Evaluation of *Beta vulgaris* as a Candidate Feedstock for Industrial Lactic Acid Production

Submitted by Naomi Gachanja to the University of Exeter as a thesis for the degree of
Masters of Science by Research in Biological Sciences in December 2018

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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ABSTRACT

An increase in global demands for fuel from renewable sources has accelerated research into alternative energy sources that are economical and can reduce greenhouse gases. These renewable fuels must also not cause further challenges by competing for agricultural land use with food sources. Therefore, lignocellulosic feedstock, which are cheap and abundant non-food materials are an emerging solution. Concurrently, there is also rising interest in sustainable and renewable based chemicals. Lactic acid is valuable in many industries and is one such chemical. In addition to use as a preservative and emulsifying agent, lactic acid is also a precursor for poly-lactic acid, a biodegradable and biocompatible polymer. Microbial fermentation with *Beta vulgaris* (sugar beet) in a low-technology process such as ensiling has the potential to provide high titers of ethanol for use as a biofuel and lactic acid for industry.

In this project, the utilisation of seven carbohydrates, sucrose, glucose, fructose, xylose, arabinose, cellobiose and D-galacturonic acid by several lactic acid bacteria that were considered potential inoculants for sugar beet ensiling was assessed. This was used to determine the optimal microbial consortia for use as an inoculant to produce high lactic acid yields. A laboratory sugar beet ensiling protocol was also developed and various factors including shredding sugar beet hypocotyls prior to ensiling, incubation between 32-37 °C, 5-7% w/v $CaCO_3$ and 5% w/v sodium chloride determined to produce 20-25 mmol g^{-1} lactic acid. Ensiling with *Saccharomyces cerevisiae*, acid and the antibiotic Lactrol resulted in high ethanol selectivity and yields. Lastly, metagenomic analysis of sugar beet samples prior and post ensiling determined the endogenous microbial community and changes to relative distributions during ensiling. Analysis showed *Carnobacterium* was the most abundant genus and most likely responsible for lactic acid production and sugar beet samples prior to ensiling had greater bacterial diversity than post-ensiled samples. Performing ensiling at optimal conditions with selected additive resulted in high titers of the desired product and reduced by-product formation demonstrating *Beta vulgaris* can be successfully ensiled.

AKNOWLEDGEMENTS

I would first like to thank Prof. John Love, my supervisor for providing me the opportunity to be involved in this unique research placement and dedicating time in his busy schedule to offer advice and guidance. I would also like to thank Dr. Sara Burton for always having a door open, her consistent encouragement and invaluable support during the writing process.

I am grateful to Shell for sponsorship and the team at Shell Technology Centre-Houston in particular David Parker, James Winkler, Jeff Fedenko and Lindsey Aston for their knowledge on the project whilst in Houston. Additionally, Carol Sempira, Ashley Baugh and Chris Dealing for their assistance in the laboratory and providing an enjoyable and welcoming workplace. I also thank Dr. Richard Tennant and Chloe Singleton of the Exeter Microbiofuels Group for assistance in data analysis and proofreading.

Finally, I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis.

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ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
bp	base pair
DSM	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
g	gram
g	relative centrifugal force
h	hour
HPLC	High Performance Liquid Chromatography
kg	kilogram
µl	microlitre
µM	micromolar
min	minute
ml	millilitre
mM	millimolar
MRS	De Man, Rogosa and Sharpe agar
nm	nanometre
PCR	Polymerase Chain Reaction
pH	potential hydrogen
rpm	revolutions per minute
s	second
STCH	Shell Technology Centre Houston
TS	Total Solid
UDS	Undissolved Solid
UV	Ultraviolet
V	volts
w/v	weight per volume
w/w	weight per weight

INTRODUCTION

1.1 Biofuels and bioethanol

The continuing use of fossil fuels and concern over the related increase in greenhouse gas emissions (Naik *et al*., 2010), in addition to declining reserves of fossil fuels has accelerated the intensified search for alternative energy sources (Atabani *et al*., 2012). Research into renewable forms of energy has made way for development and adoption of new technologies such as transport biofuels which are produced primarily from renewable plant biomass. To qualify, a renewable fuel must reduce greenhouse gas emissions by at least 20%, moreover, advanced biofuels must show a reduction of 50% and cellulosic biofuels a 60% reduction compared to fossil fuel-based fuel (Environmental Protection Agency, 2017). Bioethanol produced from biomass feedstocks can be blended with petroleum distillates in different fractions between 5-25% and utilised in flexible fuel vehicles without needing vehicular modification (Naik *et al*., 2010). The use of biofuels is thought to reduce fossil fuel consumption and greenhouse gas emissions as the amount of CO_2 released into the atmosphere when burned equals CO_2 captured in photosynthesis (Naik *et al*., 2010; Persson *et al*., 2010).

First generation biofuels are produced from crops that can also be used for human consumption, for example, *Saccharinum officinarum* (sugarcane), *Zea mays* (corn), *Brassica napus* (oilseed rape) or *Elaeis guineensis* (oil-palm) (Naik *et al*., 2010). Generally, these crops are divided into 3 categories according to the type of carbohydrate they contain:

a) Sugar containing crops e.g. sugar cane
b) Starch containing crops e.g. wheat, sweet sorghum
c) Cellulosic biomass e.g. wood waste

When using starch containing crops, the starch must first be broken down to simpler glucose components before fermentation. To begin, the feedstock is ground and mixed with water then cooked using temperature and pressure and treated with 2 enzymes. The first enzyme, amylase is used to hydrolyse starch to glucose. Glucoamylase is then used to convert dextrans to glucose, maltose and isomaltose (Naik *et al*., 2010). Before fermentation, the mixture is cooled, subsequently, under anaerobic conditions, the added yeast convert glucose to ethanol (Naik *et al*., 2010). Despite the obvious advantages, there is some opposition to the pursuit of further developments to first generation biofuels. The key arguments include increased land use, competition with food crops and the effect on biodiversity. It is therefore critical to investigate biofuel alternatives that address these concerns and provide additional benefits.

Second generation biofuels are produced using lignocellulosic feedstock, which are cheap and abundant non-food materials. Lignocellulosic material is composed of cellulose, hemicellulose and lignin (Figure 1) (Sarria, Kruyer and Peralta-Yahya, 2017).

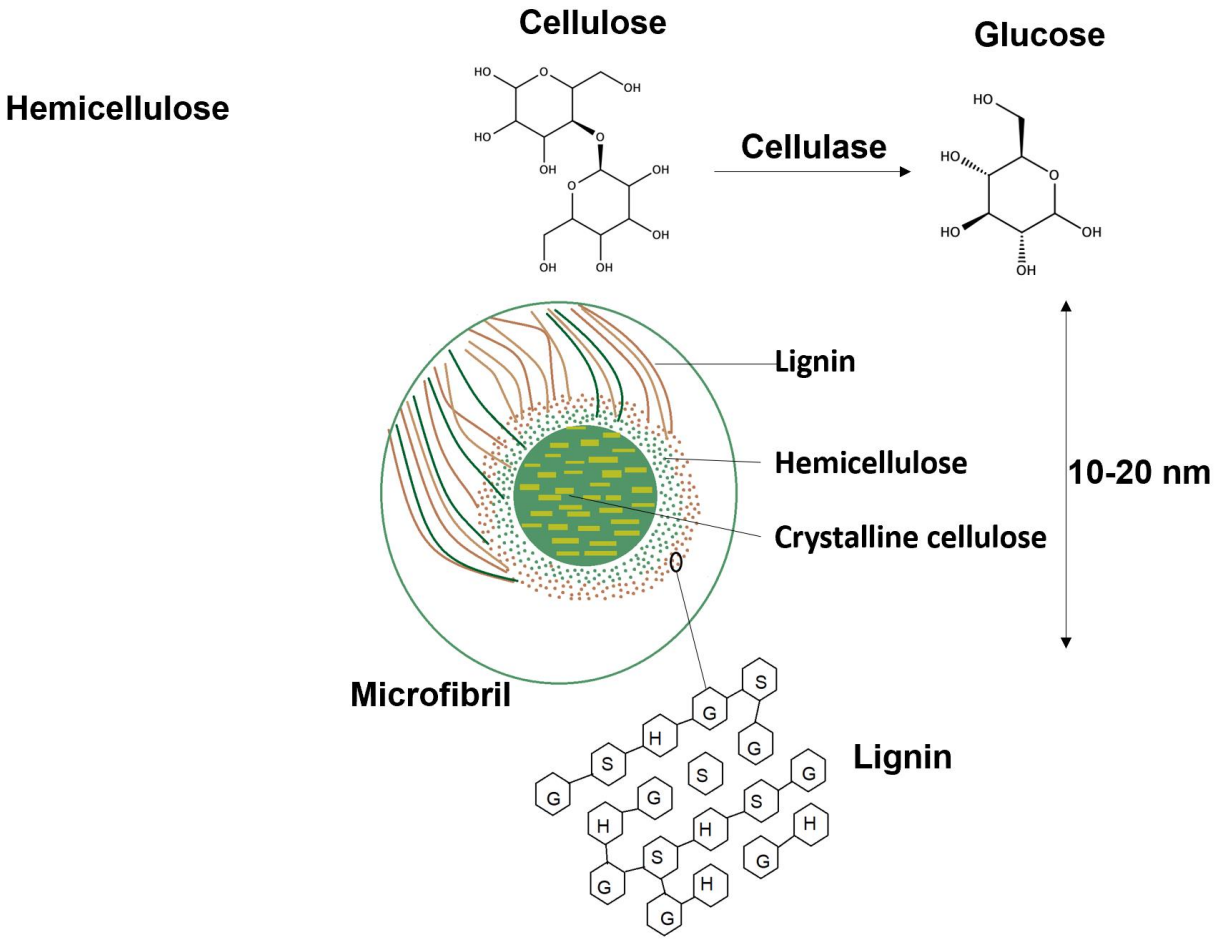


Figure 4. Structure of lignocellulose.

A crystalline structure of cellulose (light green) is surrounded by hemicellulose (dark green) and lignin (brown). The chemical structures of cellulose and its monomer glucose, hemicellulose and lignin are shown. G represents guaiacyl, H *p*-hydroxyphenyl and S syringyl.

Cellulose is a hydrophilic polymer made of 1,4-β-bonded linear glucose chains and is easily depolymerised to glucose by cellulase (Mohanty, Misra and Hinrichsen, 2000) and thus, the primary substrate for microbial fermentation as glucose is readily metabolised to a variety of products. Hemicellulose is a complex carbohydrate structure composed of several polymers such as pentoses, hexoses and sugar acids. The main function is to connect cellulose to lignin and provide rigidity to the network (Hendriks and Zeeman, 2009). Unlike cellulose, there is greater difficulty in the depolymerisation of hemicellulose due to different reactivities of the linkages found

in the polymer. For example, glucomannan in hemicellulose can only be extracted in strong alkaline environments whereas xylan can be extracted in acid or weak alkaline environments (Hendriks and Zeeman, 2009). The exact proportion of lignin monomers used to compose the polymer varies from species to species and within cultivars, tissue and environmental conditions. However, oxidative coupling of three main components *p*-hydroxycinnamyl alcohol monomers (*p*-coumaryl, coniferyl, and sinapyl alcohols) as well as other components such as hydroxycinnamic acids and aldehydes have been found to be critical to lignin synthesis (Lourenço *et al*., 2016).

Ether type linkages of lignin hydroxyls combine with the hydroxyls of cellulose to form a cross-linked network between more than one neighbouring chain molecule of cellulose. Whereas ester-type combination from alkali sensitive linkages link lignin hydroxyls and carboxyls of hemicellulose (Mohanty, Misra and Hinrichsen, 2000). Similarly to hemicellulose, lignin catabolism is also expensive, at times requiring specially engineered enzymes to break down lignin to its composite monomers (Sarria, Kruyer and Peralta-Yahya, 2017).

Bioethanol production using lignocellulosic biomass is by a 3 step process followed by separation and purification:

1. Pretreatment by steam explosion to separate xylose and lignin from the crystalline cellulose using a pressure vessel at approximately 500 °K and 15 bar (an energetically expensive process) (Naik *et al*., 2010).
2. Acid or enzymatic hydrolysis is then required to break down polysaccharides to smaller, simple sugars (Hendriks and Zeeman, 2009).
3. Fermentation of sugars by the yeast *Saccharomyces cerevisiae* to produce ethanol (Mussatto *et al*., 2010).

However, several challenges persist within the use of lignocellulosic biomass for ethanol production. For example, there are high energy costs associated with pretreatment of the biomass and ethanol separation from the rest of the reaction material. In addition, a proportion of the lignin is non-convertible to molecules valuable in downstream processes, resulting in waste. Xylose and glucose are generally not fermented simultaneously meaning less product is formed (Naik *et al*., 2010). To ensure efficient and economical production of biofuels that can be cost-competitive with traditional petroleum based fuels, further development of solutions to these challenges including separation processes such as adsorption with a molecular sieve and complete transformation of lignin is required.

1.2 Lactic Acid

In addition to finding a renewable source of ethanol, there is also increasing interest in more green, sustainable and renewable based chemicals. One key chemical is lactic acid which is utilised in various industries, for example as a preservative and emulsifying agent in the food industry, for ink-erasure in textiles and as an anticaries agent in pharmaceuticals (Cubas-Cano *et al*., 2018; Vandenbergh *et al*., 2018). Lactic acid products are also potential 'green' solvents for industrial use. Ethyl lactate can be formed by esterification of ammonium lactate and blending with biosolvents, 1,2-propanediol can be converted from lactic acid by hydrogenation and lactic acid can also be dehydrated to acrylic acid (Eiteman and Ramalingam, 2015; Datta and Henry, 2006).

The annual demand for lactic acid is estimated to be approximately 130,000-150,000 tons per year and is predicted to rise to 1.96×10^6 tons by 2020 (Zhang *et al*., 2016; Vandenbergh *et al*., 2018). The greatest emerging demand for lactic acid is as a feedstock to produce the biodegradable and biocompatible polymer poly-lactic acid, an alternative to current plastics (Abdel-Rahman, Tashiro and Sonomoto, 2011; Zhang *et al*., 2016). Poly lactic acid also widely used in the biomedical field in sutures, implants and for controlled drug delivery (Vandenbergh *et al*., 2018)

There are currently two methods used to produce lactic acid; hydrolysis of lactonitrile with strong acids that always produces a racemic mixture or biological synthesis by microbial fermentation resulting in optically pure lactic acid. Biological synthesis is considered the more advantageous method as optically pure lactic acid is important for downstream uses. For example, D-lactic acid is not metabolised by humans or animals and can cause acidosis. In addition, properties such as tensile strength and crystallinity of poly lactic acid depend on proportions of D/L-lactic acid (Vandenbergh *et al*., 2018). Microbial fermentation is also a low-cost production method as renewable carbohydrate biomass can be utilised and low temperatures are required (Abdel-Rahman, Tashiro and Sonomoto, 2011). Lactic acid bacteria are utilised as they can metabolise carbohydrate sources to lactic acid, different strains are categorised according to the metabolic route. *Lactococcus lactis* is an example of a homofermentative strain. Heterofermentative strains include *Lactobacillus brevis* and *Lactobacillus plantarum*. Homofermentative strains produce lactic acid as the main product using the glycolytic or Embden-Meyerhof-Parnas (EMP) pathway forming 2 moles of lactic acid from 1 mole of glucose. Hexose sugars are metabolised via glycolysis and strains possess the enzyme fructose-1,6-diphosphate aldolase (Figure 2). In contrast, heterofermentative strains metabolise hexose and pentose sugars via the phosphoketolase (PK) pathway (Figure 2) resulting in lactic acid (1 mole) as well as other products such as acetic acid, ethanol and carbon dioxide. Heterolactic strains utilise both the EMP pathway to metabolise hexose sugars and the PK pathway to metabolise pentose sugars (Zhang *et al*.,

2016; Vandenbergh *et al*., 2018). *L. plantarum* is commonly utilised in industry and is known to utilise glucose, fructose and arabinose making it an ideal strain for biomass fermentation to produce lactic acid (Zhang *et al*., 2016; da Silva Sabo *et al*., 2014). The US Food and Drug Administration has recognised *L. plantarum* as food grade and is therefore safe to be used in industrial products that may be consumed by animals and humans (Zotta, Parente and Ricciardi, 2017). Additionally, the complete genome of *L. plantarum* is available in Integrated Microbial Genome, a publicly accessible genome database (Zotta, Parente and Ricciardi, 2017).

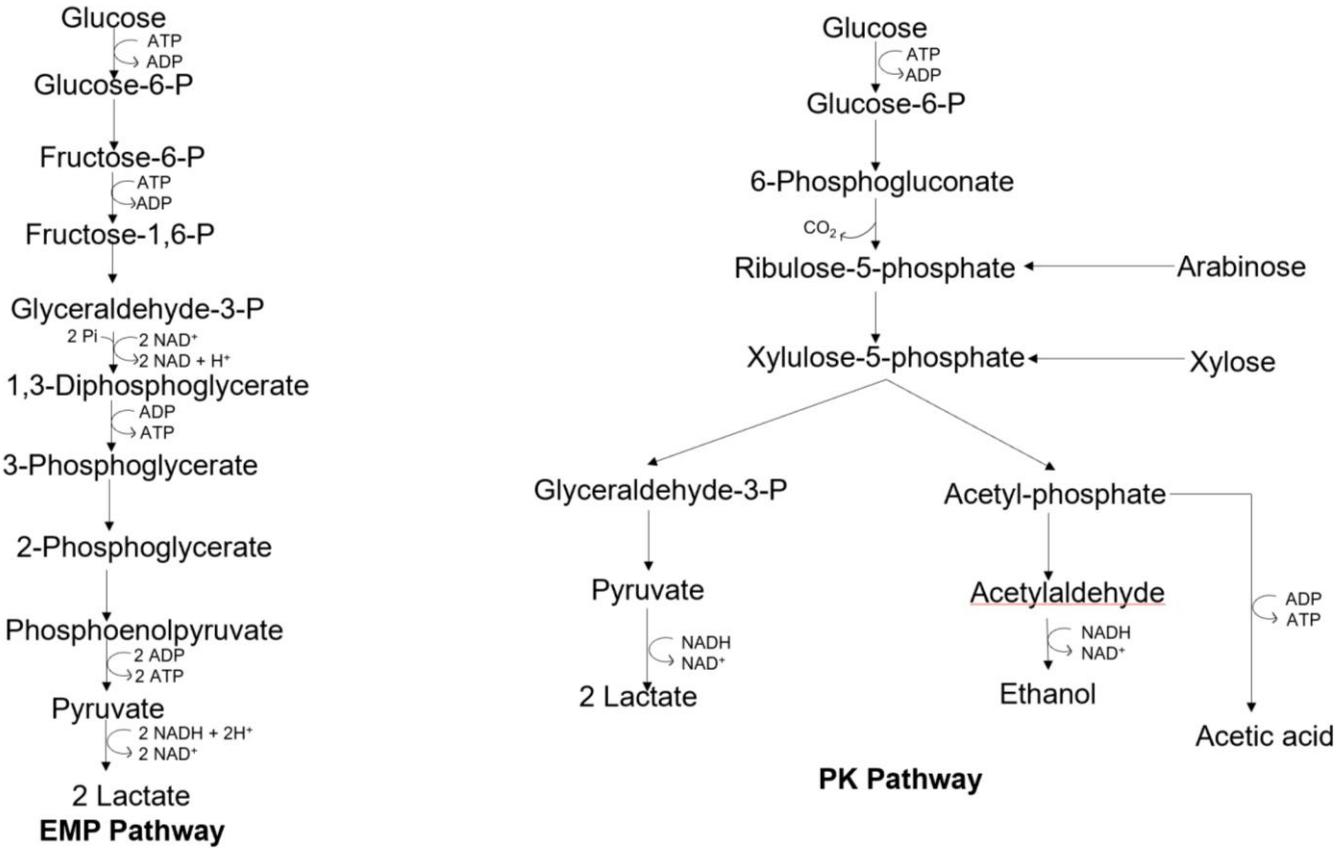


Figure 5. Lactobacillus metabolic routes. EMP pathway used by homofermentative strains to produce 2 mol of lactic acid per mol of glucose. The PK pathway is used by heterofermentative strains to produce 1 mol of lactic acid, ethanol and acetic acid from 1 mol of glucose. CO_2 is also produced in the PK pathway.

Lignocellulosic material can be utilised to produce lactic acid as it is a low cost, non-food, renewable carbohydrate source. A four step process similar to ethanol production is required involving pre-treatment, enzymatic hydrolysis, fermentation then lactic acid separation and purification (Hofvendahl and Hahn–Hägerdal, 2000). However, biological synthesis has some challenges nonetheless. One major challenge with the use of lactic acid bacteria for fermentation is carbon catabolite repression. Many lactic acid bacteria utilise glucose as the preferred carbon

and energy source and prevent the utilisation of other sugars thereby decreasing lactic acid yields (Zhang *et al*., 2016; Görke and Stülke, 2008). Furthermore, lactic acid separation is one of the high cost steps in the production process due to the addition of nutrients and buffering agents to the reaction medium and the low volatility of lactic acid (Abdel-Rahman, Tashiro and Sonomoto, 2011; Castillo Martinez *et al*., 2013). Other challenges to production include the inhibition to fermentation by acidity (pH < 5) caused by lactic acid production. Lactic acid bacteria grow optimally between pH 5-7 therefore the lactic acid produced during fermentation needs to be partially neutralised as it is formed (Castillo Martinez *et al*., 2013; Bosma, Forster and Nielsen, 2017). Commonly, calcium carbonate is added to the fermentation at the beginning although ammonium hydroxide and sodium hydroxide can also be used as alternative buffers (Castillo Martinez *et al*., 2013; Hofvendahl and Hahn–Hägerdal, 2000). This neutralisation produces additional obstacles, the dissociation of lactic acid from its salt, and the disposal or recycling of the cation can be arduous and expensive (Singhvi, Zendo and Sonomoto, 2018).

A possible solution to optimise product yields would be to use co-cultures for better carbohydrate source consumption. Strains with different preferred sugars or that are capable of fermentation of hexose and pentose concurrently could result in more consistent utilisation of the various carbohydrate sources present in the feedstock and increase product formation. Alternatively, genetically engineering strains could improve yields and lactic acid optical purity. For example, deleting D- or L-lactate dehydrogenase genes, developing strains more tolerant to low pH or inserting a xylose plasmid would allow simultaneous xylose and glucose conversion (Zhang *et al*., 2016; Vandenbergh *et al*., 2018; Castillo Martinez *et al*., 2013).

1.3 Ensiling

Traditionally, ensiling is a process used to preserve moist forage crops in containers called silos for later use as animal feed. The most commonly ensiled crops globally are whole-crop corn, alfalfa and various grasses (Weinberg and Ashbell, 2003). The main aim of ensiling is conversion of the biomass thus preventing general and nutritional deterioration, carbohydrate degradation and maintaining palatability (Zheng *et al*., 2010). Successful ensiling results in silage with a high conversion of water-soluble carbohydrates into organic acids, characterised by a low pH (Filya *et al*., 2000). The decrease in pH prevents forage spoilage by epiphytic microorganisms and maintains high nutritional value and high dry matter content (Bolsen, Ashbell and Weinberg, 1996; Filya et al., 2000). To be suitable for consumption by livestock, silage must also be low in toxic compounds, this is achieved by minimising the consumption of sugars, organic acids and other

soluble nutrients by aerobic microorganisms (Bolsen, Ashbell and Weinberg, 1996). There are four phases of ensiling:

1. Respiration and fungi and yeast activity occurs in a short aerobic phase whilst some air is still present (Weinberg and Ashbell, 2003; Herrmann, Heiermann and Idler, 2011).
2. This is followed by fermentation when lactic acid bacteria native to the biomass produce lactic acid and the pH decreases to approximately pH 4-5. The decrease in pH also impedes the growth of unfavourable species such as *Clostridia* (Herrmann, Heiermann and Idler, 2011; Oleskowicz-Popiel, Thomsen and Schmidt, 2011).
3. Subsequently, a stable phase where there is little change in the silage (Haag *et al*., 2015; Herrmann, Heiermann and Idler, 2011).
4. The final stage re-exposes the silage to air and revives aerobic microorganisms (Weinberg and Ashbell, 2003; Herrmann, Heiermann and Idler, 2011).

Ambye-Jensen *et al*., 2013 argued that biomass composition, the dry matter of the biomass and the microbial community present at fermentation had the greatest influence on the decomposition of the carbohydrate structure during ensiling. Moreover, fermentation must be strictly anaerobic to prevent crop spoilage by respiration of soluble substrates and more complex compounds, the subsequent increase in temperature and pH results in reduced nutritional value (Borreani *et al*., 2018). The addition of microbial inoculants on silage has shown a reduction in pH and increase in lactic acid production compared to uninoculated silage (Contreras-Govea *et al*., 2011).

At Shell Technology Centre Houston (STCH), primary ensiling is used to produce ethanol by yeast fermentation (*Saccharomyces cerevisiae*; Eagle® C6 Fuel, Lallemand Biofuels & Distilled Spirits) of free hexose sugars present in a biomass feedstock. The process has been studied using falcon tubes in the laboratory and in buckets and stacked piles of crop covered in plastic sheeting in the field. Additives are used to optimise ethanol yield, for example H_2SO_4 aids in lowering the pH (target of an initial pH of ~4.0) (Gallagher *et al*., 2018). The antibiotic commercial product Lactrol™ (virginiamycin) is also added to help prevent the growth of gram-positive bacteria and ethanol yield losses (Hynes *et al*., 1997). The addition of enzymes aids in the breakdown of complex, long-chained cellulose and hemicellulose molecules into their monomeric components, resulting in higher ethanol yields (Whitfield, Chinn and Veal, 2012; Xing, Chen and Han, 2009).

Thus far, ensiling efforts have concentrated on ethanol production from *Sorghum bicolor* (sweet sorghum) as the plant has high drought and salinity resistance, the stalks have a high carbohydrate content and plants have high biomass productivity (Almodares and Hadi, 2009; Gallagher *et al*., 2018). Sorghum was considered an appropriate alternative for ensiling as farming is more water efficient thereby reducing costs compared to the more traditionally used corn crop (Meeske *et al*., 1993; Amer *et al*., 2012). Also in favour of selecting sorghum is that glucose and

fructose constitute most of the soluble carbohydrates which are easily metabolised by the microbes regularly utilised in fermentation (Whitfield, Chinn and Veal, 2012). Although sorghum can be ensiled by fermenting the juice or by fermenting the chopped stalks in a solid state process, some studies showed greater conversion to ethanol when utilising chopped stalks (Regassa and Wortmann, 2014). Hence, this is the method utilised at STCH, also due to its ease in up-scaled trials.

In spite of these advantages, ensiling with sorghum remains challenging. The cellulose in sorghum stalks must be converted into glucose monomers prior to conversion to ethanol as the microorganisms involved in ensiling can only metabolize mono- or disaccharides (Whitfield, Chinn and Veal, 2012). This process requires enzymes and high temperatures (>60 °C) culminating in high costs. Another common challenge is fast sugar degradation during storage, a partial solution is air-drying to constant weight (Wu *et al*., 2010; Barros-Rios *et al*., 2014). Further research into methods to reduce costs and overcome these challenges would ensure more efficient ensiling. Alternatively, the selection of an alternative crop could eliminate the need for some of the costly steps without making fundamental alterations to the process.

Considering the second stage of ensiling involves fermentation by lactic acid bacteria, it logically followed that adjustments and optimisation of the ensiling process could lead to production of high lactic acid yields. Kromus *et al*., 2004 suggested the development of a ‘green biorefinery’ which would utilise green biomass for the manufacture of industrial products such as lactic acid in silos. Additives such as additional lactic acid bacteria and buffering agents alongside reduced particle size may increase the amount of lactic acid produced during ensiling (Haag *et al*., 2015).

1.4 Sugar beet

Beta vulgaris (sugar beet) are a sugar containing crop normally grown in temperate zones along with arid and semi-tropical locations (Evans and Messerschmidt, 2017). Sugar beet are tolerant of soil containing high levels of sodium and alkaline soil therefore can be grown in areas not suitable for other crops (Evans and Messerschmidt, 2017). As a root crop, sugar beet require less fertilizer compared to other feedstock as the long roots can permeate deep into the soil and obtain moisture and nutrients that would be otherwise inaccessible (Vargas-Ramirez *et al*., 2016).

Sugar beet (Figure 3) are a conical, white, fleshy root with a flat crown once leafy tops are removed and comprise 75% water and a high sucrose content (up to 20% by fresh weight) (Food and Agriculture Organisation of the United Nations, 2009; Panella, 2010). Thus, sugar beet are primarily used for sugar production. The abundance of sucrose makes sugar beet an ideal

feedstock for ethanol and lactic acid production as sucrose is easily fermented by yeasts, lactic acid bacteria, enterobacteria and other microorganisms (Panella, 2010; Gerlach *et al*., 2017).

Of the 25% dry matter, 5% is pulp composed of a high carbohydrate content (22-30% cellulose, 22-30% hemicellulose and 24-32% pectin) and low lignin content (1-3%) (Food and Agriculture Organisation of the United Nations, 2009; Zheng *et al*., 2011). Although not fully exploited in fermentation, pulp is still a valuable constituent and can be sold as a feed source for livestock (Gilbery, Lardy and Bauer, 2009). This extra benefit offsets the reduced ability for combustion of sugar beet pulp for heat and power production (Kühnel, Schols and Gruppen, 2011).

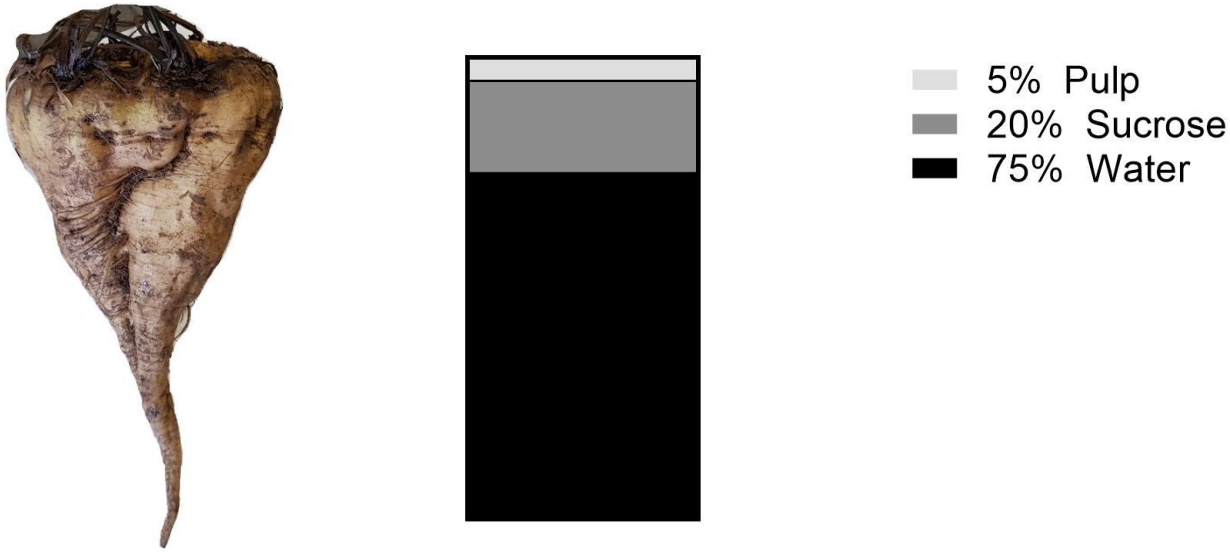


Figure 6. Indicative image and structure of a *Beta vulgaris*. Composition of a harvested sugar beet with top removed from Grand Forks, North Dakota, USA. Within the pulp component, 3% protein, 8% structural fiber, 1% lignin and 6% Ash (comprised of Si, K, Al, S, Na, P and Cl) (Figure created from NREL data analysis).

Sugar beet were proposed as a potential feedstock for lactic acid production using the Shell ensiling process for several reasons. In contrast to sorghum, sugar beet contain a lower fibre content (17% vs. 29%) and are therefore considered an easier substrate to degrade (Kumanowska *et al*., 2017). This in turn eliminates the need of pre-treatment to dissociate complex lignocellulosic polymers thereby reducing process costs (Barros-Rios *et al*., 2014). Since the largest source of soluble carbohydrates is sucrose, an easily hydrolysed sugar by various microorganisms, sugar beet have the potential to produce a range of products. Selection of microbe and ensiling conditions would lead to different fermentation processes, for example, addition of lactic acid bacteria and neutralising agent in anaerobic conditions may result in lactic acid production.

As with sorghum, there is difficulty in preventing sugar degradation in storage prior to ensiling. Sugar beet have a high moisture content which restricts long-term storage due to activation of microbial activity (Vargas-Ramirez *et al*., 2016). Other specific challenges associated with fermentation persist such as carbon catabolite repression and neutralisation in lactic acid fermentation, the use of sugar beet for ensiling would need to circumvent these issues.

Metagenomic analysis of key *Lactobacillus* species and planned genetic modification has recently emerged as a tool with the potential to decrease some process issues in lactic acid production. Bai *et al*. (2004) mutagenized *Lactobacillus lactis* using UV radiation developing the BME5-18 M strain which produced higher lactic acid concentrations than the parent strain. The increment was credited to increased glucose consumption and reduced NADH oxidase activity, lessening the effect of substrate inhibition (Bai *et al*., 2004; Abdel-Rahman and Sonomoto, 2016). There is also the possibility of redirecting metabolic pathways to decrease the formation of by-products and therefore increase lactic acid yields. For example, the introduction of two genes, *pfkA* and *fbaA* for fructose-6-phosphate kinase (PFK) and fructose-1,6-biphosphatealdolase (FBA) enzymes from *Lactobacillus rhamnosus* into *Lactobacillus brevis* resulted in metabolic redirection from the PK pathway (1 mole lactic acid from 1 mole glucose) into glycolytic (homofermentative) pathway (2 moles lactic acid from 1 mole glucose) (Guo *et al*., 2014; Abdel-Rahman and Sonomoto, 2016). Overall, an increase in lactic acid yield was obtained compared to the wild type strain (1.14 mol/mol vs. 0.74 mol/mol). Other challenges including acid tolerance of fermentation organisms, optical purity of lactic acid and utilisation of mixed sugar sources can also be tackled using metabolic engineering (Upadhyaya, DeVeaux and Christopher, 2014).

At STCH, previous ensiling trials were performed to optimise ethanol production using sorghum. As part of the ongoing long-range research program, evaluation of other feedstocks was suggested. This was with a view to convert the biomass into molecules that fit with few modifications into Shell's current market interests at high yields and titers. Thus, sugar beet was selected as a candidate feedstock to build a viable ensiling process for chemical production in the long term by determining the optimal combination of biocatalysts and conditions.

HYPOTHESIS AND AIMS

It was hypothesised that optimisation of Shell's ensiling process using *Beta vulgaris* biomass had the potential to produce high titers of lactic acid and ethanol. Given the high sucrose content and low lignin percentage found in sugar beet, it was considered there could be a more economic and greater conversion of carbohydrates to the desired product compared to *Sorghum bicolor* stems which require pre-treatment to hydrolyse cellulose, pectin and fibre.

To address this hypothesis, the aims of the project were to:

1. Screen organisms as potential biocatalysts for sugar beet ensiling
   a) Assess the utilisation of a provided main carbohydrate sources (sucrose, glucose, fructose, xylose, arabinose, cellobiose and D-galacturonic acid).
   b) Evaluate microbial catalysts as consortia by determining ensiling performance of listed strains individually and in different combinations.
2. Develop a suitable ensiling protocol for sugar beet with a focus on optimising lactic acid yields
   a) Describe sugar beet preparation.
   b) Define the process envelope (temperature, particle size, sterility).
   c) Examine and select additives to maximise lactic acid production (enzymes, pH control methods and microbial control agents).

MATERIALS AND METHODS

3.1 Bacterial Strains and Culture Conditions

Bacteria (*Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus pentosus, Lactobacillus amylovorous, Lactobacillus fermentum, Lactobacillus acidophilus, Lactobacillus sakei, Lactobacillus diolovorans, Lactococcus lactis* and *Zymomonas mobilis*) were purchased from American Type Culture Collection (ATCC) and Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSM) (Table 1). *Lactobacillus* species were cultured on De Man, Rogosa and Sharpe agar (MRS; peptone 10 g l^{-1}, beef extract 10 g l^{-1}, yeast extract 5 g l^{-1}, D- $C_6H_{12}O_6$ 20 g l^{-1}, polysorbate 80 1 g l^{-1}, $C_6H_{14}N_2O_7$ 2 g l^{-1}, CH_3COONa 5 g l^{-1}, $MgSO_4$ 0.1 g l^{-1}, $MnSO_4$ 0.05 g l^{-1}, K_2HPO_4 2 g l^{-1}, agar 15 g l^{-1}). *Lactococcus lactis* was cultured on M17 agar (tryptone 5 g l^{-1}, meat digest 5 g l^{-1}, soya peptone 5 g l^{-1}, yeast extract 2.5 g l^{-1}, $C_6H_8O_6$ 0.5 g l^{-1}, $MgSO_4$ 0.25 g l^{-1}, di-sodium-glycerophosphate 19 g l^{-1}, 10 % w/v D- $C_6H_{12}O_6$ 50 ml, agar 15 g l^{-1}) and *Zymomonas mobilis* was cultured on RM agar ($C_6H_{12}O_6$ 20 g l^{-1}, yeast extract 10 g l^{-1}, K_2HPO_4 2 g l^{-1}, agar 15 g l^{-1}). Cultures on agar plates were incubated at 37 °C for 24 h with the exception of *Z. mobilis*, *L. sakei* and *L. acidophilus* which were grown for 48 h. Planktonic cultures were grown in 25 ml of selected media as described above in a 125 ml vented, baffled flask, overnight at 37 °C, in an orbital shaker at 200 rpm.

Table 1. Details of bacterial strains used in this study.

Species	Isolate no.	Source[a]	Culture media	Type strain
Lactobacillus plantarum	793	ATCC® BAA	MRS	No
Lactobacillus acidophilus	4356	ATCC	MRS	No
Lactobacillus amylovorous	33621	ATCC	MRS	No
Lactobacillus brevis	367	ATCC	MRS	No
Lactobacillus diolivorans	14421	DSM	MRS	Yes
Lactobacillus fermentum	14931	ATCC	MRS	No
Lactococcus lactis subsp*. lactis*	19435	ATCC	M17	No
Lactobacillus pentosus	8041	ATCC	MRS	No
Lactobacillus sakei	15521	ATCC	MRS	No
Zymomonas mobilis subsp. *mobilis*	424	DSM	RM	Yes

[a]ATCC, American Type Culture Collection, Manassas, USA; DSM, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

3.2 Growth and Screening of Bacterial Carbon Source Utilisation

Carbohydrate sources (sucrose, glucose, fructose, xylose, arabinose, cellobiose and D-galacturonic acid) were prepared as 200 g l^{-1} stock solutions and sterile filtered (Rapid-Flow filter unit 0.2 µm cellulose nitrate membrane, 50mm diameter, ThermoFisher Scientific).

Bacteria were cultured in 10 ml MRS, M17 or RM media lacking glucose substituted with 250 µl/ml one of the following sugars as the sole principal carbohydrate source: sucrose, glucose, fructose, xylose, arabinose, cellobiose and D-galacturonic acid for at least 24 h, with the exception of *Z. mobilis, L. sakei* and *L. acidophilus* which were incubated for 48 h at 37 °C. A negative control containing no bacteria and media only and a carbohydrate source was also incubated.

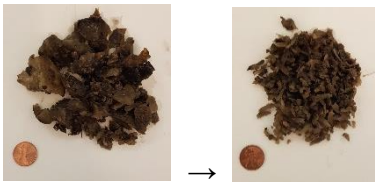
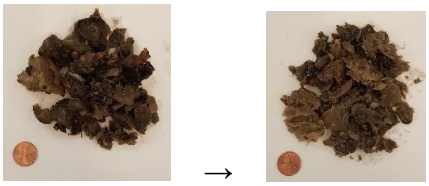
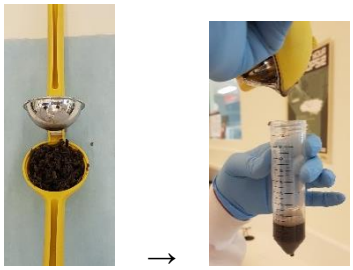
Following incubation, cells were harvested and washed in 5 ml of media minus a sole principal carbohydrate source and the resultant suspension centrifuged at 10, 000 *g* for 7 min. The supernatant was discarded and pellets resuspended in media without a carbohydrate source.

Cultures were diluted 1/100 in 10 ml of media (200 µl/ml) in a 125 ml baffled flask containing 250 µl/ml of one principal carbohydrate source and flask vents covered to prevent evaporation. After incubation for 24 h and 48 h at 37 °C and 200 rpm, samples were prepared for high performance liquid chromatography (HPLC) analysis and filtered through a 0.2 µm nylon Spin X centrifuge tube filter. The flow through was diluted 1:5 in 10 mM H_2SO_4. Optical density at 600 nm was determined to indicate growth at 0 h, 24 h and 48 h with a spectrophotometer (GENESYS 10S UV-Vis spectrophotometer, ThermoFisher Scientific, Waltham, USA). A standard curve was constructed for each strain

3.3 Sugar beet ensiling

Mature *Beta vulgaris* (sugar beets) hypocotyls were obtained from a North Dakota field site, the tops were chopped off and the hypocotyl chopped into chunks before packing and freezing for transport to STCH. The sugar beets were thawed at 4 °C for at least 18 h prior to ensiling and were used either as obtained i.e. post harvesting and chopping, after malleting for 2 min (Table 2) or shredding (KitchenAid food processor model KFP0711).

Table 2. Equipment used in the sugar beet ensiling laboratory process.

Equipment	**Use**
KitchenAid food processor model KFP0711	Shred thawed sugar beet chunks for easier compaction in Falcon tubes.   0.5: 10
Mallet	Alter surface area pre-ensiling to determine effect on lactic acid production. 
Chef'n FreshForce Citrus Juicer model 102-159-017	Squeeze post-ensiled sugar beet material, extracted liquid analysed by HPLC. 

Combinations of bacteria (Table 1) were added at approximately 8.6 x 10^9 cfu per gram of sugar beet material (estimated from standard curves at 600 nm), 50 g of biomass was packed into 50 ml Falcon tubes. Other additives, including pectic enzyme, sulfuric acid and lactrol, were added according to experimental design (Table 3) and mixed thoroughly into the sugar beet biomass.

Table 3. Ensiling additives. Additive choices were informed by previous sorghum ensiling trials (Shell Global Solutions (US) Inc., 2017)

Additive/kg sugar beets	**Concentration**
20% w/w H_2SO_4	4.2 ml kg^{-1}
20% w/w H_2SO_4	7.2 ml kg^{-1}
2.5% w/v Lactrol (virginiamycin, Phibro)	132 µL kg^{-1}
Hop oil (Kalsec)	30 µL kg^{-1}
C6 Fuel dry yeast (Lallemand)	0.42 g kg^{-1}
Wine yeast (Lalvin V1116, Lalvin EC1118, IONYSwf)	0.14 g kg^{-1}
Pectic enzyme (BSG Handcraft)	25 g kg^{-1}
3% w/v $CaCO_3$, $Ca(OH)_2$, $Mg(OH)_2$	21.4 g kg^{-1}
4% w/v CaCO3, $Ca(OH)_2$, $Mg(OH)_2$	28.5 g kg^{-1}
5% w/v $CaCO_3$, $Ca(OH)_2$, $Mg(OH)_2$	35.6 g kg^{-1}
6% w/v $CaCO_3$	42.7 g kg^{-1}
7% w/v $CaCO_3$	49.8 g kg^{-1}
8% w/v $CaCO_3$	57 g kg^{-1}
5% w/v NaCl	35.6 g kg^{-1}
Pimaricin	200 mg kg^{-1}
Sodium benzoate, Potassium sorbate	1 g kg^{-1}

The Falcon tubes were sealed with a drilled rubber stopper (Fermenthaus) and a three-piece fermentation airlock inserted into the stopper. Falcon tubes containing the biomass were incubated in a static incubator at a range of 30 °C – 42 °C. Following 6 days incubation, liquid from ensiled sugar beet material was sampled for HPLC analysis to determine lactic and acetic acid and ethanol concentrations. Liquid was extracted from ensiled sugar beet samples by hand squeezing using a citrus juicer (Chef'n FreshForce Citrus Juicer model 102-159-017) or by using a blender when specified. Exudate was clarified at 10,000 x *g* for 10 min. Supernatant was filtered through a 0.2 µm nylon Spin X centrifuge tube filter and the filtrate diluted 1:5 in 10 mM H_2SO_4 (Figure 1).

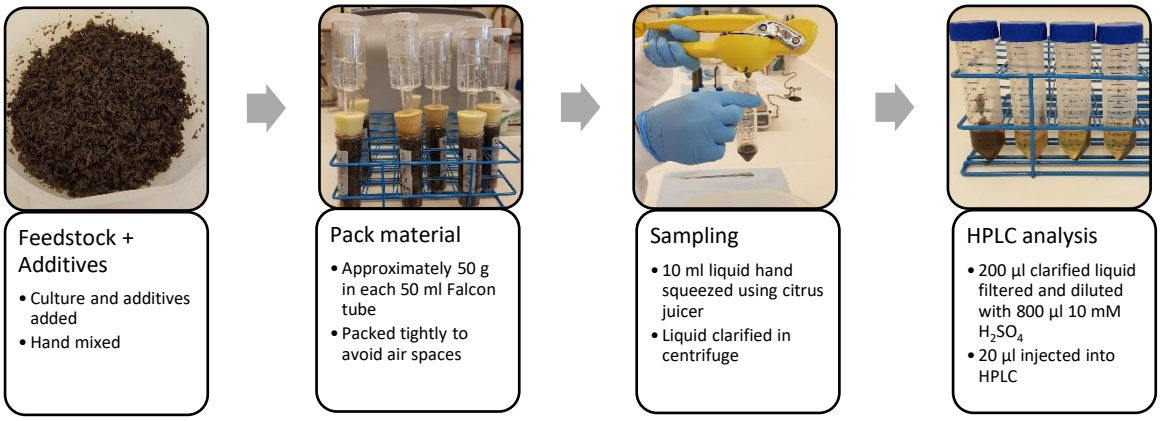


Figure 4. Workflow of sugar beet ensiling laboratory process involved in the production of lactic acid.

3.4 Rationale for use of additives

Various additives were used to optimise lactic acid or ethanol, the type and concentrations added to 1 kg sugar beets prior to ensiling were informed by previous sorghum ensiling trials and aimed to utilise methods and infrastructure already present at STCH (Shell, 2017). For example, the addition of acid was shown to lower pH thereby preventing microbial growth. Other microbial growth inhibitors such as the commercially available virginiamycin antibiotic Lactrol™ and hop oil were also used to control the growth of undesirable microorganisms. C6 fuel, a high-performance active dry yeast, Lalvin V1116, IONYSwf and Lalvin EC1118 dry yeasts used to make wine were added when ensiling to produce ethanol. Pectic enzyme was used to degrading pectin into its monomers and calcium carbonate as a buffering agent.

3.5 HPLC Analysis; Bacterial Species Carbohydrate Utilisation Screening and Post Ensiled Material

HPLC was used to detect lactic acid, acetic acid, ethanol, glucose, sucrose, fructose, arabinose, cellobiose and xylose content in ensiled samples and bacterial species cultured in various principal carbohydrate sources.

Acidified samples were analysed using a Dionex Ultimate 3000 HPLC system (ThermoFisher Scientific, 2017). A BioRad Aminex HPX-87H column (H Column) (Bio-Rad Laboratories, 2017) was used for quantification of lactic acid, acetic acid and ethanol concentration (g l^{-1}). For quantification of glucose, sucrose and fructose concentration (g l^{-1}) an Aminex HPX-87P Column (P Column) (Bio-Rad Laboratories, 2017) was used. A Rezex RMN-Carbohydrate Na^{+} column (RMN column) (Phenomenex) was also used for quantification of glucose, sucrose and fructose concentration (g l^{-1}) on an Agilent 1260 Infinity HPLC system (Agilent, 2016).

The H, P and RMN columns are pre-packed HPLC columns used for carbohydrate quantification in hydrogen form in 1-9 pH range, 9 µm particle size and 8% cross linkage. Samples were run on the H column and P column at 65 °C, with a flow rate of 0.6 ml min^{-1} for 25 min, the RMN column had a flow rate of 0.8 ml min^{-1} at 75 °C and had a duration of 9 min.

The H column had an isocratic elution of 0.005 % H_2SO_4 whilst the P column and RMN column used deionised water. Known concentration standards (1, 5, 10, 15, and 20 g l^{-1}) were used to compare concentrations of ethanol, glucose, lactic and acetic acid in ensiling and carbon utilisation samples.

From HPLC results, lactic acid, acetic acid and ethanol titers were calculated as well as the percentage of carbon consumed and the optimal lactic acid producing treatments. Lactic acid yields were calculated thus:

$$\text{Lactic acid yield} = \frac{\text{lactic acid concentration}}{\text{sucrose concentration} - \text{residual sugars}}$$

3.6 Total Solid and Undissolved Solid Determination

To determine the amount of solids remaining after heating sugar beet samples at 69 ºC to constant weight, also defined as percentage of total solids (TS) in a sample, 2 g of sugar beet biomass was weighed into a Falcon tube and gross weight recorded. The Falcon tubes were placed in a vacuum oven (69 °C ± 5 °C) for at least 40 h then reweighed and masses recorded. Percentage TS was calculated using the equation (Appendix 1A).

The moisture content (measure of the amount of water and other components volatilized at 69 °C present in a sample) was also determined using the formula in Appendix 1B.

The undissolved solids (total solids – total dissolved solids) were determined by weighing four grams of sugar beet biomass and 100 ml of deionised water was used to wash the sample through a GF-D filter (G6 glass fiber filter circles, 1.6 µm particle retention, Fisherbrand) through a buchner funnel using vacuum filtration. The glass filter containing the undissolved solids was transferred to a tared Falcon tube. The Falcon tubes and an empty Falcon with an unused filter as a control were placed in a vacuum oven (69 °C ± 5 °C) for at least 40 h then reweighed and masses recorded. UDS was calculated according to Appendix 1C.

3.7 DNA Extraction

DNA from microbial consortia found in the sugar beet samples pre and post ensiling was extracted using a washing method where samples were thawed to room temperature then 8 g of sugar beet samples were weighed into a sterile 50 ml Falcon tube and 30 ml Milli-Q water added. The samples were vortexed for 1 min with inversion every 15 s then centrifuged at 14,000 x *g* for 20 min. The supernatant was discarded and the pellet transferred to a beadbeater tube to aid with lysis before storing at -20 °C.

The DNA in the pellets was isolated using the protocol for the DNeasy Powerlyzer PowerSoil Kit DNA isolation kit (Qiagen). There was a target wet sample mass of 0.25 – 0.6 g and homogenisation was conducted using a FastPrep-24™ 5G Homogenizer (MP Biomedicals) set to: 4 ms^{-1} 30 s, rest 300 s, 4 ms^{-1} 30 s.

Amplicon polymerase chain reaction (PCR) was performed with one PCR reaction per DNA sample plus a positive and negative control of Milli-Q water. A PCR mix for 16 S bacterial sequencing was produced by adding 0.125 µl per 25 µl of the forward universal primer (U515New_F, GTG YCA GCM GCC GCG GTA A, Integrated DNA Technologies) and reverse universal primer (U926New_R, AAC TTT YRR CAA YGG ATC WCT, Integrated DNA Technologies), both to a final concentration of 0.5 µM, 11.25 µl of Ambion RT-PCR Grade Water (ThermoFisher Scientific) and 12.5 µl Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent) and 1 µl of the sample DNA (10-60 ng/µl). The 16S bacterial PCR was run for 25 cycles: 95 °C 15 s, 55 °C 30 s, 72 °C 30 s and the ITS fungal PCR was run for 27 cycles: 95 °C 15 s, 52 °C 20 s, 72 °C 30 s.

DNA was resolved on a 1% agarose gel, samples from amplicon PCR were used. DNA bands were separated electrophoretically at 100 V for 1 h and a pre-stained broad range marker (TrackIt 100 bp

DNA Ladder 10488058, ThermoFisher Scientific) was used for size comparisons. Gels were imaged using a Thermo Scientific myECL Imager (ThermoFisher Scientific, Waltham, US) by exposure to ultra violet (UV) light for 1 s.

PCR products from each sample were purified using the QIAQuick PCR purification kit (Qiagen) using the provided protocol with one modification; 30 µl Ambion RT-PCR grade H_2O was used for elution. Samples were quantified on a Qubit Fluorimeter 2.0 (ThermoFisher) using the Qubit dsDNA High Sensitivity assay (ThermoFisher). 16S amplicon DNA fragments were sequenced using a MinION DNA sequencer (Oxford Nanopore Technologies). Oxford Nanopore DNA sequencing libraries were prepared by following the SQK-LSK108 protocol (Community.nanoporetech.com, 2018) with the following amendments: a 1:1 ratio of the volume of reaction: volume of AMPure XP beads was used whenever specified, NEBNext High-Fidelity PCR Mix (New England BioLabs) was used for barcoding PCR instead of the LongTaq 2x master mix and PCR was run for 25 cycles: 98 °C 3 min, 98 °C 15 s, 62 °C 15 s, 72 °C 30 s, 72 °C 5 min.

3.8 DNA Sequence Analysis

QIIME 1.9.1 was used to analyse 16S and ITS amplicon DNA fragments sequenced using a MinION DNA. QIIME scripts are denoted using `courier`

The flow cell type and library preparation kit were identified using Albacore

```
minion_tools/read_fast5_basecaller.py --flowcell FLO-MIN107 --recursive --kit SQK-LSK108 --barcoding --output_format fastq --input data--save_path data_called --worker_threads 4
```

Porechop was used for DNA quality filtering (Wick *et al*., 2017)

```
porechop-runner.py-i—formatfastq-t4-b minion_data/data_barcoded
```

Once filtered and adapters removed from the reads, fastq files were split to fasta and qual using the input. Subsequently, long reads were truncated to 415 so as to be a similar length to the amplicon. The fasta files were then combined and each read named with a sample name. Operational Taxonomic Units (OTUs) were picked based on sequence similarity within the reads, strands that were reversed were checked and matched.

90% similarity was used for OTU calling, higher clustering (up to 97%) created an abundance of singletons that were excluded in the next step. Subsequently, a table was created using 2 OTUs per read

```
biom summarize-table -i Desktop/fastq0328/truncated/pickOTU/otu_table_mc2_w_tax.biom data
```

Communities were then summarised by taxonomic composition

```
summarize_taxa_through_plots.py -i
Desktop/fastq0328/truncated/pickOTU/otu_table_mc2_w_tax.biom data -o
Desktop/fastq0328/truncated/taxa_summary data -m
Desktop/fastq0328/truncated/mappingfile.txt data -c Treatment
```

To aid with analysis, a mapping file was created and validated for example

#Sample ID	Treatment	Sample Name	Description
BC01	37	Ensiling1	Sugar beet Ensiling *L. plantarum*, 5% $CaCO_3$
BC13	37	Ensiling2	Sugar beet Ensiling *L. plantarum*, 5% $CaCO_3$, pimaricin
BC25	37	Ensiling3	Sugar beet Ensiling *L. pentosus*

3.9 Statistical Analysis

Student t-tests and one way analysis of variance (ANOVA) with a Tukey test were used to determine the difference and significance (if any) in means of lactic acid, acetic acid and ethanol data. The tests were used to compare the mean product concentration for different treatments to the mean of the base-case treatment, p-values of below .05 were considered significant. The Tukey test allowed for pairwise comparison of replicate means and determined differences between two means greater than the expected standard error.

RESULTS

4.1 Carbohydrate source utilisation patterns

Various species were grown in a planktonic culture with a main carbohydrate source (sucrose, glucose, fructose, xylose, arabinose, cellobiose and D-galacturonic acid) added for 48 h. The remaining carbohydrate source as well as end metabolites (lactic and acetic acid and ethanol) were calculated, a threshold lactic acid value for test positivity was determined as ≥1 g/L. The resulting carbon distribution was used to identify which lactic acid bacteria species would be best suited as an inoculant for sugar beet ensiling.

L. plantarum, considered the 'control' species was found to be able to convert most of the supplied sucrose, glucose and fructose to lactic acid where 5.8 g l^{-1} lactic was produced from sucrose and glucose and 6.1 g l^{-1} using fructose from the supplied 10 g l^{-1}. Cellobiose and arabinose were also suitably utilised, producing 3.3 g l^{-1} and 4.1 g l^{-1} lactic acid respectively (Figure 5A). However, incubation with xylose and D- galacturonic (DGA) resulted in minimal lactic acid production. For all carbohydrate sources except xylose and DGA, a higher amount of lactic acid was produced compared to acetic acid indicating the supplied carbohydrate sources were preferentially converted to lactic acid. Ethanol production was low and unquantifiable by HPLC therefore not considered consequential.

Lactic acid titers for *L. brevis* were generally negligible with all the supplied carbohydrate sources except arabinose and fructose, 2.2 g l^{-1} and 1.1 g l^{-1} produced respectively. Generally, poor growth was observed for *L. brevis* in the planktonic cultures, thought to be the cause of low lactic acid production. To try and overcome these complex nutritional requirements, *L. brevis* was cultured in MRS which contained both xylose and glucose as the predominant carbohydrate sources. In this case, lactic acid titers improved to 3 g l^{-1} compared to the previous experiment which xylose and glucose were added individually (0.1 g l^{-1} and 0.2 g l^{-1} lactic acid respectively) (Figure 5B). Interestingly, *L. brevis* produced high titers of acetic acid (1.2-4.2 g l^{-1}) when cultured with all the various carbohydrate sources except sucrose (0.8 g l^{-1}). Addition of glucose resulted in the highest production of acetic acid, 4.2 g l^{-1} followed by arabinose, 3.8 g l^{-1}.

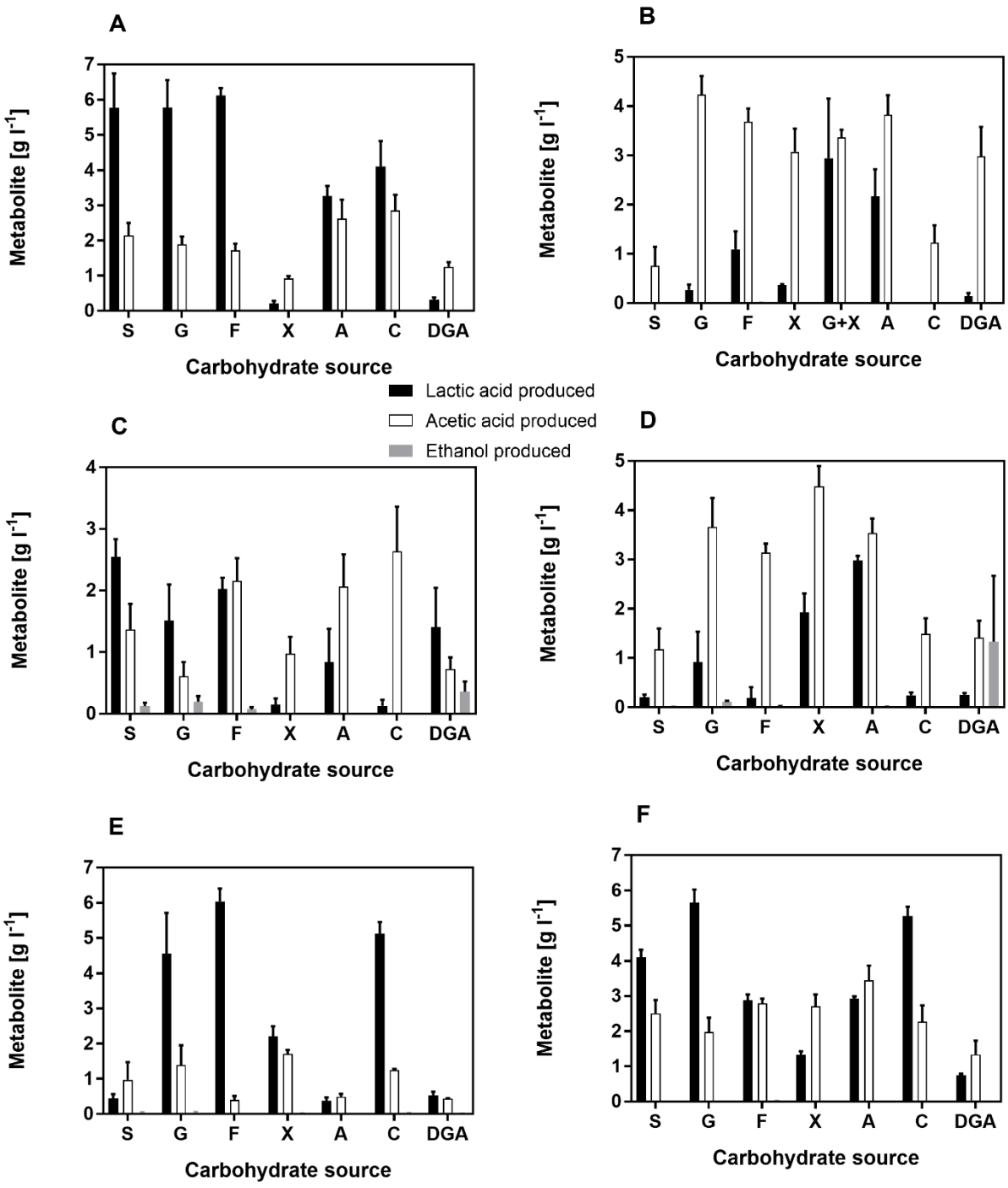

A
B
C
D
E
F
Metabolite [g l-1]
Carbohydrate source
S
G
F
X
G+X
A
C
DGA
Lactic acid produced
Acetic acid produced
Ethanol produced

Figure 5. Product formation of A: *L. plantarum*, B: *L. brevis*, C: *L. fermentum*, D: *L. diolivorans*, E: *Lc. Lactis* and F: *L. pentosus* after incubation for 48 h at 37 °C on various carbohydrate sources added at 10 g l^{-1}, glucose and xylose mixed carbohydrate source was added at 20 g l^{-1}.

S: Sucrose

G: Glucose

F: Fructose

X: Xylose

G+X: Glucose and xylose

A: Arabinose

C: Cellobiose

DGA: D- galacturonic acid

Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. Bars represent the mean of *n=6* replicates with error bars shown.

Xylose and arabinose were the only tested carbohydrate sources that produced a positive lactic acid titer result according to the determined threshold value, 1.9 g l^{-1} and 3 g l^{-1} for *L. diolivorans* (Figure 5C). Sucrose, glucose, fructose, arabinose, cellobiose and DGA only produced approximately 0.2 g l^{-1} lactic acid. *L. diolovorans* fermented all the supplied sugars to acetic acid as the major product in each case. Xylose fermentation provided the highest yields of acetic acid (4.5 g l^{-1}), arabinose, fructose and glucose also produced high acetic acid titers (3.6 g l^{-1}, 3.1 g l^{-1} and 3.5 g l^{-1}). DGA was the only carbohydrate source that was fermented to produce ethanol, with a similar titer produced for acetic acid,1.5 g l^{-1}.

Similarly to the *L. plantarum* control*, L. fermentum* showed adequate conversion of sucrose, glucose and fructose to lactic acid, 2.5 g l^{-1}, 1.5 g^{-1} and 2 g l^{-1} produced respectively (Figure 5D). As with many of the other species, there was poor utilisation of xylose producing only 0.2 g l^{-1} lactic acid, a similar titer was observed when cellobiose was provided. *L. fermentum* was the only species tested able to utilise the provided D- galacturonic acid and yielded 1.4 g l^{-1} lactic acid.

Lc. lactis was one of a few species in our studies to utilise xylose effectively for lactic and acetic acid production, 2.2 g l^{-1} and 1.7 g l^{-1} (Figure 5E). Sucrose, the predominant sugar in sugar beet showed poor product formation, 1.5 g l^{-1} of products were detected (Lactic and acetic acid, ethanol). However, incubation with glucose and cellobiose produced 4.6 g l^{-1} and 5.1 g l^{-1} lactic acid.

Approximately 8 g l^{-1} of the provided 10 g l^{-1} glucose was converted to lactic acid (6 g l^{-1}) and acetic acid (2 g l^{-1}) by *L. pentosus* (Figure 5F). Cellobiose produced 5 g l^{-1} of lactic acid and utilisation of sucrose yielded 4 g l^{-1}. In addition to these high lactic acid titers, arabinose and fructose yielded approximately 3 g l^{-1} of lactic acid and 3 g l^{-1} acetic acid each, and xylose was largely converted to acetic acid (3 g l^{-1}).

In the case of *L. acidophilus* and *L. amylovorous,* fermentation of sucrose, glucose and fructose to lactic acid was similar to *L. plantarum* (Appendix 2A, 2B). *L. sakei* largely yielded acetic acid (approximately 2 g l^{-1}) when fermenting xylose, arabinose, cellobiose and DGA (Appendix 2C). Lactic acid production when *L. sakei* was provided with these carbohydrates was negligible but effective with sucrose, glucose and fructose (1.7 g l^{-1}, 5.2 g l^{-1} and 4.5 g l^{-1}). *Z. mobilis* poorly fermented all the

supplied carbohydrate sources and produced low titers of lactic and acetic acid, glucose and fructose showed the highest production of ethanol of approximately 0.1 g l^{-1} (Appendix 2D).

4.2 Sugar beet ensiling

Surface area manipulation

Sugar beet hypocotyl chunks were subjected to different physical treatments to alter the surface area and the effect on lactic acid production examined. Sugar beet biomass was either hand- malleted, shredded using a food processor or used as chunks prior to ensiling (Table 2), *L. plantarum* was added at approximately 8.6 x 10^9 cfu.

The corresponding titers of lactic acid, acetic acid and ethanol after the 6-day ensiling process are displayed in Figure 6A. An average of 7 mmol g^{-1} of lactic acid was produced between the different treatments and there was no significant difference in yields of lactic acid and ethanol when malleted or shredded compared to the chunks (malleted vs. chopped: P = 0.3, shredded vs. chopped: P = 0.6, Student's two-tailed T test). Interestingly, acetic acid production was minimal, and a considerable amount of the carbon present was diverted to ethanol production. Further kinetic studies showed a considerable increase in ethanol production between 2 days, 0.5 mmol g^{-1} and 8 days, 29 mmol g^{-1} (Figure 6B).

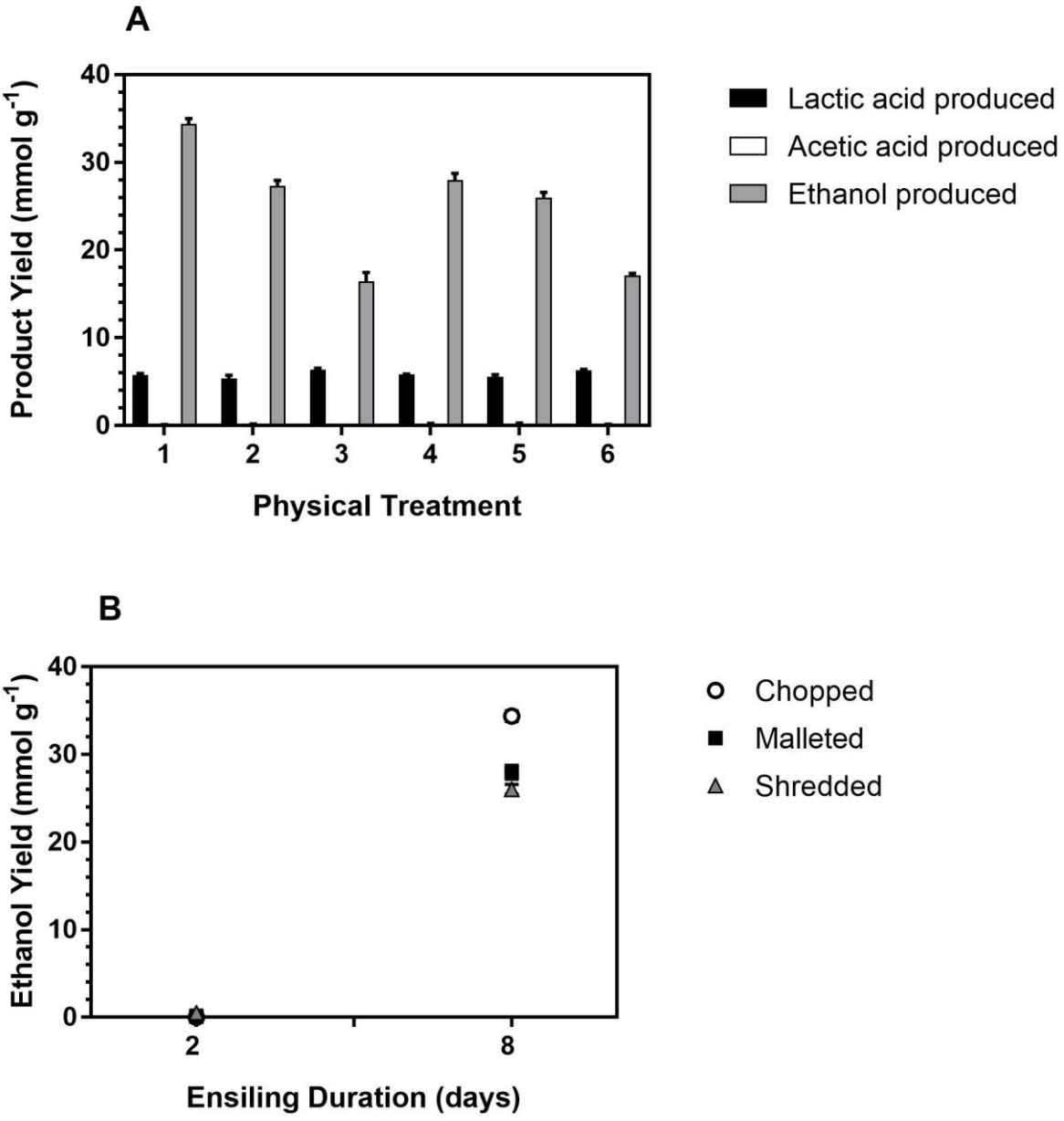

A
Product Yield (mmol g-1)
40
30
20
10
0
1
2
3
4
5
6
Physical Treatment
Lactic acid produced
Acetic acid produced
Ethanol produced
B
Ethanol Yield (mmol g-1)
40
30
20
10
0
2
8
Ensiling Duration (days)
Chopped
Malleted
Shredded

Figure 6. Product yields in ensiled *Beta vulgaris* biomass.

A: Final component concentrations in ensiled sugar beet (mallet-beaten, shredded in a food processor or chopped post-harvesting) after 6 days, less amount at T=0. Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. Bars represent the mean of *n=5* replicates with error bars shown.

1: Unhandled

2: Chopped

3: Chopped (blended)

4: Malleted

5: Shredded

6: Shredded (blended)

B: Kinetics of ethanol produced in ensiled sugar beet biomass at 2 days and 8 days. Chopped sugar beet are represented by circles with a solid black line, malleted by a square with a dotted line and shredded by a triangle with a dashed line.

An additional ensiling experiment was conducted to determine if sucrose was retained within the interior of the biomass at the end of ensiling and was incompletely extracted using hand juicing, resulting in potentially inflated product yields on a sucrose basis. For this case, four different treatments were evaluated: sugar beet chunks as obtained post harvesting and chopping, beets shredded in a food processor and at the end of the six days, HPLC samples were obtained by either blending with a juicer or hand squeezing using a citrus juicer (Table 2).

As per the previous experiment, there was nominal difference in lactic acid titers between blended samples and hand juiced samples. However, there was a large decrease in ethanol concentration when blending compared to hand squeezing was observed; from 27.3 mmol g^{-1} to 16.4 mmol g^{-1} in sugar beet chunks and 26 mmol g^{-1} to 17 mmol g^{-1} in shredded sugar beet (Figure 6A). This loss in ethanol could be attributed to evaporation during the blending process, visible in the unaffected lactic acid concentrations for the three conditions regardless of sampling method.

Temperature

Given that temperature significantly affects the kinetics of ensiling and enzymatic reactions, incubation temperature was varied as part of the efforts to determine the optimal conditions for lactic acid production. Ensiling sugar beet at 4 °C was concluded to result in the reduced metabolic activity of *L. plantarum* and the natural microbiome for lactic acid production due to low titers, 0.4 mmol g^{-1} (Figure 7). There was a consistent yield of between 5.1-6.2 mmol g^{-1} of lactic acid produced between 30 °C, 32 °C, 35 °C and 37 °C and there was no significant difference in lactic acid produced (P= 0.01, ANOVA Single factor test). 42 °C resulted in the lowest lactic acid yield, 4.9 mmol g^{-1}, thought to reflect the reduced metabolic activity of *L. plantarum* at this temperature. Compared to 42 °C, ensiling at 30 °C, 32 °C and 37 °C decreased acetic acid production (0.5 mmol g^{-1} vs. 0 mmol g^{-1}). The higher yield at 42 °C was considered to suggest the presence of an epiphytic species on the sugar beet that could convert sucrose to acetic acid at the higher temperature. Titers of ethanol were approximately 32 mmol g^{-1} at 32- 37 °C but 42 °C resulted in a decreased yield (13.4 mmol g^{-1}). The optimal ensiling temperature for lactic acid production was determined as 37 °C (with 5% w/v $CaCO_3$ added) due to high lactic acid titers and reduced by-product formation. However, given the small difference in titers between 21 °C and 37 °C, there is potential for optimization of ensiling at a lower temperature. This would be advantageous in colder climates, potentially increasing the number of geographic areas ensiling of sugar beet can be carried out.

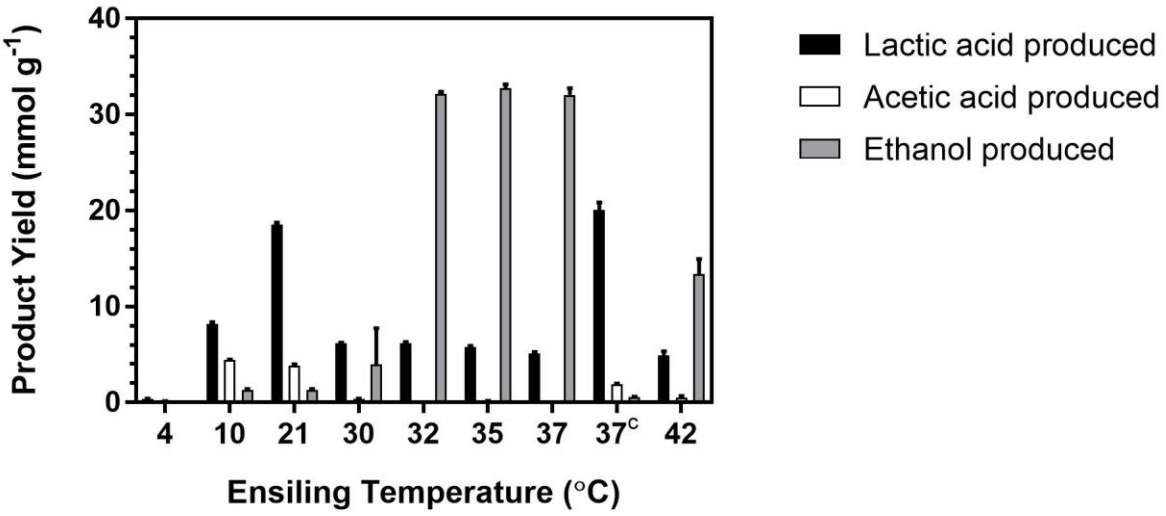


Figure 7. Effect of ensiling incubation temperature variation on lactic acid, acetic acid and ethanol yields from *Beta vulgaris* ensiled biomass. Data were produced from the ensiling of *Beta vulgaris* with standard additive loadings of *L. plantarum* and 5% w/v $CaCO_3$ when specified. Ensiling temperature was varied. 5% w/v $CaCO_3$ was added to samples prior to ensiling at 4, 10, 21 and 37^c °C. Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. Bars represent the mean of *n=5* replicates with error bars shown.

When lower temperatures (4, 10, 21 and 37 °C) with 5% w/v $CaCO_3$ added were tested, the general observed trend was that as the temperature increased, the lactic acid titer also increased (Figure 7). The lowest lactic acid was produced at 4 °C and at 21 °C the lactic acid titer was lower than expected. In contrast, 37 °C showed the highest lactic acid yields (52% vs. 5% at 4 °C, 21% at 10 °C and 45% at 21 °C) and titer (20.1 mmol g^{-1}). Ethanol production was low at all temperatures, ≤1.5 mmol g^{-1}, typical when $CaCO_3$ is added as it increases the selectivity for lactic acid over ethanol. Given that lactic acid titers were poor at 4 °C and 10 °C but were not strongly affected between 32 °C and 37 °C, it was concluded sugar beet could be successfully ensiled in this temperature range using *L. plantarum*.

Optimal biocatalyst

Different lactic acid bacteria were added to sugar beet prior to ensiling individually and in different combinations, when stated 5 % w/v $CaCO_3$ was also added. Lactic acid yields as well as concentrations of the by-products acetic acid and ethanol were examined to evaluate the species for their suitability to sugar beet ensiling.

Addition of *L. acidophilus* resulted in comparable lactic acid production to *L. plantarum*, approximately 5 mmol g^{-1} (Figure 8). Although more acetic acid was generated when ensiling with *L. acidophilus*, 1.6 mmol g^{-1}, ethanol titers were higher when using *L. plantarum* (24 mmol g^{-1} vs. 0.1 mmol g^{-1}). Ensiling with *L. amylovorous* also yielded similar lactic acid titers to *L. plantarum*, approximately 5 mmol g^{-1} but more ethanol was produced with *L. amylovorous* (29 mmol g^{-1} vs. 23.7 mmol g^{-1}). Acetic acid concentration for both species were negligible. When *L. brevis* and *L. fermentum* were evaluated for their suitability for sugar beet ensiling, *L. fermentum* showed a low ethanol titer, 4.5 mmol g^{-1} followed by *L. brevis*, 8.8 mmol g^{-1}, *L. plantarum* had the highest measured titer 29 mmol g^{-1}. In contrast, *L. brevis* and *L. fermentum* both produced higher amounts of acetic acid compared to *L. plantarum*, 2 mmol g^{-1} and 0 mmol g^{-1} respectively.

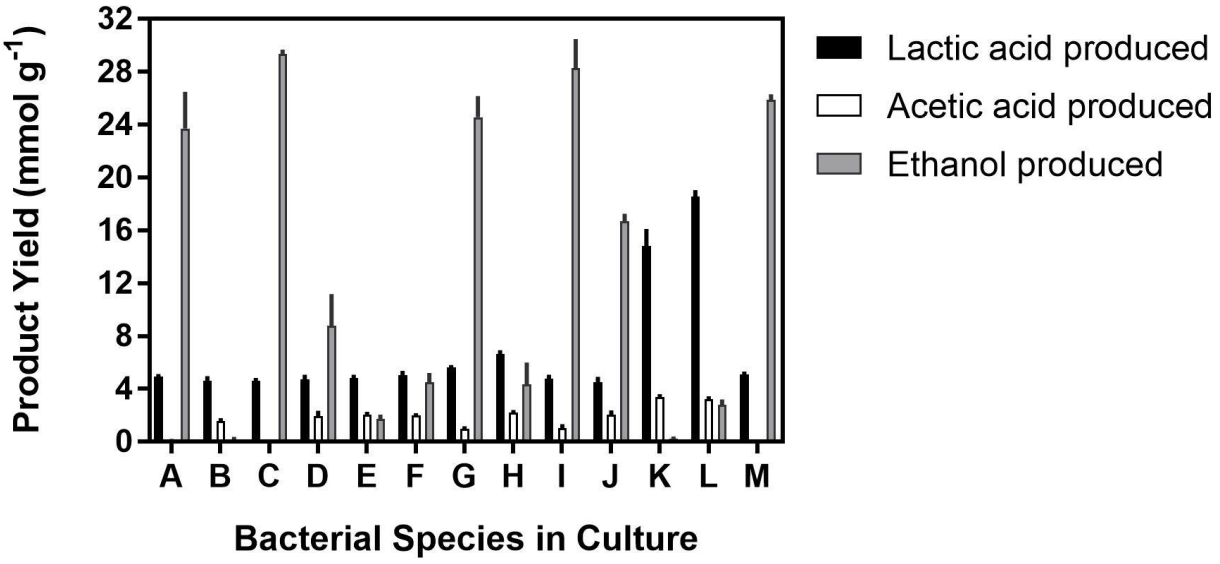


Figure 8. Final component concentrations in ensiled *Beta vulgaris* with various lactic acid bacteria and yeast (*Saccharomyces cerevisiae*), when specified 5% w/v $CaCO_3$ was also added. Concentrations are less amount present at T=0. Lactic acid bacteria and additive combination variables were:

A: *L. plantarum*

B: *L. acidophilus*

C: *L. amylovorous*

D: *L. brevis*

E: *L. diolivorans*

F: *L. fermentum*

G: *Lc. Lactis*

H: *L. pentosus*

I: *L. sakei*

J: *Z. mobilis*

K: *L. plantarum* + 5% w/v $CaCO_3$

L: *L. plantarum* + *L. diolivorans* + 5% w/v $CaCO_3$

M: *L. plantarum* + C6 Fuel

Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. Bars represent the mean of *n=5* replicates with error bars shown.

L. pentosus was the only species tested which showed a higher lactic acid titer, 6.7 mmol g^{-1} than the *L. plantarum* control, 5 mmol g^{-1} (Figure 8). Additionally, there was also considerably more acetic acid produced in samples ensiled with *L. pentosus* compared to *L. plantarum* (2.2 mmol g^{-1} vs. 0 mmol g^{-1}). On the other hand, concentrations of ethanol were higher when sugar beet were ensiled with *L. plantarum*, 24 mmol g^{-1} than in samples ensiled with *L. pentosus,* 4.3 mmol g^{-1}. Samples ensiled with *L. diolivorans, L. sakei* and *Lc. lactis* showed similar lactic acid titers, approximately 5.1 mmol g^{-1}. Similar ethanol titers were determined [1] for *L. sakei* and *Lc. lactis*, approximately 26 mmol g^{-1}. Acetic acid levels were similar to the other species tested (1.5 mmol g^{-1}). *L. plantarum*-treated beets outperformed those with added *Z. mobilis* in both lactic acid production (5 mmol g^{-1} vs. 4.5 mmol g^{-1}) and production of ethanol (23.7 mmol g^{-1} vs. 16.7 mmol g^{-1}).

Combinations of *L. plantarum* and other alternative biocatalysts were also evaluated for their capability of alternative sugar degradation or production formation. *L. plantarum*, *L. diolivorans* and 5% w/v $CaCO_3$ ensiling resulted in a high lactic acid titer (18.5 mmol g^{-1}). This result was slightly higher than titers obtained when using *L. plantarum* and $CaCO_3$ alone (14.8), indicating there was no additional benefit of ensiling with *L. diolovorans* in terms of lactic acid titer uplift. As expected, ethanol production when ensiling with *L. plantarum* and C6 Fuel yielded a high ethanol titer, 25.9 mmol g^{-1} but no improvement in lactic acid concentrations (5.1 mmol g^{-1}).

Sterilised Ensiling

The production of ethanol in sugar beet ensiling was considered an undesired diversion of carbon away from the primary target molecule, lactic acid. To assess whether the generated ethanol was a result of an endogenous ethanologen or a contaminant from processing, the ensiling performance of sugar beet biomass sterilized at 116 °C for 10 minutes or unsterilized, inoculated with *L. plantarum* and either incubated at 37 °C and 42 °C were compared.

Lactic acid production was greatest in unsterilised material at 37 °C and least in sterilised samples at 42 °C (Figure 9). A notable increase was observed in lactic acid production between sterilised and unsterilised material at 37 °C, 3 mmol g^{-1} to 5.3 mmol g^{-1}. In contrast, ethanol production was greatest in sterilised samples at 37 °C compared to unsterilised samples, but the difference in ethanol titers was not considered significant as there was high sample to sample variability. At 42 °C, ethanol titers were greater in the unsterilised samples (12.2 mmol g^{-1}) compared to the sterilised samples (4.3 mmol g^{-1}), thought to indicate possible introduction of laboratory ethanologens as opposed to native microbial flora.

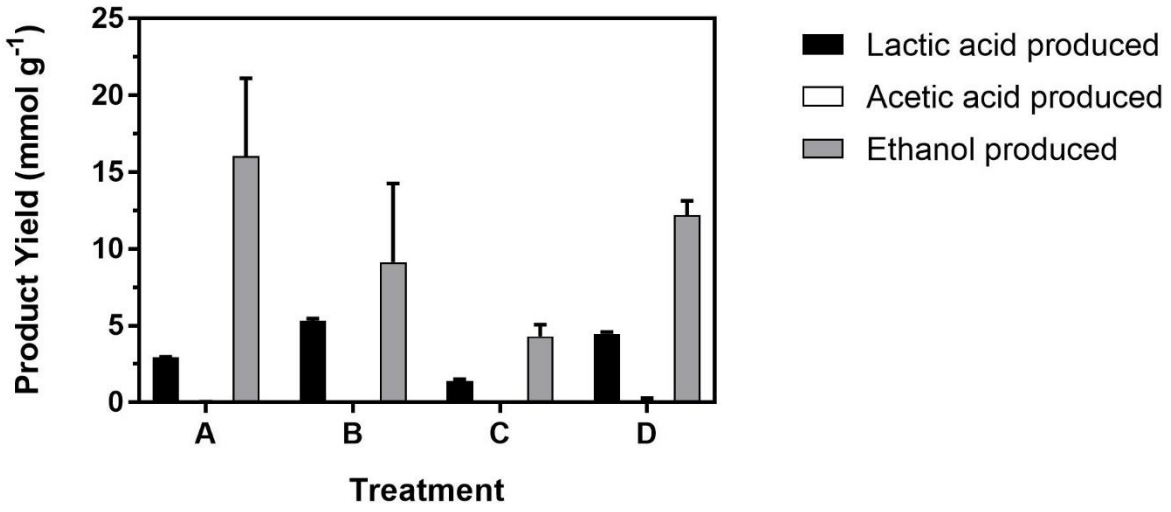


Figure 9. Final product yields in ensiled *Beta vulgaris* subjected to various sterilisation treatments.

A: Sterilised, 37 °C

B: Unsterilised, 37 °C

C: Sterilised, 42 °C

D: Unsterilised, 42 °C

Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. Bars represent the mean of *n=3* replicates with error bars shown.

Ethanol Ensiling

Further to evaluating lactic acid production by various lactic acid bacteria, different yeasts were also considered for their suitability for sugar beet ensiling to produce ethanol, currently used as a low-carbon intensity biofuel. C6 Fuel previously showed success in field trials and Shell in-house ensiling of *Sorghum bicolor*. Lalvin V1116, IONYSwf and Lalvin EC1118 have high alcohol tolerance and have been utilised for making wine at lower temperatures (Lalvin V1116: 18%, 10-35 °C, Lalvin EC1118: 18%, 10-30 °C and IONYSwf: 15.5%, 25-28 °C).

Of the four yeast strains used, the largest quantity of ethanol was produced when C6 Fuel, acid and lactrol were in combination, 35 mmol g^{-1} (Figure 10). Acid addition with EC1118 and lactrol also yielded a high amount of ethanol, 34.5 mmol g^{-1}, in both cases lactic acid and ethanol production was minimal (≤ 1 mmol g^{-1}). Acetic acid production wherein only wine yeasts were added were the highest observed (approximately 3.5 mmol g^{-1}). Compared to the control with no additives (6 mmol g^{-1}), ensiling with yeasts produced less lactic acid (approximately 5 mmol g^{-1}). Addition of acid yielded lower lactic acid titers, 0.4-3.8 mmol g^{-1} attributed to inhibition of native lactic acid bacteria.

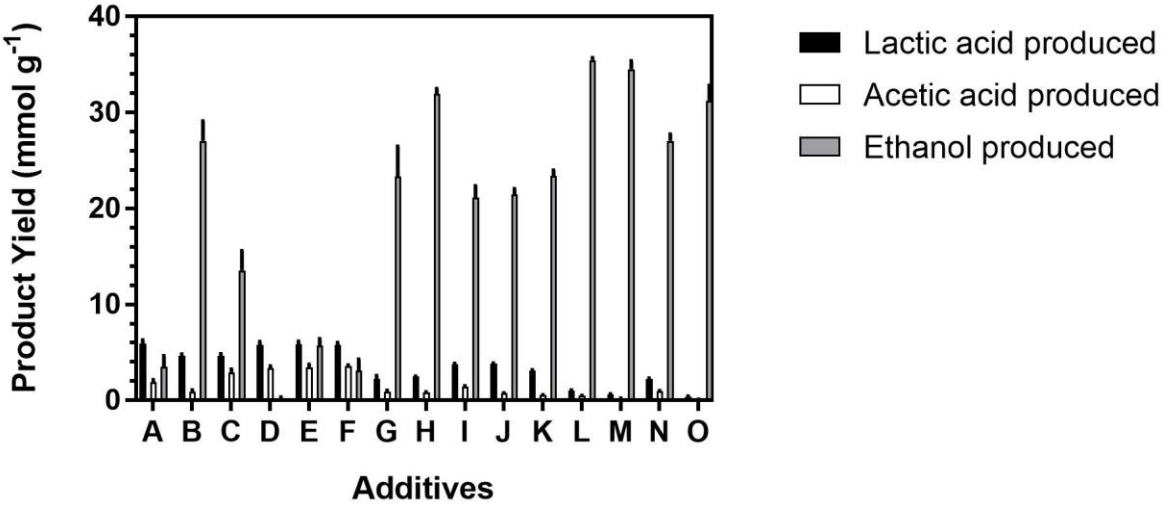


Figure 10: Effect of additive variation on ethanol, lactic acid and acetic acid yields from *Beta vulgaris* ensiling. Ensiling yeast loading and additive combination variables were:

A: N/A

B: Acid

C: C6 Fuel

D: EC1118

E: V1116

F: IONYS wf

G: Lactrol

H: Acid + C6 Fuel

I: Acid + EC1118

J: Acid + IONYS wf

K: Acid + V1116

L: Acid + C6 Fuel + Lactrol

M: Acid + EC1118 + Lactrol

N: Acid + IONYS wf + Lactrol

O: Acid + V1116 + Lactrol

Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. Bars represent the mean of *n=5* replicates with error bars shown.

Microbial Inhibition

Preservatives were chosen based on their ability to control the endogenous microbial flora without impairing lactic acid bacteria function. Common food preservatives sodium benzoate and potassium sorbate were dosed at 1 g kg^{-1} sugar beet, the osmotic stressor sodium chloride was used at 5 % w/v and the fungal specific antibiotic pimaricin dosed at 200 mg kg^{-1} sugar beet. Tubes were ensiled with our standard dose of *L. plantarum* and 5 % w/v $CaCO_3$ except the control condition where no $CaCO_3$ was added.

Product titers, minus contributions present at T=0, are shown in Figure 11 below; all conditions with added inhibitor produced approximately 20 mmol g^{-1} lactic acid and there was no significant difference between potassium sorbate, sodium benzoate and pimaricin addition trials in lactic acid production (potassium sorbate vs. sodium benzoate vs. pimaricin P= 0.78, ANOVA Single factor test). Although samples with no inhibitor contained a low lactic acid titer, there was no $CaCO_3$ added and therefore 5.8 mmol g^{-1} was typical for the condition. In the case of samples with added NaCl, the calculated lactic acid yield was 83 %, one of the highest observed in any of the ensiling trials.

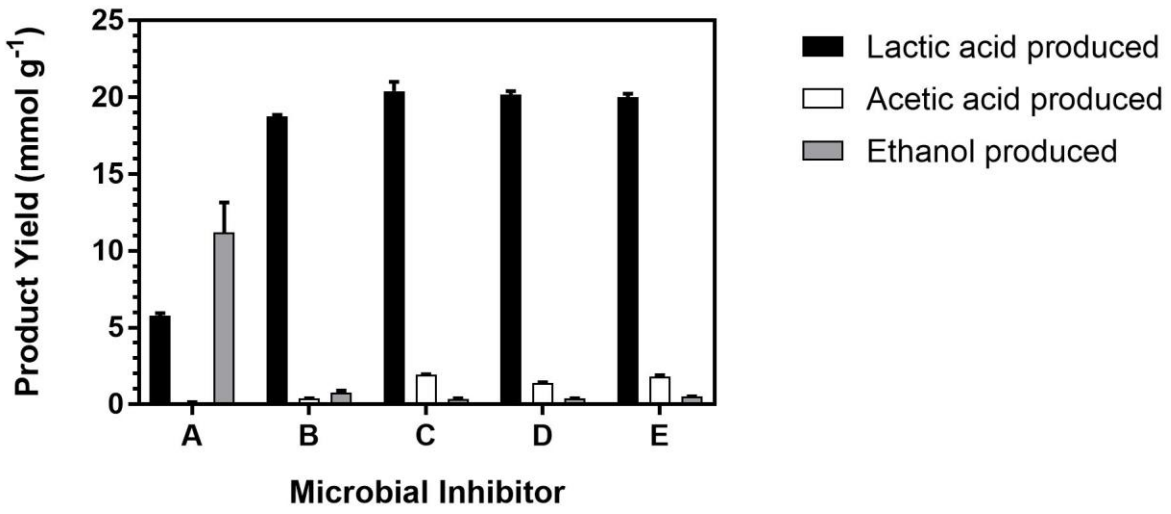


Figure 11. Product titers, minus contributions at T=0 from *Beta vulgaris* ensiling with various microbial inhibitors.

A: Control

B: 5% w/v NaCl

C: 1g kg^{-1} Potassium sorbate

D: 1g kg^{-1} Sodium benzoate

E: 200 mg kg^{-1} Pimaricin

Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. Bars represent the mean of *n=5* replicates with error bars shown.

Acetic acid was produced in small quantities in samples with no inhibitor added, typical of our other ensiling experiments (0.1 mmol g^{-1}). However, addition of potassium sorbate yielded the highest amount of acetic acid, 2 mmol g^{-1}. Ethanol was also produced in small quantities, approximately 0.5 mmol g^{-1} when an inhibitor was added. The highest titer, 11.2 mmol g^{-1} was in the control condition.

Overall, none of the inhibitors had a significant effect on the amount of lactic acid produced. However, the high lactic acid yield observed with added NaCl suggests further optimisation and combination with other high yielding conditions could be promising.

$CaCO_3$ Loading Optimisation

Previous ensiling trials showed addition of 5% $CaCO_3$ tripled the final lactic acid titer (Figure 12), likely due to the increase in pH from 3.7 to 4.3 allowing *L. plantarum* to continue fermenting sucrose to lactic acid for longer. However, as *L. plantarum* has an optimum pH of 4.5 or above for optimal growth, buffering of the environment needed to be improved for optimum yields. The ensiling performance of 6 different $CaCO_3$ concentrations were tested to assess the buffering capabilities of these economically viable and practical doses. In all cases, sugar beet were ensiled with our standard dose of *L. plantarum,* at 37°C and samples were taken at 6 days.

The addition of $CaCO_3$ increased the pH in all conditions (up to pH 4.4 at 7% w/v) reducing the organic acid stress on *L. plantarum* thus resulting in increased production of lactic acid (6 mmol g^{-1} lactic acid in 0% w/v $CaCO_3$ vs. 15 mmol g^{-1} lactic acid in 3% w/v $CaCO_3$) (Figure 12A). Lactic acid titers increased at a linear rate (R^2= 0.95; Figure 9B); as $CaCO_3$ concentration increased, lactic acid produced also increased. Compared to the control 0% w/v $CaCO_3$, 7% w/v $CaCO_3$ had a 60% lactic acid yield (26.5 mmol g^{-1}), the highest recorded in all experiments likely because *L. plantarum* was not acid stress limited in this environment (average pH 4.6). Acetic acid titers increased to a maximum of 2.7 mmol g^{-1} at 4% w/v $CaCO_3$, then decreased to 2.5 mmol g^{-1} and 1.9 mmol g^{-1} with 5% w/v and 7% w/v respectively, 8% w/v $CaCO_3$ also yielded a high acetic acid titer (2.3 mmol g^{-1}). Ethanol production decreased in a linear manner with highest production at 0% w/v $CaCO_3$ (32.8 mmol g^{-1}) falling to approximately 1 mmol g^{-1} in the range 4-8% $CaCO_3$.

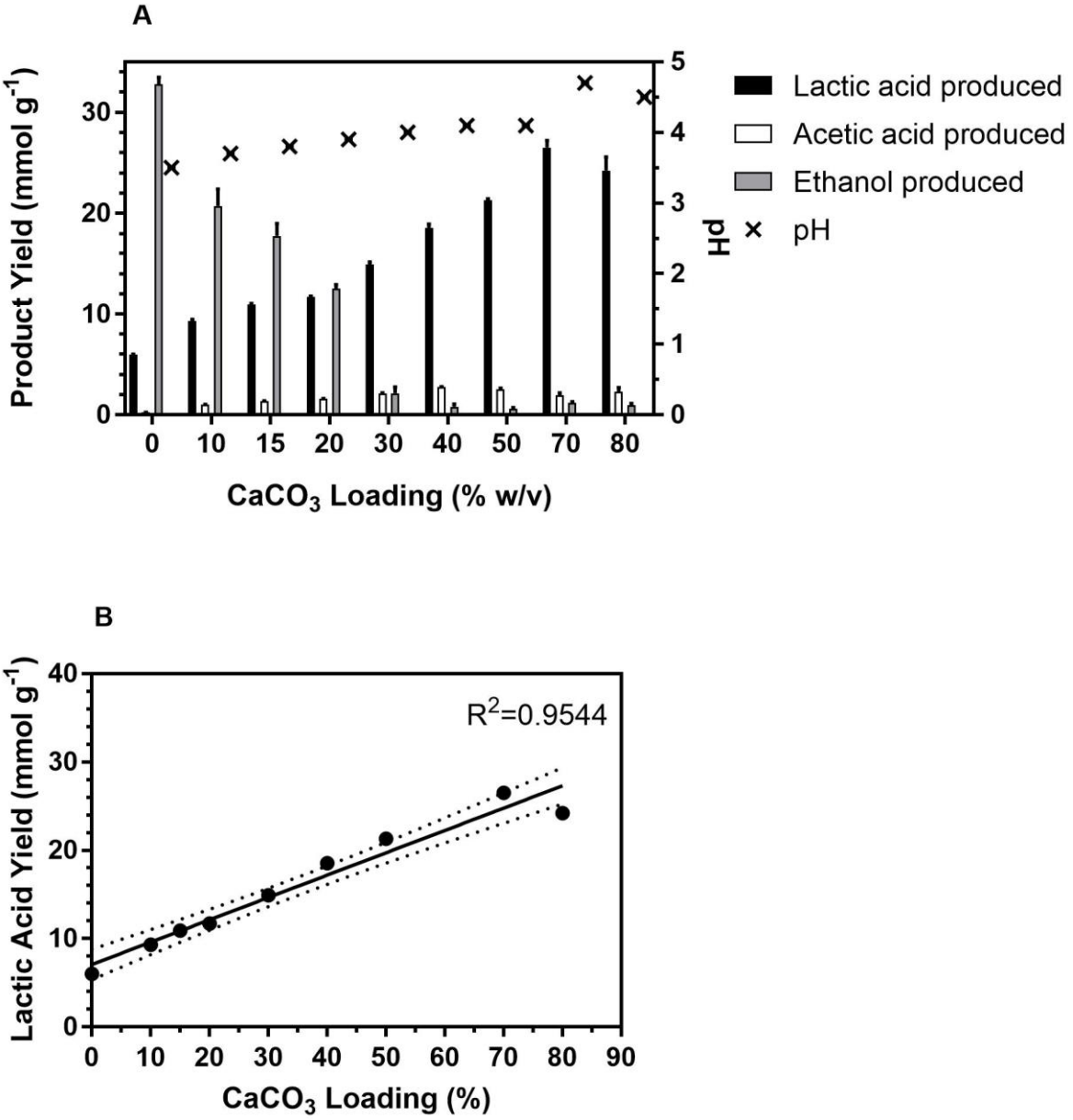


Figure 12. Effect of additive variation on lactic acid, acetic acid and ethanol yields from ensiled *Beta vulgaris* biomass. Data were produced from the ensiling of *Beta vulgaris* with standard additive loadings of *L. plantarum* and various $CaCO_3$ loadings.

A: Various $CaCO_3$ loadings effect on ensiling product yields

B: Linear regression of $CaCO_3$ loadings with 90% confidence intervals.

Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. pH is represented by black crosses. Bars represent the mean of *n=5* replicates with error bars shown.

pH adjustment

In addition to evaluating different chemicals for buffering in our ensiling experiments, we also conducted a study to determine the limits of buffering our system. The setup involved using $CaCO_3$ and evaluating the effect on lactic acid yields by using enhanced buffering. Ensiling tubes were set up as per our normal procedure with 5% w/v $CaCO_3$, pH was adjusted up to approximately 6.5 after 24 h, 48 h and 72 h using 1N KOH, final sampling was after 6 days.

Lactic acid titers increased from 6.5 mmol g^{-1} to 23.6 mmol g^{-1} in samples where there was no pH adjustment but had $CaCO_3$ added (Figure 13). The same effect, to a lesser extent was also observed when samples were pH adjusted without the addition of $CaCO_3$ (6.5 mmol g^{-1} to 10.5 mmol g^{-1}). However, in samples with added $CaCO_3$ with and without pH adjustment, there was minimal difference in the amount of lactic acid produced (23.2 mmol g^{-1} vs. 23.6 mmol g^{-1}). This correlated well with the measured pH values at 6 days, where the average pH of samples with $CaCO_3$ and no pH adjustment was 4.2 and the average pH of samples with $CaCO_3$ and pH adjustment was 4.3. As previously observed, ethanol production was low in samples with added $CaCO_3$ compared to samples where buffering was by pH adjustment alone, due to the increased selectivity for lactic acid over ethanol. Interestingly, ensiling with $CaCO_3$ produced a higher amount of acetic acid compared to samples without (2-2.4 mmol g^{-1} vs. 0-0.3 mmol g^{-1}). This suggests the initial buffering by $CaCO_3$ allows for greater activity of species responsible for acetic acid production. Generally, initial buffering has the greatest effect on lactic acid production, any subsequent buffering attempts have lesser influence.

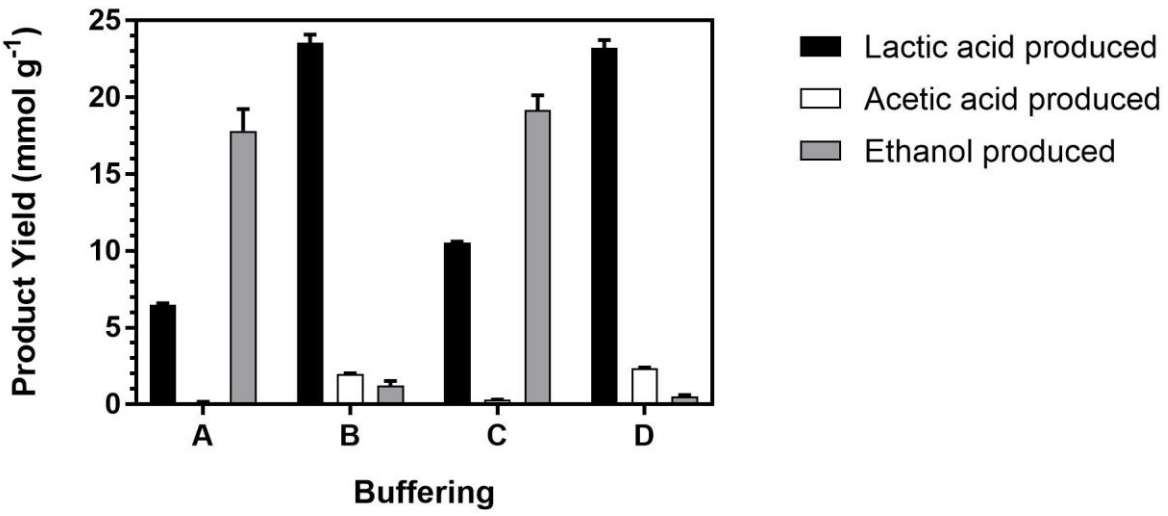


Figure 13. Effect of additional pH adjustment on lactic acid, acetic acid and ethanol yields from *Beta vulgaris* ensiled biomass. Data were produced from the ensiling of *Beta vulgaris* with standard additive loadings of *L. plantarum* and 5% w/v $CaCO_3$ when specified, there was additional daily pH adjustment to pH 6.5 by 1N KOH.

A: No $CaCO_3$ + no pH adjustment

B: 5% w/v $CaCO_3$ + no pH adjustment

C: No $CaCO_3$ + daily pH adjustment

D: 5% $CaCO_3$ + daily pH adjustment

Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. Bars represent the mean of *n=5* replicates with error bars shown.

Alternative Base Loadings

The formation of the insoluble salt calcium lactate and production of high levels of CO_2 thought to reduce lactic acid yields associated with $CaCO_3$ use led to the evaluation of different concentrations of more soluble bases as an alternative to 5% w/v $CaCO_3$.

5% w/v $Ca(OH)_2$ yielded the lowest lactic acid titer of 0.2 mmol g^{-1} related to the high pH of 10.5-11 recorded (Figure 14). Generally, as the concentration of $Ca(OH)_2$ increased, so did the lactic acid titer produced with a maximum at 3% w/v $Ca(OH)_2$, 16.4 mmol g^{-1} (approximately pH 4.05). At 4% w/v $Ca(OH)_2$ the pH increased above pH 4 and lead to a decrease in produced lactic acid (12.8 mmol g^{-1}). Compared to $Ca(OH)_2$ and CaCO3, ensiling with $Mg(OH)_2$ decreased lactic acid production with titers between 10.2-12.3 mmol g^{-1}. Ensiling with 3-4% w/v $Ca(OH)_2$ showed the highest yield of lactic acid observed in any of the experiments (80-100%).

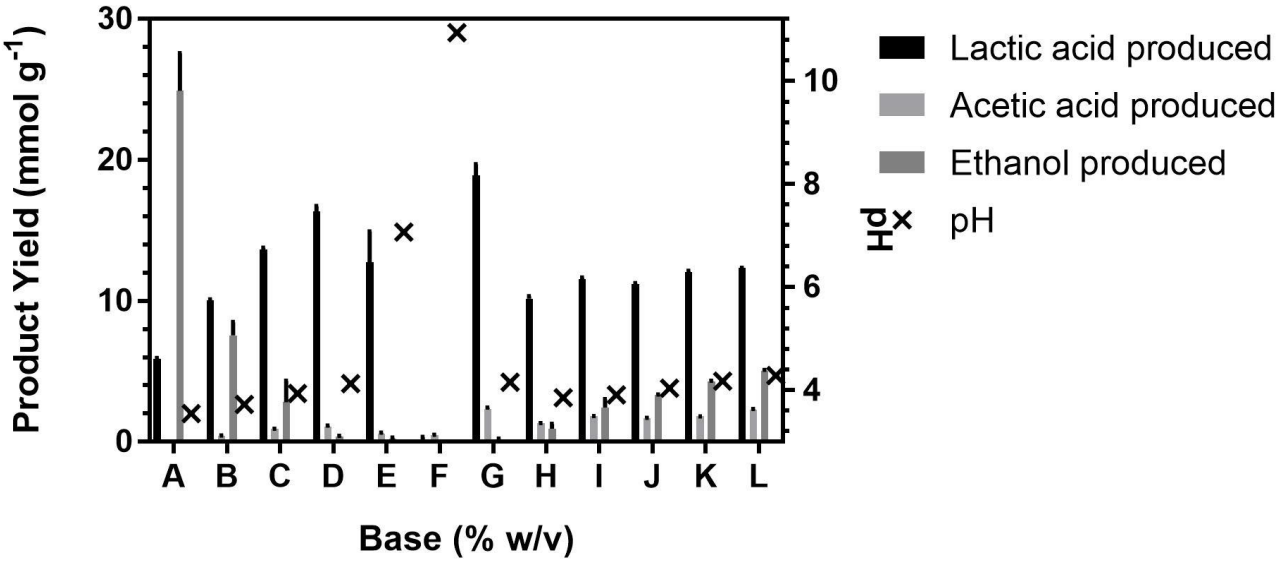


Figure 14. Effect of various base doses on pH, lactic acid, acetic acid and ethanol yields from *Beta vulgaris* ensiled biomass. Data were produced from the ensiling of *Beta vulgaris* with standard additive loadings of *L. plantarum* and various bases.

A: No base

B: 1% w/v $Ca(OH)_2$

C: 2% w/v $Ca(OH)_2$

D: 3% w/v $Ca(OH)_2$

E: 4% w/v $Ca(OH)_2$

F: 5% w/v $Ca(OH)_2$

G: 5% w/v $CaCO_3$

H: 1% w/v $Mg(OH)_2$

I: 2% w/v $Mg(OH)_2$

J: 3% w/v $Mg(OH)_2$

K: 4% w/v $Mg(OH)_2$

L: 5% w/v $Mg(OH)_2$

Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. pH is represented by black crosses. Bars represent the mean of *n=5* replicates with error bars shown.

4.3 TS Determination

Feedstock moisture content is critical to the ensiling process as it affects harvest and transportation costs, heating and steam requirements for the feedstock within large scale ensiling plants and wastewater production. Therefore, the moisture content of pre-ensiled sugar beet was tested. Previous laboratory analysis of moisture content in biomass samples was based on sorghum varieties, consequently, a new method for testing sugar beet was developed requiring a drying time of at least 40 h.

Moisture content of 74.6 % was the average for unhandled T=0 sugar beet material (Figure 15). Interestingly, altering the surface area of the sugar beet by physical manipulation also altered the measured moisture content. The highest value recorded was 80.9 % when chunks were shredded using a food processor. However, this condition also showed the greatest variability between replicates (σ=2.3). Likewise, hand malleted sugar beet had a higher moisture content compared to unhandled chunks, 78.6 %. $CaCO_3$ addition resulted in a lower measured moisture content, 72 % most likely as a result of adding $CaCO_3$ as a powder.

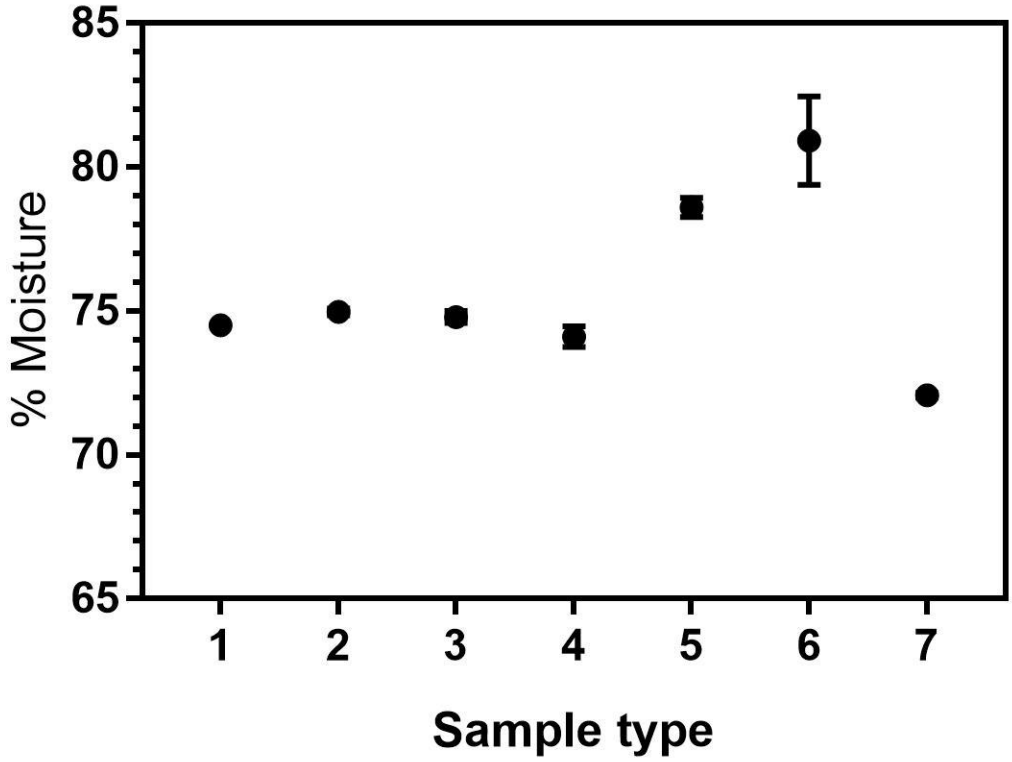


Figure 15. Pre-ensiled *Beta vulgaris* feedstock moisture content as determined according to a standard vacuum oven method. Samples were dried at 69 °C for at least 40 h.

Samples 1-4: T=0 material

Sample 5: T=0 malleted material

Sample 6: T=0 shredded material

Sample 7: T=0 + 5% w/v $CaCO_3$ material

Data points represent the mean of *n=3* replicates with error bars shown.

4.4 Metagenomic Analysis

During ensiling trials, there was a higher than expected concentration of ethanol measured in final samples. Addition of *L. plantarum* and restrictive conditions such as anaerobic ensiling chosen in an effort to increase lactic acid production over ethanol resulted in latent ethanol production nevertheless. Furthermore, a white, mould-like growth was observed in samples at the end of the ensiling period. Thus, some samples were selected for metagenomic analysis based on unexpected ethanol titers or inspection of mould growth. The aim was to identify key species endogenously present on the sugar beet and any changes in their distribution post ensiling to inform of the ideal consortia to optimise lactic acid yields for sugar beet ensiling.

Samples of fresh sugar beet showed the greatest bacterial diversity of the samples tested (Figure 16). The bacterial species found in the highest amounts were *Helicobacteraceae*, *Thermomicrobium*, *Methylococcaceae* and *Bacillus*, the prevalence of these species is evidence of their ubiquitous nature. However, many of the identified genera were found in different distributions compared to post-ensiled samples, for example the percentage of *Carnobacterium* measured was up to 36% lower. In addition, most of the common species found in the post-ensiled samples e.g. *Verrucomicrobium* and *Acidiphilium* were only determined to be in small quantities (<1%). The most commonly found genus in post ensiled samples was *Carnobacterium* (28-39%, Figure 13). Interestingly, there was a small amount of *Lactobacillaceae* detected in these samples (0.02-0.06% of all bacteria) despite inoculation with *L. plantarum* cultured in the laboratory. The next largest group of bacteria identified were *Mycobacterium* (14-16%) followed by *Acidiphilium* (approximately 10%). Of the identified bacteria, a small percentage (2%) were *Verrucomicrobium*, *Brevibacterium* and Peptococcus. There were minimal differences in bacterial composition with increasing addition of $CaCO_3$ concentration, this explains the similar concentrations of lactic acid and ethanol produced in these cases.

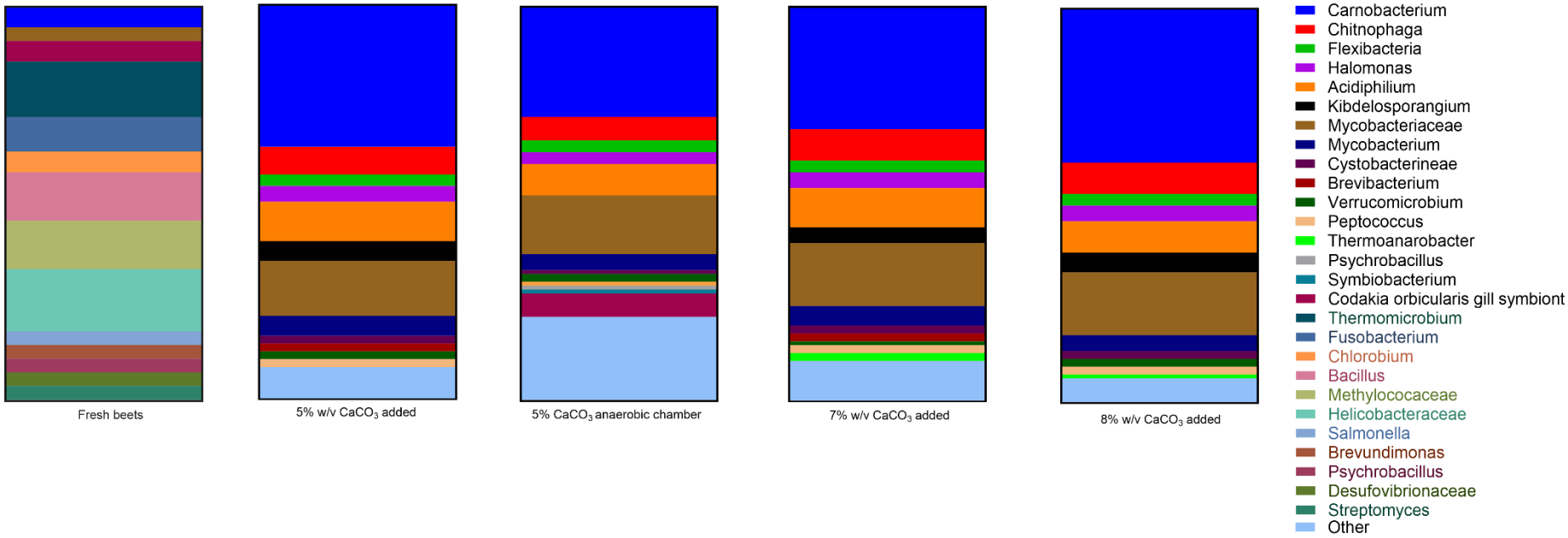


Figure 16. Bacterial community composition in Fresh beets and post-ensiled sugar beet with either 5% w/v $CaCO_3$ added, 5% w/v $CaCO_3$ ensiled in an anaerobic chamber, 7% w/v $CaCO_3$ added and 8% w/v $CaCO_3$ added. The height of each bar was determined using quantitative PCR of the 16S rRNA gene, taxa not designated to a genus are appointed to their family name. The bars are coloured according to the genus identified by amplicon sequencing of the 16S rRNA gene.

DISCUSSION

Previous Shell research involved enzymatic hydrolysis of *Sorghum bicolor* to produce monomeric sugars for fermentation by *Saccharomyces cerevisiae* to produce ethanol (Almodares and Hadi, 2009; Gallagher *et al*., 2018). However, lactic acid, a source of sustainable and renewable based chemicals has a potential commercial value (Abdel-Rahman, Tashiro and Sonomoto, 2011). Thus, development of an efficient and financially advantageous lactic acid producing process is crucial. As sorghum ensiling requires enzymes and high temperatures with high costs, *Beta vulgaris* with a lower fibre content may be a preferred substrate as an alternative. The aim of this project was to develop a suitable ensiling protocol for sugar beet to optimise lactic acid yields and screen organisms as suitable potential biocatalysts. To address this hypothesis, carbohydrate source utilisation of various bacteria were assessed and the optimal combination of lactic acid bacteria strains best suited as an inoculant for sugar beet ensiling were identified. Key process variables such as temperature and sugar beet surface area were also tested to determine the effect on lactic acid yields. Trials to increase lactic acid yields included use of preservatives to control the endogenous microbial flora and addition of bases as neutralising agents.

5.1 Identification of consumption of a main carbohydrate source

Lactic acid bacteria are known to have different metabolic pathways for sugar metabolism resulting in varying yields of lactic acid (Vandenbergh *et al*., 2018). *L. plantarum* was found to utilise sucrose, the most abundant sugar in sugar beet to produce lactic acid. The high titers of acetic acid produced were typical of *L. plantarum*'s heterofermentative nature and the PK pathway (Zhang *et al*., 2016). Interestingly, there was minimal production of ethanol (unquantifiable by HPLC), an expected product.

Despite successful utilisation of most of the supplied carbohydrate sources, the poor lactic acid titers when *L. plantarum* was incubated with DGA (a common pectin monomer), suggested addition of another species may increase final lactic acid production in sugar beet ensiling (Dranca and Oroian, 2018). The obligately heterofermentative *L. diolovorans* consumed pentose sugars (arabinose and xylose) more readily than hexose sugars (Krooneman *et al*., 2002). This suggests that *L. diolovorans* is not suitable for ensiling alone, however if combined with an additional lactic acid bacteria (likely *L. plantarum*) lactic acid titers may increase as this combination would have the ability to consume the common substrates present in sugar beet: sucrose, cellobiose, glucose, xylose and arabinose. *L. pentosus*, a pentose consuming species produced high titers of acetic

acid when incubated with various carbohydrate sources (Buyondo and Liu, 2011). As acetic acid is an undesirable product which will lower the pH and slow production of lactic acid by diverting carbon (Vandenbergh *et al*., 2018), using *L. pentosus* in isolation would therefore be unsuitable for sugar beet ensiling. However, there was consumption of arabinose and high lactic acid yields on most sugars tested. Thus, if ensiled in an environment designed to encourage homofermentation as opposed to heterofermentation, *L. pentosus* may be a favourable inoculum.

According to these findings, sugar beet ensiling with inoculations of *L. plantarum*, *L. diolivorans* and *L. pentosus* has the potential to utilise most of the main substrates for lactic acid production. The formation of by-products would be limited by ensuring strict anaerobic conditions. To evaluate this, future work should conduct a laboratory ensiling trial with of *L. plantarum*, *L. diolivorans* and *L. pentosus* to examine the effect on lactic acid production.

5.2 Determination of optimal operating ranges

Altering the surface area of sugar beet chunks by different physical treatments showed no significant difference in yields of lactic acid and ethanol when malleted or shredded compared to the chunks. Final pH values of approximately 3.5 measured for the ensiled material suggested that acidification led to the suspension of lactic acid production by *L. plantarum* fermentation followed by ethanol production using residual sucrose. It was thought the ethanologen was likely, an acid resistant microbe that is naturally associated with sugar beet. The large drop in apparent ethanol concentration when blending compared to hand squeezing attributed to evaporation during the blending process also suggested ethanol might be retained in the fibrous material left over after blending rather than squeezed out when using hand juicing. Based on this, hand squeezing was considered the better sampling method for all subsequent experiments.

Sugar beet ensiling at 42 °C resulted in reduced ethanol and lactic acid titers, this was considered to reflect the reduced metabolic activity of *L. plantarum* and any incidental ethanologens. However, lactic acid yields were not strongly affected between 32 °C and 37 °C, therefore implying sugar beet can be successfully ensiled in this temperature range using *L. plantarum*. Ensiling at 4 °C and 10 °C yielded poor lactic acid titers, this was not considered disadvantageous as large-scale trials in piles has shown that compaction leads to a ‘self-heating’ effect to approximately 40 °C (Shell Global Solutions (US) Inc., 2017).

Based on these trials, optimal lactic acid production can be achieved regardless of sugar beet particle size when ensiled between 32 °C and 37 °C with addition of a base. As there is no requirement for pre-treatment, and to prevent a rise in temperature during pile assembly, large-

scale ensiling can be performed without the need of additional infrastructure in already developed plants/facilities. This also has the extra benefit of reducing transportation costs ($17.66 per planted acre in 2005 in the USA) as ensiling plants would be located in North Dakota and Minnesota where there is already sugar beet production in the Red River Valley region (16,506 metric tons in 2006) (Gilbery, Lardy and Bauer, 2009; United States Department of Agriculture, 2006).

5.3 Determination of an optimal biocatalyst

Various lactic acid bacteria selected for several potential benefits for sugar beet ensiling for example *L. brevis* and *L. fermentum* both previously found to be present in fermenting sorghum biomass in metagenomic analysis (Gallagher *et al*., 2018) and *L. amylovorous*, an amylose consumer (Giraud and Cuny, 1997) were tested to evaluate their suitability for sugar beet ensiling. Alongside the carbon source utilisation work, these individual and combination biocatalyst evaluation experiments would be used in the future to design LAB consortia to effectively degrade a majority of the target small molecule sugars present in sugar beet biomass.

The higher amounts of acetic acid produced by both *L. brevis* and *L. fermentum* compared to *L. plantarum w*ere expected due to the heterofermentative metabolism of both strains (Cubas-Cano *et al*., 2018). This may also account for the significantly reduced ethanol production observed in samples ensiled with these species due to the increased inhibitory effects at the lower pH encountered during ensiling. The observed low product formation when ensiling with *L. fermentum* was in line with results from the carbon utilisation studies indicating that *L. fermentum* fermentation kinetics appear to be slower than the other species. There was a significant increase in lactic acid titers and less ethanol produced in samples ensiled with *L. pentosus* compared to *L.* plantarum, this suggests *L. pentosus* may be able to select for lactic acid production and preferentially produce it over ethanol.

Z. mobilis, selected for its ability to ferment sugar to ethanol with an extremely high ethanol tolerance; up to 16% (v/v) (Gunasekaran and Chandra Raj, 1999) was outperformed by *L. plantarum* in both lactic acid and ethanol production. This collaborated carbon utilization tests where there was also minimal product formation. Within the context of ensiling, it is possible the high concentrations of acetic acid, produced by native microbes on the sugar beet inhibited the acetic acid intolerable *Z. mobilis* thereby preventing additional formation of lactic acid and ethanol.

An increase in lactic acid production between sterilised and unsterilised material at 37 °C was observed suggesting that a portion of the lactic acid production during ensiling may be due to endogenous microorganisms, or due to syntrophic interactions between *L. plantarum* and the native sugar beet microbiome. The similar result at 42 °C also suggests native microbes are responsible for products generated during higher temperature ensiling. In contrast, ethanol production at 37 °C was higher in sterilised samples but at 42 °C the reverse was observed. Given that it is unlikely that much of native microbial flora survived autoclaving, this result suggests that laboratory-introduced ethanologens were responsible for the observed ethanol production. Although it will be difficult to control all sources of possible contamination in a field-scale ensiling trial, lactic acid itself may prove to be an effective agent for reducing the growth of unwanted microbes; improved lactic acid yields appear to be correlated with suppression of ethanol formation (Figure 12, $CaCO_3$ optimisation).

Testing C6 Fuel (*Saccharomyces cerevisiae* active dry yeast package), which has successfully produced high yields of ethanol in sorghum field trials and other yeasts used to make at lower temperatures with a high alcohol tolerance showed that addition of C6 Fuel, acid and lactrol yielded the highest amount of ethanol. Generally, the largest quantity of ethanol was produced in all conditions containing yeast C6 Fuel. However, it is possible the wine yeasts were not operating at optimum as these experiments were conducted at 37 °C, this was supported by the production of large amounts of glucose (presumably from sucrose) not consumed, implying that additional ensiling time was needed compared to C6 Fuel. Another ensiling trial would evaluate the effectiveness of the wine yeast whilst at lower temperatures.

Inoculation of sugar beet biomass with *L. plantarum* and a consortium of other lactic acid bacteria, addition of $CaCO_3$ between 32- 37 °C could potentially produce >23 mmol g^{-1} of lactic acid. The addition of bacteria to the biomass in the laboratory by culturing then harvesting cells by centrifuging and resuspending the pellet would be impractical and economically unviable in large scale trials. Future trials should evaluate the success of commercially available powdered lactic acid bacteria, for example Fermentation “plus”™ dry granular silage inoculant (Midwestern BioAg, 2018) at producing similar lactic acid titers. Using a low-cost powder such as the one described would allow ensiling of up to 50 tons in a single trial and production of high lactic acid titers.

There is also the possibility of having a lactic acid-ethanol pipeline to utilise as much sugar from the sugar beet as possible and also increase profits from the process. The theoretical yield of ethanol from sucrose is 163 gallons per ton, with an estimated 90% of that yield obtainable in the laboratory in ideal conditions (United States Department of Agriculture, 2006). Ethanol ensiling in this study achieved between 78-99% yield when acid, yeast, and lactrol were combined, given these yields, the $2.35 estimated cost of ethanol production in the United States and $2.89 in the

European Union (United States Department of Agriculture, 2006) still allow for an economically viable process. Further optimisation studies to fine-tune additive doses would ensure yields are always >90 % and there was maximum ethanol production.

5.4 Identification of effective methods to increase lactic acid yields

Addition of preservatives sodium benzoate (1 g kg^{-1}), potassium sorbate (1 g kg^{-1}), sodium chloride (5 % v/v) and pimaricin (200 mg kg^{-1}) with the aim of limiting growth and function of the endogenous microbial flora without affecting lactic acid bacteria function resulted in comparable lactic acid titers in ensiling trials with only 5 % w/v $CaCO_3$ added. However, a high lactic acid yield of 83 % was achieved in samples with added NaCl but some residual sucrose remained, allowing for further optimisation and increased yield.

Ensiling sugar beet with different $CaCO_3$ loadings showed increasing lactic acid titers as $CaCO_3$ concentration increased associated with decreased organic acid stress on *L. plantarum*. The decrease in titer at 8 % w/v $CaCO_3$ was considered a consequence of loss of airlocks and material, the production of CO_2 was the most likely product responsible for the forced-out airlocks. Despite the high, 26.5 mmol g^{-1} lactic acid titer achieved when 7 % w/v $CaCO_3$, addition of this amount of $CaCO_3$ is impractical in large-scale trials. Further buffering attempts using KOH for pH adjustment to pH6.5 revealed initial buffering had the greatest effect on lactic acid production. As seen with $CaCO_3$, increasing loadings of $Ca(OH)_2$ also increased produced lactic acid titers with a maximum measured at 3% w/v $Ca(OH)_2$. This was in part because $Ca(OH)_2$ was able to reduce the pH to approximately pH 4 thereby maintaining lactic acid bacteria activity. Although $CaCO_3$ produced a higher lactic acid titer, ensiling with 3-4% w/v $Ca(OH)_2$ produced a 80-100% yield of lactic acid. One reason for this could be that $Ca(OH)_2$ is more soluble than $CaCO_3$ therefore there was less salt and CO_2 production, leading to a reduced impact on lactic acid yields.

The necessary addition of a neutralising agent for buffering introduces the further challenge of separating any produced salts such as calcium lactate. Solvent extraction, a commonly used method extracts lactic acid from the mixture using an extractant followed by back extraction of lactic acid into another solvent (Othman *et al*., 2017). Activated carbon, a porous material with a specific affinity to organic materials could be a low-cost alternative. Gao *et al*., (2011) found that acetone was an efficient solvent for lactic acid desorption from activated carbon but there should be caution as activated carbon was unable to adsorb lactic acid at pH >4.

5.5 TS determination

On average, a moisture content of 74.6 % was determined for T=0 sugar beet chunks. This value is typical for sugar beet with other studies reporting 70-78 % moisture (Food and Agriculture Organisation of the United Nations, 2009; Vargas-Ramirez *et al*., 2016). As mentioned in the introduction, the high moisture content in sugar beet often impedes long-term storage due to activation of microbial activity, for example moisture content of <70 % and pH >5 leads to *Clostridia* growth, which ferments sugars and lactic acid to butyric acid (Vargas-Ramirez *et al*., 2016). Therefore, ensiling should be performed soon after harvesting. Alternatively, to obtain the required weight of sugar beet for large-scale ensiling, harvested roots would need to be frozen or stored in low-temperature controlled bunkers. Vargas-Ramirez *et al* (2016) also found that an increased moisture content had an adverse effect on sugar retention. Since the sugar beet tested in this project had a high moisture content, it would be advisable to add an inhibitor that can limit *Clostridia* growth and a neutralising agent to maintain the pH between 4-5.

5.6 Identification of microbial species present on sugar beet

Fresh sugar beet had a greater bacterial diversity than post-ensiled sugar beet samples. It was expected that additive and *Lactobacillus* inoculation would have an effect on the microbial make-up as product yields varied. The hypothesis was that as lactic acid was produced and pH decreased, the growth of some microbial organisms would be limited. In contrast, species that were acid tolerant would appear in the same quantities post-ensiling. The most abundant bacterial genus was *Carnobacterium*, a ubiquitous group of lactic acid bacteria consisting of 9 species which are commonly found in terrestrial environments such as compost and manure (Leisner *et al*., 2007). These species are most likely responsible for the lactic acid produced in the ensiled sugar beet samples. However, there was only a small percentage of *Lactobacillaceae* detected in post-ensiled samples. It is possible most of the added *Lactobacillus* cells were inhibited and were not viable at the end of the ensiling period due to high concentrations of the produced lactic acid. Aellen *et al*., (2006) found a proportional increase in yields of amplified 16 rRNA genes and bacterial mass during logarithmic growth, there was also correlation between the quantities of amplicons and viable counts with drug-induced killing during penicillin treatment. The increased abundance in *Mycobacterium* from fresh sugar beet samples to post-ensiled samples (2% to 15%) could explain the presence of the observed white growth as mycobacteria are reported to be capable of forming spores (Singh *et al*., 2010). Therefore, addition of a *Mycobacterium* inhibitor prior to ensiling would prevent growth and allow for more of the available sugars in the sugar beet to be converted to lactic acid thus increasing yields.

CONCLUSION

The main aim of this project was to develop a suitable ensiling protocol for sugar beet including screening potential inoculants and biocatalysts by assessing the utilisation of carbohydrate sources. The focus was to produce high titers of lactic acid and ethanol for use as a precursor for renewable based chemicals and biofuels respectively. Carbohydrate utilisation trials identified that a consortia of *L. plantarum*, *L. diolivorans* and *L. pentosus* had the potential ability to consume common sugar substrates present in sugar beet. Optimum laboratory ensiling conditions were determined as shredding sugar beet chunks prior to ensiling, incubation between 32 °C and 37 °C and hand squeezing for sampling at the end of the ensiling period. Addition of a *L. plantarum* inoculation and $CaCO_3$ consistently yielded >23 mmol g^{-1} of lactic acid. Conversely, 35 mmol g^{-1} ethanol production and selectivity over lactic acid required *S. cerevisiae*, acid and lactrol. Investigations into additional methods to increase lactic acid yields through additional trials investigating the effect of NaCl as a microbial inhibitor and $Ca(OH)_2$ as a neutralising agent were required. More research also needs to be conducted to identify an economical and effective method to separate and extract lactic acid from the fermentation mixture. Metagenomic analysis of sugar beet samples prior to ensiling and post ensiling showed an increased abundance in *Mycobacterium* thought be responsible for the observed spores and decreased lactic aid yields. Utilising an inhibitor may reduce the diversion of sugar beet carbohydrates to *Mycobacterium* growth and therefore increase lactic acid yields.

Ultimately, this work shows sugar beet ensiling has the potential to produce high yields of lactic acid and ethanol. However, exact dosage of additives and a product extraction method need to be resolved before it can be determined if the process is profitable.

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APPENDIX

Appendix 1: Equations used for Total Solid and Undissolved Solid Determination.

A:

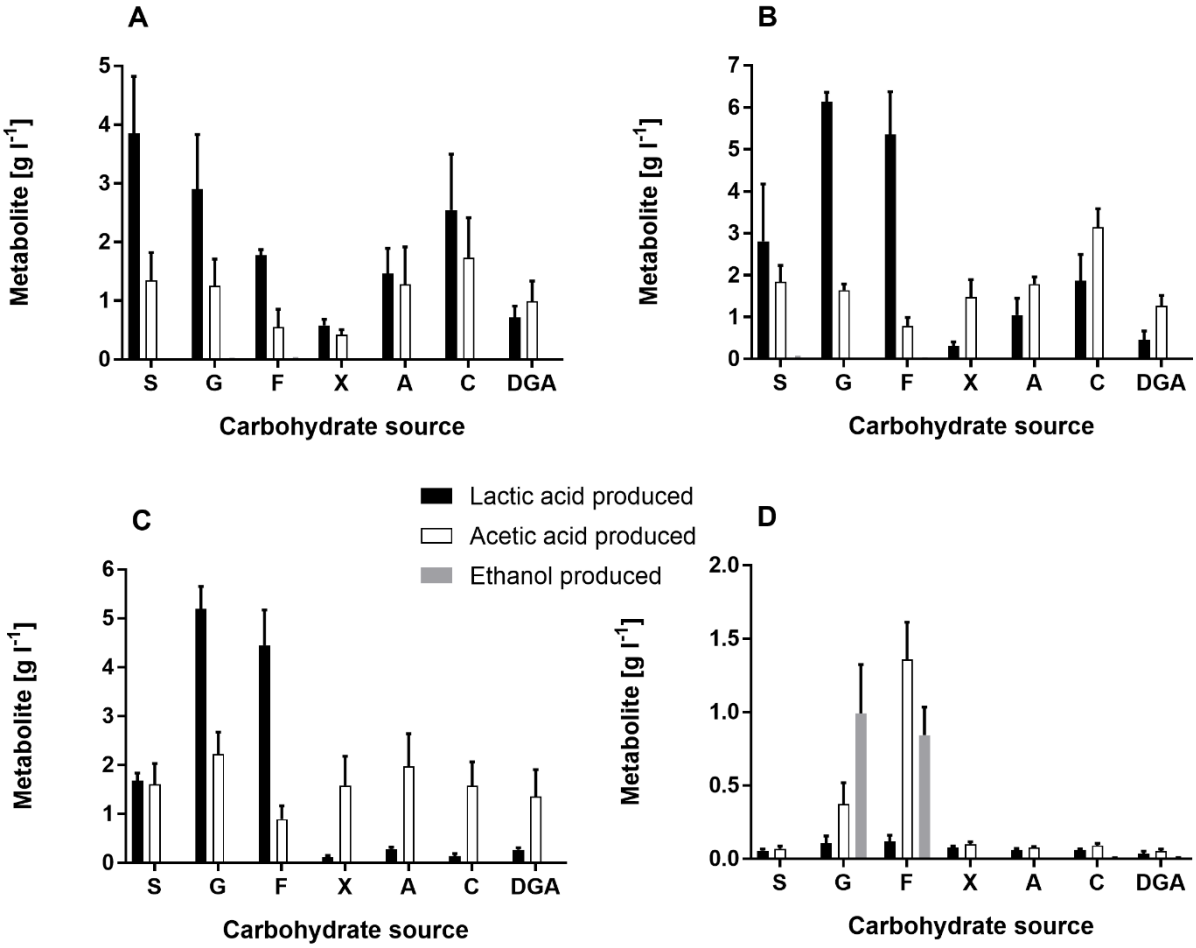
$$\%TS = \frac{\text{Weight}_{\text{dry Falcon + dry substrate}} - \text{Weight}_{\text{Falcon}} + \text{Weight difference}_{\text{empty Falcon}}}{\text{Weight}_{\text{wet substrate}}} \times 100$$

B:

$$\%\text{Moisture} = 100 - \frac{\text{Weight}_{\text{dry Falcon + dry substrate}} - \text{Weight}_{\text{Falcon}} + \text{Weight difference}_{\text{empty Falcon}}}{\text{Weight}_{\text{wet substrate}}} \times 100$$

C:

$$\%UDS = \frac{\text{Weight}_{\text{dry Falcon + dry filter + dry substrate}} - \text{Weight}_{\text{Falcon + filter}} + \text{Weight difference}_{\text{empty Falcon}}}{\text{Weight}_{\text{wet substrate}}} \times 100$$



Appendix 2. Product formation of A: *L. acidophilus*, B: *L. amylovorous*, C: *L. sakei*, D: *Z. mobilis*, after incubation for 48 h at 37 °C on various carbohydrate sources added at 10 g l^{-1}.

S: Sucrose

G: Glucose

F: Fructose

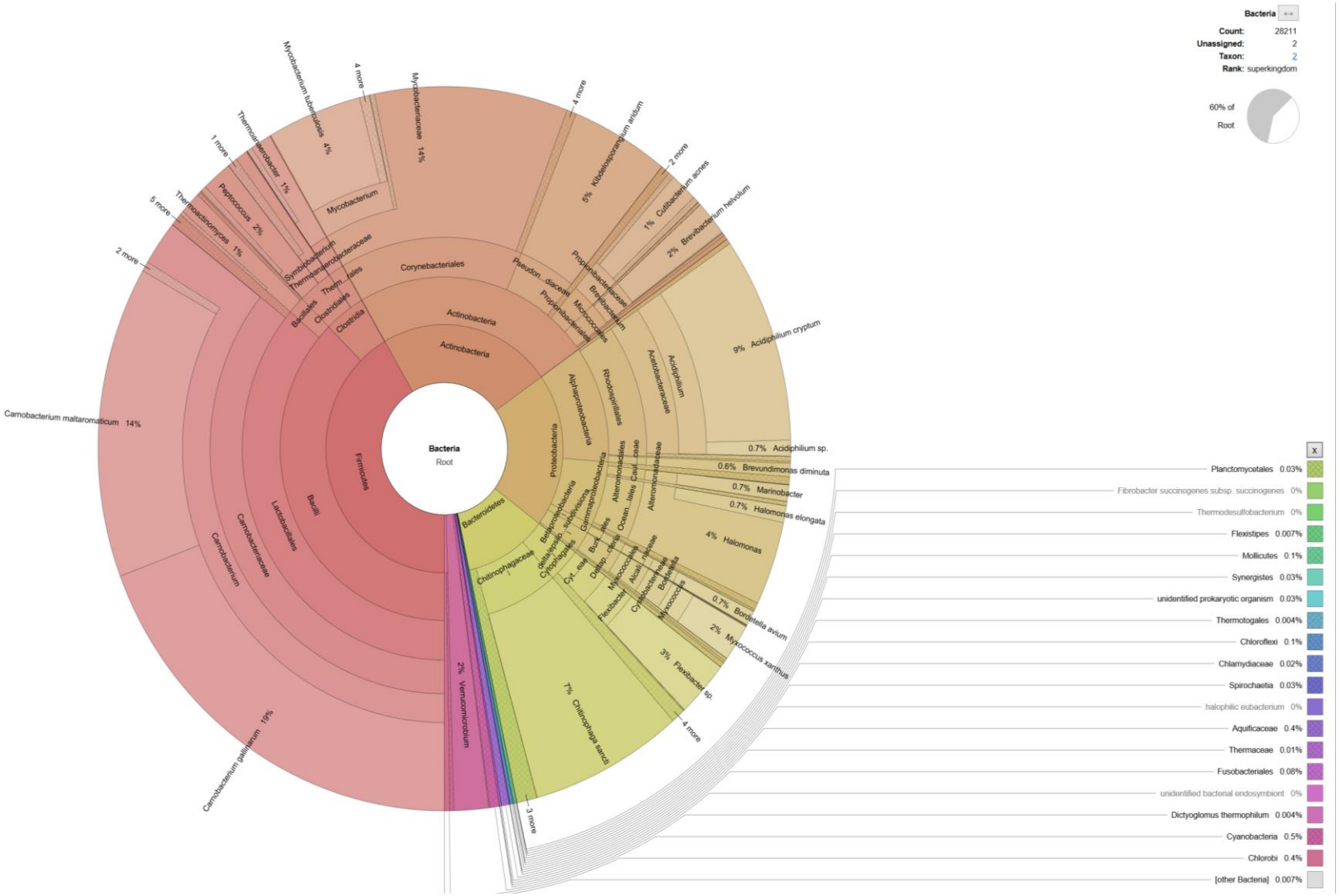
X: Xylose

A: Arabinose

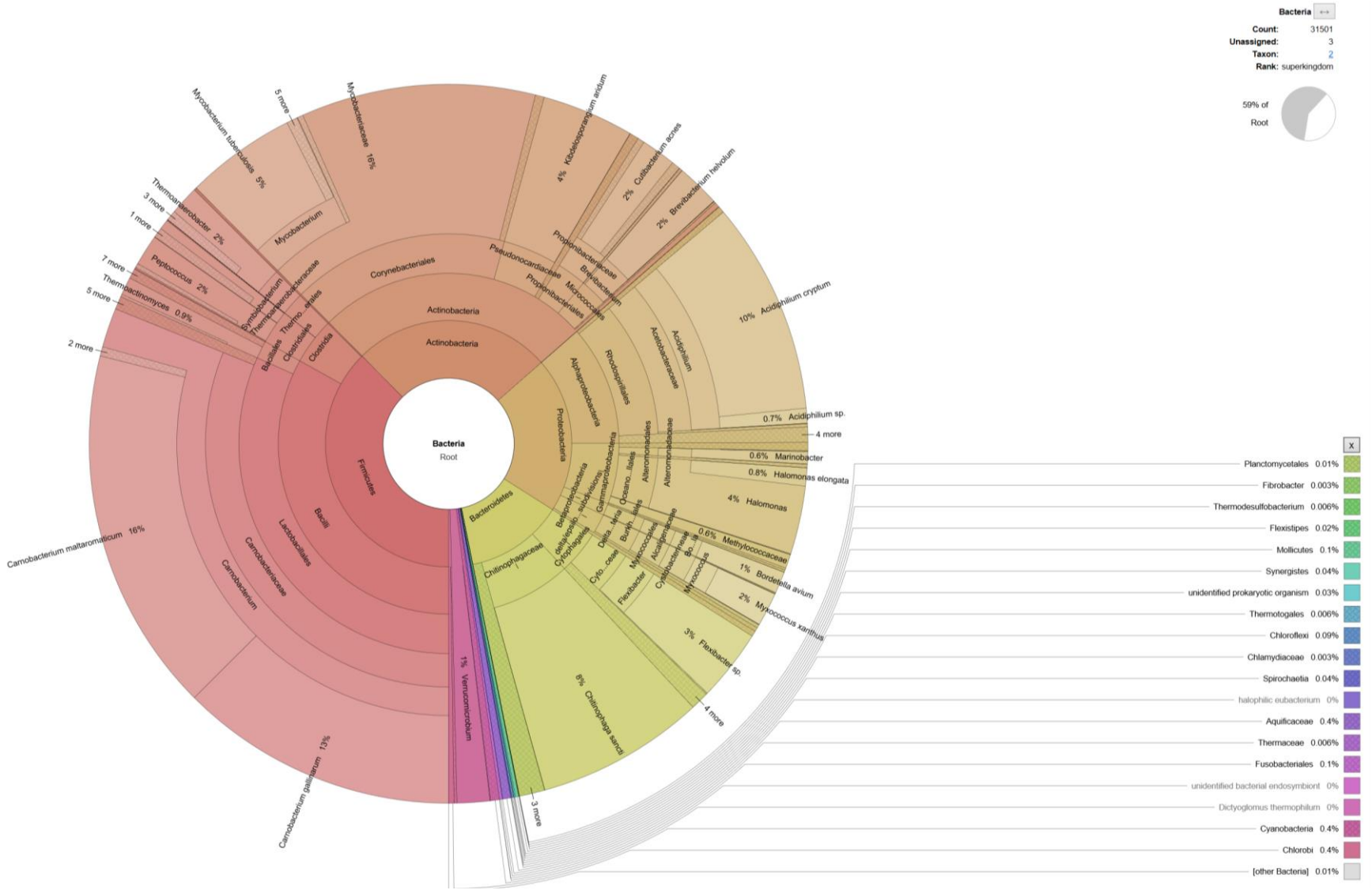
C: Cellobiose

DGA: D- galacturonic acid

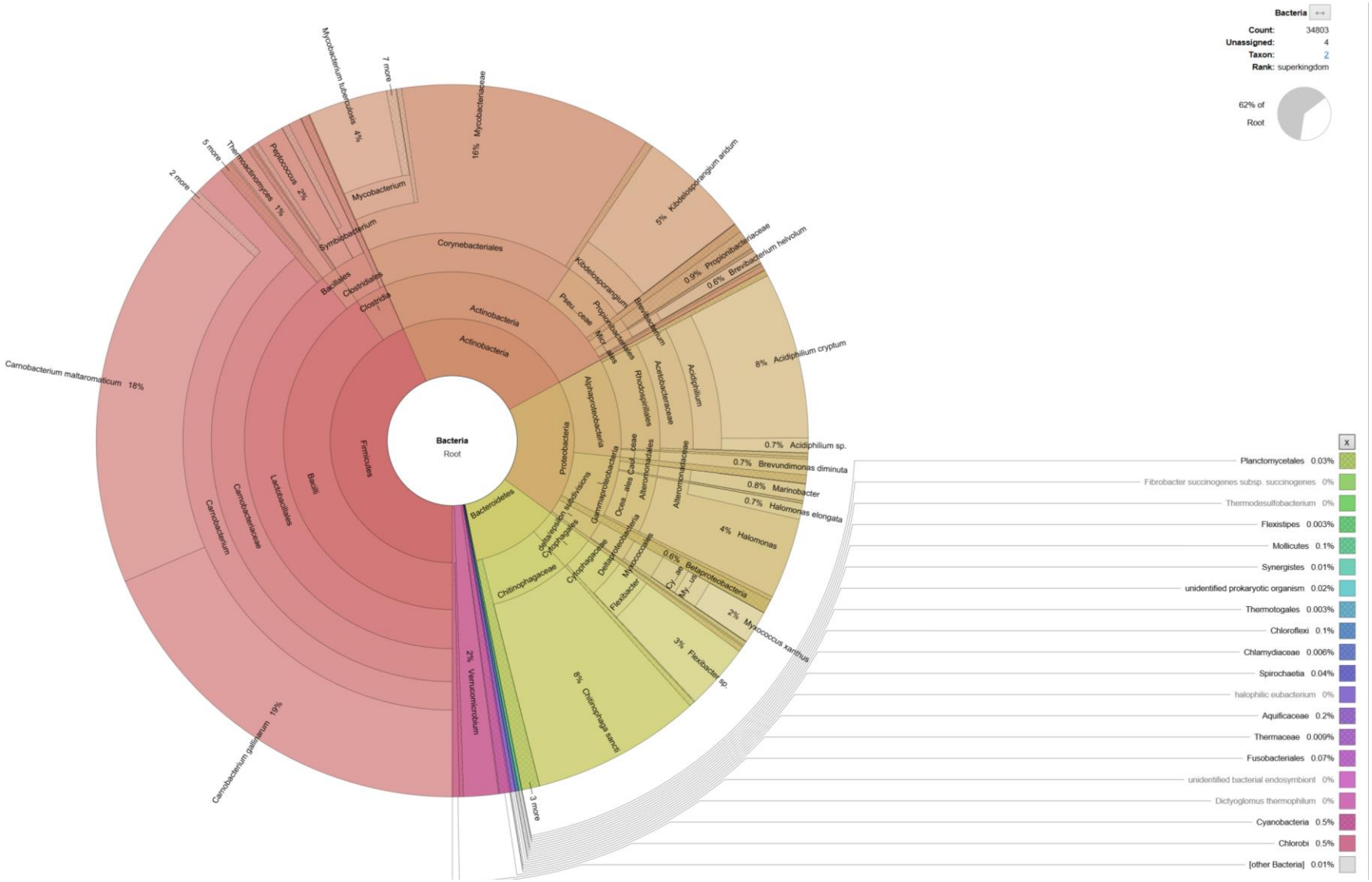
Lactic acid is represented by black bars, acetic acid by white bars and ethanol by grey bars. Bars represent the mean of *n=6* replicates with error bars shown.



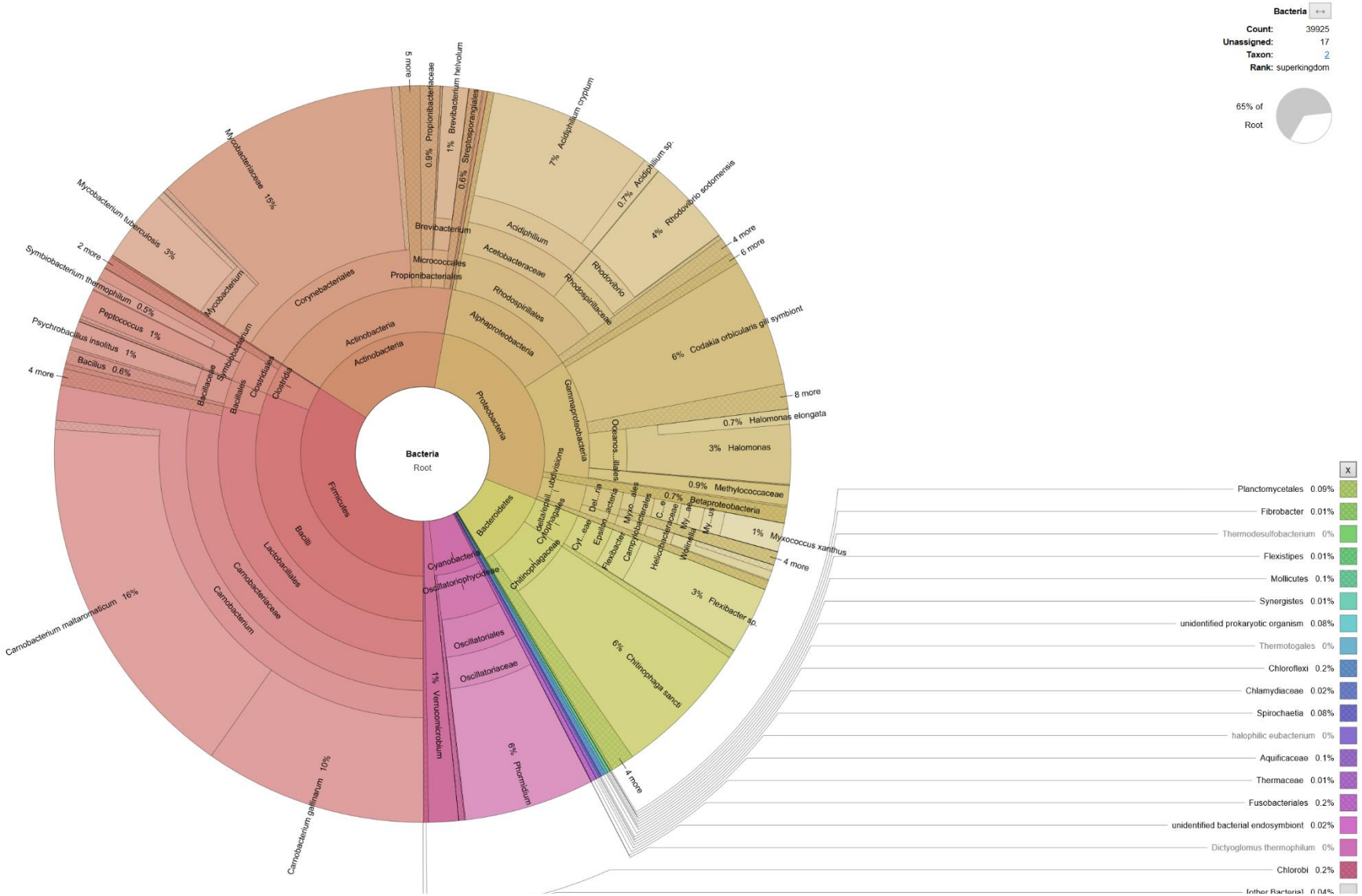
Appendix 3. Bacterial community composition in post-ensiled sugar beet with 5% w/v $CaCO_3$ added.



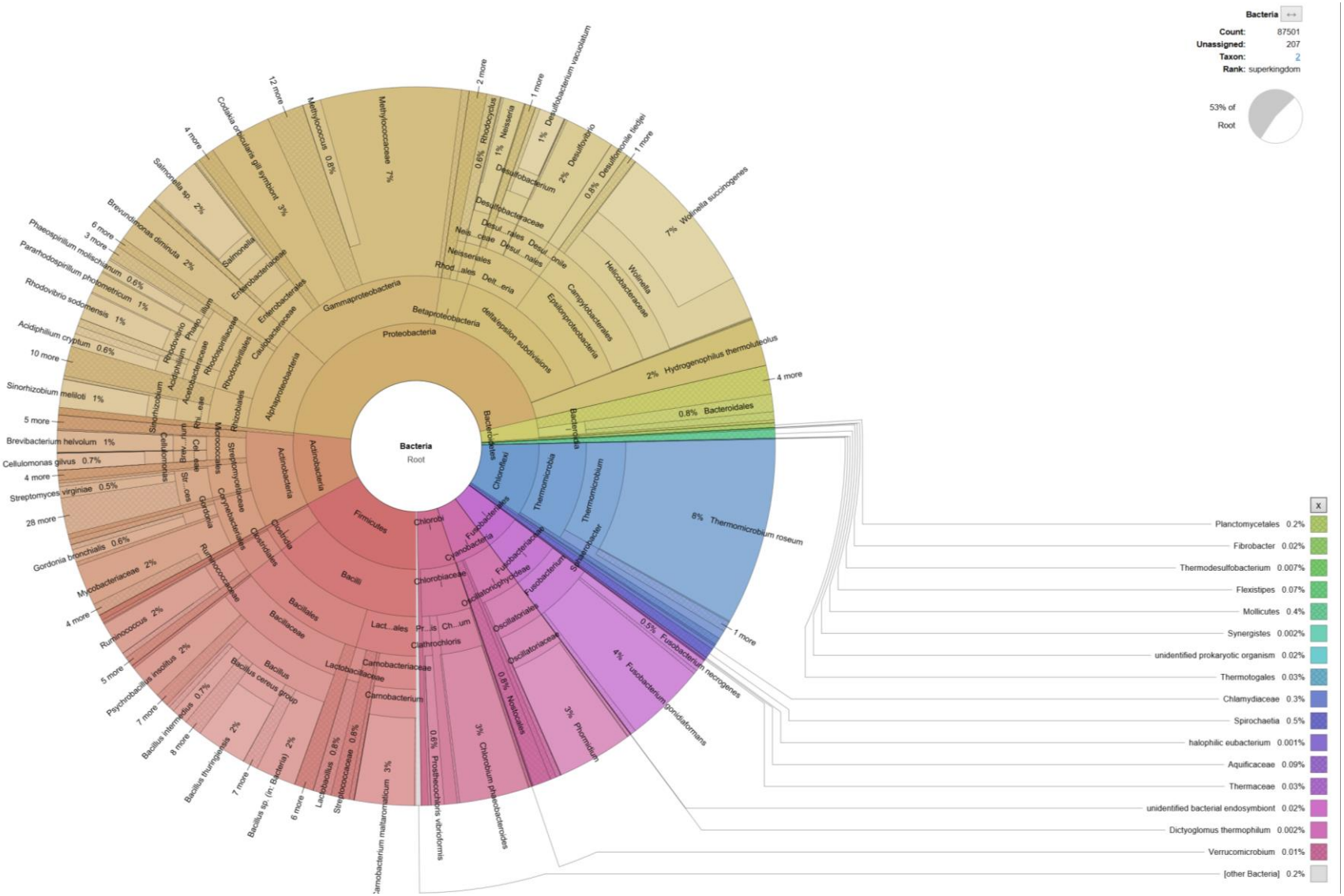
Appendix 4. Bacterial community composition in post-ensiled sugar beet with 7% w/v $CaCO_3$ added.



Appendix 5. Bacterial community composition in post-ensiled sugar beet with 8% w/v $CaCO_3$ added.



Appendix 6. Bacterial community composition in post-ensiled sugar beet with 5% w/v $CaCO_3$ added and ensiled in an anaerobic chamber.



Appendix 7. Bacterial community composition in fresh sugar beet.