**Title**

Anaerobic fermentation results in loss of viability of *Fasciola hepatica* metacercariae in grass silage.

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**Abstract**

The parasitic liver fluke, *Fasciola hepatica,* has a detrimental impact on food security and poses a welfare concern to ruminant livestock. *F. hepatica* metacercariae, shed from an intermediate mud snail host, encyst on vegetation and present a source of infection to grazing livestock. Feeding grass silage to ruminants is a common practice, however the role it plays in the transmission of *F. hepatica* remains largely unknown. Our current understanding relies on historical studies that are not representative of current silage production and did not apply molecular methods to detect *F. hepatica* DNA persistence within silages. This study determined the impact of specific fermentation factors, including grass dry matter (DM) content (20, 30 & 40%), length of ensiling period and maintaining an anaerobic environment on *F. hepatica* metacercariae viability. *In vitro* excystment assays demonstrated that regardless of grass DM content, metacercariae ensiled under anaerobic conditions were not viable from two weeks post-sealing. Metacercariae recovered from ensiled grass of 20% DM content subjected to aerobic spoilage, remained viable for up to 10 weeks. DNA of *F. hepatica* remained detectable for up to 10 weeks in both anaerobic and spoiled silages. This study highlights i) the importance of maintaining an anaerobic ensiling environment to eliminate the risk of *F. hepatica* transmission from silage and ii) an inverse relationship between grass DM content and duration of metacercariae survival within spoiled silages. Improving our understanding of trematode metacercariae survival rates within silages, especially of highly pathogenic species such as *F. hepatica*, allows farmers to make informed decisions regarding on-farm parasite control.

**Keywords**

*Fasciola hepatica*; metacercariae; trematode; silage; aerobic spoilage

1. **Introduction**

*Fasciola hepatica*, is a parasitic trematode commonly referred to as liver fluke. It is associated with temperate climates and primarily infects sheep and cattle. Acute fasciolosis, when large numbers of immature fluke migrate through the liver, is typically observed in sheep and is characterised by haemorrhage and sudden death (Fiss et al., 2013). Cattle often experience chronic disease indicated by anaemia, biliary obstruction and weight loss (Boray et al., 2007). The impact of sub-clinical *F. hepatica* infection on growth rate and milk yield is estimated to cost the UK cattle industry £40.4 million annually (Bennett and Ijpelaar, 2005). Additional economic costs include those related to veterinary diagnostics and anthelmintic treatment, implementing biosecurity measures and loss of carcass value. Climate change and emerging anthelmintic drug resistance, particularly to triclabendazole, have been associated with an increase in disease prevalence (Fox et al., 2011; Kelley et al., 2016).

The majority of grazing systems within temperate areas require silage provision for some of the year as pasture grazing is often not viable during winter months; preserved forages are also important in zero-grazing dairy units. Within Western Europe, approximately 10 million hectares of grassland are harvested for silage production annually (Wilkinson and Toivonen, 2003). Ensiling utilises the anaerobic fermentation of naturally occurring lactic acid bacteria which convert water soluble carbohydrates present within cut grass into lactic acid. Acidic conditions limit microbial activity and undesirable microorganisms gradually decline, providing anaerobic conditions are maintained. Achieving a pH between 3.8 and 4.5 within stored forage is desirable to limit coliform activity, a factor which is dependent on grass Dry Matter (DM) content. The introduction of oxygen to ensiled forages through poor consolidation, inadequate sealing or careless feed-out regimens reduces silage digestibility and nutritional benefit (Whitlock et al., 2000). During aerobic spoilage, proliferating yeasts produce ethanol which oxidises lactic acid. The subsequent rise in pH provides favourable conditions for bacilli and mould growth which negatively impact livestock health (Wilkinson and Davies, 2013).

*F. hepatica* metacercariae encysted on vegetation are the principle source of infection to grazing livestock. Survival of metacercariae on pasture is dependent on moisture and moderate temperatures, although they have been observed to survive repeated freeze-thaw (Boray and Enigk, 1964) and remain viable for 50 days within dried hay (Enigk and Hildebrandt, 1964). Our knowledge of the survival of *F. hepatica* metacercariae within silage is limited and relies on historic publications. A series of laboratory-based ensiling experiments conducted at 20° C and 40° C demonstrated loss of viability by 12 and 6 days storage, respectively (Enigk et al., 1964). Wikerhauser and Brglez (1961) found irreversible changes to the colour and shape of ensiled metacercariae. Metacercariae were unviable following 35 days ensiling, determined by unsuccessful experimental infection in 24 guinea pigs. Following experimental infection in rabbits, one animal developed hepatic lesions after infection with metacercariae which were seeded onto silage for 24 hrs (Tarczyñski and Podkówka, 1964). However, in thirty rabbits infected with metacercariae exposed to silage for 72 hrs no pathological signs of fasciolosis were observed; leading the authors to conclude that loss of metacercariae viability was due to lactic acid production during early fermentation. More recent work demonstrates that *F. hepatica* metacercariae incubated in lactic acid (pH 4) have a significantly lower *in vitro* excystment rate compared with metacercariae incubated in water and at pH 6 (p<0.01 and p<0.05, respectively) (Cuthill et al., 2017).

Silage production and forage management practices have advanced since the earliest literature was published and the current risk from feeding silage contaminated with *F. hepatica* metacercariae is unknown. Similarly, the impact, if any, of specific fermentation factors such as aerobic spoilage, silage pH, grass dry matter (DM) content and duration of ensiling on metacercariae viability requires further investigation (John et al., 2019). Following advancements in the detection of low quantities of *F. hepatica* DNA (Cwiklinski et al., 2015), there is potential to detect parasite contamination within the environment (Jones et al., 2018). The aim of this study was to determine if ensiled metacercariae pose an infection risk to livestock by i) understanding the impact of specific fermentation factors, including aerobic spoilage, on metacercariae viability and ii) evaluating the effectiveness of PCR as a diagnostic tool for detecting metacercariae contamination within silage.

1. **Materials and methodology**
	1. *Seeding of F. hepatica metacercariae onto grass and laboratory ensiling*

Grass was harvested under dry conditions from an established ley at a beef farm based in North West England. Wilting was conducted at 21° C, Dry Matter (DM) content was measured at regular intervals by microwave oven drying 10 g fresh sample (wet weight) and reweighing (dry weight) until all residual water was removed. Percentage DM (%) content was calculated as follows:

($Dry Weight \left(g\right)/Wet Weight (g))×100 = DM (\%) content$

Grass samples of 20, 30 and 40% DM content were produced, to represent a range of ensiling conditions typically found on farm. Grass was shredded into lengths of approximately 3 cm to encourage consolidation and packed into 150 ml glass boiling tubes. No silage additive was used. This convenient laboratory-based ensiling method supported a fermentation similar to that occurring in field-based silos (Autrey et al., 1947; Meiske et al., 1975) and allowed accurate sampling of multiple conditions and replicates which would not be possible if using commercial sized silos (Cherney et al., 2004). The assay was conducted in duplicate for 20% DM content grass (Trial A), quintuplicate for 30% DM content grass (Trial B) and triplicate for 40% DM content grass (Trial C) (Figure 1).

*G. truncatula* were experimentally infected with *F. hepatica* miracidia and stimulated to shed cercariae onto visking tubing as described previously (Hodgkinson et al., 2018). Cercarial encystment is facilitated by oral sucker attachment and resultant metacercariae strongly adhere to visking tubing. Metacercariae were at least eight weeks post shed, prior to ensiling. Encysted metacercariae were batched into groups of approximately 30 – 50 cysts. To ensure standardised exposure of metacercariae to fermentation compounds, two pieces of visking tubing containing metacercariae were inoculated 5 cm from the base and another two visking pieces were inoculated 5 cm from the top of each ensiling vessel (Figure 2). As a control for both the storage period and the *in vitro* excystment assay, one piece of visking tubing of ~30 metacercariae was submerged in ddH2O and kept at either 4° C or room temperature, for each experimental condition.

Vessels were anaerobically sealed using paraffin fermentation locks or covered with perforated cling film wrap to facilitate onset of aerobic spoilage. All vessels were stored in darkness at 21° C and ~70% relative humidity for either two, six or 10 weeks (Fig 1). The impact of delayed aerobic spoilage on metacercariae viability was also studied in duplicate: one week post-sealing with anaerobic fermentation locks, vessels were resealed with perforated cling film and sampled nine weeks later (Fig 1: Trial D). In total therefore, 19 different conditions were tested.

Wet chemistry analysis of key fermentation compounds to ascertain silage quality was conducted on batches of silage not inoculated with metacercariae (see section 2.4.).

* 1. *Assessment of metacercariae viability using in vitro excystment assays*

One visking piece was taken from each end of the ensiling vessel (Fig 2). Ensiled and control metacercariae were detached from visking using a scalpel and subjected to an *in vitro* excystment assay. ,The outer cyst wall was manually removed with tweezers and metacercariae were incubated at 37° C for 1 hour in a prepared solution containing bile salts and hydrochloric acid (McVeigh et al., 2014). Excystment activity was noted at 30 minute intervals for a maximum of 3 hours. Metacercariae were considered viable if motile juveniles developed with or without complete excystment.

* 1. *Detection of F. hepatica DNA from ensiled metacercariae*

One visking piece was taken from each end of the vessel and subjected to washing and DNA detection by PCR (Fig 2). Metacercariae were removed from visking tubing by suspension in bleach solution (30 ml sodium hypochlorite (5%) and 270 ml ddH2O) which was agitated in a magnetised stirring beaker for 2 hours. Beakers were coated with foetal calf serum (FCS) to prevent adhesion of metacercariae. Washings were filtered through a NalgeneTM column (Thermo ScientificTM, Loughborough, UK) and 10µM Millipore nylon net filter, both of which had been lined with FCS. Filters were retained for DNA extraction using the DNeasy Blood and Tissue kit (Qiagen™, Manchester, UK) according to the manufacturer’s instructions but with the following volume modifications (AL = 44 µl, Proteinase K = 40 µl, AE = 60 µl, molecular grade ethanol (Sigma-Aldrich) = 60 µl). ITS-2 and COX1 coding regions were amplified via PCR according to the literature (Cucher et al., 2006; Novobilský et al., 2013) but with the following modifications: (1) Biomix Red (Bioline) was used as the reaction mix and (2) template DNA was 4 µl of eluted *F. hepatica* DNA obtained from DNA extraction diluted to 1:10. PCR products were visualised using SYBR Safe DNA stain (Life Technologies) on a 1.5% agarose gel.

* 1. *Wet chemistry analysis of silages*

One hundred millilitre water extracts taken from 10 g of fresh control silages, were submitted for chemical analyses (Sciantec, UK) of acetic acid, n-Butyric Acid, lactic acid and ammonia-N content (Table 2). Post-ensiling DM was calculated (see section 2.1.), where silage DM content could not be calculated due to low recovery volume, pre-ensiling grass DM content was used when converting wet chemistry analyses data from mg/L to g/kg/DM. Silage pH was measured by agitating a 10 g sample in 90 ml ddH2O for 2 minutes, triplicate measurements were taken using a calibrated pH probe.

* 1. *Statistical analysis*

A chi square test was used to compare the number of viable control metacercariae from samples stored at 4° C and at room temperature. Exact binomial confidence intervals were calculated for the number of viable metacercariae recovered from each ensiling condition. Analysis was carried out in RStudio (RStudio Team, 2020) using packages “binom” (Dorai-Raj, 2014) and “ggplot2” (Wickham, 2016).

1. **Results**
	1. *Metacercariae recovery*

A range of 10 – 161 metacercariae were recovered from each ensiling vessel (Tables 1 and 2). Lower numbers were recovered from 20% DM silage as the visking tubing had partially disintegrated, presumably due to the wet conditions. A range of 18 – 32 metacercariae were recovered from controls stored at room temperature, alongside 21 – 55 metacercariae recovered from controls stored at 4° C.

* 1. *Viability of metacercariae in control assays*

Viable metacercariae, determined by excystment of newly excysted juvenile (NEJ) parasites, were observed in all control samples stored either at room temperature or at 4o C; excepting those used at 6 weeks for anaerobic and aerobic 40% DM conditions which were removed from subsequent analyses. Percentage viability ranged from 3 to 67%, with no statistical difference between those stored at room temperature and those stored at 4° C (χ-squared = 2.2345, df = 1, p-value = 0.135). Percentage viability of control metacercariae stored at room temperature was 21%, 26% and 42% at two, six and 10 weeks, respectively, showing no decline in viability over the duration of the experiment. Likewise, percentage viability of control metacercariae stored at 4° C was 23%, 11% and 23% at two, six and 10 weeks, respectively.

* 1. *Viability of metacercariae in anaerobic conditions*

Regardless of grass DM content, all metacercariae lost viability by two weeks when ensiled under anaerobic conditions, with loss of viability confirmed at six and 10 week timepoints (Figure 3).

*F. hepatica* DNA was detected in all anaerobic silages except in the 20% DM content grass ensiled for two weeks (Table 1).

The mean pH of anaerobic silages, regardless of grass DM content and ensiling period was 5.78 (range pH 5.08 – 6.14) (Table 3). Silages originating from grass of 20% DM content grass were high in n-butyric acid with undetectable levels of lactic acid. Grass of 30% DM content produced silages with high levels of acetic acid and undetectable levels of n-butyric acid. Similarly, silages originating from grass of 40% DM content were dominated by lactic acid with undetectable levels of n-butyric acid.

* 1. *Viability of metacercariae in aerobic conditions*

Under aerobic spoilage conditions, metacercariae remained viable post ensiling, with a range of values for each DM content and timepoint (Figure 4). For 20% DM content grass, viable metacercariae were recovered at every time point (two, six and 10 weeks). However, at 30% DM viable metacercariae were only recovered at two and six weeks and at 40% DM, viable metacercariae were recovered at two weeks only. When delayed spoilage was simulated at one week post-sealing, metacercariae were not viable at 10 weeks.

*F. hepatica* DNA was detected in all aerobic silages, except from 40% DM content at 10 weeks and the 40% DM content subjected to delayed aerobic spoilage (Table 2).

The mean pH of aerobic silages (excluding delayed spoilage samples), regardless of grass DM content and ensiling period was 9.16 (range pH 9.03 – 9.36) (Table 4). In general, chemical analyses for fermentation end-products indicated that aerobic silages lacked lactic acid, experienced low levels of acetic acid and relatively low levels of ammonia-N.

1. **Discussion**

This study demonstrates that there is no risk of *F. hepatica* transmission from anaerobically fermented silages, even when fed as soon as two weeks post-sealing. These results build upon previous work which concluded metacercariae viability was lost at 35 days post-sealing (Wikerhauser and Brglez, 1961). By contrast, under aerobic conditions, there is a risk of metacercariae survival for up to 10 weeks in silages made from grass of 20% DM content and two weeks in silages made from grass of 40% DM content. This novel finding demonstrates that spoiled forages may present a risk of *F. hepatica* transmission to livestock. Metacercariae recovered from silage subjected to delayed spoilage at one week post-sealing were not viable at 10 weeks post-storage; which reinforces the importance of sufficient anaerobic sealing at the start of the storage process, to destroy metacercariae. Other factors affecting metacercariae viability, including oxygen saturation are also likely to be important. The importance of adequate grass wilting to encourage optimal fermentation and prevent persistence of viable metacercariae within forage masses for long durations is highlighted. Metacercariae aerobically stored in grass of 20% DM content survived for 10 weeks; as DM content increased to 30% and 40% the duration for which metacercariae remained viable within spoiled silages decreased to six and two weeks, respectively. The relatively short survival period of metacercariae within aerobically stored grass of 40% DM content may be due to desiccation. Additional grass wilting following storage, which can occur under field conditions, resulted in silage DM content increasing to 85%. *Fasciola* spp. metacercariae have previously demonstrated limited resistance to drying (Ono et al., 1954) and a positive relationship between relative humidity and duration of survival (Suhardono et al., 2006). For silage making, a rapid wilting period between 24 – 48 hrs is advised to achieve a recommended DM content of 28 – 32% with minimal loss of water-soluble carbohydrates and protein. Within this timeframe, it is unlikely that metacercariae would perish prior to ensiling, as they can remain viable within dried hay for 50 days (Enigk and Hildebrandt, 1964).

*F. hepatica* DNA remained detectable within aerobic silages originating from grass of 20 and 30% DM content for up to 10 weeks, yet only detectable for up to two weeks within grass of 40% DM content. Four ensiling conditions were negative for *F. hepatica* DNA using both PCR protocols, despite metacercariae being successfully recovered from these silages. When anaerobically ensiling grass of 20% DM content, *F. hepatica* DNA was not detected by either ITS-2 or COX1 PCR protocol at two weeks; yet DNA was detected from the samples recovered at six and 10 weeks. This inconsistency in the PCR results may be due to variation in the quality of extracted DNA and presence of PCR inhibitors in some reactions, such as residual bleach from silage washing (Arifin et al., 2016).Our results suggest that using PCR as a reliable diagnostic tool for evaluating the potential *F. hepatica* infection risk from silages is not appropriate. In practice, *F. hepatica* DNA from other life cycle stages including eggs and miracidia may be amplified suggesting successful amplification of *F. hepatica* DNA from silage does not necessarily indicate that viable metacercariae are present.

Silages undergoing aerobic spoilage experience a reduction in acidity due to the oxidation of lactic acid by proliferating yeasts (Wilkinson and Davies, 2013), which is consistent with our results. The recommended pH of clamped grass silage originating from grass of 30% DM content ranges between pH 3.5 – 4.2. Despite metacercariae being killed as early as two weeks under anaerobic conditions, the pH values of anaerobic silages produced were above recommendation, indicating a sub-optimal fermentation. As such, the exact relationship between acidity and metacercariae viability proposed by other workers (Cuthill et al., 2017; Tarczyñski and Podkówka, 1964) was not investigated here.. Silages were not treated with additives or inoculants, meaning the fermentation relied on the epiphytic microorganism populations on the grass. Natural lactic acid bacteria populations may not have produced sufficient acid to inhibit clostridial metabolism; resulting in the secondary fermentation and proteolysis observed.

Chemical analyses of fermentation end-products demonstrate that a variety of fermentation types observed on farm were reproduced within our laboratory silos. As a generalisation, analyses follow those described by (Kung Jr et al., 2018) in terms of silage quality measures. Spoiled silages lacked lactic acid (occasionally n-butyric acid), experienced low levels of acetic acid and ammonia-N. Such data are typical of silages from the marginal regions of a clamp where some oxygen is present throughout storage. Anaerobic fermentation of 20 and 30% DM content grass had evidence of extensive secondary clostridial fermentation, reflected by increased ammonia-N levels. This type of fermentation is characteristic of peripheral regions of lower DM silages (Wilkinson, 2005). Anaerobically ensiling 40% DM content grass produced silages dominated by lactic acid with reduced levels of ammonia-N signifying minimal protein degradation. However, a high grass DM limited the extent of fermentation reducing the amount of acid produced overall.

1. **Conclusion**

Our results show that there isno risk of liver fluke infection to sheep and cattle from silage that has been ensiled for two weeks, providing anaerobic conditions are maintained during the silage making process. Under conditions associated with aerobic spoilage, metacercariae remain viable for up to 10 weeks in silages originating from grass of 20% DM content. Our findings also demonstrate that PCR is not a reliable diagnostic tool for evaluating potential contamination of silage with *F. hepatica*. This research has reinforced the importance of basic forage management practices and provides important new information regarding on-farm parasite control.

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**Tables**

Table 1: Molecular analyses for anaerobic ensiling conditions, number of metacercariae recovered and viability (%) following *in vitro* excystment assay.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Grass DM content (%) | Weeks Ensiling | ITS-2 PCR | COX1 PCR | No. of metacercariae recovered | No. of viable metacercariae  | Viability (%) | 95% CI |
| 20 | 2 | - | - | 10 | 0 | 0 | 0.00-30.85 |
| 6 | - | + | 60 | 0 | 0 | 0.00-5.96 |
| 10 | + | - | 30 | 0 | 0 | 0.00-11.57 |
| 30 | 2 | + | + | 74 | 0 | 0 | 0.00-4.86 |
| 6 | - | + | 108 | 0 | 0 | 0.00-3.36 |
| 10 | + | - | 161 | 0 | 0 | 0.00-2.27 |
| 40 | 2 | + | + | 85 | 0 | 0 | 0.00-4.25 |
| 10 | + | - | 140 | 0 | 0 | 0.00-2.60 |

Table 2: Molecular analyses for aerobic ensiling conditions (DS = delayed spoilage), metacercariae recovery and viability (%) following *in vitro* excystment assay.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Grass DM content (%) | Weeks Ensiling | ITS-2 PCR | COX1 PCR | No. metacercariae recovered | No. of viable metacercariae | Viability (%) | 95% CI |
| 20 | 2 | + | + | 27 | 26 | 96 | 81.03-99.91 |
| 6 | + | + | 19 | 1 | 5 | 0.13-26.03 |
| 10 | + | + | 21 | 6  | 29 | 11.28-52.18 |
| 30 | 2 | + | + | 114 | 41 | 36 | 27.19-45.49 |
| 6 | + | + | 62 | 8 | 13 | 5.74-23.85 |
| 10 | + | - | 47 | 0 | 0 | 0.00-7.55 |
| 40 | 2 | + | + | 111 | 12 | 11 | 5.71-18.12 |
| 10 | - | - | 112 | 0 | 0 | 0.00-3.24 |
| 40 | DS | - | - | 24 | 0 | 0 | 0.00-14.25 |

Table 3: Post-ensiling analysis of anaerobic silagesa, b.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Grass DM content (%) | Weeks Ensiling | pH | Silage DM content (%) | Lactic Acid (g/kg/DM) | Acetic Acid (g/kg/DM) | n-Butyric Acid (g/kg/DM) | Ammoniacal Nitrogen (g/kg/DM) | Total Acid (g/kg/DM) |
| 20 | 2 | 6.06 | 18 | \* | 25.22 | 24.88 | 6.99 | 57.09 |
| 6 | 6.14 | 19 | \* | 5.62 | 1.67 | 6.18 | 13.47 |
| 10 | 6.09 | 17 | \* | 13.78 | 12.81 | 3.10 | 29.69 |
| 30 | 2 | 5.62 | 22 | \* | 12.48 | \* | 3.87 | 16.35 |
| 6 | 5.35 | 22 | \* | 27.10 | \* | 6.57 | 33.67 |
| 10 | 5.93 | 21.5 | \* | 25.49 | \* | 6.98 | 32.47 |
| 40 | 2 | 5.83 | 36 | 14.47 | 5.99 | \* | 1.96 | 22.42 |
| 6 | 5.08 | 40 | 12.90 | 7.27 | \* | 1.73 | 21.90 |
| 10 | 5.96 | \* | \* | 12.49 | \* | 2.59 | 15.08 |

a Detection limits for analyses: 25 mg/L Acetic Acid, 25 mg/L n-Butyric Acid, 1000mg/L Lactic Acid, 50mg/kg Ammoniacal Nitrogen.

b Below detection limit = \*.

Table 4: Post-ensiling analysis of aerobic silages (DS = delayed spoilage) a, b.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Grass DM content (%) | Weeks Ensiling | pH | Silage DM content (%) | Lactic Acid (g/kg/DM) | Acetic Acid (g/kg/DM) | n-Butyric Acid (g/kg/DM) | Ammoniacal Nitrogen (g/kg/DM) | Total Acid (g/kg/DM) |
| 20 | 2 | 9.17 | 21 | \* | 3.63 | 0.66 | 4.11 | 8.40 |
| 6 | 9.08 | 15 | \* | 7.24 | \* | 4.24 | 11.48 |
| 10 | 9.05 | 19 | \* | 3.37 | 0.68 | 4.20 | 8.25 |
| 30 | 2 | 9.07 | 19 | \* | 3.66 | \* | 5.98 | 9.64 |
| 6 | 9.19 | 20.5 | \* | 3.67 | \* | 5.77 | 9.44 |
| 10 | 9.23 | 67 | \* | 1.66 | \* | 0.98 | 2.64 |
| 40 | 2 | 9.36 | 85 | \* | 0.61 | \* | 0.59 | 1.20 |
| 6 | 9.23 | \* | \* | 1.32 | \* | 1.32 | 2.64 |
| 10 | 9.03 | \* | \* | 3.84 | \* | \* | 3.84 |
| 40 | DS | 7.84 |  |  |  |  |  |  |

a Detection limits for analyses: 25 mg/L Acetic Acid, 25 mg/L n-Butyric Acid, 1000mg/L Lactic Acid, 50mg/kg Ammoniacal Nitrogen.

b Below detection limit = \*.

**Figure captions**

Figure 1: Experimental workflow showing grass samples of 20% (Trial A), 30% (Trial B) and 40% (Trial C) DM content being inoculated with *F. hepatica* metacercariae. Metacercariae were ensiled for two, six and 10 weeks under anaerobic (shaded) or aerobic (non-shaded) conditions alongside a delayed aerobic spoilage scenario (Trial D). Following ensiling, visking pieces were recovered and either used for molecular detection of *F. hepatica* DNA via PCR or processed for an *in vitro* excystment assay to determine metacercariae viability.

Figure 2: Diagram of an ensiling vessel containing four visking pieces stratified across two marked points (5 cm from the vessels top and base). Following ensiling, one visking piece was taken from each marked point and subjected to washing and PCR, remaining visking pieces were processed for an *in vitro* excystment assay to determine metacercariae viability.

Figure 3: Percentage (%) of *F. hepatica* metacercariae recovered from anaerobic ensiling conditions which were found to be viable by an *in vitro* excystment assay, with 95% CI.

Figure 4: Percentage (%) of *F. hepatica* metacercariae recovered from aerobic ensiling conditions which were found to be viable by an *in vitro* excystment assay, with 95% CI.