Upcycling dairy industry byproducts: Transformation of distillery stillage into fertilizer

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ABSTRACT

The dairy industry is dealing with the disposal of a costly by-product, called milk permeate, when creating a variety of dairy products such as cheese, yogurt and butter. Milk permeate is a solution high in lactose that is costly to dispose of because of its high biological oxygen demand (BOD) and chemical oxygen demand (COD).

We addressed this issue in two parts; one by using milk permeate with a lactose-fermenting yeast, *Kluyveromyces Marxianus,* to create ethanol to be sold vodka, by fermentation and distillation processes. The fermentation and distillation eliminates the lactose from solution, but it creates a secondary waste product that is high in yeast biomass, therefor still contains high BOD and COD. This secondary by-product, distillery stillage, was repurposed into a fertilizer and tested in greenhouse cultivation of *Capsicum annuum* (bell pepper).

The transformation of milk permeate was successful at creating a profitable ethanol product and reducing waste, after fermentation with *K.marxianus*. The usage of repurposed distillery stillage as a fertilizer shows promise as it was applied to *C. annuum* plants in a greenhouse setting and showed results comparable to commercial fertilizer available on the market.

The work accomplished demonstrated how transformation and upcycling successfully reduced waste in the dairy industry by applying it to two different waste products, milk permeate, followed by distillery stillage. This has reduced the environmental footprint of dairy waste streams and created new revenue opportunities while promoting sustainable agriculture.

Résumé

L'industrie laitière doit gérer l'élimination d'un sous-produit coûteux, appelé perméat de lait, lors de la fabrication de divers produits laitiers tels que le fromage, le yaourt et le beurre. Le perméat de lait est une solution riche en lactose dont l'élimination est coûteuse en raison de sa demande biologique en oxygène (DBO) et de sa demande chimique en oxygène (DCO) élevées.

Nous avons abordé cette question en deux parties : d'une part, en utilisant le perméat de lait avec une levure fermentant le lactose, *Kluyveromyces Marxianus*, pour créer de l'éthanol destiné à être vendu sous forme de vodka, par des processus de fermentation et de distillation. La fermentation et la distillation éliminent le lactose de la solution, mais elles créent un déchet secondaire riche en biomasse de levure, qui contient donc toujours une DBO et une DCO élevées. Ce sous-produit secondaire, la vignasse de distillerie, a été réutilisé comme engrais et testé dans la culture en serre de *Capsicum annuum* (poivron).

La transformation du perméat de lait a permis de créer un produit éthanol rentable et de réduire les déchets, après fermentation avec *K. marxianus*. L'utilisation de la vignasse de distillerie recyclé comme engrais s'avère prometteuse, car il a été appliqué à des plants de *C. annuum* dans une serre et a donné des résultats comparables à ceux des engrais commerciaux disponibles sur le marché.

Les travaux réalisés ont démontré comment la transformation et le surcyclage ont permis de réduire efficacement les déchets dans l'industrie laitière en les appliquant à deux types de déchets différents, le premier le perméat de lait, puis la vignasses de distillerie. Cela a permis de réduire l'empreinte environnementale des flux de déchets laitiers et de créer de nouvelles sources de revenus tout en favorisant l'agriculture durable.

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ABBREVIATIONS

BOD -Biological oxygen demand
CF – Conventional fertilizer
CFIA -Canadian Food Inspection Agency
COD -Chemical oxygen demand
DPT -Days post-transplant
DRW- Dry root weight
DSW-Dry shoot weight
FSW -Fresh shoot weight
ICP-MS -Inductively coupled plasma mass spectrometry
LDM – Leaves dry mass
ORS- Octopole Reaction System
SDM – Stem dry mass
SF – Stillage fertilizer
WPT- Week post-transplant

CHAPTER ONE

Project Introduction

1.1 Thesis objective

The Canadian dairy industry is faced with the problematic waste treatment of milk permeate: a milk by-product containing high concentrations of the sugar lactose. This waste is created when milk is transformed into cheese, yogurt, and butter and contains around 20% lactose (w/w). Due to its high sugar concentration, milk permeate is associated with a high biological oxygen demand (BOD) and must be disposed of safely so as not to contribute to the development of anoxic zones in ecosystems where it is disposed. Legislative regulations have become increasingly strict over the last three decades as the polluting effect of this waste has been brought to public attention (Smithers, 2008).

Currently, proper treatment of milk permeate consists of using digesters aiming at reducing the BOD, which costs the dairy industry hundreds of thousands of dollars per year due to the large volume of permeate produced. For example, the milk permeate that we acquired comes from the Parmalat dairy plant in Winchester, ON. It is the biggest plant in Canada and creates 100 000 liters of milk permeate per day. One possible valorization of this waste is using fermentation and distillation to transform milk permeate into ethanol.

The process of transforming milk permeate into ethanol is a fermentation followed by distillation. During distillation, high purity ethanol is separated by evaporation from the rest of the liquid. It is the left-over liquid after distillation that is called distillery stillage. Stillage contains all the dead yeast cells from the fermentation process and other nutrients from the initial solution, in this case the milk permeate. Stillage is a high oxygen demanding waste product that contains relatively high phosphorus (P) and potassium (K) (Ramana et al., 2002). In the literature “stillage” and “vinasse” can be used interchangeably.

This project is rooted in an industrial partnership between the University of Ottawa and our partners at Dairy Distillery. In our partnership, the two problems addressed are the transformations of by-products, firstly with milk permeate into ethanol, followed by the transformation of distillery stillage into a fertilizer. The Dairy Distillery is now a distillery in the town of Almonte, Ontario that uses milk permeate to create vodka.

Currently at the Dairy Distillery, the ethanol is profitable when sold as a high-quality spirit, but every distillation run creates approximately 30 000 liters of stillage which must be disposed. Dairy Distillery currently incurs costs to dispose of stillage in its municipal sewer system. With future plans to venture into biofuel, the increased production of stillage associated with this expansion would exceed the municipal sewer system capacities, forcing the distillery to install a waste treatment center. To minimize the significant cost this will entail, we proposed to transform the stillage into a fertilizer to enhance plant growth, while also reducing the carbon footprint of our partners and once again giving a new life to a waste product.

1.2 Transformation of milk permeate into ethanol

Worldwide, fermentation has been commonly used to transform a sugar source into ethanol. *Saccharomyces cerevisiae,* the most thoroughly researched yeast, is capable of withstanding high ethanol concentrations as well as producing a high sugar to ethanol yield (Guimarães et al., 2010). However, this species cannot metabolize lactose. Researchers have investigated different methods for using *S. cerevisiae* to ferment lactose such as a construction of a lactose-consuming *S. cerevisiae* strain through genetic manipulation (Guimarães et al., 2010).

Still, there are some yeasts capable of fermenting lactose directly, though they have been far less studied than *S. cerevisiae* (Guimarães et al., 2010). One of these yeasts is *Kluveromyces marxianus*. *K. marxianus* contains the genes *LAC12* and *LAC4*. The *LAC12* gene allows for transport of lactose into the cell by a lactose permease (Rubio-Texeira., 2006). After entering the cell, the lactose is hydrolyzed into its two monomers, glucose and galactose, by the *B-galactosidase* enzyme encoded by *LAC4* gene (Guimarães et al., 2010). From there, glucose undergoes glycolysis while galactose goes through the Leloir pathway, converting it to glucose. The variability in the efficiency of lactose uptake by different strains of the *K. marxianus* yeast has been associated with polymorphisms in its *LAC12* gene (Varela et al., 2019).

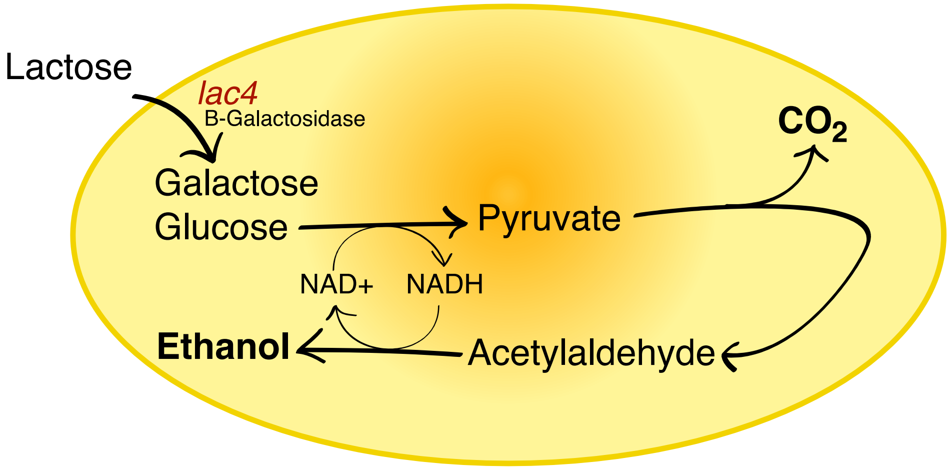

Figure 1.1 Lactose metabolic pathway in *K. marxianus*.

Its ability to ferment lactose makes using *K. marxianus* possible for a variety of biotechnological applications (Lane & Morissey, 2010). Industrial applications include ethanol production, biosynthesis of endogenous enzymes such as B-galactosidase and inulinase, and environmental application such as wastewater treatment (Fonseca et al., 2008). *K. marxianus* also has the ability to withstand high temperatures, a favourable trait that lowers the cost of cooling during industrial fermentations (Lertwattanasakul et al., 2015).

However, it is important to note that most literature investigating lactose fermentation has used a solution containing whey as their base (Guimarães et al., 2010; Silveira et al., 2005; Kargi & Ozmihci, 2006; Diniz, Silveira & Passos, 2012; Menchik et al., 2019. As mentioned before, milk permeate is the resulting liquid created after transforming milk into other dairy products. Another byproduct of this process is whey. Large scale production of whey and milk permeate are both the result of an increase in demand for high protein foods. Although they are both considered "waste" and share many similarities, lactose concentration can greatly differ between them. One study comparing acid whey from yogurt, acid whey from cheese, and milk permeate, found that milk permeate had almost three times the amount of lactose as the whey products. This resulted in the BOD and chemical oxygen demand (COD) of milk permeate being 3 and 2 times higher, respectively (Menchik et al., 2019). Still, a 2015 report by the U.S. Dairy Council found that the differences in lactose concentration between whey permeate and milk permeate were insignificant (Burrington, 2015). These varying results indicate that composition of whey and permeate may greatly vary between dairy plants.

Some whey and permeate solutions may have too low of an initial lactose concentration to be considered as viable substrates for successful fermentation from an economic point of view, since low lactose concentrations will translate to low ethanol yields. The process of ultrafiltration increases initial sugar concentrations (Guimarães et al., 2010). This is the process that is employed at the Parmalat dairy plant in Winchester, allowing the milk permeate to have a high initial lactose concentration. This process has drawbacks however, because all solutes become more concentrated, and in some cases, this translates to a very high salt concentration (Guimarães et al., 2010).

First, I was tasked to optimize the transformation of milk permeate into ethanol, which I performed successfully. The next step of this partnership, and the topic of this thesis, was investigating the possibility of using stillage, the waste from the ethanol fermentation and distillation, as a fertilizer.

Some of the information needed to discuss the process of turning milk permeate into ethanol is sensitive proprietary information to Dairy Distillery, and for that reason the focus of the thesis will be on the transformation of distillery stillage into fertilizer.

1.3 Transformation of distillery stillage into fertilizer

The three main elements found in conventional fertilizer are nitrogen (N), phosphorus (P), and potassium (K), usually given as a NPK ratio. In their review, Tadeu & Loureiro (2014) state that the use of stillage can reduce NPK requirements in common fertilizer applications up to 25, 80 and 50%, respectively, because of its high nutrient content. Distillery effluents have previously been transformed into fertilizer, but these distilleries were based upon molasses or sugarcane fermentation (Tadeu & Loureiro, 2014; Christofoletti et al., 2013; España -Gamboa et al., 2011; Oliveira et al., 2013). The stillage used in my project is dairy based, and this raw material may or may not perform differently than those already studied.

Research on distillery stillage as a fertilizer has examined how it affects seed germination. One study showed that seed germination in soil fertilized with different stillage concentrations varied between the five vegetable crops used: cucumber, chili, onion, bottle gourd and tomato, showing the possibility of stillage having a crop specific effect. (Ramana et al., 2002). In another study, the germination of mung beans, *Vigna radiata*, was negatively affected by stillage concentrations diluted 20 times, and the lower dilutions tested, 5x and 10x, had even more of a negative impact on germination (Kannan & Upreti, 2008). Percent seed germination of fenugreek, *Trigonella foenugraecu*, decreased steadily as the stillage concentration in the soil increased from 0, 5, 10, 25, 50, 75, and 100% (Kumar & Chopra, 2012). The authors believed this effect was due to the low pH and high salt concentration in undiluted stillage (Kumar & Chopra, 2012).

Distillery stillage contains high levels of salts, complex organic compounds, and is slightly acidic. As these characteristics have the potential to adversely impact seed germination, and seedling growth, we wished to assess the effect of exposing bell pepper seeds to our stillage fertilizer during germination.

These studies can yield some insight into which crops may be better suited for fertilization with stillage, and which dilutions to test initially, but the effect of stillage on seed germination might not be the same as the effect of stillage as a fertilizer for adult plants. After 3 applications in a 90-day growing period, one study saw an increase of 30% yield in mustard seeds, *Brassica compestris,* with diluted molasses stillage compared to regular fertilizer (Kumari et al., 2016). The stillage was diluted down before use for fertigation, the process of applying fertilizer to crops by an irrigation system, and although they do not mention how they diluted it, the COD and BOD were around 300 times lower than the raw stillage (Kumari et al., 2016). In their study, Kumar and Chopra

(2012) examined the growth of fenugreek from germination to maturity. They found that seed germination decreases with increasing stillage concentrations. Growth indexes such as shoot and root length, dry weight, and crop yield of the plant increased with the stillage concentration up to 50%; afterwards, increasing stillage concentration had a negative impact on plant growth (Kumar & Chopra, 2012). The metal content also increased in both the soil and the plants with the higher stillage concentrations, but no metals concentrated at levels above legal guidelines, which is important for plants grown for human consumption (Kumar & Chopra, 2012).

After researching the literature on distillery stillage used as a fertilizer, stillage from Dairy Distillery is used to examine the possibility of turning the byproduct into a fertilizer, comparable to those already on the market. Specifically, I conducted the experiment in a greenhouse setting with bell peppers, *Capsicum annuum*. This crop was chosen as it is a common greenhouse crop in Canada, with 169,857 metric tonnes produced in 2023 (Pest Management Centre Division of Agriculture and Agri-Food Canada, 2024). Greenhouses are growing in popularity for many different crops, including pepper cultivation, because they provide a controlled environment that can produce crops year-round. To date, a majority of research has examined the application of stillage fertilizers to agricultural soils, with relatively few studies reporting their use on horticultural crops cultivated in controlled environments, such as greenhouses.

Based on chemical analyses of Dairy Distillery stillage (**Table 2.1**), we predicted that the addition of two ingredients, water and a source of nitrogen, would create a stillage-based fertilizer of equivalent potential to that of conventional fertilizers. To test our hypothesis, we assessed the impact of amended dairy-based distillery stillage on the germination, growth, and productivity of bell pepper cultivated under greenhouse conditions.

Chapter 2

Publication manuscript

Dairy-based distillery stillage supports greenhouse bell pepper (*Capsicum anuum*) production

2.1. Introduction

Ethanol fermentation generates substantial quantities of stillage, an acidic and saline wastewater by-product that is rich in complex organic compounds. Traditionally, ethanol production relies on the fermentation of plant-based sugars derived from various sources, including fruits (e.g., grapes), grains (e.g., barley, sorghum, and rye malts), and molasses (e.g., sugarcane). Recent demand for ethanol as a biofuel has seen the emergence of a valuable corn-based ethanol fermentation industry that presently utilizes almost half of all corn produced in the United States (U.S. Department of Agriculture, 2023), as well as novel fermentation processes that are based upon non-plant substrates such as milk permeate.

Milk permeate is a lactose-rich byproduct obtained from the ultrafiltration of milk and is a promising substrate for ethanol fermentation. When fermented, milk permeate yields a distillery stillage, which contains a variety of valuable nutrients and salts, including nitrogen, phosphorus, and potassium, which are essential for plant growth.

Distillery stillage management is a crucial aspect of the distillery industry, prompting ongoing endeavors to investigate environmentally sustainable methods of stillage utilization, including transformation into biogas, fertilizers, and animal feed, to minimize its environmental impact and, where possible, extract value from this waste byproduct. Distillery stillage often contains significant concentrations of nitrogen, phosphorus, and potassium, important plant macronutrients, along with various essential micronutrients. In certain countries, the management of stillage disposal involves direct application to soils for enhancing soil nutrient levels through fertigation, an irrigation system containing both water and fertilizer (Alotaibi et al., 2014; Mikucka and Zielińska, 2020; Rusecki et al., 2019). However, the release of untreated stillage into the environment poses an environmental threat due to its potential to generate oxygen-depleted dead zones in downstream aquatic ecosystems, increase soil salinity, and introduce metals such as copper, lead, and cadmium that may surpass recommended guidelines for toxicity, among other concerns (Fuess and Garcia, 2014; Mohana et al., 2009; Pant and Adholeya, 2007). Addressing these risks through treatment of distillery stillage prior to release can be challenging, expensive, and ineffective, depending on the specific physical and chemical properties of the stillage in question (Mikucka and Zielińska, 2020). To date, a majority of research has examined the application of stillage fertilizers to agricultural soils, with relatively few studies reporting their use on horticultural crops cultivated in controlled environments, such as greenhouses.

The distillery stillage was sourced from Dairy Distillery, a Canadian company that specializes in producing ethanol from milk permeate. Dairy Distillery generates approximately 16 liters of distillery stillage for every liter of ethanol produced, yet the potential of this byproduct as a fertilizer has not been fully explored in scientific research.

Bell peppers are a widely produced, high-value, nutrient dense vegetable crop and represent one of the most valuable Canadian commodities farmed in greenhouses year-round (Crops and Horticulture Division of Agriculture and Agri-Food Canada, 2020). In Canada, bell peppers represent almost one quarter (21.1% share; by volume) of all greenhouse-grown vegetables (Statistics Canada, 2023). Notably, bell peppers are categorized as salt-sensitive (Ayers and Westcot, 1985; Cha et al., 2021; Erel et al., 2020; Lycoskoufis et al., 2005; Maas and Hoffman, 1977), making them an ideal crop for evaluating potential impacts of salt-stress induced by stillage fertilizer (SF).

Given the environmental concerns associated with traditional fertilizer use and the need for sustainable alternatives, it is crucial to explore innovative methods for repurposing distillery stillage. In this study, we demonstrate that dairy-based distillery stillage, when appropriately diluted and amended, has significant potential as a sustainable fertilizer. This alternative not only reduces the environmental impact of stillage disposal but also provides a cost-effective solution for enhancing plant growth with minimal risks to human health and environmental safety.

2.2.0 Materials and methods

2.2.1.1 Stillage composition

Stillage was collected every two weeks from a distillation plant in Almonte, Ontario, Canada. Six independent samples of raw stillage were first submitted for chemical analysis. Similar to the chemical profile of distillery stillage from other sources, stillage produced from milk permeate fermentation is characterized by an acidic pH (6.20-6.45), high levels of salinity (16.2 dS/m), and relatively high P and K content (Mikucka and Zielińska, 2020; Ramana et al., 2002), although the relative N content of our dairy-based stillage is lower than most plant-derived stillages. The results from the chemical analysis of stillage show that our stillage has an NPK ratio of 1-10-20. Based on the physiochemical analysis, we predicted that dilution of the stillage with water to a 10% (v/v) would reduce salinity while providing an appropriate amount of P and K optimal for plant growth. The diluted stillage solution was amended with 40 mM (128 mg/L) of ammonium nitrate to improve the capacity to support plant growth. The NPK of the amended stillage fertilizer (SF) was 10-1-3.

2.2.1.2 Seed germination

Distillery stillage contains high levels of salts, complex organic compounds, and is slightly acidic. As these characteristics have the potential to adversely impact seed germination, and seedling growth, we wished to assess the effect of exposing bell pepper seeds to our stillage fertilizer during germination. Assessment of germination was performed following directions outlined in the Canadian Food Inspection Agency (CFIA) Canadian Methods and Procedures for Testing Seed (2012). Two pieces of Whatman 11 cm filter paper were placed inside sterile polystyrene petri plates (140mm diameter; Thermo Fisher Scientific, MA, USA), and were soaked in 10 mL of each treatment (autoclaved water or SF). Twenty-five bell pepper (*Capsicum anuum* cv. California Wonder, McKenzie Seeds) seeds were positioned on the filter paper in replicates of four. Seeds were placed inside a Conviron MTR30 growth chamber (25°C day, 20°C night, 50% humidity) in the absence of light. The number of germinated seedlings was tallied every day until germination ceased for three days in a row. To keep seeds moist, an additional 5 mL of each treatment was applied after four days. Germination was defined as the emergence of the radicle 2 mm beyond the seed coat.

2.2.2 Plant growth

2.2.2.1 Seeding and transplanting

Bell pepper (*Capsicum anuum* cv. California Wonder) seeds acquired from McKenzie Seeds (Brandon, MB, Canada) were sown 2 mm deep in moistened ProMix BX soil (Saint-Hubert, QC, Canada) and covered with a moistened plastic dome for increased humidity. After three weeks, 24 seedlings of uniform size were transferred to 2.25 Gallon pots filled with moistened soil.

2.2.2.2 SF preparation and pepper fertilization

Each week, SF was prepared by diluting distillery stillage 10-fold and supplementing with ammonium nitrate to a final concentration of 400 mg/L. Miracle-Gro (TM) (Ohio, USA) Water Soluble All-Purpose Plant Food (NPK 24-8-16, pH 6) was diluted according to package directions (1 tbsp/gallon) and used as the conventional fertilizer (CF) control. Miracle-Gro is widely accessible, cost-effective, easy-to-use and initial testing demonstrated that it is suitable to support bell pepper growth under greenhouse conditions.

Beginning at one-week post-transplant (WPT), plants were fertilized twice a week with 100 mL of each treatment (water, CF or SF). Every two weeks, the treatment volume was increased 50 mL to a maximum of 300 mL to align with increasing plant nutrient requirements. Each treatment was replicated six times and positioned in a greenhouse (20-hour light/4-hour dark, 20°C constant) using a random design.

2.2.3 Biometric measurements

At 28-, 42-, and 56-days post-transplant (DPT), leaf chlorophyll content, number of leaves, number of pepper fruit, stem height, and stem diameter were recorded for each plant. The leaf chlorophyll content was measured using Apogee Instruments Chlorophyll Concentration Meter (Model MC-100). The chlorophyll concentration was determined by averaging six areas of the adaxial lamina for each replicate. Two areas at the leaf base and one area at the leaf apex were recorded for the second leaf from the top and the sixth leaf from the top. Leaves greater than 5 cm in length were included in the leaf count. The stem height was measured from the soil to the tallest node. The stem diameter was measured at 10 cm above the soil.

2.2.4 Harvest

Pepper plants were destructively harvested 56 DPT and the leaf chlorophyll content, number of leaves, number of pepper fruit, stem height, stem diameter was measured. The fresh (FSW) and dry shoot weight (DSW) were recorded for the leaves and stems separately. The dry root weight (DRW) was recorded for each plant. Tissues were dried at 60°C until constant weight before recording mass. Upon harvest, each fruit was weighed, and the average was calculated for each treatment.

2.2.5 Metal analyses

Five bell peppers were randomly selected from each treatment group, crushed using liquid nitrogen and freeze-dried for four days at -50ºC using a Labconco FreeZone 2.5 freeze drier (Labconco, MO, U.S.A). Freeze-dried samples were weighed in duplicate (500 mg) and placed into 50 mL digestion tubes (DigiPrep, QC, Canada). A diluted nitric acid solution (1:1, 6 mL) was added to each sample for acid digestion in a DigiPREP MS heating block (SCP Science, Quebec, Canada) for 240 minutes at 100ºC. Evaporation was prevented using a 50 mL watch glass (SCP Science, Quebec, Canada). Hydrogen peroxide (2 mL) was added to each sample before further digestion for 40 minutes at 95°C using a DigiPREP MS heating block (SCP Science, QC, Canada). Deionized water was added to a final volume of 20 mL. Vacuum filtration was carried out using 0.45 µm DigiFILTERs for 50 mL conical tubes (SCP Science, Quebec, Canada) before analysis. Trace metals in the stillage and digested plant materials were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) (7700x ICP-MS, Agilent Technologies, Japan). The system was equipped with low flow borosilicate glass MicroMist concentric nebulizer and quartz, Scott-type double pass spray chamber. Interface consisted of 1mm diameter Ni-typped sampling and 0.4mm diameter Ni-typped skimmer cone. Octopole Reaction System (ORS) was used as interference removal in Helium mode. An Agilent ASX-500 was used as an ICP-MS auto sampler. The instrument Calibration standard (Spex CertiPrep Cat#:CL-CAL2) stock solution was diluted in 1% nitric acid and used to provide a working calibration curve of at least five points. For analytical quality controls and method validation an element quality control standard stock (High-Purity Standards, Cat# QCS-19) and different certified reference materials, NIST1515 (Apple Leaves) and NIST1568b (Rice Flour) from National Institute of standard and Technology of U.S Department of Commerce were used. *M/z* ratio monitored were 23Na, 24Mg, 39K, 43Ca, 56Fe, 55Mn, 59Co, 60Ni, 63Cu, 66Zn, 75As, 95Mo, 111Cd and 208Pb. The results for both standard and reference materials were within 10-20% of expected values.

2.3.0 Results

2.3.1 Dairy-based stillage treatment does not impact bell pepper seed germination

Percent seed germination was recorded on a daily basis for 15 days to assess the impact of SF on bell pepper seeds, using seeds germinated in the presence of water only as a comparison. As seed germination was only used as an indicator of possible toxicity from stillage to seed growth, no conventional fertilizer treatment was used. The germination rate did not differ between treatment groups and the percent germination of the seedlings was equivalent after 15 days **(Figure 2.1).**

2.3.2 Dairy-based stillage promotes bell pepper growth and fruit yield

At 28 and 42 DPT, plant height did not differ significantly between treatment groups. At 56 DPT, plant height was significantly greater in plants treated with CF or SF compared to water (**Figure 2.2A**). Stem diameter did not differ significantly between treatment groups at any time point measured (**Figure 2.2B).** The number of leaves produced (of a size greater than 5 cm) did not differ significantly between treatments at 28 DPT. At 42 and 56 DPT, the CF and SF treatment groups had a statistically significantly greater number of leaves than the water control (**Figure 2.2C**). At 28 DPT, chlorophyll content was comparable between water- and CF-treated plants, as well as between SF- and CF-treatment groups, but was significantly higher in SF-treated plants compared to water. By 42 and 56 DPT, chlorophyll content in CF- and SF-treatments remained statistically similar to one another, and both were significantly greater than that observed in water-treated plants. (**Figure 2.2D**).

Biomass production is an important indicator of plant health (Younginger et al., 2017). Accordingly, Dry Shoot Weight (DSW), Dry Root Weight (DRW) and fruit mass per plant were evaluated for each treatment group at harvest. The dry mass of leaves (LDM) and stems (SDM) were measured separately, with their combined values equating to the DSW. Both LDM and SDM were significantly higher ($p<0.05$) in CF and SF treatment groups compared to water, reflecting increased growth of aerial tissues (**Figure 2.3A**). The dry root biomass did not significantly differ between any treatment (**Figure 2.3B**. Importantly, bell pepper fruit yield is more than two-fold higher in plants of both fertilized treatment groups (**Figure 2.4A**). The mean fruit number per plant is also significantly higher for both CF and SF treatments (**Figure 2.4B**).

2.3.3 Metal analysis of bell pepper fruit from plants treated with dairy-based stillage

To evaluate the nutritional composition and assess the presence of metals with potential health implications, the concentrations of 14 key metals were analyzed in bell pepper fruits harvested from plants subjected to each treatment group (**Table 2.2 & Table 2.3)**. SF treated bell pepper did have a significantly lower concentration of calcium and cadmium when compared to water. There is no statistically significant differences observed between SF and CF treatments in the concentrations of any metals analyzed.

2.4.0 Discussion

Milk permeate is a by-product generated during the ultrafiltration of milk, predominantly composed of water, lactose, minerals, and trace amounts of vitamins. As a carbohydrate-rich liquid, it offers a suitable substrate for fermentation, producing ethanol and a distillery stillage that is light-colored, acidic, and rich in salts such as potassium and phosphorus. Approximately 16 liters of distillery stillage are produced for every liter of ethanol, making the repurposing of this nutrient-rich byproduct highly desirable. Given its properties, we hypothesized that dairy-based stillage could be reformulated into a fertilizer with the potential to enhance plant growth, an approach that has proven successful for the upcycling of other stillages (Alotaibi et al., 2014; Qian et al., 2011; Rusecki et al., 2019). To test this hypothesis, we evaluated the impact of a dairy-based distillery stillage fertilizer on the germination, vegetative growth, and fruit yield of bell peppers (*Capsicum anuum* cv. California Wonder) under controlled greenhouse conditions. Distillery stillage from Dairy Distillery in Almonte, Ontario was used as the starting material for the plant fertilizer described herein.

Distillery stillage can be reformulated into fertilizer through methods such as dilution with water to lower salinity and acidity, adding nitrogen sources like ammonium or urea to balance nutrients, and (if required) treatments to remove or neutralize potential contaminants. Chemical analysis of the dairy-based distillery stillage indicated a relatively low nitrogen content, which we attribute to the low nitrogen concentration of milk permeate, the raw material used for ethanol production from dairy sources. The ultrafiltration process of milk removes much of the protein and nitrogen content, resulting in a waste byproduct that is rich in carbohydrates like lactose and minerals, but lacking in nitrogen. As a result, our dairy-based distillery stillage required supplementation with an additional nitrogen source to be effective in promoting plant growth. Ammonium nitrate (NH_4NO_3) is a widely used nitrogen source in fertilizers due to its high solubility and efficiency in delivering both ammonium (NH_4^+) and nitrate (NO_3^-) forms of nitrogen. It is often used in combination with other nutrients to enhance plant growth, and we amended the dairy-based stillage via the addition of supplemental ammonium nitrate. Alternative nitrogen sources that could be used to supplement dairy-based distillery stillage include urea, ammonium sulfate, and organic nitrogen sources such as composted manure or legume-based fertilizers (e.g., alfalfa meal). Our analyses did not identify significant concentrations of heavy metals or potentially toxic substances in the stillage (Table 1) or in the pepper fruit collected at harvest (Table 2.1 & Table 2.2). While there are no specific Health Canada guidelines for the maximum acceptable level (ML) of all metals in whole fruits, the concentration of arsenic in the SF-treated bell pepper is below the ML of 1 mg/kg for edible bone meal, and the concentration of lead is below the ML of 1.5 mg/kg for whole tomatoes (Health Canada, 2024).

To evaluate the effectiveness of our stillage fertilizer (SF) on promoting plant growth, we assessed multiple biometric parameters to compare the growth and development of bell pepper treated with water, CF or SF. Not surprisingly, we found that water alone does not adequately support bell pepper growth particularly in the fruiting stage of plant development, as key macro- and micro-nutrients are lacking, and plants within this treatment group were significantly shorter (Figure 2A), produced fewer leaves (Figure 2C), and had much reduced levels of chlorophyll (Figure 2D). In contrast, treatment with either CF or SF supplies the mineral elements necessary to support plant growth and development, including key micronutrients such as calcium, magnesium, and trace elements, which are present in dairy-based stillage (**Table 2.1**). Bell pepper treated with SF produced an increased dry shoot weight (DSW) compared to water-only plants, and an increased balance between dry root weight (DRW) and DSW, a parameter used as a proxy for plant productivity (Agren and Ingestad, 1987)

Biomass measurements are often used to estimate plant health and fitness (Younginger et al., 2017). Importantly, bell pepper plants treated with CF or SF exhibited more than a two-fold increase in fruit production, indicating a significant enhancement in crop yield and productivity. Taken together, our results indicate that fertilization of bell pepper with SF performs equally as well as conventional fertilizer.

Seed germination toxicity tests are used to assess the toxicity of a substance by placing seeds in an aqueous solution of interest and recording germination data, a method commonly used to assess compost quality (Luo et al., 2018). In addition to sending undiluted SF for chemical analysis (**Table 2.1**), we tested for potential adverse effects of SF treatment on seed germination rate and percentage (**Figure 2.1**). As seedlings are particularly sensitive to toxic substances, any such compounds within SF might be expected to inhibit the germination of seeds. Our results show that SF did not adversely affect bell pepper seed germination, providing independent support to our chemical analyses that indicate the stillage to be free of potentially toxic substances that might pose a threat to plant health or human safety.

While there is a strong preference to upcycle waste byproducts whenever possible, there are potential environmental concerns associated with the application of raw stillage to soils (Fuess and Garcia, 2014). Indeed, ongoing application of raw stillage has the potential to increase soil and groundwater salinity, disrupt the balanced uptake of mineral nutrients, and may acidify the soil, any of which may negatively impact crop growth over time (Rusecki et al., 2019). However, a 10-fold dilution in tap water decreased the electrical conductivity of our SF to an acceptable level (FAO, 1992). This is further evidenced by a lack of symptoms associated with salinity stress in our SF treated bell pepper, which are considered a salt-sensitive species (Ayers and Westcot, 1985; Cha et al., 2021; Erel et al., 2020; Maas and Hoffman, 1977)(**Figures 2.2-2.4).**

While amended distillery stillage has a positive impact on plant growth parameters, less is known about the impacts it might have on soil microbial community, highlighting a crucial gap in knowledge. The amendment of treated soils with SF has the potential to alter microbial communities and alter plant-microbe interactions, thereby indirectly impacting plant health. Dairy-based distillery stillage is high in organic carbon levels, which may be expected to stimulate microbial growth, which in turn may also indirectly benefit plant growth. Additionally, application of carbon-rich fertilizers may also contribute towards carbon soil sequestration, conferring additional environmental benefits to the upcycling of our stillage.

More research is needed to understand these dynamics fully and assess the long-term sustainability of using stillage in agricultural systems. Experimental characterization of microbial communities before and after the addition of stillage should be assessed in field conditions.

2.5 Conclusion

The mining and production of conventional plant fertilizers impart a significant negative environmental impact to many different ecosystems. By utilizing nutrient-rich waste products, a global food production stream can be reduced. In this study, we amended distillery stillage to produce a fertilizer that successfully supports bell pepper plant growth and fruit production equal to that of conventional fertilizers. A waste stream is diverted, and the need for potassium and phosphorus mining is reduced. In this study, we altered distillery stillage to address salinity concerns, while also amending with ammonium nitrate as a nitrogen source. Consequently, raw stillage was converted into a sustainable alternative to conventional fertilizers for local plant growers, thereby diverting a waste stream, reducing operational costs, and supporting crop growth in an environmentally conscious manner.

2.6.0 Tables and Figures

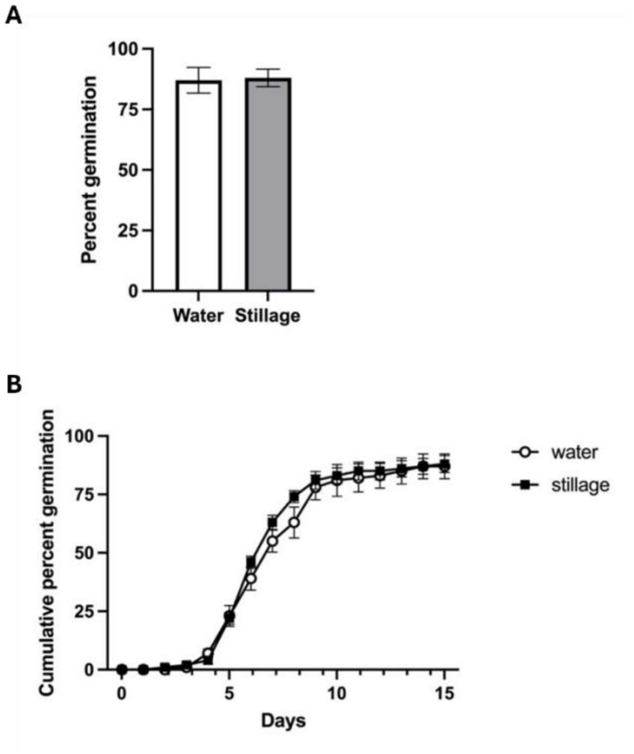

Figure 2.1 The effect of stillage fertilizer (SF) on bell pepper (Capsicum annuum) seed germination. Seeds were germinated in either SF or water-only control conditions, with daily monitoring of germination rates over 15 days.

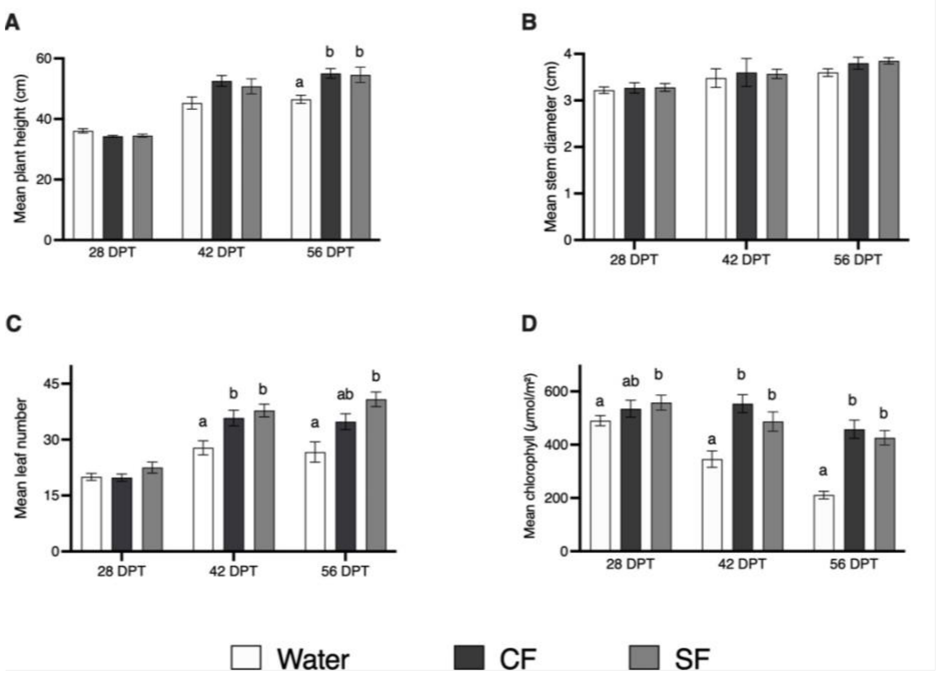

Figure 2.2 Biometric measurements of greenhouse grown bell pepper treated with water, conventional fertilizer (CF), or stillage fertilizer (SF) at 28, 42 and 56 DPT. A) plant height, B) stem diameter, C) leaf number, D) mean chlorophyll content. Data shown is mean ± SE. Statistical significance analyzed using one-way ANOVA and Tukey's HSD, $p<0.05$. n=6. Different letters indicate significant differences ($p<0.05$) within each time point.

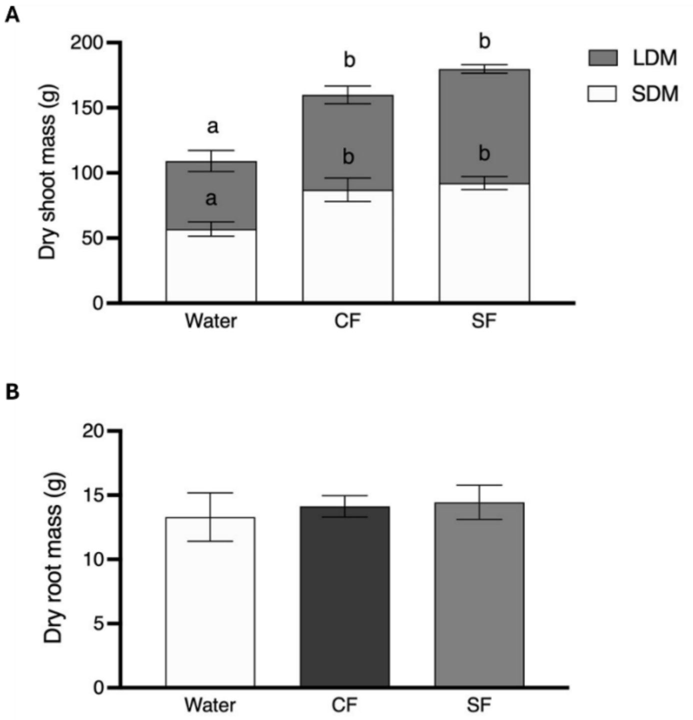

Figure 2.3 Biomass of greenhouse grown bell pepper treated with water, conventional fertilizer (CF), or stillage fertilizer (SF) at 56 DPT. A) dry shoot biomass, the sum of leaf dry mass (LDM) and stem dry mass (SDM) B) dry root biomass. Data shown is mean ± SE. Statistical significance analyzed using one-way ANOVA and Tukey’s HSD, $p<0.05$. $n=6$. Different letters indicate significant differences ($p<0.05$).

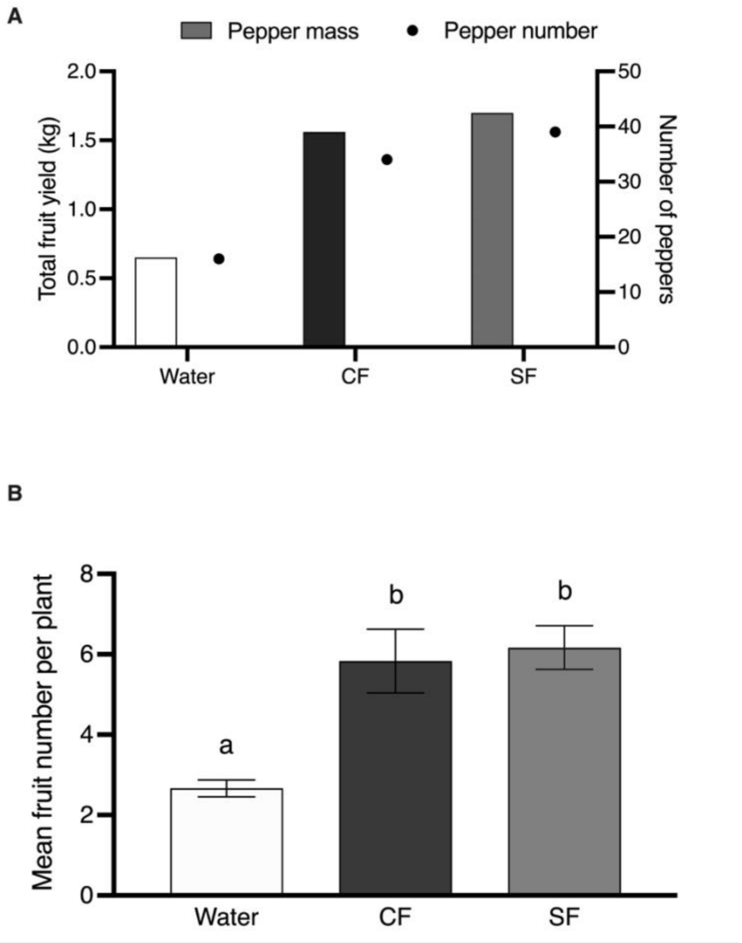

Figure 2.4 Fruit yield and number of greenhouse bell peppers treated with water, conventional fertilizer (CF), or stillage fertilizer (SF) at harvest. A) total fruit yield and total fruit number B) fruit number per plant. Data shown in panel B is mean ± SE. Statistical significance analyzed using one-way ANOVA and Tukey's HSD, $p<0.05$. $n=6$.

	Average ± SEM (mg/L)	
Inorganics	**Raw stillage (n = 7)**	**CF (n=2)**
Ammonia-n	98 ± 11	58
Organic Carbon	8792 ± 3325	186 ± 16
Orthophosphate (P)	906 ± 47	60 ± 2
Phosphorus	1424 ± 236	57.25 ± 0.8
Sulphate	338 ± 10	206.5 ± 28.5
Chloride	2858 ± 48	154.5 ± 39
Nitrite (N)	0	0
Nitrate (N)	3 ± 2	3.775 ± 3.8
Metals		
Boron	1	0.313
Calcium	537 ± 68	6 ± 6
Copper	6 ± 4	1
Magnesium	203 ± 4	1 ± 1
Phosphorus	1051 ± 51	53 ± 1
Potassium	4450 ± 96	198 ± 16.5
Sodium	1108 ± 28	13 ± 12.3
Titanium	0	0.0062
Zinc	0	1 ± 0.1

Table 2.1 Composition analysis of raw distillery stillage obtained from Dairy Distillery (SF) and Miracle-Gro (CF). Concentrations are shown as mean ± SE. n=7 and n=2, respectively.

	Major metals mean concentration (mg/kg)		
Element	**Water (n=6)**	**CF (n=6)**	**SF (n=5)**
Na	178 ± 12^{a}	117 ± 11^{b}	163 ± 17^{ab}
Mg	$1.58 \times 10^{3} \pm 48^{a}$	$1.32 \times 10^{3} \pm 30^{b}$	$1.41 \times 10^{3} \pm 1.1 \times 10^{2ab}$
K	$2.73 \times 10^{4} \pm 4.2 \times 10^{2a}$	$2.34 \times 10^{4} \pm 0.52^{b}$	$2.61 \times 10^{4} \pm 1.59 \times 10^{2\ ab}$
Ca	734 ± 74^{a}	496 ± 51^{b}	467 ± 43^{b}
Fe	273 ± 31	291 ± 52	$402 \pm 1.1 \times 10^{2}$

Table 2.2 Concentration of major (mg/kg) metals found in fruit tissue analyzed using ICPMS ± SD. The tissue was taken from the fruit of the bell pepper plant during harvest. Concentrations are shown as mean ± SE. Statistical significance analyzed using one-way ANOVA and Tukey's HSD, $p<0.05$. n=5.

	Minor metals mean concentration (ug/kg)		
Element	**Water (n=6)**	**CF (n=6)**	**SF (n=5)**
As	44.0 ± 7.60	31.2 ± 1.90	43.8 ± 4.00
Cd	53.5 ± 3.0[a]	38.0 ± 3.80[b]	38.0 ± 2.50[b]
Co	85.8 ± 14	89.1 ± 13.0	121 ± 16.0
Cu	$4.25 \times 10^3 \pm 1.10 \times 10^3$	$7.40 \times 10^3 \pm 2.40 \times 10^3$	$1.78 \times 10^3 \pm 460$
Mn	$1.17 \times 10^4 \pm 57.0$	$1.13 \times 10^4 \pm 2.80 \times 10^3$	$1.20 \times 10^3 \pm 920$
Mo	221 ± 34.0	174 ± 39.0	123 ± 20.0
Ni	$4.25 \times 10^3 \pm 1.1 \times 10^3$	$7.41 \times 10^3 \pm 2.40 \times 10^3$	$1.78 \times 10^3 \pm 4.60 \times 10^3$
Pb	507 ± 380	118 ± 12.0	108 ± 14.0
Zn	$4.26 \times 10^3 \pm 1.1 \times 10^3$	$7.40 \times 10^3 \pm 2.40 \times 10^3$	$1.78 \times 10^3 \pm 460$

Table 2.3 Concentration of minor (µg/kg) metals found in fruit tissue analyzed using ICPMS. The tissue was taken from the fruit of the bell pepper plant during harvest. Concentrations are shown as mean ± SE. Statistical significance analyzed using one-way ANOVA and Tukey's HSD, $p<0.05$. n=6, n=6 and n=5, respectively.

Chapter 3

Conclusion

3.0 Conclusion

This project is a successful collaboration between academia and industry. This partnership has identified two different processes to reuse byproducts from the dairy industry, firstly done with the transformation of milk permeate into vodka, followed by the transformation of distillery stillage into fertilizer, as discussed here. This approach not only lowers the cost of disposal to the dairy industry, but it also has the potential to become an economically feasible product itself. This has been shown by the success of transforming milk permeate into ethanol which has become a profitable market product being sold across Canada.

The transformation of distillery stillage into fertilizer shows promise, but to become a commercially feasible product, there are further steps needed. The next steps would be to expand the scope of this research, including using variety of different crops and exploring the potential impact of stillage fertilizer on the composition and function of soil microbial communities.

In conclusion, my research was an important first step to demonstrate that fertilizer from stillage can be as effective as conventional fertilizer in one crop, bell pepper, within a greenhouse setting. The possible use of stillage fertilizer would help reduce waste by utilizing an already made solution, reduce cost by having it already contain potassium and phosphorus, and provide an alternative to conventional fertilizers that is capable of supporting crop growth in an environmentally conscious manner.

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