

If the tissues were to be homogenized, the result from the localization study might have been different. Having the correct equipment to homogenize the tissues could have made it easier to both plate and enumerate the *C. jejuni* on the agar plates. Assessing whether *C. jejuni* translocate differently in the different chicken treatments would be interesting and might provide further insights into the effect of laminarin.

These are however still based on plating and enumeration. Plating and enumeration of bacteria require live bacteria. In this study, after the samples were plated, they were placed at 4°C until the samples were counted, after which they were placed in -70°C. This is not an optimal environment for the bacteria, which might die. Using the same experimental approach using the same samples would not be possible, but quantification by molecular methods such as qPCR could be a possible way to analyse the samples already taken further.

Since no statistically significant differences were found using a concentration of 300 ppm of laminarin supplemented to the feed, it might be interesting to assess if a difference in dosage could influence the colonization capability of the *C. jejuni.* This could be assessed in further studies where the chickens were raised on feed supplemented with different amounts of laminarin. The effect of other algae extracts could also be assessed.

3.3. Conclusion

This study proved that there was no difference in how well two different *C. jejuni* strains of different host origin colonized the intestines of chickens raised on feed supplemented with laminarin as compared to conventional feed. Even if this result was what we expected, it is still interesting. Supplementing the feed with laminarin has been proven to increase the growth of chickens. Having a supplement that increases growth, while also does not increase the load of *C. jejuni* in chickens could be highly beneficial to the chicken industry. An increased interest in the field of aquatic agriculture could provide basis for increased research and development of new and novel ways of using marine algae for a sustainable future.

This study also showed that there was no difference in colonization capability between the strain of *C. jejuni* isolated from chickens and the strain isolated from thrush, in contrast to previous findings for the same strains in experimental infection of mallards.

4. Methods and materials

4.1. Research animals

The research animal ethical permit was given by the Uppsala regional research animal ethics board and was given the permit number 5.8.18-10572/2019.

The animals used were chicken of the breed Ross 308. The chickens were kept four and four in 24 separate cages. The chicken had constant access to feed and water. The ambient environment was humidified to prevent dust formation and the temperature was raised to 25°C to keep the chickens warm.

4.2. Treatments

45 hens obtained at the start of the project at SLU. These were divided into three groups. The three different groups of hens were given either conventional control feed, conventional feed with whole algae or conventional feed with laminarin extract. These hens laid approx. 120 eggs each. 19 days before inoculation, the eggs were hatched. The chickens were assigned either a conventional control feed or a conventional feed with 300 ppm laminarin extract. 48 animals in total were used in this study, where 24 got the control treatment and 24 the laminarin treatment. The 48 chickens were hatched from hens that also got different treatments A table of which treatment every chicken belonged to can be found in **Supplementary 1**.

Laminarin was extracted from the algae *Saccharina latissima* cultivated at the Sven Lovén Centre for Marine Science at the Swedish West Coast. Dried algae was washed with 0.3 M hydrochloric acid followed by precipitation by ethanol. The extract contained 40% laminarin, determined enzymatically by measuring of the β 1,3/1,6-glucan content (K-YBGL 12/16, Megazyme). The experimental diets were thereby optimized to contain 250 ppm laminarin. The feed was based on a combination of wheat and soy protein. The exact composition of the two feeds used can be found in **Supplementary 2**.

4.3. Inoculate

C. jejuni isolated from chicken (*Gallus gallus domesticus*) (*C. jejuni* 65) and *C. jejuni* isolated from song thrush (*Turdus philomelus*) (*C. jejuni* 4:628) used in the previous experiments by Atterby were thawed and streaked onto mCCDA-plates (CM0739 Campylobacter Blood-Free Selective Agar Base, Oxoid with CCDA Selective Supplement, Oxoid). The plates were incubated at 42°C in a microaerobic environment using microaerophilic bags (Campygen 2.5l, Oxoid**)** for 30 hours. The colonies were streaked and placed in brucella broth (Uppsala Akademiska hospital) and incubated at 42°C in a microaerobic environment for 30 hours. The brucella broth contains 10 g/l tryptone, 10 g/l peptone, 2 g/l yeast extract, 1 g/l dextrose, 5 g/l sodium chloride, and 0.1 g/l sodium bisulphite. The optical density of the brucella medium was observed to get a rough count of the cfu/ml of *C. jejuni* in the brucella broth. Before measuring, both inoculates were diluted 1:10 in fresh brucella broth. The optical density of the brucella broth with *C. jejuni* 65 was 0.284, which corresponds to a cfu/ml of about 2.48×10^8, according to previous measurements. The optical density of the brucella broth with *C. jejuni* 4:628 was 0.106, which corresponds to a cfu/ml of about 8.74×10^7, also according to previous measurements. The inoculates were then diluted to a lower cfu/ml in PBS (Phosphate buffered saline tablet in water, Medicago). 2x50 ml tubes of each *C. jejuni* inoculate was used for the inoculation of the chickens.

On the day of inoculation, the chicken stable was divided into two separate parts to keep the two groups isolated from each other. The chickens on one side of the stable were inoculated intraoesophagically with 1 ml of PBS containing 231 cfu/ml of *C. jejuni* 65, and the other side with 1 ml of PBS containing 400 cfu/ml of *C. jejuni* 4:628.

4.4. Collection of faecal samples

The sampling was carried out by placing the chickens in cardboard boxes lined with A4 paper. The chickens were left in the boxes for approx. one hour until they had left a faecal sample in the bottom of the box. About 100 mg of the faeces was swabbed with cotton swabs and placed in tubes containing 1 ml Luria Broth containing 20% glycerol (Uppsala Akademiska hospital). In the cases where the chickens did not leave a faecal sample, the cloaca of the chickens were swabbed with the cotton swabs instead, before placing the swabs in the LB-Glycerol. After swabbing, the tubes were placed on ice.

4.5. Plating of faeces

The 6x6 drop plate method works by doing dilutions in a 96 well plate using a multi-channel pipette. 180 µl of PBS is added to each well, except two of the bottom rows. In the first column, 20 µl of the sample is added to each well, resulting in six duplicates from the same faecal sample being loaded. The samples are mixed by pipetting up and down ten times with a multi-channel pipette followed by a change of pipette tips and a transfer of the sample in the first column to the second column. The samples in the second row are mixed by pipetting up and down ten times followed by a change of pipette tips. This procedure is repeated until the sixth column has sample added. 10 µl of the contents of the wells in column six is transferred onto an agar plate using a multi-channel pipette followed by the contents in column five, four, three, two and one. This results in 36 samples being loaded onto the agar plate in a 6x6 drop pattern. After loading the first sample in duplicates and dilutions, the second sample is loaded into the seventh column of the 96 well plate and the same procedure is repeated for all the samples.

Each of the samples were vortexed for 15 seconds twice followed by a brief sedimentation of the particulates. 20 µl of sample was loaded into each of the six first wells in the first and the seventh column of a non-treated 96-well plates (WVR) preloaded with 180 µl of PBS in each well, as seen in **Figure 10**. Six tenfold dilutions with six replicates were then made using a multi-channel pipette (Finnpipette).

10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-

Figure 10. *A 96-well plate map showing how the dilutions were made. The grey shaded wells were left empty, while all the other wells had 180 µl of PBS. The orange shaded wells were loaded with 20 µl of one sample, while the blue shaded wells were loaded with 20 µl of one sample. The two samples on the same 96-well plate came from the same module.*

The multi-channel pipette was then used again to transfer 10 µl of one column of sample and placing it dropwise onto a mCCDA-plate. This was repeated to create a 6x6 grid on the mCCDA-plate with the different dilutions and replicates, as seen on **Figure 11**. The plates were left to dry for approx. 10 minutes before incubating them at 42°C for approx. 30 hours in a microaerophilic environment.

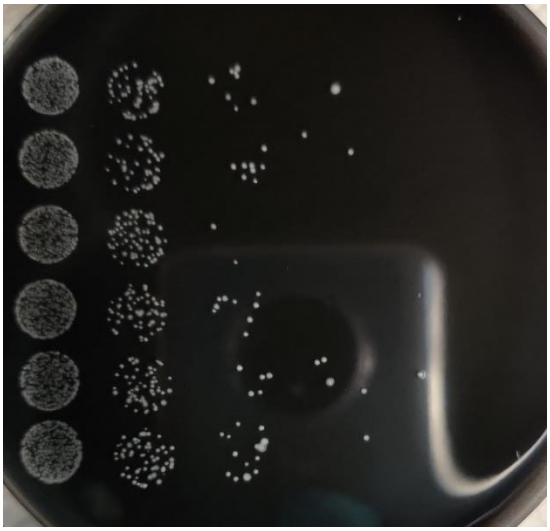

Figure 11. *Colonies of C. jejuni on mCCDA agar using the 6x6 drop plate method. Six tenfold dilutions were made using six replicates of the same sample. The plates were incubated in a microaerophilic environment at 42°C for approx. 30 h. followed by counting of the colonies.*

4.6. Plating of organs

The livers and spleens of the chickens were dissected out by the staff at SLU and transported on ice in separate Stomacher bags to the lab on the same day. 10 ml of PBS was added to each of the bags containing livers and 5 ml of PBS was added to each of the bags containing spleens. The tissues were then homogenized by hand.

100 µl of the homogenate was added onto mCCDA-plates. Some of the mCCDA-plates used for the plating of the organs were obtained from Uppsala Akademiska hospital, since the mCCDA-plates made in the lab were not enough. (Oxoid and Uppsala Akademiska hospital) and spread out using five glass beads to cover the entire plate. The plates were placed in a microaerophilic environment at 42°C for 48 hours.

5. Acknowledgements

I would like to thank my supervisor Patrik Ellström at the Department of Medical Biochemistry and Microbiology, Zoonosis Science Center, Uppsala University, Uppsala, Sweden for the supervision during this project.

I would also like to thank Emma Ivarsson, at the Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden who was the researcher responsible for the animal experiments and who has assisted us greatly with sampling at the chicken house.

Furthermore, I would like to acknowledge all my colleagues in the research group at the Zoonosis Science Lab, for helping me with the lab work.

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7. Supplementary 1

Chicken ID	Hen treatment	Chicken treatment	*C. jejuni* strain
1:2	Control	Laminarin	*C. jejuni* 4:628 – Thrush
1:7	Laminarin	Laminarin	*C. jejuni* 4:628 – Thrush
2:9	Algae	Control	*C. jejuni* 4:628 – Thrush
2:13	Laminarin	Control	*C. jejuni* 4:628 – Thrush
3:18	Control	Laminarin	*C. jejuni* 4:628 – Thrush
3:23	Laminarin	Laminarin	*C. jejuni* 4:628 – Thrush
4:24	Algae	Control	*C. jejuni* 4:628 – Thrush
4:27	Laminarin	Control	*C. jejuni* 4:628 – Thrush
5:30	Algae	Laminarin	*C. jejuni* 4:628 – Thrush
5:35	Laminarin	Laminarin	*C. jejuni* 4:628 – Thrush
6:39	Algae	Laminarin	*C. jejuni* 4:628 – Thrush
6:44	Control	Laminarin	*C. jejuni* 4:628 – Thrush
7:50	Control	Control	*C. jejuni* 4:628 – Thrush
7:55	Laminarin	Control	*C. jejuni* 4:628 – Thrush
8:59	Algae	Control	*C. jejuni* 4:628 – Thrush
8:67	Laminarin	Control	*C. jejuni* 4:628 – Thrush
9:70	Control	Laminarin	*C. jejuni* 4:628 – Thrush
9:74	Laminarin	Laminarin	*C. jejuni* 4:628 – Thrush
10:78	Algae	Control	*C. jejuni* 4:628 – Thrush
10:81	Control	Control	*C. jejuni* 4:628 – Thrush
11:86	Algae	Control	*C. jejuni* 4:628 – Thrush
11:88	Control	Control	*C. jejuni* 4:628 – Thrush
12:94	Algae	Laminarin	*C. jejuni* 4:628 – Thrush
12:99	Laminarin	Laminarin	*C. jejuni* 4:628 – Thrush
13:101	Algae	Control	*C. jejuni* 65 – Chicken
13:114	Laminarin	Control	*C. jejuni* 65 – Chicken
14:118	Control	Control	*C. jejuni* 65 – Chicken
14:122	Laminarin	Control	*C. jejuni* 65 – Chicken
15:125	Algae	Laminarin	*C. jejuni* 65 – Chicken
15:128	Control	Laminarin	*C. jejuni* 65 – Chicken
16:139	Laminarin	Control	*C. jejuni* 65 – Chicken
16:140	Laminarin	Control	*C. jejuni* 65 – Chicken
17:144	Algae	Control	*C. jejuni* 65 – Chicken
17:146	Control	Control	*C. jejuni* 65 – Chicken
18:150	Algae	Laminarin	*C. jejuni* 65 – Chicken
18:158	Laminarin	Laminarin	*C. jejuni* 65 – Chicken
19:163	Control	Laminarin	*C. jejuni* 65 – Chicken
19:166	Laminarin	Laminarin	*C. jejuni* 65 – Chicken
20:167	Algae	Control	*C. jejuni* 65 – Chicken
20:173	Laminarin	Control	*C. jejuni* 65 – Chicken
21:179	Algae	Laminarin	*C. jejuni* 65 – Chicken
21:202	Control	Laminarin	*C. jejuni* 65 – Chicken
22:210	Algae	Laminarin	*C. jejuni* 65 – Chicken
22:211	Control	Laminarin	*C. jejuni* 65 – Chicken
23:223	Control	Laminarin	*C. jejuni* 65 – Chicken
22:226	Laminarin	Laminarin	*C. jejuni* 65 – Chicken
24:228	Algae	Control	*C. jejuni* 65 – Chicken
24:232	Laminarin	Control	*C. jejuni* 65 – Chicken

8. Supplementary 2

Ingredient	Control (%)	Treatment (%)
Wheat	64.949	64.878
Soy 49% rp	27.223	27.223
Rapeseed oil	2.7	2.7
Chalk 0.0-0.5 mm KÖ BK	1.908	1.908
Monocalcium phosphate bk	1.008	1.008
Lysin BB	0.477	0.477
Sodium bicarbonate BB	0.383	0.383
Methionine BB	0.372	0.372
PRX Kyck UN3077(9) SS	0.250	0.250
Threonine BB	0.228	0.228
Vacuum salt bulk	0.100	0.100
Valine BB	0.008	0.008
Laminarin	-	0.075