GRASS SILAGE FOR BIOREFINERY-

DIGESTION OF PROCESSED GRASS SILAGE FIBRE

Thesis Report

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Preface and acknowledgements

I am currently a 4th year student at the Aeres University of Applied Sciences in Dronten, The Netherlands. To validate my bachelor diploma in Livestock production with a major in Dairy Management, I got the great opportunity to carry out my final placement at the Natural Resources Institute (Luke) Finland in the department of Animal production. During this internship, I had the privilege to participate to some experiments but also, to conduct my own experiment with the expertise of researchers. The research centre is specialized in renewable natural resources and sustainable feed production. My subject was in linked with the interests of the institute. In that extend, during these fourth-months internship, I have been working on "Grass silage for biorefinery and in vitro digestion for dairy cows" which is one of the main research topics of Luke.

Throughout this report, it provides a bibliography on green biorefinery and in vitro process. An experiment was carried out according to the analysis of bibliography. Results and discussion are also detailed in this document.

This rapport is written for the module AGWP Dairy Production at Aeres University of applied sciences in Dronten.

I want to express my sincere gratitude to Marcia Franco, my supervisor for her guidance and unfailing support during my internship.

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I would like to thank you all the team of Animal Production of Luke for their kindness and their goodwill.

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Summary

The concept of green biorefinery is based on the processing of green biomass into range of innovative products. The basic idea is to use the green biomass in the most optimal way to produce energy and marketable products. Grass silage has versatile properties as raw material for green biorefinery with the advantage of being available all year round in Finland. Usually the biorefinery starts with mechanical separation of liquid and solid fractions, which can be used as animal feeds (solid part for ruminants and liquid part for ruminants but also for monogastric animals).Some studies showed the positive impact of using processed grass silage on milk production. The *in vitro* approach is a suitable method to evaluate rate of digestibility considering factors such as labour, costs, efficiency and time compared with *In vivo* experiments which are very time-consuming and expensive, The objective of the current study was to compare fibre composition of intact and processed grass and red clover material and to determine the digestibility of fibre fractions from biorefinery process, using an *in vitro* gas production method.

An *in vitro* gas production study was conducted at the Natural Resources Institute Finland to estimate the digestion rate of grass silage, fibre fraction and reconstituted silages processed by laboratory (Angel) and farm-scale (Haarslev) twin-screw presses, and red clover silage, fibre and reconstituted silage processed by Angel. Samples were incubated as fresh, but the weights were adjusted to 1 g of dry matter. Samples were incubated in two replicates within run and there were three separated runs that lasted for 72 h each one. A statistical analysis was carried out using SAS program. Results indicated that red clover silage had greater digestion rate than grass silage, while reconstituted red clover silage from Angel press produced less gas than original red clover silage during the incubation time. The reconstitution of the silage after processing with the Angel press for both grass and red clover did not result in any differences in digestion rate when compared to the original silages. We hypothesized that processing of fibre would improve the rate of its digestion, but the current experiment could not confirm it.

Chapter 1: Introduction

1- Background

Cattle are very important for human survival due to their capacity to digest fibrous feeds (like grass) and convert them into meat and milk (Gidlund, 2017). Moreover, given the expected increase in human population, especially in the developing countries, and because Finland has the objective to stop using soybean as animal feed by 2025 (Luke, 2019), an increasing demand for sustainable protein production from grass is more and more expected, as well as systems based on locally produced feeds.

Sustainable feed production aims to combine social responsibility, ecologically friendly and economically fair ways of feed production. In recent years, this concept is gaining importance due to environmental and natural resources utilization issues (Damborg et al., 2019). There is also increasing interest of the dairy industry in novel feed sources, which are mainly produced on farms and reinforce the consumer's confidence in terms of product quality as well as animal welfare (Hetta, 2004).

Northern European countries such as Finland have a humid temperate climate which is good for growing grasses. These areas have a capacity for high biomass production compared with other annual crops. Forages from grasses (especially short-term grassland) are the most important source of feed in Finland (Kuoppala, 2008). Some legumes such as red clover have very high production potential in these conditions. Furthermore high protein yields, with a low impact on environment can be achieved with these crops (Papendiek et al, 2016; Damborg et al, 2019).

For many years in Finland (at the Natural Resources Institute, Luke) intensive studies are carried out on red clover and grass silage as feed for dairy cows. Popularization and further development in the green biorefinery processes opens new possibilities for utilization of these feeds. The mechanical separation of these feeds during green biorefinery to liquid and fibre fraction allow to study the possibility to use these plant materials (red clover and grass silage) or part of it as feed for monogastric animals and for ruminants.

An experiment was set up by Rinne et al (2018) which put the emphasis on palatability of silage juice for growing pigs and lactating cows. For monogastric animal (as pigs) feeding silage juice would have positive benefits on the intestinal health of pigs (thanks to a lower pH of silage juice which help in stabilizing the pig digesta as well as the liquid feed). The daily growth rate was also improved. Furthermore, the utilization of silage juice as liquid feed should increase feed self-sufficiency of pig farms. In fact, grass fibre can also be used for bioenergy in a biogas farm combined with pig's slurry.

For dairy cows, researchers suggested that the TMR of high productive dairy cows could be fortified thanks to silage juice to increase the amount of on-farm produced grass in the diet. Moreover, the study showed that the soluble elements (sugars, amino acids) could partly escape the rumen degradation due to fast passage rate in the liquid fraction of silage. Parts of

mechanical separations can be diverted to quite a few groups of cows. For instance, the most fibrous press residue could be given to heifers and dry cows which need lower nutrient requirements. In that extend, biorefinery could give technical solutions for farmers at a regional, national and EU level. The trial also indicated that silage juice (which is the liquid part of the mechanical separation process) was very well consumed by cows (they could drink it as water).

2- Green biorefinery as a part of sustainable solution in feed production

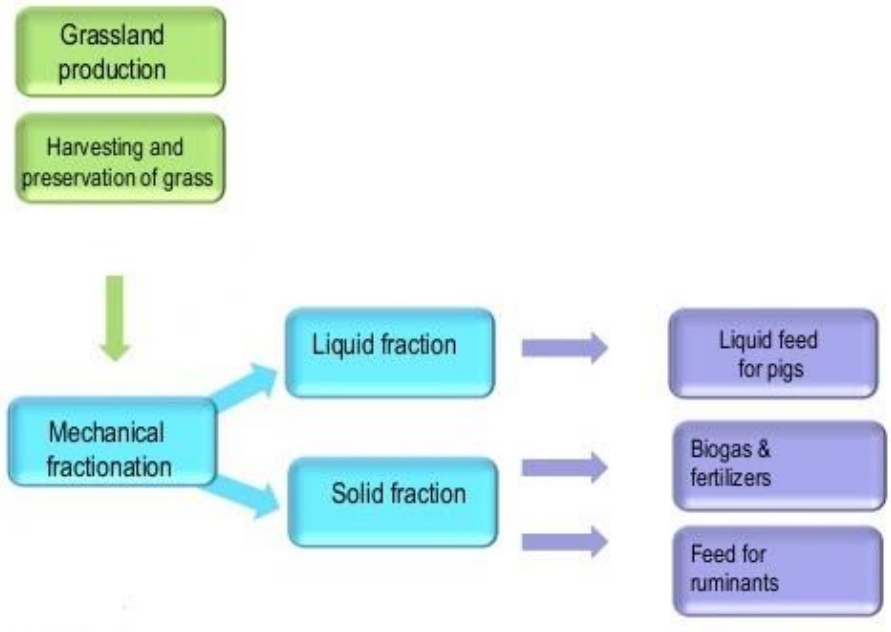

Figure 1: Concept of Green Biorefinery (source: Rinne et al, 2018)

The concept of green biorefinery (Figure 1) is based on the processing of green biomass into a range of innovative products (Damborg et al, 2019). Biorefinery can be used to improve the utilization of all sorts of wet biomass as agricultural crops (grass, clover, lucerne, cereals and other agricultural products) and cultivation residues (corn leaves, sugar beet leaves and by-products) (Damborg et al, 2019). These plants are considered as a natural chemical factory and are source of proteins, carbohydrates, lipids and lignin. They are also rich in vitamins and minerals (Kuoppala, 2008). The purpose of biorefinery is to use the green biomass in the most optimal way to produce energy and marketable products (for example solid fraction can be used for insulation boards or hydrolysed into simple sugars for other/further processes).

Usually the biorefinery starts with mechanical separation of liquid and solid fractions (Stefański et al, 2018), which can be used as animal feeds. The solid part can be used in ruminant diets, biogas, insulation boards, hydrolysed into simple sugars for further processes. The liquid part can be used for feeding pigs and ruminants (Rinne et al, 2018). Due to the utilisation of every fraction of nutrients, waste and environmental pollution are minimized (Damborg et al, 2019).

Different methods can be used to separate solid and liquid parts.

The separation method has impact on liquid and solid composition. Stefański et al (2018) reported that the solid fraction of separated silage showed a higher NDF and DM concentrations as well as a lower ash and CP than the original silage. Another experiment carried out by Wachendorf et al (2009) demonstrated that solid fraction from separated silage presented a lower ash concentration and as well a higher fibre concentration than the original silage (Stefański et al, 2018). Regarding liquid fraction, Stefanski et al (2018) indicated an impact on liquid DM concentration and liquid yield due to the separation method of silage. Moreover, the experiment resulted in a significant impact on the CP, DM and ash concentration in liquid. For the study presented in this report the silages were separated to solid and liquid fraction using farm scale twin screw press (FTS; Haarslev Industries A) and laboratory scale twin screw press (LTS; Angel Juicer Ltd).
Separation of liquid and solid fraction affects the value of the end product as animal feed, when compared to unprocessed silage. A study reported by Savonen (2020) demonstrated a higher milk production of cows which were feeding with diets composing solid fraction than cows which were feeding with intact silage.

3- Estimation of digestibility

The value of the end-product as animal feed is related to its digestibility. Usually to determine digestibility, *in vitro* experiments are conducted as the first step of evaluation feed value for new feed components and then, to confirm the results, *in vivo* tests can be conducted. In this study, the interest is to determine the gas production which is a good indicator of ruminal feed digestion. The *in vitro* gas production method is widely used by scientists because it provides good estimates of feed degradability at low cost and has high capacity.

In vitro method

The *in vitro* gas production method is often used as a first step in estimating digestibility. The objective of this procedure is to evaluate the gas production during microbial degradation of samples. The rate of gas production is used to estimate the rate of degradation.

The *in vitro* procedure involves anaerobic incubation of samples with rumen fluid and buffer. In other words, the objective is to incubate the substrate of interest in a biological medium for imitating ruminal conditions (Vaga, 2017). *In vitro* measurements are quick and easy to carry out compared to *in vivo* measurements. Moreover, the *in vitro* experiment provides the opportunity of rapidly analysing a high number of samples and only small quantities of feedstuffs are required. The *in vitro* and *in situ* methods have the advantage that they can examine separately feedstuffs, which is not often possible with the *in vivo* methods, because most of the time, the diet consist of a mixture of feedstuffs. Also *in vivo* experiments are resource demanding, and very labour intensive. *In vitro* and in situ methods are used for estimating digestibility using less resources.

Based on this background information, the *in vitro* approach is a suitable method to evaluate rate of digestibility considering factors such as labour, costs, efficiency and time.

Moreover, to evaluate digestibility, some studies (Savonen, 2020) demonstrated that physical form of forage is very important and will also have an important impact on nutritive value. This physical form will affect (for ruminants) dry matter consumption, rumen function, milk production and composition. As mentioned above, biorefinery has an impact on physical properties of silage (because of separation of liquid and solid parts). It this current study, the objective was to compare the particle size of intact and processed silage and the impact on fibre digestibility and therefore the main research question of this study is:

How does biorefinery mechanical separation affect the in vitro fibre digestibility of processed grass or clover for dairy cows?

To better understanding this main question, some sub questions were formulated to split the work and give a clearer picture of the research purpose:

Sub question 1: How will biorefinery mechanical separation affect compositions of the fibre fraction when intact and processed silage are compared?

Sub question 2: What is the impact (positive or negative) of processing red clover/ grass silage on the in vitro fibre digestion?

Sub question 3: To what extend does processing of red clover/ grass silage impact the particle size distribution?

The aim of the experiment was to compare fibre composition of intact and processed grass and red clover material and to determine the digestibility of fibre fractions from biorefinery process, using an *in vitro* gas production method. In other words, the study wants to determine if processing silage during screw-pressing would result in a faster rate of digestion in vitro. It's interesting to observe if processing silage would be a viable and suitable solution for future compared with intact silage. In this study, the digestibility can be calculated using the rate of digestion in a rumen model. In order to accomplish the objectives, two types of raw material were used: red clover silage and timothy grass silages. Red clover is the most important forage legume in Finland. This plant plays an important increasing role in future silage (Kuoppala, 2008). Silages were compared to determine the fibre digestibility. Moreover, the study will evaluate the effect of green biorefinery on liquid and solid yield as well as the composition and retained compounds of these raw materials.

Chapter 2: Materials and Methods

In this study, fibre digestibility of two types of raw materials were evaluated: the red clover silage and grass silage. The experimentation was conducted at the Natural Resources Institute Finland (Luke) in Jokioinen, Finland.

1- Experimental design

Samples of red clover silage and timothy grass silage came from the harvest of summer 2017 and were stored frozen at -20 C degrees. Also, frozen (at -20) samples of fibre and liquid fractions from a previous *in vivo* experiment (Grass silage solid fraction from a biorefinery as a feed for dairy cows in 2017, Savonen et al., 2020) where the grass silage was processed by Haarslev twin screw press was used (picture 2 and 3). One week before the experiment starts, all samples were thawed at 5 °C for 48 h, and dry matter (DM) for all samples was determined. The samples of silages were processed by Angel press, and the yield and DM of fibre and liquid fractions were measured. To decrease the particle size of intact silages and fibre fractions to facilitate weighing a homogenous sample for the in vitro incubations, the samples were chopped by scissors. The samples for analyses were taken, and the remaining samples were divided into tree batches (one for each *in vitro* run) and refrozen at – 20 °C. One day before each *in vitro* run, one batch of samples was thawed at 5 °C. In the afternoon, after thorough mixing, fresh matter samples equal to 1 g DM were weighed into the 250 ml glass bottles, the liquid and fibre fraction for reconstituted treatment were weighed into the same glass bottles.

The *in vitro* incubation lasted for 72 h and gas production measurements were recorded every 1 min. Cumulative gas curves were produced and the amount of gas from the inoculum was reduced from the curves. A 2-pool Gompertz function was fitted to the curve and the model parameters was used in a rumen model to yield rate of digestion and digestibility of the feeds as described by Rinne et al. (2016).

Picture 2: Angel Press

Photograph: Léa Piou

Picture 3 : Haarslev press

Photograph: Marketta Rinne

2- *In vitro* procedure

The Ankom RF Gas production system (see attachement 1 for details) was used for measuring and monitoring gas production. The samples were incubated in 250 ml bottles equipped with pressure sensor modules. One pressure sensor module was used to measure ambient pressure.

Every bottle is equipped with a module which is communicating with computer via wireless connection. The module measures pressure in the bottle and releases it automatically when the chosen range is reached. The cumulative gas production (GP) is recorded at a chosen interval in the computer. In fermentation studies with rumen fluid as the inoculum, the pressure is measured every 1 minute during 72 to 96 hours.

The current setup of the *in vitro* GP apparatus at Luke (Rinne et al. 2016) consists of 2 units (water baths) of 16 bottles each. Two blank bottles (buffer and rumen fluid with no sample) were used per each water bath. The blanks are run in every experiment to correct for the gas produced by the inoculum and the gas lost by slight permeability of CO_2 through the elastomeric components of the system (gasket, silicon tube and connections). The standard bottles contain standard samples with known digestibility and are used to check the inoculum quality and the overall 'quality of the run'. During the experiment, if the digestibility of the standard samples varies more than 10 % from the know digestibility (average from many runs) the run is repeated.

3- Treatments for *in vitro* gas production

In order to evaluate the *in vitro* gas production, eight treatments were tested additionally to the inoculum and control as follow:

I- Intact red clover silage
II- Intact timothy grass silage
III- Timothy grass silage fibre from the Angel (solid part)
IV- Reconstituted grass silage processed by Angel press (solid + liquid part)
V- Timothy grass silage fibre from Haarslev press (solid part)
VI- Reconstituted grass silage processed by Haarslev press (solid + liquid part)
VII- Red clover silage fibre from the Angel (solid part)
VIII- Reconstituted red clover silage processed by Angel (solid + liquid part)

The samples were produced in Finland and they vary in silage processing method and intensity.

4- Particle Size and Wet-Sieving

The mean particle size of both silages and solid fractions was determined by wet sieving (attachment 2)

5- Analysis of samples

The samples were analyzed using the *in vitro* gas production method according to the existing routine procedure (see Rinne et al. 2016). All samples are incubated in two replicates within the runs and there were three runs.

The DM, neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), ash and nitrogen were determined for intact silages and solid fractions. Crude protein (CP) was calculated as 6.25 × N. For liquid samples, DM, ash and CP were determined.

During separation of the silage into liquid and solid fractions, the amount of liquid was quantitated to calculate the yield (separation efficiency) of the fractions.

6- Calculation of results

The results of GP were analysed using the GLM procedure (SAS Inc. 2002-2012, Release 9.4; SAS Inst., Inc., Cary, NC) of SAS. A mean of the analytical replicates from the same run was used in statistical analysis. Least squares mean and standard error of the means was reported per treatment and differences among treatment means it is declared significant at 5% of probability. A pairwise comparison among treatment means was performed using a Tukey´s test when the overall effect of treatment is found significant. Comparisons were also done using orthogonal contrasts.

7- Donor Animal

Three Nordic Red milking cows which were equipped with rumen cannula of Luke Jokioinen were used. They were in different stage of lactation, to increase the versatility of inoculum. Cows were fed diet based on grass silage and concentrate. The rumen fluid was collected 2 h after morning feeding. The experiment was carried out in accordance with the laws and regulations controlling experiments performed with live animal in Finland

Chapter 3: Results

1- Composition of fibre fraction

The DM concentration of original grass silage and original red clover silage were 221 and 258 g/kg, respectively (Table 1). Both plants were processed by a laboratory scale twin-screw press, separating the liquid and solid fraction (fibre and liquid). Table 1 presents the chemical composition of original silages, fibre and liquid fractions. It can be noticed that the dry matter concentration of original silage for both plants are lower than grass silage fibre and red clover fibre produced by Haarslev press as well as Angel press (Table 1). The dry matter concentration of liquid fraction for grass silage and red clover processed by Angel or Haarslev presses ranged from 70 to 117 g/Kg and are the lowest compared with all results.

The ash concentration of original red clover silage (106 g/kg) is higher than the ash concentration of original grass silage (70 g/kg). It can be observed that this difference didn't change with both plants which were processed by Angel or Haarslev press. The Ash concentration is highest for red clover independent of presses (for also Ash in fibre).

2- In vitro digestibility

Total gas production, digested fraction of the potentially digestible dry matter and digestion rate are shown in Figure 1. It can be noticed that the total gas production of grass silages is higher than red clover silages ($P<0.05$; Table 2).
Results showed that there were no statistical differences for the total gas production between original grass silage and grass silage reconstituted by Haarslev press as well as between grass silage from Haarslev and grass silage from Angel ($P>0.05$; Figure 1B and Table 2). There were also no statistical differences between grass silage fibre from the Angel and grass silage fibre from the Haarslev press. Nevertheless, it can be observed that grass silage fibre from the Haarslev and Angel presses resulted in a significantly lower total gas production than the grass silage reconstituted grass silages from Angel and Haarslev press. Furthermore, it indicates that fibre fraction, regardless of plant species or press type, resulted in a lower gas production ($P<0.05$) than silage and reconstituted silages.
It can be noticed that the total gas production of grass silages is higher than red clover silages ($P<0.05$; Table 2). Furthermore, red clover fibre from Angel press has the lower gas production ($P<0.05$; Figure 1A and Table 2). However, red clover from the Angel has the higher amount of fraction which can be digested.

There were no statistical differences for the digested fraction of the potentially digestible dry matter ($P>0.05$; Figure 1B and Table 2) according to the different plant species, press method and biorefined silage fractions. Highest digestion rate ($P<0.05$) was found for the fibre fraction of red clover pressed by Angel, followed by red clover silage and reconstituted red clover silage (Figure 1B). It can be noticed a small difference between red clover silage and reconstituted red

clover by Angel (P<0.05; Figure 1B and Table 2). Grass fibre fraction resulted in lower digestion rate than red clover fibre processed by the Angel press. Red clover silage had greater digestion rate than grass silage (Table 2). Reconstituted silages from Angel press for both grass and red clover did not differ in digestion rate when compared to the original silages.

3- Particle size distribution

The last step of the experiment was to determine the particle size distribution by wet sieving. According to results, (Table 1) it can be noticed that biorefinery process impacted fibre particle size in comparison with original silage. Moreover, the Angel press resulted in the smallest particle size when compared to original silage and Haarslev press. In that extend with this current study, biorefinery has at least an impact on the physical properties of silage.

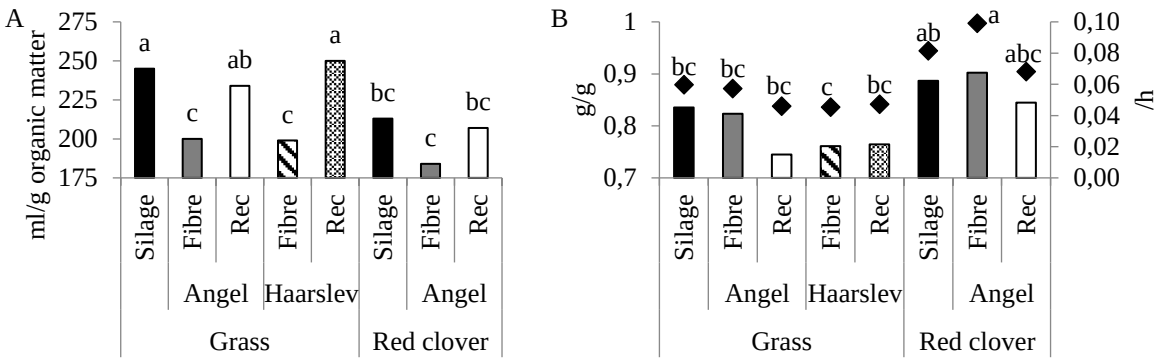

Figure 1. Total gas production (ml/g organic matter; A), and digested fraction of the potentially digestible dry matter (bars, g/g; B) and digestion rate (dots, k_d, /h; B) of grass and red clover silages, fibre and reconstituted (Rec) silages processed by Angel or Haarslev presses. Values with same letter are not significantly different at 5% Tukey test.

Table 1. Chemical composition of original silages, fibre and liquid fractions

	Grass					Red clover		
	Silage	Haarslev press[1]		Angel press[2]		Silage	Angel press[3]	
		Fibre	Liquid	Fibre	Liquid		Fibre	Liquid
Dry matter (DM), g/kg	221	449	70	492	84	258	418	117
Ash, g/kg DM	70	43	180	45	148	106	74	230
Crude protein, g/kg DM	136	105	277	97		173	160	
Neutral detergent fibre, g/kg DM	573	699	-		-			-
Acid detergent fibre, g/kg DM								
Acid detergent lignin, g/kg DM								
Particle size, mm	6.0	4.5		0.4		12.7	0.3	

[1]Farm scale twin screw press, liquid yield 0.605; [2]Laboratory scale twin screw press, liquid yield 0.587; [3]Laboratory scale twin screw press, liquid yield 0.421.

Table 2. P-values for the contrasts in Figure 1

	Contrasts							SEM
	GvsRC	GvsGRA	GvsGRH	GFAvsGFH	RCvsRCRA	GFAvsRCFA	GRAvsRCRA	
Total gas	<0.01	0.28	0.64	0.93	0.50	0.11	<0.01	6.9
Digested	0.36	0.09	0.15	0.26	0.57	0.24	0.19	0.068
k_d	0.04	0.16	0.18	0.26	0.35	<0.01	0.12	0.012

G, grass silage; RC, red clover silage; GRA, grass silage reconstituted from Angel press; GRH, grass silage reconstituted from Haarslev press; GFA, grass silage fibre processed by Angel press; GFH, grass silage fibre processed by Haarslev press; RC, red clover silage; RCRA, red clover silage reconstituted from Angel press; RCFA, red clover silage fibre processed by Angel press.

Chapter 4: Discussion

The main aim of this study was to determine the digestibility of original silages and fibre fractions from biorefinery process using an *in vitro* method. In other words, the study wants to show if processing silage would be suitable for future compared with intact silage. We studied also the effect of biorefinery process on particle size distribution in the fibre fractions of processed material.
It was hypothesized that processing of the fibre with twin-screw presses would aid the microbial digestion of it and thus would result in a faster rate of *in vitro* digestion, and that the biorefinery process will affect the particle size distribution in the fibre fractions.

Impact of biorefinery process

The processing of silage by biorefinery modified the fermentation parameters in an *in vitro* system.
In the current study, the biorefinery has indeed a significant impact on particle size especially with fibre processed with the Angel press. The laboratory press (Angel) can significantly reduce the particle size of silage. There is limiting literature on the effect of biorefinery on particle size of silage. Literature showed that small particles can pass out of the rumen more rapidly than bigger silage particles (Li et al, 2012). Small particles size may also result in faster microbial attachment to the fibre and provide easier access to it. Faster passage rate would decrease digestibility. but since closed bottles were used during the experiment, it's not possible to mimic effects of rate of passage, only rate of digestion. Thus, it could be hypothesized that processing silage with Angel would result in a better digestion rate than intact silage. In other words, processing silage with the Angel press would have a positive impact on fibre digestion.

However, results demonstrated that press methods had minor effect on the fermentation dynamics of silage, fibre fraction and reconstituted silage. The reconstitution of the silage after processing with the Angel press for both grass and red clover did not result in any differences in digestion rate when compared to the intact silages. Nevertheless, the trial could not totally confirm that the process of forage fibre with screw-pressing would result in a faster rate of in vitro digestion and would have a significant positive impact on fibre digestibility.
There are limited studies on the impact of fractionation on nutritive value due to press methods. Stefanski et al (2018) reported that there was not significant difference between both presses on liquid fraction. It was also noticed that the separation of silage into liquid and solid fractions had an impact on DM, CP and ash concentration as well as fibre content. The composition of silage and fibre are thus affected by biorefinery. According to literature, it would result in an impact on the milk production.

The experiment indicated that species behaved differently with greater digestion rate for red clover than timothy grass silage. Hetta et al (2004) also reported that red clover resulted in a faster digestion rate of pdNDF for red clover than grass silage. Moreover, red clover resulted

in a higher fractional degradation rate for forage but a lower fractional degradation rates for neutral detergent soluble components than timothy grass silage.

Chapter 5: Conclusion and recommendations

The goal of this study carried out at the Natural Resources Institute Finland wanted to evaluate the effect of green biorefinery on liquid and solid fraction compared with original silage. Two press methods were used to measure the fibre fraction of Grass silage and red clover (two plants which are very used in Finland). The experiment did not show that press methods clearly affect the fermentation dynamics of silage, reconstituted silage and fibre. But it could be noticed that plant species behaved differently with greater digestion rate for red clover than grass silage. The study indicated that processed silage had a slight impact on fibre composition. Literature put the emphasis that processing silage results in a positive impact on fibre digestion for ruminants. Nevertheless, the current experiment didn't indicate that processing of silage fibre during screw-pressing would result in a faster rate of digestion *in vitro.*

Regarding results of the experiment, it would be interesting to follow up the work with an i*n sacco* method. This method consists of inserting nylon bags (with samples inside) through the canula and thus, to evaluate the fibre digestibility in the rumen. This procedure would give us a more accurate estimation of the fibre digestibility in the rumen and could confirm results.

Furthermore, as mentioned in the literature, gas production is the result of microbial activity. Nevertheless, microbial activity may be influenced by time of feeding. Few studies showed the effect of microbial activity according to the feeding time and put the emphasis on a better microbial activity before morning feeding (Li et al, 2012). In this current study, rumen fluid was sampled 2 hours after morning feeding (8.00 am). In other words, if I would have the opportunity to repeat the experience again, it would be interested to evaluate this parameter on total gas production. The objective would be to compare the microbial activity before and after morning feeding. I would make 2 rumen sampling times: one before morning feeding (at 6.00 am) and one after morning feeding (around 8 am). Nevertheless, making samples at 6.00 am would have been too much constraining (preparation of the experiment needs 3 hours before sampling: bottle samples, catching cows, in vitro materials, etc.).

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Attachment 1:

Preparation of Ankom gas measurement equipment for *in vitro* experiment (Rinne et al, 2016)

There are some important instructions to follow for preparing a study with the ANKOM Gas Production System

Step 1: Start the GPM Software

The first step is to start the program by clicking on the GPM icon on the computer monitor. Moreover, the reference Module 0 which is used to measures the ambient pressure (there is no glass bottle attached to it) has to be started.

Step 2: Testing that each Module is communicating with the computer

The communication between the Modules and the Computer need to be tested.

First of all, the base coordinator needs to be connected to the computer. A battery packs is put into each Modules which must be tested. The battery packs within each module must be connected from the male connector on the battery pack to the female connector on the circuit board. Then, it just needs to verify that the GPM software recognizes each module (the GPM screen will display a battery voltage for each module that it recognizes).

Step 3: Test the vent valves operation for each Module

On the GPM screen, live interval is changed to 1 second and the recording interval to 0.50 second, and the valve opening pressure is set to 0.4 psi. To start, the record button on the computer is press for recording pressure data.

Then, the glass bottle is attached to the Module (the rubber gasket and silicone gel is used to seal the connection).

A luer check valve is joined to luer port of each Module which must be tested.

The syringe is filled with air and attached to the end of the luer check valve. The glass bottle is pressurized by pushing slowly the syringe plunger. When the pressure exceeds 0.4 psi the vent valve will be open to release the gas.

The aim is to check that the Module recorded the pressure changing and that the vent valve works properly.

Modules with failing pieces

After the test operation, some modules:

- do not measure the pressure change,

- measured the pressure but the vent valve do not open,

-or the module measured pressure, the vent valve open but the gas was not released.

The not working Modules and the working modules are separated.

Modules which have problems are repaired by removing and replacing the failing pieces.

Attachment 2:

Protocol of Wet sieving

I- Intact Grass silage
II- Grass silage fibre from the Angle (solid part)
III- Grass silage fibre from Haarslev press (solid part)
IV- Red clover silage fibre from the Angle (solid part)

Preparations:

- Particle size distribution is determined at minimum as two replicates
- After the first sievings, the distribution of particulate matter on the sieves is checked and possible modifications on the use of sieves can be done, if the distribution is very uneven.
- For each sample and sieve, a dried (overnight at 60 □C) nylon bag (pore size 38 µm) is weighed
- Frozen samples are taken into cool storage well in advance and placed in buckets (the two samples from each cow and period per bucket, because plastic bags may be broken)
- Melted sample is thorougly mixed and 30 g is weighed for each sieving. The rest of the sample is refrozen. Representative sampling of especially ruminal samples is critical!
- Sample is transfered to a bigger container, approximately 1 l of water is added and the sample is stirred until all lumps have disappeared.
- The sieves are installed into the machine: 2500 µm, 1250 µm, (possibly 630 µm,) 315 µm and 160 µm (if especially for faecal samples, there is very much material in the nylon bag, it may be better to include the 80 µm sieve).
- A nylon bag (38 µm) is attached to the outlet of the sieving machine to collect the smallest particles.

Sieving:

- Decant the sample on the top sieve and flush the container carefully with water to remove all particles
- Fasten the lid of the sieving machine
- Open the water tap. The water flow is controlled by the nozzle and is approximately 3.5 l/min (could be measured to check the current value)
- Connect the intermittent shaking
- Set amplitude so that the optic scale is at approximately 2
- If the water does not flow well through the sieves (no water is coming to the outlet), increase the amplitude for a moment
- Sieve for 10 minutes

Treatment of particles

- Flush the particles to tared nylon bags using a funnel. ALL particles are needed!
- Dry the nylon bags at 60 □C for 2 days, place in exicator and weigh

- Collect the samples from each sieve to plastic containers, mill, and weigh to nylon bags: