# REMOVAL OF CYANOGENIC GLYCOSIDES IN WHOLE FLAXSEED VIA LACTOBCILLACEAE FERMENTATION

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By

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# ABSTRACT

Flaxseed is considered as a functional food because it enriches with omega-3, dietary fiber, lignan and protein. Whole flaxseed can be added to bakery products including breads, buns, and cookies to increase the health benefits of these foods. However, flaxseed contains cyanogenic glycosides (CGs), including linamarin, linustatin, lotaustralin and neolinustatin. Cyanogenic glycosides are metabolized to cyanohydrins by the action of the enzymes beta-glucosidase and hydroxynitrile lyase which first cleave glucose from CG then release hydrogen cyanide (HCN) from the cyanohydrin, respectively. Hydrogen cyanide can affect mammalian respiratory systems and can also influence nutrient absorption from food. Thus, CGs should be removed from flaxseed. In this study, whole flaxseed (35 g, 340 g and 1 kg) was fermented with a consortium culture of *Lactobacillaceae* at 30 °C over 72 h. Concentrations of CGs (total HCN, linustatin and neolinustatin), percent oil, percent secoisolariciresinol diglucoside (SDG) and fatty acid profiles in whole flaxseed were measured using $^{1}$H-NMR. The results showed that CGs decreased to below detection limits over 72 h for both bench-scale and scale-up bacterial fermentation experiments. The consortium culture was largely from members of the family *Lactobacillaceae*. Furthermore, fatty acid composition remained unchanged during fermentation, while concentration of flaxseed oil and SDG significantly improved from 41.20% to 54.40% and 2.26% to 4.13%, respectively, after fermentation. Altogether, whole flaxseed treated *via* *Lactobacillaceae* fermentation was depleted in CGs, while nutritional compounds were maintained.

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| | |
|---|---|
| **1,3-PD** | 1,3-Propanediol |
| **ALA** | α-Linolenic Fatty Acids |
| **CAGR** | Compound Annual Growth Rate |
| **CGs** | Cyanogenic Glycosides |
| **DCM** | Methylene Chloride |
| **DMF** | N,*N*-dimethyl Formamide |
| **DMSO** | Dimethyl Sulfoxide |
| **GC** | Gas Chromatography |
| **GRAS** | Generally Regarded as Safe |
| **HCN** | Hydrogen Cyanide |
| **$^{1}$H NMR** | Proton Nuclear Magnetic Resonance Spectroscopy |
| **HPLC** | High-Performance Liquid Chromatography |
| **LAB** | Lactic Acid Bacteria |
| **SDG** | Secoisolariciresinol Diglucoside |
| **TLC** | Thin-Layer Chromatography |
| **TSF** | Two-Stage Fermentation |
| **W-TS** | Wheat-based Thin Stillage |

;

# 1. INTRODUCTION

Flaxseed is considered a functional food due to its high content of free-fatty acids, oil, lignans, and protein (Singh et al. 2011). It is used in food products such as flaxseed bread, oil, and meal to enhance human health benefits. The flaxseed market is expected to have a compound annual growth rate (CAGR) of 11.8% from 2022 to 2027, according to Mordor Intelligence (2023).

The trend of consuming whole flaxseed for optimal nutrition is limited by its naturally occurring antinutrients, such as cyanogenic glycosides (CGs), phytic acid, and linatine (Bekhit et al. 2018; Dzuvor et al. 2018). CGs are converted to hydrogen cyanide (HCN) by the enzyme hydroxynitrile lyase in the presence of beta-glucosidase (Yamashita et al. 2007). HCN can harm respiratory systems and impair nutrient absorption from food (Singh et al. 2011).

Antinutrients in flaxseed must be removed or deactivated before consumption by humans or animals (Dzuvor et al. 2018). The depletion of cyanogenic glycosides (CGs) is often necessary for market entry in countries like Japan and South Korea (Thompson et al. 2015). Finding effective methods to remove CGs in whole flaxseed is a priority (Dzuvor et al. 2018).

Previous methods for removing CGs from flaxseed include heating treatments (Feng et al. 2003; Yang et al. 2004), chemical extractions (Bacela and Barthet 2010; Wanasundara et al. 1993; Waszkowiak et al. 2015), and biological treatments (Lei et al. 1999; Yamashita et al. 2007; Wu et al. 2012). Heating and chemical extractions can only partially remove CGs from flaxseed meal while also reducing its nutritional content (protein, lignans, and fatty acids) (Yamashita et al. 2007). In contrast, biological treatments not only deplete CGs but also preserve the nutritional components of flaxseed meal (Yamashita et al. 2007). Moreover, biological treatments have a minimal impact on the environment because of their low wastes production (Wu et al. 2012).

## 1.1 Project Motivation and Knowledge Gap

Lei et al. (1999) successfully removed cyanogenic glycosides (CGs) from flaxseed meal using fermentation with an inoculation of *Lactobacillus plantarum* LP1 (a species of the

*Lactobacillaceae* family). *Lactobacillaceae* have been demonstrated to produce enzymes that effectively hydrolyze cyanide in cassava flour (Nwokoro 2016). With the growing use of whole flaxseed in food products (cookies, breads, energy bars), a method to remove CGs from whole flaxseed must be researched. The *Lactobacillaceae* fermentation process has not been applied to whole flaxseed yet, but investigating it may lead to a new and promising technology to deplete CGs without milling or grinding.

## 1.2 Hypothesis

Researchers (Lei et al. 1999; Wu et al. 2012; Yamashita et al. 2007) have shown that *Lactobacillaceae* fermentation can remove cyanogenic glycosides (CGs) from flaxseed meal. It is proposed that using *Lactobacillaceae* fermentation could offer a nontoxic and innovative approach to depleting CGs from whole flaxseed.

## 1.3 Research Objectives

The overall objective of my study is to deplete CGs from whole flaxseed using *Lactobacillaceae* fermentation. The specific objectives are:

1) to develop a method to remove CGs from whole flaxseed using fermentation (bioprocessing) with an in-house inoculant of *Lactobacillaceae.*

2) to monitor the concentration of major CGs (linustatin and neolinustatin, as well as total HCN) during fermentation;

3) to identify the microorganisms in the fermentation culture; and

4) to compare the nutritional components of the treated flaxseed with untreated flaxseed

## 1.4 Organization of Thesis

This thesis is structured as a manuscript-style work, comprising five chapters. Chapter 1 outlines the motivation for the project, identifies the knowledge gap, presents the hypothesis, outlines the research objectives, and provides an overview of the thesis organization. Chapter 2 is a literature review, providing background information on flaxseed, cyanogenic glycosides, methods of removing CGs from flaxseed, and *Lactobacillaceae* fermentation. Chapter 3 features the

manuscript titled "Depletion of Cyanogenic Glycosides in Whole Flaxseed via *Lactobacillaceae* Fermentation." Chapter 4 provides a general discussion, including insights not covered in the manuscript. Conclusions and recommendations are presented in Chapter 5. The references are listed at the end of the thesis, followed by the appendix, which includes supplementary data and graphs.

# 2. LITERATURE REVIEW

## 2.1 Flaxseed and Nutritional Value

This section provides a comprehensive exploration of flaxseed, encompassing its physical and chemical properties, nutritional composition, associated health benefits, and an examination of the market for flaxseed consumption.

### 2.1.1 Physical Structure and Properties of Flaxseed

Flaxseed is the seed from the flax plant (*Linum usitatissimum L.*). It is native to West Asia and the Mediterranean and is currently grown in countries such as Kazakhstan, Russia, Canada, China, Argentina, America, and India (Saleem et al. 2020). Flaxseeds have a flat, oval shape with a pointed tip and can be brown (Figure 2.1), olive, or yellow in color. The moisture content of flaxseeds ranges from 6.09% to 16.81% (d.b.). With an increase of moisture content, the weight of 1000 seeds increase from 4.79 to 5.32 g, the true density increases from 1000 to 1111 kg/$m^3$ and porosity increases from 27.34 to 57.44%, but bulk density decreases from 726.6 to 555.6 kg/$m^3$. The length, width, thickness (Figure 2.2), arithmetic mean diameter, and geometric mean diameter vary from 4.27 to 4.64 mm, 2.22 to 2.38 mm, 0.85 to 0.88 mm, 2.45 to 2.63 mm, and 2.00 to 2.12 mm, respectively, with an increase in moisture content (Coşkuner and Karababa 2007).

**Figure 2.1** Photo of CDC Sorrel flaxseed

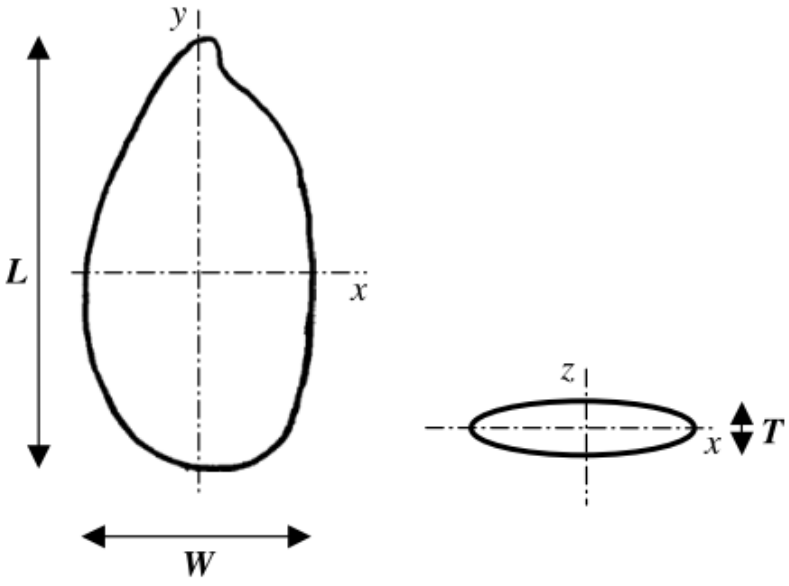


**Figure 2.2** Characteristic dimensions of flaxseed: L, W and T are the length, width and thickness (Coşkuner and Karababa 2007) used in accordance with a University of Saskatchewan library license agreement

### 2.1.2 Chemical Composition of Flaxseed

Flaxseed is considered a functional food ingredient due to its high content of omega-3, proteins, dietary fiber, vitamins, and lignans. Table 2.1 shows the detail of chemical composition of flaxseed (Bernacchia et al. 2014).

Flaxseed contains 45% lipid on a dry basis, of which 70% are polyunsaturated fatty acids, including 55% alpha-linolenic acid (ALA) and 14% linoleic acid. The remaining 20% of lipids are monounsaturated fatty acids (mainly oleic acid), and 9-10% are saturated fatty acids (palmitic and stearic) (Daun et al. 2003).

The protein content of flaxseeds estimated by nitrogen content ranges from 20-30%. A portion of the nitrogen content in flaxseed is in the form of non-protein nitrogen compounds such as certain vitamins, sinapine, choline, and cyanogenic glycosides. Only 18% of the protein in flaxseed is considered true protein, and amino acids including asparagine, glutamic acid, leucine, and arginine (Bekhit et al. 2018).

The dietary fiber content of flaxseed is approximately 28% by weight of the whole seed, with a ratio of soluble to insoluble fiber ranging from 20:80 to 40:60 (Martinchik et al. 2012). Soluble flaxseed fiber, also known as flaxseed mucilage or flaxseed gum, is found in the outer layer of the flaxseed and can be easily extracted through soaking the seed in water. The insoluble part of the flaxseed fiber includes cellulose and lignans (Vaisey-Genser and Morris 2003).

Flaxseed contains various vitamins and minerals, including calcium, magnesium, and phosphorus (Bernacchia et al. 2014). The primary form of vitamin E in flaxseeds is gamma-tocopherol, which constitutes 9.2 mg/100 g of seed. Additionally, flaxseed is the richest vegetable source of lignan, with a content of up to 0.7-1.5% of the dry weight of the seed. The main lignan precursor found in flaxseed is secoisolariciresinol diglucoside (Martinchik et al. 2012).

**Table 2.1** Chemical composition of flaxseed based on data from (Bernacchia et al. 2014)

| Chemical Compounds | g/100g of flaxseed |
|---|---|
| α-linolenic acid | 22.8 |
| Soluble fibers | 4.3-8.6 |
| Insoluble fibers | 12.8-17.1 |
| Calcium | 0.236 |
| Magnesium | 0.431 |
| Phosphorus | 0.622 |
| Secoisolariciresinol Diglucoside (a lignan) | 0.165 |

### 2.1.3 Nutritional Values of Flaxseed

Flaxseed has long been recognized as a healthy food, with potential benefits of regular consumption including reduced risks of heart disease, cancer, stroke, and diabetes. The consumption of alpha-linolenic acid (ALA) from flaxseed has been shown to reduce the risk of cardiovascular disease (Ruxton et al. 2007). Additionally, the consumption of dietary flaxseed has been shown to lower plasma triglycerides, cholesterol, and low-density lipoprotein cholesterol in rat, as well as plasma cholesterol and low-density lipoprotein cholesterol in human, indicating that regular consumption of flaxseed protects against the development of atherosclerosis (Ridges et al. 2001). Furthermore, studies have indicated that lignan, a significant family of dietary polyphenols found in high concentrations in food sources such as flaxseed, may have potential benefits in cancer prevention and treatment (De Silva and Alcorn 2019). Another report suggests that secoisolariciresinol diglucoside (SDG) isolated from flaxseed may have potential in reducing the incidence of type 1 diabetes and delaying the onset of type 2 diabetes in humans (Prasad and Dhar 2016).

### 2.1.4 Current Flaxseed Consumption and Market

Flaxseed is widely available in the international market in whole, roasted, sprouted, and ground forms. It can also be processed to produce flax meal and oil, which are used to make food products for both humans and animals. Additionally, flax oil is frequently used in the production of ink, varnish, and linoleum. Asia-Pacific region is the largest manufacturer of flaxseed products globally, with China, India, Australia, Kazakhstan, and Iraq being the top producers and consumers in the region (Research and Markets 2022).

The flaxseed market is predicted to grow at a CAGR of 11.8% from 2022 to 2027 as consumers become more interested in eating healthy foods, as their use in industrial applications and animal feed increases, and as flaxseed is a more affordable alternative to chia and quinoa seeds (Mordor Intelligence 2023).

Due to customers' growing health consciousness, there has been a remarkable increase in demand for flaxseed during COVID-19. Because of the disruptions in the supply chain and a shortage of seed during the pandemic outbreak, it has been challenging to meet the unexpected spike in demand. The flaxseed market was therefore significantly impacted by the COVID-19 (Fortune Business Insights 2023).

Ingestion of flaxseed can lead to the decomposition of cyanogenic glycosides (CGs) into hydrogen cyanide (HCN), which can be harmful to human and animal neurological systems. As a result, some countries, such as Japan and South Korea, have imposed strict regulations on the CG content of imported flaxseed (Thompson et al. 2015). These regulations may affect the flaxseed export market.

## 2.2 Cyanogenic Glycosides and Health Impact

In this section, it includes the topics of antinutrients in flaxseed, the structure and degradation of cyanogenic glycosides, the potential health impact of hydrogen cyanide, quantifying cyanogenic glycosides, and assessing the content of cyanogenic glycosides in flaxseed.

### 2.2.1 Antinutrients in Flaxseed

Flaxseed contains antinutrients such as cyanogenic glycosides, phytic acid, and linatine, which can inhibit nutrient absorption and lead to neurological problems and difficulty breathing in humans and animals (Dzuvor et al. 2018).

#### 2.2.1.1 Cyanogenic Glycosides

Cyanogenic glycosides are nitrogenous compounds found in over 2,500 plant species. They are thought to serve as an evolutionary defense mechanism (Bak et al. 2006) or as a storage deposit of reduced nitrogen and sugar for seedling development (Selmar et al. 1990). Flaxseed contains four types of cyanogenic glycosides: linustatin, neolinustatin, linamarin, and lotaustralin. When hydrolyzed by acid or an enzyme, these compounds release hydrogen cyanide (HCN), a toxic substance that impairs endocrine, cardiovascular, and neurological systems and restricts breathing. It is estimated that 100g of flaxseed can release 19-100 mg of HCN (Daun et al. 2003).

#### *2.2.1.2 Phytic Acid*

Phytic acid is a phosphorus-rich compound that plays an important role in germination and seedling growth (Bekhit et al. 2018). According to a study by Oomah et al. (1996), flaxseed meal contains 23-33g/kg of phytic acid. Its strong chelating properties can bind to mono- or divalent mineral cations and affect the absorption of calcium, potassium, magnesium, iron, and zinc (Kajla et al. 2015). However, a study found that feeding rat a small amount of flaxseed did not result in significant zinc deficiency (Ratnayake et al. 1992). The negative effect of phytic acid on human and animals depends on the intake dose of flaxseed and may become a concern if high amounts are consumed regularly (Bekhit et al. 2018).

#### *2.2.1.3 Linatine*

Linatine is a pyrrolidine monocarboxylic acid, which is a natural component of flaxseed cotyledons. It is present in flaxseed at a level of about 100 ppm. Some studies have suggested that feeding flaxseed to chicks can lead to symptoms of vitamin B6 deficiency, such as decreased appetite and poor growth (Kratzer 1946). However, no such effect was observed in humans who consumed 50 g of ground flaxseed per day (Ratnayake et al. 1992). To minimize the

antinutritional effects of linatine, it is recommended to have an adequate intake of vitamin B6 in the diet for those who consume large amounts of flaxseed or flaxseed meal (Bekhit et al. 2018).

### 2.2.2 Structure and Degradation of Cyanogenic Glycosides

Flaxseed contains cyanogenic glycosides which are undesirable in food product. There are four types of CGs in flaxseed: linustatin, neolinustatin, linamarin, and lotautralin (Figure 2.3). Linustatin and neolinustatin are diglycosides while linamarin and lotaustralin are monoglycosides (Bekhit et al. 2018). The monoglycosides linamarin and lotaustralin are generally found in trace amounts in mature seed (Bacala and Barthet 2007).

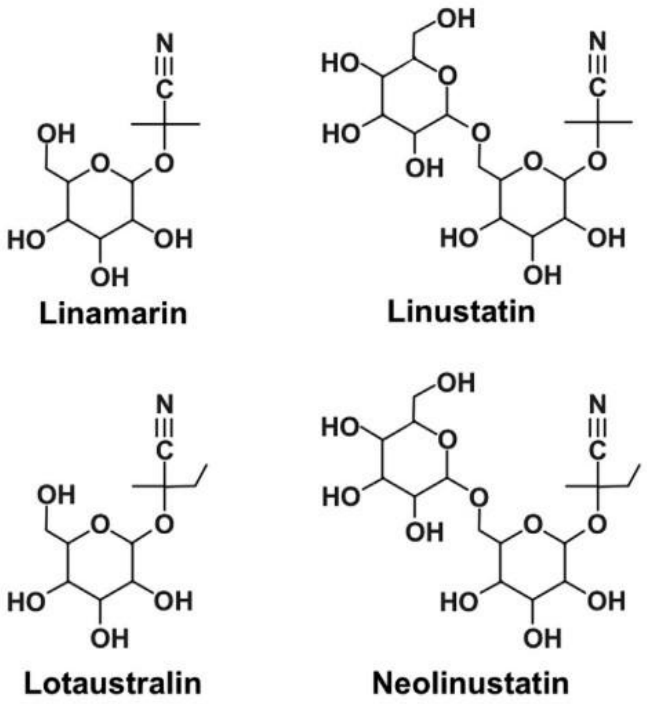


**Figure 2.3** Structure of four cyanogenic glycosides in flaxseed (Shim et al. 2014) used in accordance with a University of Saskatchewan library license agreement

Cyanogenic glycosides are hydrolyzed by intestinal β-glycosidases or by the acidic condition in the digestive tract to release HCN (Bacala and Barthet 2007). Cyanogenic glycosides can also be degraded by endogenous seed borne β-glycosidases. Chewing or grinding flaxseed disrupts cellular structures and facilitates the contact between endogenous β-glycosidases located in the cell wall with the cyanogenic glycosides in the cytoplasm (Mkpong et al. 1990).

The degradation process of cyanogenic glycosides in flaxseed is depicted in Figure 2.4 (Bekhit et al. 2018). Flaxseed naturally contains the endogenous enzymes linamarase and linustatinase (Fan and Conn 1985). Linustatinase converts neolinustatin to lotaustralin and linustatin to linamarin. The linamarinase enzyme then hydrolyzes the monoglycosides into cyanohydrin and glucose. Lotaustralin hydrolysis results in butanone cyanohydrin, while linamarin hydrolysis results in acetone cyanohydrin (Custer et al. 2003). These cyanohydrins are then broken down into D-glucose, acetone, or 2-butanone. At neutral or alkaline pH levels, these cyanohydrin intermediates are unstable and can spontaneously decompose (Cooke 1978). For example, acetone cyanohydrin spontaneously degrades into acetone and HCN at temperatures above 35°C and a pH of 5.0 (White et al. 1998). Endogenous hydroxynitrile lyase can also accelerate the breakdown of cyanohydrins (Hickel et al. 1996).

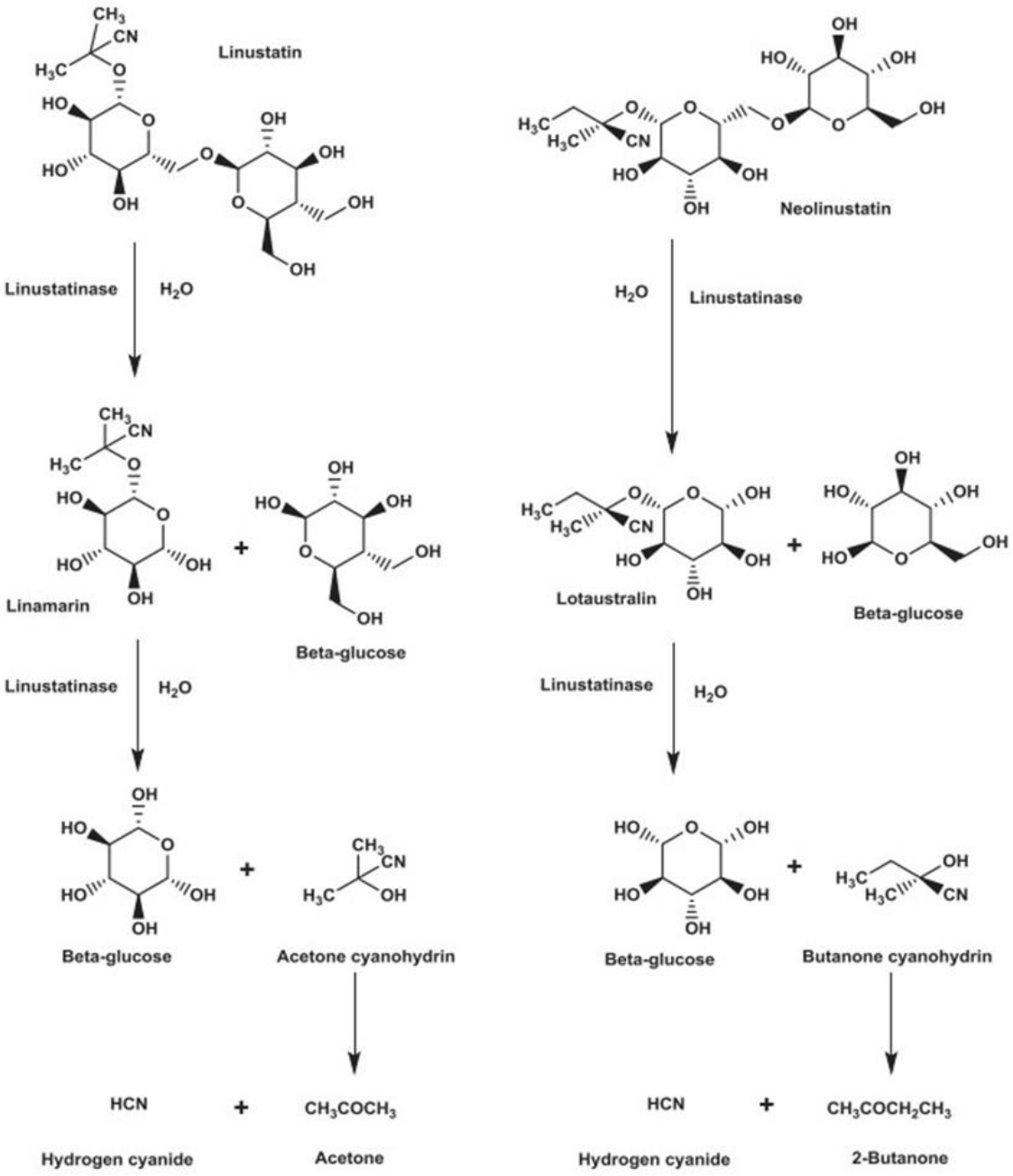


**Figure 2.4** Cyanogenic glycosides in flaxseed and their degradation by endogenous β-glycosidases (Bekhit et al. 2018) used in accordance with a University of Saskatchewan library license agreement.

### 2.2.3 Health Impact of Hydrogen Cyanide

Cyanogenic glycosides can be further decomposed into aliphatic ketones and hydrogen cyanide (HCN). Chronic exposure to HCN can lead to defects in the neurological, respiratory, cardiovascular system, and thyroid gland (Dhas et al. 2011). The World Health Organization (WHO) has also proven that long-term exposure to cyanide from a rich-in-cyanogenic-plant diet is responsible for a human central nervous syndrome called Konzo (Simeonova and Fishbein 2004; WHO 2011).

The adult acute toxic dose of inorganic cyanide is 50-60 mg, and humans can detoxify cyanide levels below 30-100 mg/day (Roseling 1994). Therefore, consuming 1-2 tablespoons of flaxseed, which contain approximately 5-10 mg of hydrogen cyanide, is not considered a lethal dose for humans (Bekhit et al. 2018). However, the safety of consuming flaxseed on a regular and high proportion basis is still questionable (Arslanoglu and Aytaç 2020), especially for flaxseed meal where the cyanogenic glycosides content increases due to oil removal. A similar amount of flaxseed meal contains twice the amount of HCN compared to whole flaxseed and only 6 tablespoons of flaxseed meal can reach the acute toxicity level in adults (Bekhit et al. 2018).

The presence of cyanogenic glycosides in flaxseed meal has traditionally imposed limitations on its utilization in animal feed (Oomah et al. 1992) and hindered its marketing in international markets (Thompson et al. 2015). To illustrate, according to Commission Regulation (EU) 2022/1364, the maximum allowable level of hydrocyanic acid, including hydrocyanic acid bound in cyanogenic glycosides, in unprocessed whole, ground milled, cracked, and chopped flaxseed is set at 250 mg/kg, while the permissible limit for the end consumer is 150 mg/kg (European Commission 2022).

### 2.2.4 Quantification of Cyanogenic Glycosides

Cyanogenic glycosides present in flaxseed can be broken down by its endogenous enzymes to release hydrogen cyanide (HCN), which is toxic to the endocrine, cardiovascular, and neurological systems of humans and animals. It is important for manufacturers and plant breeders to have a clear understanding of the cyanogenic glycoside content in flaxseed or processed flaxseed. Various techniques have been explored by researchers to measure the presence of

cyanogenic glycosides in flaxseed meal and whole flaxseed (Bacala and Barthet 2007; Roulard et al. 2017; Shim et al. 2016).

The limitations of chemical methods, enzymatic methods, and thin-layer chromatography (TLC) in determining the CG content in flax products has been noted. Chemical methods have low sensitivity and lack specificity, while enzymatic methods only provide the total amount of CGs without information on the specific CGs present or the variation in enzyme activity. Thin-layer chromatography is only a semi-quantitative method (Bacala and Barthet 2007).

Gas chromatography (GC) or high-performance liquid chromatography (HPLC) are preferred methods for analysis of flaxseed cyanogenic glycosides based on the methods high resolving power, direct quantitation of analytes, and automation capabilities. Bacala and Barthet (2007) developed a protocol for extracting cyanogenic glycosides from flaxseed and a GC method for measuring them, with the ability to process up to 90 samples per day. The GC method demonstrated reliable linearity and repeatable precision.

The analysis of cyanogenic glycosides (CGs) in flaxseed using proton nuclear magnetic resonance spectroscopy ($^{1}$H NMR) was first reported by Shim et al. (2016). Their study showed that $^{1}$H NMR is a fast, accurate, and sensitive method for quantifying CGs. This method has the advantage of being non-destructive as it does not involve a derivatization process or heating the samples, unlike GC. Roulard et al. (2017) developed a routine method that could quantify linustatin and neolinustatin in flaxseeds in less than 20 minutes using $^{1}$H NMR. The research demonstrated that $^{1}$H NMR can be used for varietal selection of flaxseed based on CG content.

### 2.2.5 Cyanogenic Glycosides Content in Flaxseed

The content and composition of cyanogenic glycosides in flaxseed are dependent on factors such as seed age, cultivar, location, and environmental conditions during growth. Oomah et al. (1992) used HPLC to identify various types of CGs in different flaxseed cultivars. The study found that the primary CGs present in flaxseed are linustatin, neolinustatin, and linamarin. Linustatin was found to range from 213 to 352 mg/100 g of seed, followed by neolinustatin (91 to 203 mg/100 g seed), while linamarin was found to be present in smaller amounts (<32 mg/100g) (Oomah et al. 1992).

Roulard et al. (2017) used $^{1}$H NMR to quantify the CGs content in flaxseeds. They found that the neolinustatin content and total CGs were higher in the hull, while a greater amount of linustatin was present in hull free fraction (cotyledons and germ). They analyzed seven different cultivars grown in two locations over the course of three years and found that differences in CGs content among samples were as high as 70% with standard deviation variations of less than 6%. The linustatin, neolinustatin, and total CGs content in the samples ranged from 91-267 mg/100 g, 78-272 mg/100 g, and 198-513 mg/100 g dry weight, respectively.

## 2.3 Removal of Cyanogenic Glycosides

Removing cyanogenic glycosides (CGs) from flaxseed can make it safe for consumption and improve nutrient absorption. CG-free flaxseed can also be exported to markets with CG content restrictions.

CGs are degraded through enzymatic hydrolysis to a cyanohydrin intermediate and further decomposed to hydrogen cyanide (HCN), which can then be volatilized under acidic conditions. The effectiveness of each step during food processing affects the detoxification of flaxseed. Three catabolic steps have different pH requirements: (1) enzymatic hydrolysis of CGs to cyanohydrin requires near-neutral pH, (2) breakdown of cyanohydrin to release HCN requires alkaline conditions ($pH \geq 6$), and (3) volatilization of HCN occurs best under acidic conditions. (Sanders 2016; WHO 2012)

Different methods have been explored for removing CGs from flaxseed, including heat treatment (Feng et al. 2003; Yang et al. 2004), chemical extraction (Bacela and Barthet 2010; Wanasundara et al. 1993; Waszkowiak et al. 2015), and biological treatment (Lei et al. 1999; Wu et al. 2012;Yamashita et al. 2007). The strengths and weaknesses of each method are summarized in Table 2.2.

**Table 2.2** Summary of the strengths and weakness of different processing methods for removing CGs from flaxseed

| Method | Strengths | Weakness | Reference |
|---|---|---|---|
| Heating | Detoxifying the food to acceptable detection limits (82%-83.3%) | Loss of nutritional constituents (lignans and fatty acids), high temperature | Feng et al. (2003), Yang et al. (2004) |
| Chemical Extraction | Traditional, high removal efficiency (83-90%) | Not able to extract CG completely and reduces protein, fibre, fat, and lignan content | Wanasundara et al. (1993), Bacela and Barthet (2010), Waszkowiak et al. (2015) |
| Biological Treatment (Enzyme) | Cheap, the cyanide-free flaxseed meal retains the beneficial nutrients at the same level as untreated flaxseed meal | Long reaction time (18 hours), Need to exhaust HCN directly into the air by steam heating at 120 °C for 2 hours | Wu et al. (2012), Yamashita et al. (2007) |
| Biological Treatment (Bacterial Fermentation/bioprocessing) | Effective, lower energy consumption and no environmental pollution | Long reaction time (48-72 hours), degraded CG from ground flaxseed | Lei et al. (1999) |

### 2.3.1 Heat Treatment

In 2003, Feng et al. studied the efficacy of various processing methods such as microwave heating and pelleting in reducing hydrogen cyanide content in flaxseed for animal feed. The concentration of HCN in microwave-roasted flaxseed decreased by 83.3%, likely due to the increased evaporation of newly formed HCN as evidenced by the 5.7% water loss during the 4-minute roasting process. In contrast, pelleting flaxseed once resulted in only a 13.3% reduction in

HCN content. However, when the flaxseed was mixed with other ingredients and pelleted twice, the greatest reduction in HCN content was achieved at 73.8%. This decrease may be due to the deactivation of glycosidase or evaporation of HCN produced from cyanogenic glycosides. The study indicated that increasing the quantity of pelleting and the temperature of the final product led to a higher reduction in HCN content (Feng et al. 2003).

In 2004, Yang et al. studied the removal of CGs from flaxseed using heat, solvent extraction, autoclaving, microwave, and boiling. The results indicated that microwaving reduced the HCN content by 82%, autoclaving reduced it by 27%, a single solvent extraction reduced it by 52%, a double extraction by 80%, and a triple extraction by 89%, while boiling reduced HCN content by 100% (Yang et al. 2004).

The investigations (Feng et al. 2003; Yang et al. 2004) demonstrated that heat treatments cannot completely remove HCN content in flaxseed. It is necessary to look into alternative techniques for depleting CGs from flaxseed.

### 2.3.2 Chemical Treatment

Both boiling and wet autoclaving methods for removing cyanogenic glycosides (CG) from flaxseed have a drawback of requiring high temperatures that negatively impact meal quality and result in low protein recovery. Wanasundara et al. (1993) used a two-phase solvent extraction system consisting of alkanol-ammonia and hexane to prepare high-protein flaxseed meal with reduced CGs. The extraction process with alkanol-ammonia-water/hexane not only removed CGs but also enhanced the apparent crude protein content of the meal. The crude protein content of the extracted meal was found to be between 43.5% to 48.6%, compared to 41.2% for hexane-extracted meals. Over 90% of linustatin (4.42 mg/g) and neolinustatin (1.90 mg/g) were removed from the meals under optimum conditions using methanolic solutions (Wanasundara et al. 1993).

Bacala and Barthet (2010) studied the extraction efficiency of CG from flaxseed meal using methanol. The reference method involved grinding the samples using a high-speed (at 18000 rpm) impact mill, followed by triple extraction with a 1.0 mm sieve in a sonicating water bath at 40°C for 30 minutes using 75% methanol. The routine method involved grinding the samples using a coffee mill and a single extraction. The extraction efficiencies using 70% and 80% methanol solutions were found to be equal and superior to other combinations of 50-100%

aqueous ethanol or methanol. The routine method had 87.9 ± 2.0% (linustatin) and 87.6 ± 1.9% (neolinustatin) extraction efficiencies, as compared to the reference method, based on four composite samples from multiple cultivars grown in two different crop years (Bacala and Barthet 2010).

Waszkowiak et al. (2015) studied the impact of different extractions on the composition and content of cyanogenic glucosides in flaxseed extracts. The results showed that extraction with ethanol was more selective for phenolics compared to aqueous extraction. Thus, this method could provide flaxseed extracts with high antioxidant activity, potentially as a replacement for synthetic antioxidants in food applications. However, it is important to monitor the content of cyanogenic glucosides in the extracts, as their concentration tends to be higher after ethanolic than aqueous extraction. The total content of cyanogenic glucosides in the extracts was reduced by 96-98% compared to their initial content in defatted flaxseed meal.

While chemical methods for removing cyanogenic glucosides from flaxseed can be effective, they also decrease the content of important nutrients such as protein, fiber, fat, and lignans. Therefore, it is important to explore a more environmentally friendly and food-safe method for removing cyanogenic glucosides from flaxseed.

### 2.3.3 Biological Treatment

A few researchers have studied biological methods for removing CGs from flaxseed meal. The use of a biological approach in the degradation of hydrogen cyanide is a relatively less expensive and safer method in food processing (Dzuvor et al. 2018). Commercial enzymes such as linamarase, xylanase, and cellulase have been shown to effectively degrade CGs in cassava and cassava-based food products to acceptable levels (Sornyotha et al. 2010).

Lei et al. (1999) attempted to remove CGs from flaxseed meal using lactic acid bacteria (LAB) in traditional fermentation systems, as these LAB have been shown to produce enzymes that can detoxify cyanogens (Lei et al. 1999; Nwokoro 2016). These microorganisms were able to degrade the CGs in flaxseed, but not all the cyanogens, as a toxic cyanohydrin intermediate breakdown product remained. This toxic intermediate could not be removed because cyanohydrins are stabilized at the low pH achieved through LAB fermentation; the levels of cyanohydrin and HCN

remained at nearly 50% of the original cyanogen level after 70 hours of fermentation (Sanders 2016).

The second fermentation study by Yamashita et al. (2007) utilized an incubation period of 18 h at 30°C, which allowed sufficient time for enzymatic release of hydrogen cyanide (HCN) and its ultimate removal through steam evaporation. However, this method has the drawback of being energy intensive. An industrial-scale steam-heated oven and steam room equipped with a recovery system for steam vapor to absorb the generated HCN gas was deemed necessary.

Wu et al. (2012) developed a response surface method to optimize a fermentation process for removing cyanogenic glucosides from flaxseed powder. The optimized fermentation process used 12.5% β-glucosidase and 8.9% cyanide hydratase enzymes to reduce cyanide concentration from 1.156 to 0.015 mg/g after 48 h. However, the low level of cyanogenic glycosides initially present in the flaxseed meal samples (2 to 3 times lower than levels reported by other researchers) raises concerns, and the authors did not provide any remarks regarding the differences in cyanogenic glycoside levels or the method's ability to perform well with flaxseed containing normal levels of cyanogenic glycosides.

After weighing the advantages and disadvantages of various technologies for removing CGs from flaxseeds, it has been determined that bacterial fermentation is the most effective method. It is cost-effective, easy to implement, and has minimal negative impact on the environment. Further research should focus on the use of LAB fermentation to remove CGs from whole flaxseed and to identify the specific microorganisms involved in the process.

## 2.4 Lactobacillaceae Fermentation in Food Processing Industry

This section provides an introduction to *Lactobacillaceae,* exploring their characteristics and discussing their applications in food industry.

### 2.4.1 Introduction of *Lactobacillaceae*

The *Lactobacillaceae* family is composed of lactic acid bacteria, including the genera *Lactobacillus, Paralactobacillus, and Pediococcus.* The largest group within the family is the genus *Lactobacillus*, with 261 species as of March 2020 (Zheng et al. 2020). Lactobacillus are

rod-shaped, gram-positive bacteria that grow well in anaerobic conditions and are found in animal feeds, silage, manure, and milk and milk products. (Makarova et al. 2006).

*Lactobacillus* bacteria produce lactic acid during glucose metabolism, with the amount varying among species. Some, like *L. acidophilus, L. casei, and L. plantarum*, primarily produce lactic acid (85% or more) in a process known as homofermentative metabolism. Others, like *L. brevis* and *L. fermentum*, have a heterofermentative form of glucose metabolism where lactic acid makes up about 50% of metabolic by-products, with the rest being ethanol, acetic acid, and carbon dioxide. (Giraffa et al. 2010)

*Lactobacillaceae* play a vital role in the production of beta-galactosidase enzyme and fermented dairy products (Zacharof 2010), and produce bacteriocins, which act as a natural barrier against pathogens and food spoilage (Zacharof and Lovtii 2012). Fermented food using *Lactobacillaceae* is considered safe by the European Food Safety Authority and the US Food and Drug Administration (Koutsoumanis et al. 2020).

### 2.4.2 *Lactobacillaceae* Application in the Food Industry

In this section, the multifaceted applications of *Lactobacillaceae* are discussed, which serves not only as a crucial starter culture in food production (Rafique et al. 2022) but also holds immense promise in revolutionizing and augmenting diverse food products through the process of microbial fermentation (Lei et al. 1999; Ratanapariyanuch et al. 2016, 2017; Tse et al. 2020).

#### *2.4.2.1 General applications of Lactobacillaceae in Food*

*Lactobacillaceae* are used commercially to make sour milks, cheeses, and yogurt. Examples of the species of *Lactobacillaceae* naturally presented or added in dairy products are shown in Table 2.3. The diversity of *Lactobacillaceae* that can be employed as starter cultures in various dairy products as mentioned in earlier literature was summarised by Rafique et al. (2022).

*Lactobacillaceae* play an important role in the production of fermented vegetables, beverages, and sourdough breads. Both *L. plantarum* and *L. brevis* are used to make sauerkraut (Plengvidhya et al. 2004). During the fermentation process of wine, the following *Lactobacillaceae* species are naturally present or are added as a starter*: L. plantarum* (Spano et al. 2005), *L. brevis, S. collinoides* (formerly *L. collinoides*), *L. hilgardii, L. paracasei, L. pentosus, L. plantarum, and L.*

*mali* (Rodas et al. 2005). The addition of *Lactobacillus (eg. L. plantarum)* during fermentation of Kombucha increases the lactic acid content of the tea beverage (Cvetkovic et al. 2019). Species of *Lactobacillaceae* such as *F. sanfranciscensis* (formerly *L. sanfranciscensis*), *L. reuteri and L. pontis* are presented in sourdough (Vogel et al. 1999).

**Table 2.3** Examples of the species of Lactobacillus presented or added in dairy products based from (Rafique et al. 2022) used in accordance with the Creative Commons Attribution 3.0 license.

| Products | Diversity of Lactobacillus |
|---|---|
| Unpasteurized milk | *L. casei; L. paracasei; L. rhamnosus; L. plantarum; L. fermentum; L. brevis; L. buchneri; L. curvatus; L. acidophilus; L. pentosus* |
| Fermented milk | *L. kefiri* (formerly *L. kefir)* |
| Cheese | *L. helveticus; L. delbrueckii; L. casei; L. paracasei; L. rhamnosus* |
| Natural Greek style yoghurt | *L. acidophilus; L. bulgaricus* |
| Probiotic yoghurt drink | *L. acidophilus; and L. casei* |
| Ice cream | *L. acidophilus; L. johnsonii* |

*2.4.2.2 Lactobacillaceae Applications in Food Processing*

There are many applications for *Lactobacillaceae* fermentation in food processing (Lei et al. 1999; Ratanapariyanuch et al. 2016, 2017; Tse et al. 2020).

*Lactobacillaceae* fermentation has been used in removing the antinutrients from flaxseed meal. Lei et al. (1999) discovered the efficient ability to degrade CGs in the strains of *L. plantarum* and *Leuconostoc mesenteroides (*both bacteria are members of the *Lactobcillaceae* family). Also, they

found good correlation between the β-glucosidase activity in the API zym (Bio Merieux) screening for those two species. The study suggested that *Lactobcillaceae* fermentation could deplete CGs from flaxseed meal because the enzyme produced by bacteria accelerated the process of degrading CGs to HCN.

*Lactobacillaceae* fermentation also has potential to convert low-cost glycerol to valuable products (vitamins and concentrate proteins). A wheat-based thin stillage (W-TS) is a dilute liquid co-product of wheat ethanol production that contains organic solutes, microorganisms, proteins, dissolved salts, and particle. Ratanapariyanuch et al. (2016) developed a consortium of endemic organisms in W-TS from a commercial ethanol production, and converted the thin stillage compounds using endemic bacteria with *Lactobacillus panis* PM1B to improve the fermentation rate and extent. Ratanapariyanuch et al. (2017) continuedly used a subsequent fermentation of W-TS (two-stage fermentation, TSF) with endemic bacteria decreased glycerol and lactic acid concentrations, but increased 1,3-propanediol (1,3-PD), acetic acid and protein concentrates. The predominant endemic bacteria in W-TS were *L. panis, L. gallinarum, and L. helveticus*.

## 2.5 Summary

As mentioned in this chapter, bacterial fermentation is the most effective technique to remove CGs from flaxseed because of its low cost and minimal negative impact on the environment. Since previous studies (Lei et al. 1999; Wu et al. 2012; Yamashita et al. 2007) used LAB fermentation on flaxseed meal, it is necessary to develop a fermentation method for whole flaxseed processing applications.

In this study, we used fermentation inoculated with the in-house consortium of *Lactobacillaceae* bacteria, which was obtained from Tse et al. (2020), to remove CGs from whole flaxseed. The primary reason using an in-house consortium was because the previous studies demonstrated its capability in upgrading and enriching a variety of crop products (e.g., thin stillage) and food products (e.g., sour dough production) (Ratanapariyanuch et al. 2017, Tse et al. 2020).

The bacterial consortium of Tse et al. (2020) was previously cultured for >80 inoculations on W-TS and was used to enrich and upgrade the W-TS (Tse et al. 2020). In addition, Tse et al. (2020) indicated that the predominant *Lactobacillus* community in the culture generated bacteriocins and

acidified the fermentation broth, which may inhibit the growth of competitive organisms and become a natural antimicrobial food preservative. This well-characterized consortium of *Lactobacillaceae* could be used in food processing as they are generally regarded as safe (GRAS) organisms. As Lei et al. (1999) suggested that *Lactobacillaceae* can degrade CGs from flaxseed, it is hypothesized fermentation using the in-house consortium of *Lactobacillaceae* could create an innovative, food-safe, and ecofriendly approach for depleting CGs from whole flaxseed.

# 3. DEPLETION OF CYANGENIC GYLCOSIDES IN WHOLE FLAXSEED VIA LACTOBACILLACEAE FERMENTATION

Contribution of the MSc student:

Experiments were designed and performed by Chao Huang with the guidance provided by Dr. Reaney and Dr. Purdy. Formal analysis was done by Chao Huang with assistance by Dr. Shen and Dr. Tse. Writing of the manuscript was prepared by Chao Huang, with revision provided by Dr. Tse, Dr. Chicilo and Dr. Reaney. Dr. Meda also provided consultation during the entire experimental period as well as thesis preparation.

Contribution of this Chapter to the Overall Study

The chapter includes the manuscript that is published on *Food Chemistry* (Huang et al. 2023). The published manuscript has been reformatted from the original version for inclusion in the thesis. All co-authors have agreed to include this manuscript in the thesis.

In this chapter, cyanogenic glycosides in whole flaxseed fermented with a consortium culture of *Lactobacillaceae* was depleted within 72 h in both bench-scale and scale-up studies. In addition, fatty acid composition in flaxseed was maintained, while the concentrations of flaxseed oil, and SDG in flaxseed were increased after the fermentation.

## 3.1 Abstract

Flaxseed is categorized as a functional food due to its abundance in oil, α-linolenic acid, dietary fibres, and lignans. However, flaxseed contains cyanogenic glycosides (CGs). Ingestion of CGs can influence nutrient absorption and induce adverse health effects. Due to the presence of CGs in flaxseed many countries prohibit the import and sale of flaxseed and flaxseed-based foods. In this study, whole flaxseed was fermented with a mixed culture of *Lactobacillaceae* (i.e., Lactobacillusm Limosilactobacillus, Lactiplantibacillus) and the concentration of CG was determined. This process succeeded in completely removing CGs within 72 h in both bench-scale and scale-up studies. In addition, fatty acid composition in flaxseed remained unchanged and concentrations of flaxseed oil, and SDG in flaxseed were increased after fermentation. CG-free flaxseed products are beneficial, as they can be sold as health product ingredients, or as animal feed in markets that currently restrict the use of materials that contain CGs.

***Keywords***

Cyanogenic glycosides, Removal, Whole flaxseed, Fermentation, *Lactobacillaceae*

## 3.2 Introduction

Flax (*Linum usitatissimum* L.) is an important oilseed crop grown in India, Russia, Kazakhstan, Canada, China, the United States, and Ethiopia (Singh et al. 2011). Flaxseed is considered a functional food due to the high content of α-linolenic acid (ALA), oil, carbohydrates, lignans, dietary fibre, vitamins, cyclic peptides, and minerals (Dzuvor et al. 2018). The presence of these different compounds with beneficial nutritional properties led to the broad utility of flaxseed products as bioactive ingredients, condiments, bakery ingredients, food thickeners (Lee et al. 2021), or as whole seed in foods.

Flaxseed soluble fiber (i.e., mucilage or gum) is composed primarily of high molecular weight polysaccharides and conlinin protein (Liu et al. 2018), while flaxseed hull contains substantial amounts of lignan (1–26 mg/g) (Muir 2006), in the form of a co-polymer of secoisolariciresinol diglucoside (SDG) with hydroxymethylglutaric acid (HMGA) (Shim et al. 2015). Consumption of the lignan co-polymer induces hypocholesterolemic effects and, thus, has been investigated as an alternative to pharmacotherapy in the treatment of cardiovascular diseases (Felmlee et al. 2009). More recently, the pharmacokinetics (Yang et al. 2021) and efficacy of SDG (Guo et al. 2021), and its co-polymer with HMGA, have been investigated where it was observed that consuming either the co-polymer or the enriched SDG were successful in mitigating hypercholesterolemia. SDG also has exhibited benefits that might lead to its application for the prevention and treatment of cancer (De Silva and Alcorn 2019). Overall, the abundance and bioavailability of flaxseed lignans can add significant value to this agricultural product (Tse et al. 2022).

Beyond the presence of dietary fibers and lignans, flaxseed is also rich in oil, which is often extracted by cold-pressing (Shim et al. 2015). Flaxseed oil is typically an excellent source of α-linolenic acid (45 to 70%), an omega-3 fatty acid, although the nutritional composition of flaxseed can differ among cultivars (Booker 2019). Omega-3 fatty acids have anti-inflammatory, anti-thrombotic, and anti-arrhythmic properties (Singh et al. 2011). Because of the health benefits associated with the high content of lignan, fiber, and linolenic acids in flaxseed, Health Canada allows the labeling of flaxseed and flaxseed containing products as nutritional foods that lower blood cholesterol (Health Canada 2014).

A limiting factor to the commercial use of flaxseed is the presence of precursors that are converted to cyanide, or cyanogenic glycosides (CGs) (Yamashita et al. 2007). The primary

cyanogenic glycosides found in flaxseed are linustatin, neolinustatin, and linamarin, with concentrations varying 213 - 352 mg/100 g of seed, 91 - 203 mg/ 100 g of seed, and < 32 mg/100 g of seed, respectively (Oomah et al. 1992).

Hydrogen cyanide (HCN) is highly toxic to the mammalian respiratory, nervous, and endocrine systems, and the presence of HCN may also influence nutrient absorption from food products (Singh et al. 2011). Cyanide is acutely toxic in humans with a lethal dose reported to be 0.5-3.5 mg/kg body weight (Koutsoumanis et al. 2020). Because of the presence of toxic CGs in untreated seeds, many regulatory agencies restrict these products from being marketed. It is possible that removing CGs through processing could enhance the health benefits associated with flaxseed consumption. In a recommended daily serving of approximately 1-2 tablespoons of flaxseed, up to 5-10 mg of hydrogen cyanide is released (Hall et al. 2006). This amount is below the 30-100 mg/day levels of hydrogen cyanide humans can detoxify but there are no definitive "safe" levels of CG, and flaxseed might be dangerous if consumed in larger quantities. A harmful dose would depend on the consumers weight and health. Furthermore, the presence of CG can limit the desirability of flaxseed products to international markets. Japan and South Korea, for example, have regulations on the import and sale of foods that contain CG, and the CG content should be depleted before such foods can be sold in these markets (Thompson et al 2015).

Heat treatments (e.g., microwave roasting, boiling water) of flaxseed have been investigated for their effect on CG concentration. The best reported treatments reduced CG by 80% (Yang et al. 2004). Extrusion of full-fat flaxseed also reduced CGs by as much as 90% (Imran et al. 2013). However, the use of heat treatments on CG removal could lead to the degradation of sugars and lignans, as well as triglyceride hydrolysis. This can further result in the release of undesirable fatty acids, oxidative destruction of essential amino acids (e.g., methionine and tryptophan), loss of vitamins and antioxidant capacity, and a decrease in the availability of omega-3 and omega-6 fatty acids (Dzuvor et al. 2018).

Chemical treatments (e.g., solvent extractions) have also been employed to remove CGs from flaxseed meal (Wanasundara et al. 1993). For example, flaxseed meal treated with a solution of alkanol-ammonia-water/hexanes resulted in a 90% reduction in CGs (Wanasundara et al. 1993), where the alkanols were isopropyl alcohol, ethyl alcohol, and methyl alcohol. A major drawback of these processes is the use of reagents which can be toxic, and might reduce the content of beneficial ingredients from flaxseed and its fractions (Yamashita et al. 2007). To

maintain the nutritional components in flaxseed meals during CG removal, enzymatic technologies have been developed as potential treatments (Yamashita et al. 2007). However, such treatments can require undesirable energy inputs (e.g., addition of water and downstream drying) and can also generate substantial process waste.

Aside from chemical treatments, bacterial inoculation (e.g., with *Lactobacillaceae*) has also been investigated as an approach for degrading CGs in milled flaxseed (Lei et al. 1999). This method removed CGs effectively and minimized environmental impacts through the minimization of process waste and energy inputs (e.g., high heat drying). It is hypothesized that fermentation using a culture of *Lactobacillaceae* will offer a novel and nontoxic approach in removing CGs from flaxseed, as these organisms are often considered GRAS (Generally Recognized as Safe). This process has not yet been applied to whole flaxseed, but implementation of such technologies can drastically reduce processing time (e.g., milling). With the growing utilization of whole flaxseed in food, health, and animal products, it is essential to investigate efficient methods for removal of CGs from whole flaxseed to minimize health risk and ensure flaxseed and flaxseed products can be sold to international markets. Therefore, the objectives of this study were to: (1) develop a fermentation method using an in-house inoculant of *Lactobacillaceae* to remove CGs from whole flaxseed; (2) monitor the reduction of major CGs (linustatin and neolinustatin, as well as total HCN) during fermentation; (3) compare the nutritional value of the treated flaxseed with untreated flaxseed; and (4) investigate changes in SDG content during fermentation.

## 3.3 Materials and Methods

### 3.3.1 Materials

Sorrel flaxseed was purchased from G.A & Robin Fenton Farm (Tisdale, SK) and G&G Edmunds Farm (Eldersley, SK). Deuterated chloroform (99.8%; $CDCl_3$) was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, Massachusetts). Reagents, deuterium oxide ($D_2O$; 99.9 atom% D), potassium hydroxide (KOH; reagent grade), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Oakville, Ontario). Analytical-grade methylene chloride (DCM) and methanol, and N,*N*-dimethyl formamide (DMF; ultrapure, GC, ≥99.5%) were purchased from Thermo Fisher Scientific (Ottawa, Ontario).

### 3.3.2 Bench-scale flaxseed fermentation

A consortium of *Lactobacillaceae*, cultured in-house and previously isolated from wheat-based thin stillage (Tse et al. 2020), was inoculated (5 mL) in a solution containing 35 g of whole flaxseed, 5 mL of commercial white vinegar (3% acetic acid v/v; pH ~ 3) and adjusted to 250 mL with the addition of distilled water in a VWR® square narrow mouth polypropylene fermentation bottle (Radnor, PA, USA) equipped with a gas trap. Commercial white vinegar was used to provide an optimum pH environment for the *Lactobacillaceae*. Inoculated fermentation vessels were incubated at 30 ℃ using a Büchi water bath B-490 (Flawil, Switzerland) for 72 h. After 72 h, 175 mL of the fermentation media was poured into a VWR® 12" 200µm test sieve, rinsed with tap water, and dried overnight at 60 °C in a VWR® forced air oven (Radnor, PA, USA). Dried flaxseed (5 g) was then ground in a Master Chef coffee grinder (Model #043-1008-6) prior to analysis.

The bacterial consortium was previously cultured for >80 inoculations on wheat-based thin stillage and has been used to enrich and upgrade thin stillage (Tse et al. 2020), as well as used in food production (e.g., sourdough production) (T. Tse, personal communication, University of Saskatchewan). This well-characterized consortium has potential application to food processing applications, along with the current example of removal of CG from whole flaxseed.

Like Tse et al. (2020), this culture of *Lactobacillaceae* was re-inoculated on fresh whole flaxseed media after every 48 h of fermentation (Figure S1). Briefly, 75 mL of the previous fermentation media was used to culture the next batch of flaxseed, and the volume was again increased to 250 mL using distilled water. Fermentations were then conducted under identical conditions. The *Lactobacillaceae* community was recultured to maintain a continuous culture that could then be used for the downstream scale-up, feasibility experiments, described below.

### 3.3.3 Scale-up experiment

The scale-up experiments were conducted at two volumes, 4 L and 8 L containing 340 g and 1 kg of CDC Sorrel flaxseed and 750 mL and 2250 mL of bacterial inoculant, respectively. Fermentation was conducted in a VWR 1390FM mechanical oven incubator (Radnor, PA, USA) at 30 ℃ for 72 h. After incubation, the flaxseed was rinsed with tap water and the gum was separated from the flaxseed using a Robot Coupe C80 automatic sieve (Moncton, NB, Canada).

Degummed flaxseed was spread on silicone sheets and dried overnight in a Cabela's® 160 L commercial-grade food dehydrator (Sidney, Nebraska, USA) at 60 ℃. The dried flaxseed was then collected for analysis (i.e., moisture content, mass balance, and cyanogenic glycosides).

### 3.3.4 Analytical Measurement

#### *3.3.4.1 Moisture content*

Moisture content of pre- and post-fermentation flaxseed samples were conducted at Prairie Tide Diversified Inc. following AOCS Recommended Practice Ak 4-95.

#### *3.3.4.2 Linustatin, and neolinustatin*

Ground flaxseed (0.5 g) was extracted with 10 mL of methanol and the content of linustatin, and neolinustatin were determined in the extracts. For extraction, the flaxseed methanol mixtures were shaken overnight at 21 °C using a Heidolph Synthesis 1 shaker (Schwabach, Germany). After mixing, the solution was centrifuged at 3850*g* for 5 min using a Beckman Coulter Allegra X-22K centrifuge (Brea, CA, USA), and the supernatant was collected and concentrated under vacuum, using a Büchi rotavapor R-210 at 48 °C. The dried residue was diluted with 2 mL $D_2O$, and 1 drop (~50 mg) internal standard DMF was added prior to $^1$H-NMR analysis. Linustatin and neolinustatin contents were determined by comparing to the DMF internal standard (Shim et al. 2016). Total HCN production potential was then calculated from the CG contents. The concentration of CGs in each of sample was calculated using the Equations 3.1, 3.2, 3.3:

$$C_f = \frac{Vi\ X\ Ci}{V_f} \quad (3.1)$$

$$N = \frac{A}{n} \quad (3.2)$$

$$C_{CGs} = \frac{N_{CGs}\ X\ C_f}{N_{DMF}} \quad (3.3)$$

where $C_f$ is the final concentration of DMF in sample, $V_i$ is the initial volume of DMF in the sample, $C_i$ is the initial concentration of DMF in the sample, $V_f$ is the total volume of the sample, N is the area of the integrated peak of interest A in the $^1$H NMR spectra divided by the nuclei contributing to the peak in each CG molecule (for linustatin n=6; for neolinustatin n=3), $C_{CGs}$ is the concentration of CGs in each sample, $N_{CGs}$ is the sum of the A/n of CGs observed by $^1$H

NMR spectra, and $N_{DMF}$ is the integrated peak area of the amide proton of DMF standard in the $^{1}H$ NMR.

*3.3.4.3 Percent oil and fatty acid profile*

Oil and fatty acid content were determined by mixing ground flaxseed (0.5 g) with 10 mL of DCM in an Erlenmeyer flask for 5 minutes. The extracts were subsequently filtered through a cotton plug in a glass pipette and the eluent was collected and weighed. To ensure complete oil removal, the flask was rinsed with an additional 5 mL of DCM. The eluent was again collected and weighed. After the second filtration, DCM was removed from the oil fraction using a Büchi rotavapor R-210 operating at 60 °C. After evaporation, the flask (with residue) was weighed and the residual oil content $W_3$ was determined following Equation 3.4:

$$W_3\,(\%\ \text{oil}) = (W_2 - W_1) / 0.5 \times 100 \qquad (3.4)$$

where, $W_1$ is the weight of the empty Erlenmeyer flask; $W_2$ is the residue and weight of flask after evaporation; and $W_3$ is the residual oil content (%). The oil residue was then dissolved in 2 mL of $CDCl_3$, and 1 drop (~50 mg) of DMF internal standard was added to the vial prior to $^{1}H$-NMR analysis.

*3.3.4.4 Secoisolariciresinol diglucoside*

SDG was extracted from flaxseed powder (0.5 g) by mixing with 10 mL of methanol. The solution was shaken overnight using a Heidoph Synthesis 1 shaker, followed by centrifugation at 3850*g* for 5 min at room temperature (21 °C) The supernatant was retained and further mixed with 0.5 mL of 5% KOH for 1 h. The solution was centrifuged again for 5 min at 3850*g*, and concentrated via evaporation using a rotary evaporator (Büchi rotavapor R-210) at 60 °C. The dried residue was then diluted with 2 mL distilled water, and ~50 mg of 2% DMSO. This mixture was then mixed with $D_2O$ at a 1:1 ratio prior to $^{1}H$-NMR spectroscopy. Identification and quantification of SDG was performed according to Shim et al. (2016).

*3.3.4.5 $^{1}H$-NMR spectroscopy*

Quantification of linustatin and neolinustatin were accomplished using $^{1}H$-NMR analyses following previously reported methods (Shim et al. 2016). All analyses were collected using a Bruker Avance III HD 500 MHz spectrometer (Mississauga, ON, Canada) with 16 scans per

spectrum. The NMR spectra were analysed using TopSpin v2.6.2 (Bruker BioSpin GmbH, Billerica, MA, USA).

*3.3.4.6 Mass Balance*

The mass of all fractions was determined at each stage of processing to determine the mass balance. Seed weight loss was determined after 72 h of fermentation, degumming, and drying (60 °C) processes. The total seed weight loss was determined according to Equation 3.5:

$$\text{Seed weight loss (\%)} = (W_0 - W_1) / W_0 \times 100 \quad (3.5)$$

Where $W_0$ is the initial flaxseed weight and $W_1$ is the dried flaxseed weight after 72 h of fermentation, degumming, and drying process. For larger scale fermentation, seed was washed four times and the weight of flaxseed recorded after each wash. The first wash consisted of 1 kg flaxseed and fermentation medium, the second, third, and forth wash used distilled water. The mass of distilled water used was 150%, 50% and 25% of the dry weight of flaxseed for each subsequent wash.

*3.3.4.7 DNA extraction and metagenomic sequencing*

To ensure the *Lactobacillaceae* consortium remained similar from our in-house stock culture, 16S amplicon sequencing was employed. DNA extraction was similar to Tse et al. (2020). Briefly, aliquots (2 mL) were taken from the flax fermentation media and centrifuged at 7000*g* for 20 min. The supernatant was decanted, and the pellet (~ 0.2 g) was retained. DNA extraction was completed using E.Z.N.A. Soil DNA Kits (Omega Bio-tek, Norcross, GA) in accordance with manufacturer specifications, and samples were stored at -80 °C until further analysis.

Universal bacterial primers B341F (CCTACGGGNBGGCWGCAG) and B806RB (GGACTACNVGGGTWTCTAAT) were used as forward and reverse primers, respectively. These primers were modified with attached Illumina bar code overhangs to amplify the 490 bp fragment of the V3-V4 regions of the 16S rRNA subunit. Library preparations were completed following Illumina's 16S Metagenomic Sequencing Library Preparation (Part# 15044223 Rev. B), and sample libraries were pooled prior to paired-end multiplex sequencing performed on a MiSeq Desktop Sequencer (Illumina, California, USA). The quality of demultiplexed 16S rRNA amplicon sequences were checked using *mothur* to remove ambiguous bases and homopolymer lengths greater than 4 from the dataset (Tse et al., 2018). Verified 16S rRNA amplicon sequences

were then aligned against the SILVAngs 16S/18S (v. 138.1) database to taxonomically classify at the 98% identity level.

*3.3.4.8 Statistical analysis for DNA sequencing*

Statistical analyses for community diversity and gene abundances were determined using the R software v3.3.1. (R Development Core Team 2016) according to Tse et al. (2018). The breakpoint (threshold) analysis for the number of bacterial OTUs versus the fermentation sample (regular vs. acidified treatments) were performed using segmented linear regression (SegReg software) (Oosterbaan et al. 1990) to observe changes in the α-diversity. Meanwhile, the β-diversity was calculated using the Bray−Curtis dissimilarity index *via* the ordinate function of the phyllodes package (McMurdie and Holmes 2013) and visualized with a non-metric multidimensional scaling plot by the plot ordination function of phyloSeq, ellipse (Murdoch et al. 2007) and ggplot (Wickham 2009) packages. The permutational multivariate analysis of variance test (PERMANOVA, n = 9999) was subsequently employed to assess the statistical significance of the β-diversity among fermentation treatments using the vegan package (Oksanen et al. 2018).

## 3.4 Results and discussion

### 3.4.1 Bench-scale fermentation for removing CGs

The presence of linustatin (identified by a single resonance peak at 1.5 ppm) and neolinustatin (identified by two resonance peaks at 1.5 ppm and 0.9 ppm) were observed via $^{1}$H-NMR spectroscopy of extracts in untreated flaxseed before fermentation (Figure 3.1). After 24 h of fermentation, linustatin and neolinustatin in extracts decreased noticeably (Figure 3.2). Both compounds were below detection limits (< 0.05%) after 48 h. Total HCN, based on the concentration of these compounds, was < 10mg/kg (Figure 3.3). The reduction in CGs could be attributed to enzymatic processes from some *Lactobacillaceae* species. For example, β-glucosidase and cyanide hydratase enzymes are capable of degrading CGs (Wu et al. 2012), and have also been identified in some *Lactiplantibacillus sp.* (Michlmayr and Kneifel 2014; Wilson et al. 2014), some of which were previously identified in the culture used for inoculation (Tse et al. 2020).

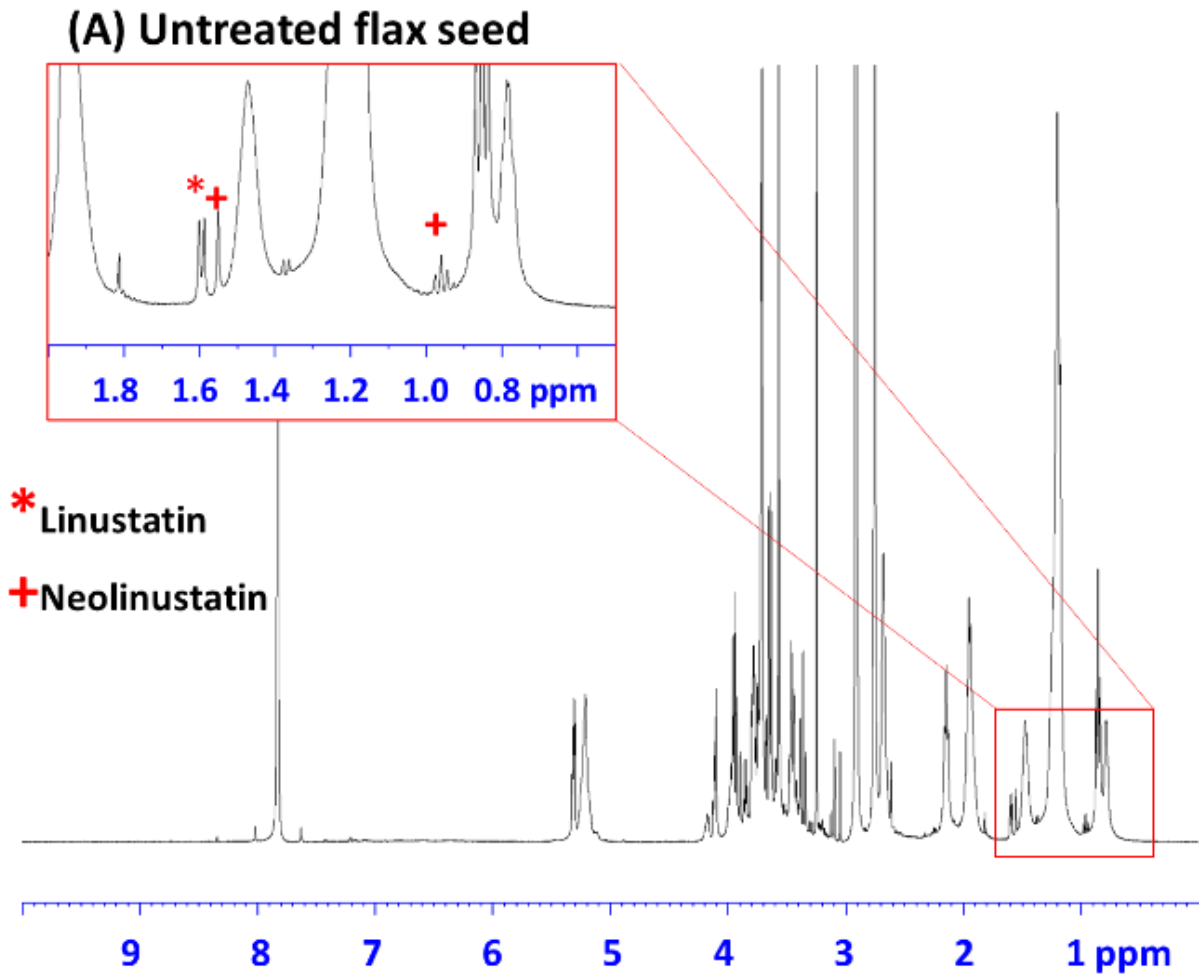


**Figure 3.1** $^{1}$H-NMR spectra of untreated flaxseed without fermentation: linustatin (one resonance at 1.5 ppm) and neolinustatin (two resonances at 1.5 ppm and 0.9 ppm) are both presented

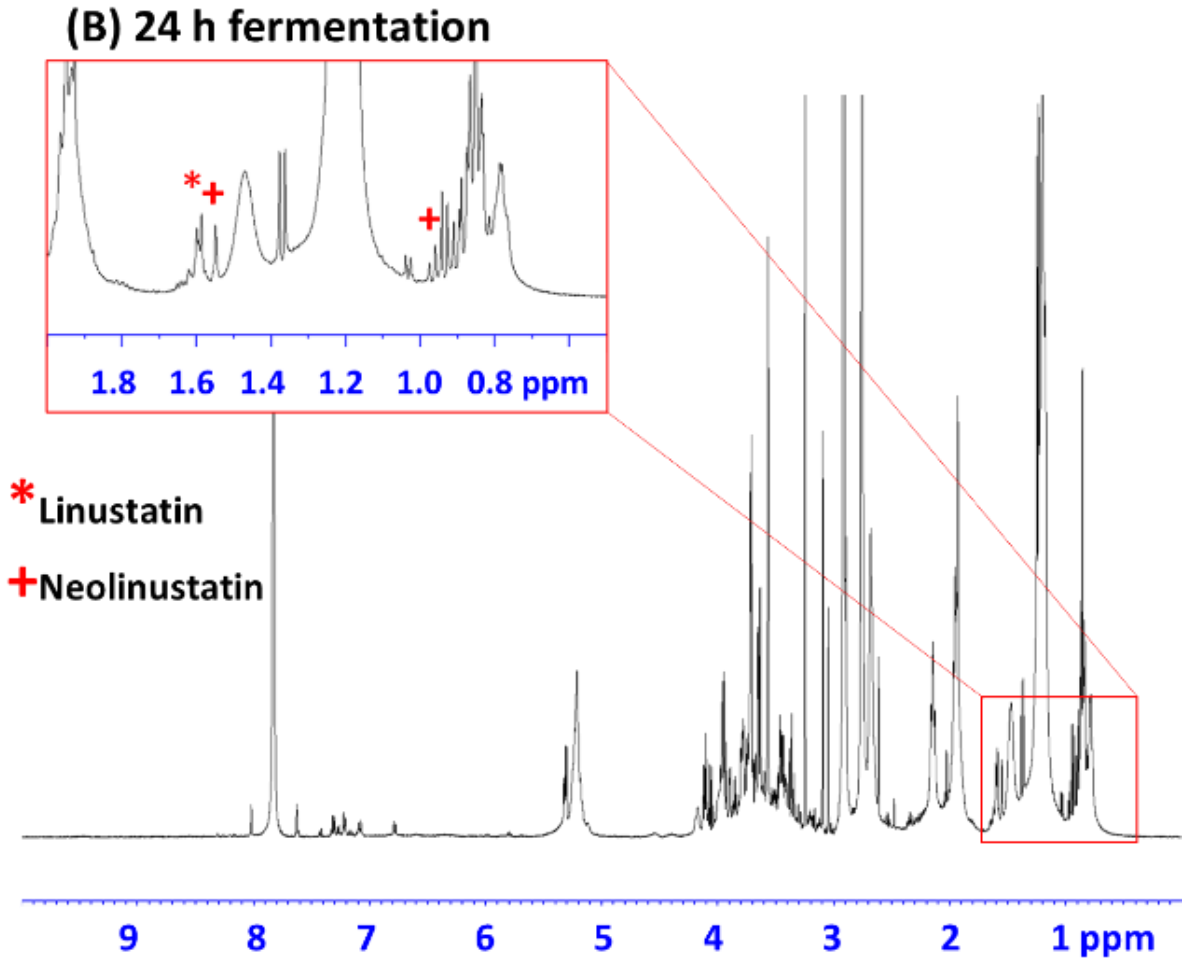


**Figure 3.2** $^{1}$H-NMR spectra of flaxseed treated by *Lactobacillaceae* after 24 h fermentation

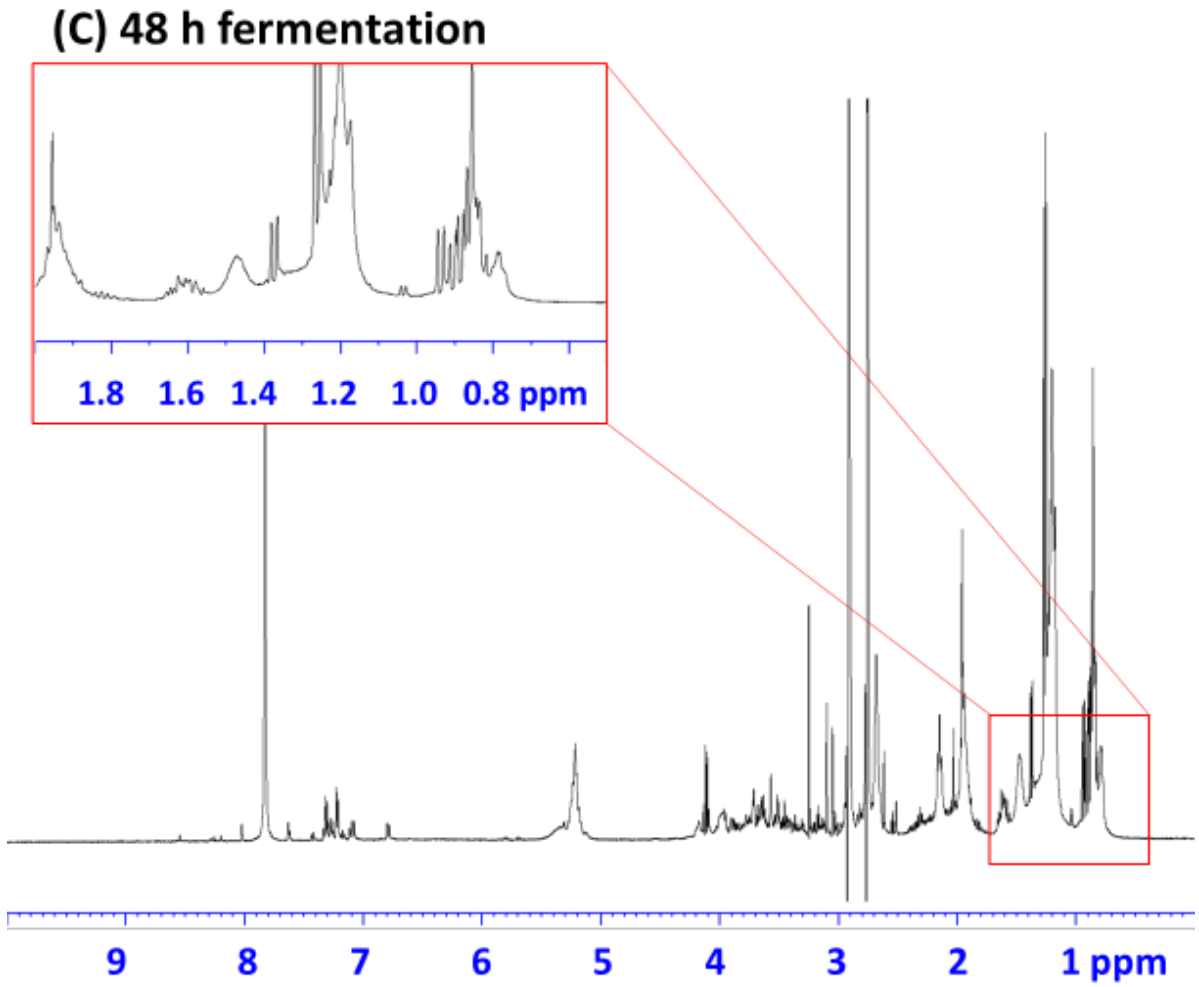


**Figure 3.3** $^{1}$H-NMR spectra of flaxseed treated by *Lactobacillaceae* after 48 h fermentation: linustatin and neolinustatin were below detection limits.

### 3.4.2 Scale-up experiments for removing CGs

Larger scale experiments (340 g and 1 kg) and bench tests (35 g) all demonstrated similar results regarding the depletion of cyanogenic glycosides (inferred from the loss of linustatin, and neolinustatin) within 72 h of fermentation (Figure 3.4). Initial values for total HCN in untreated flaxseed was 351 mg/kg. After inoculation with the *Lactobacillaceae* culture, an observable reduction in cyanogenic glycoside concentrations was evident after 48 h of bacterial fermentation, and total HCN was below detection limits after 72 h of fermentation (preformed in triplicate, Table 3.1). In bench scale experiments (~35 g of flaxseed) CG was removed after 48 h, whereas in scale-up experiments (1kg flaxseed), this removal of CG did not occur until 72 h had elapsed. This could be due to a larger volume of flaxseed requiring additional time for some species of *Lactobacillaceae* to interact with seeds, and a longer time for temperature to normalize. This issue may be solved by improved heat exchange and mixing during the fermentation process. Differences of HCN content amongst flaxseed cultivars was not investigated in this study, as the goal is to demonstrate the effectiveness of *Lactobacillaceae* fermentation. Studies of different cultivars and method refinements are planned for future work.

**Table 3.1** Flaxseed cyanogenic glycoside concentration during *Lactobacillaceae* fermentations.

| **Treatment** | **Time (h)** | **Linustatin (mg/kg)** | **Neolinustatin (mg/kg)** | **Total HCN (mg/kg)** |
|---|---|---|---|---|
| Untreated Flax | --- | 2659.3 ± 45.7 | 2761.4 ± 16.8 | 351.8 ± 2.5 |
| 1 kg | 72 | < 500 | < 500 | < 10 |

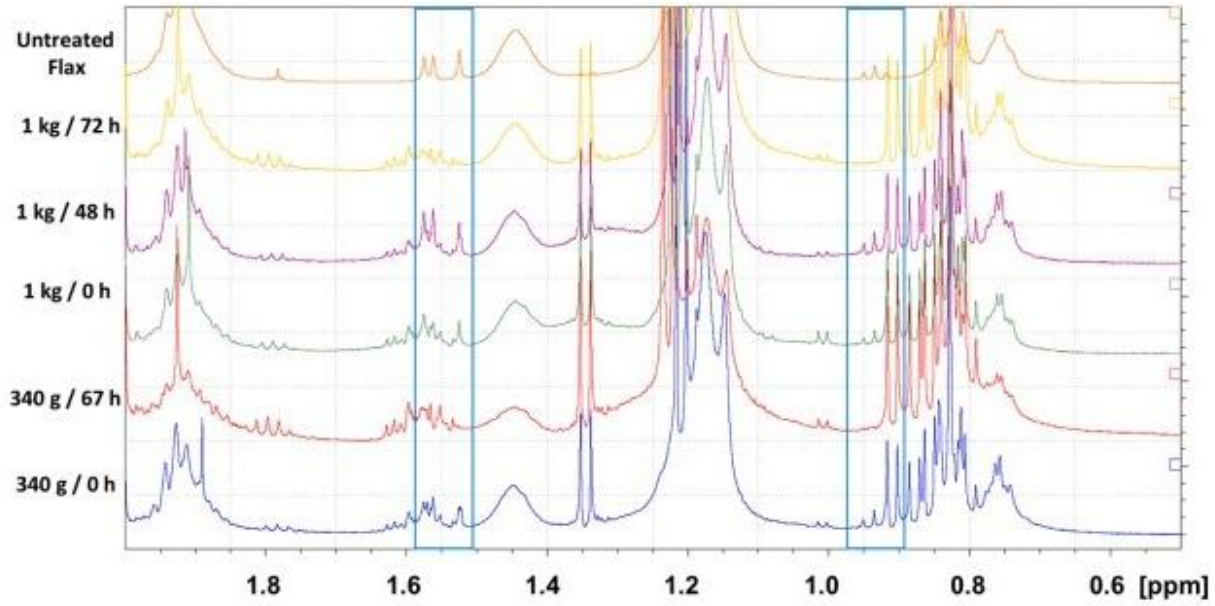


**Figure 3.4** $^{1}$H-NMR spectra of flaxseed treated by soaking in a solution of Lactobacillaceae. Linustatin is represented by the one resonance at 1.5 ppm, and neolinustatin is represented by two resonances at 1.5 ppm and 0.9 ppm.

### 3.4.3 Bacteria community composition

Metagenomic sequencing of the 16S rRNA region confirmed that the community composition remained like that reported in an earlier study (Tse et al. 2020), where the predominant genus identified were Lactobacillus, Limosilactobacillus, Lacctiplantibacillus, Lacticaseibacillus, Levilactobacillus, and Lentilactobacillus (formerly collectively grouped as Lactobacillus) (Zheng et al. 2020). (Figure 3.5). Likewise, the α-diversity of the bacterial culture were similar between treatment groups and the initial culture inoculant (Figure S2). Interestingly, the beta-diversity between these two fermentation conditions indicated some dissimilarity (Figure S3), with an increase in *Lactobacillaceae* relative abundance when cultured in the presence of acetic acid (Figure 3.5). The *Lactobacillaceae* identified in this culture previously belonged to the genera Lactobacillus, Limosilactobacillus, Levilactobacillus, Lentilactobacillus, Lacticaseibacillus, and Lactiplantibacillus (Tse et al. 2020).

*Lactobacillaceae* are resistant to acidic environments (Tannock 2004), tolerating pH as low as 1.0 (Jin et al. 1998). The increase in the relative abundance of *Lactobacillaceae* could be attributed to ideal growth conditions at lower pH values. As this culture was originally selected by fermentation on wheat-based thin stillage with a pH ranging between 3.8 and 4.6 (Tse et al. 2020), whole flaxseed fermented in the presence of acetic acid (e.g., white vinegar) was employed to provide favorable growth conditions for the *Lactobacillaceae* culture (pH of 5). However, subsequent continual fermentations were later conducted in the absence of acetic acid, as reductions in CGs were similar in both treatment groups. Interestingly, *Enterococcus* were not previously present in this culture, and might be introduced with bacteria from insects or birds during flax harvest or storage (Channaiah et al. 2010). In addition, *Pediococcus* microorganisms were absent, possibly due to the acidic conditions of the fermentation (Department of Energy Joint Genome Project 2002). Nonetheless, the fermentation of whole flaxseed using *Lactobacillaceae* successfully removed CG within 72 h, in both the presence and absence of acetic acid, as well as in the scale-up experiments.

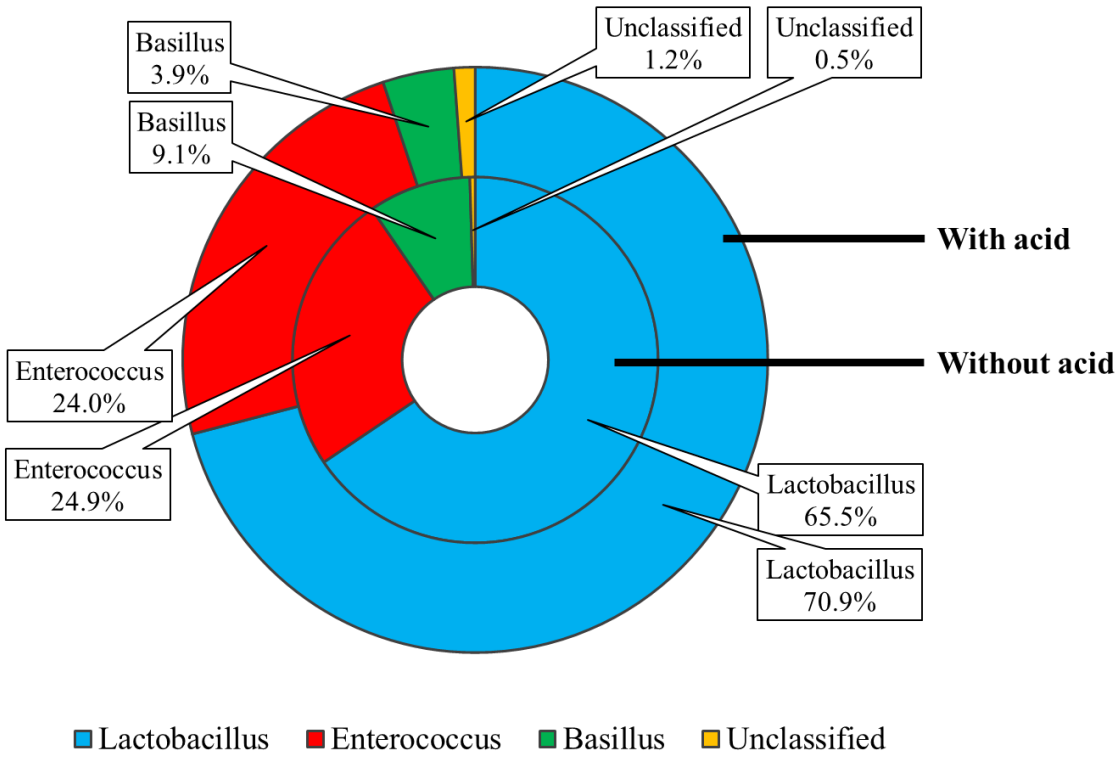


**Figure 3.5** Community composition of bacterial culture used in flaxseed fermentation without the presence of acid and in the presence of acid.

### 3.4.4 Oil content, SDG content and fatty acid composition

Fatty acid composition remained similar between untreated and treated flaxseed (Table 3.2), particularly, C18:3, C18:2, C18:1, C16:0, C18:0 which represented approximately 60%, 15%, 16%, 5% and 3%, respectively, for both untreated and treated flaxseed. This result indicates that the *Lactobacillaceae* fermentation of whole flaxseed reduces CG values but does not significantly alter flaxseed oil fatty acid composition. Additionally, oil content was observed to increase significantly ($p$-value < 0.05) in treated flaxseed (54.40%) compared to untreated flaxseed (41.20%) (Table 3.2). This increase is likely attributed to the loss of polar solutes from the flaxseed. The flaxseed would lose carbohydrates, amino acids, inorganic ions, flax gum, and conlinin during the fermentation (Liu et al. 2016).

After fermentation, SDG content increased to 4.13% ($p$-value < 0.05) compared to untreated flaxseed, 2.26% (Table 3.2). Similarly, this marked increase in SDG content could be caused by mass lost from the flaxseed, as described above, or attributed to bacterial metabolism (e.g., enzymatic degradation) that released SDG from its co-polymer structure. Enzymatic hydrolysis with esterase can release SDG from the SDG polymer, and further hydrolysis with β-glucosidase can be present in some *Lactobacillus sp.* (Michlmayr and Kneifel 2014) which can then cleave the glucose groups from SDG, yielding secoisolariciresinol (Tse et al. 2022). It is not known if SDG is polymerized with other molecules and releasable by bacterial fermentation.

### 3.4.5 Moisture content

Low-field NMR analyses revealed that the moisture content in dry fermented flaxseed, and untreated flaxseed were 9.82% and 7.55%, respectively. In addition, moisture content increased in whole flaxseed during fermentation.

**Table 3.2** Oil content (%), secoisolariciresinol diglucoside (SDG) content (%) and fatty acid composition (mean ± standard deviation) in untreated flaxseed and flaxseed treated *via* bacterial fermentation.

| | Untreated Flaxseed (mean ± SD) | Treated Flaxseed (mean ± SD) |
|---|---|---|
| SDG (%) | 2.26 ± 0.13 | 4.13 ± 0.12 |
| Oil (%) | 41.20 ± 0.00 | 54.40 ± 1.70 |
| C18:3 (% in oil) | 59.02 ± 0.46 | 58.39 ± 0.29 |
| C18:2 (% in oil) | 15.93 ± 0.47 | 15.22 ± 0.28 |
| C18:1 (% in oil) | 15.97 ± 0.63 | 16.04 ± 0.49 |
| C16:0 (% in oil) | 5.16 ± 0.10 | 5.12 ± 0.08 |
| C18:0 (% in oil) | 3.07 ± 0.05 | 3.07 ± 0.02 |

### 3.4.6 Mass balance

Mass balance of whole flaxseed was determined by weighing the flaxseed at each stage of processing. The weight of flaxseed increased after fermentation and after 1st degumming due to water absorption by the seed (Figure 3.6). This was followed by a decrease in weight during the degumming process, owing to the removal of flaxseed gum and associated water. Drying of the seed then removed the residual water, resulting in an overall weight loss. Although, minimal mass loss was observed after 72 h of fermentation, there was appreciable mass loss observed during the degumming and drying processes. This loss of mass observed in flaxseed is due to the removal of gum, conlinin, small water-soluble molecules, and water. Because of the processing time and seed quantity, the mass balance experiment was performed in an unreplicated study but is meant to demonstrate the benefits of multiple degumming stages for both flaxseed and extracted flax gum. The extracted flaxseed gum can further be concentrated by ethanol and marketed as a value-added product (Liu et al. 2018), which has usage as a food thickener, emulsifier, and foaming agent (Hu et al. 2020).

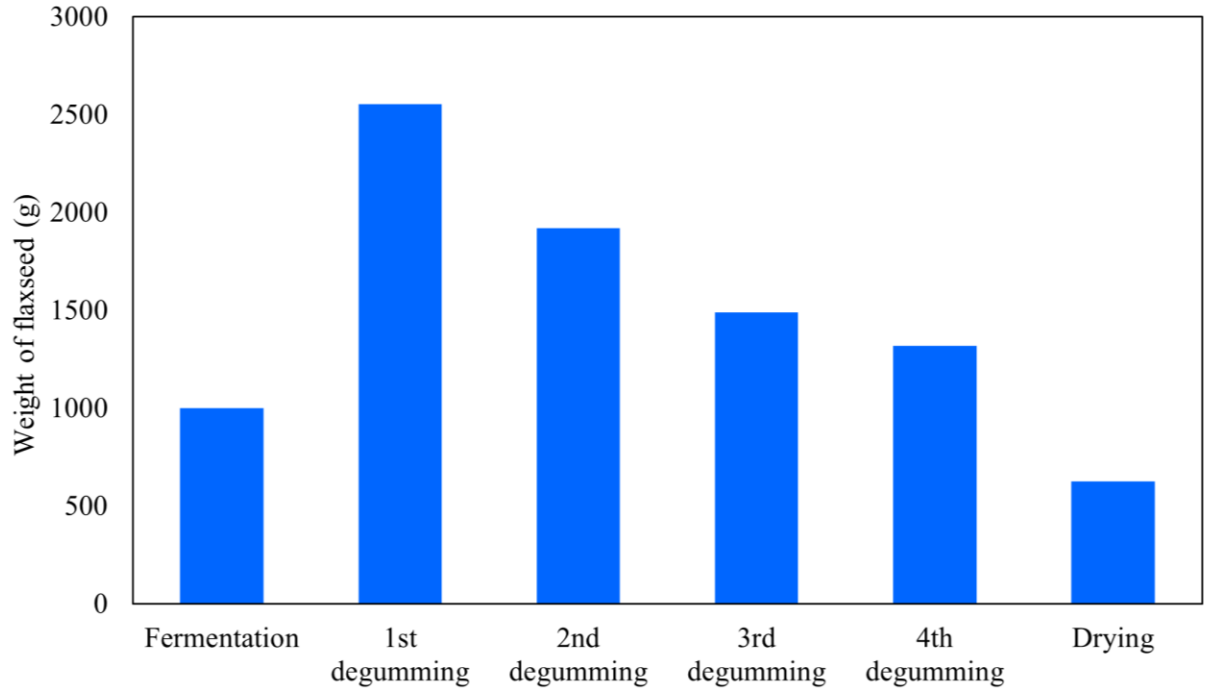


**Figure 3.6** Changes in flaxseed weight during the degumming process.

## 3.5 Conclusion

Fermentation of flaxseed using the lactic acid bacteria, *Lactobacillaceae*, showed successful removal of cyanogenic glycosides, while maintaining the levels of beneficial flaxseed nutritional components (oil, SDG, and fatty acids). Removal of CG from flaxseed offers a safer product that can be used in the production of health and natural food products. Some countries, including Japan and South Korea, have strict regulations regarding CG content of imported flaxseed; therefore, CG-free flaxseed could help improve the value and trade of this commodity. This research shows that fermentation of flaxseed, using a consortium of *Lactobacillaceae*, was able to remove HCN in 1 kg of flaxseed from 351.82 mg/kg to below the detection limits in 72 h. This method offers an economically and environmentally friendly alternative in removing CGs from whole flaxseed. The proposed methodology can be readily implemented during flaxseed processing, as demonstrated in our pilot-scale feasibility study, and can further developed to produce higher quality flaxseed that can be safely and readily used in the production of food, feed, and natural health products.

## 3.6 CRediT authorship contribution statement

**Chao Huang:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing-Original Draft, Visualization. **Timothy J. Tse**: Methodology, Formal analysis, Data Curation, Writing-Original Draft, Visualization. **Sarah K. Purdy:** Conceptualization, Methodology, Investigation, Supervision, Project administration, Funding acquisition. **Farley Chicilo:** Validation, Writing-Review & Editing. **Jianheng Shen:** Methodology, Formal analysis, Investigation, Resources, Visualization. **Venkatesh Meda:** Conceptualization, Supervision, Writing-Review & Editing. **Martin J. T. Reaney:** Conceptualization, Supervision, Resources, Writing-Review & Editing, Funding acquisition.

## 3.7 Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Martin J. T. Reaney is the founder of and has an equity interest in, Prairie Tide Diversified Inc. (PTD, Saskatoon, SK, Canada: previous company name is Prairie Tide Chemicals Inc.).

## 3.8 Data availability

The data that has been used is confidential.

## 3.9 Acknowledgement

The authors wish to thank: the Plant Sciences department at the University of Saskatchewan for use of their labs to conduct this study, and to Ms. Alana Weber for her assistance with Metagenomic Sequencing. The Saskatchewan Structural Sciences Centre (SSSC) is acknowledged for providing facilities and support to conduct this research. The authors express their gratitude for the funding support to Saskatchewan Agricultural Development Fund (grant numbers 20190155, 20190154, 20180281, 20180248, 20180255, 20170133); National Sciences and Engineering Research Council of Canada Discovery Grant (grant number RGPIN-2018-

06631); Mitacs (grant numbers IT19882, IT19122, IT16156); and Brain Pool Programs (grant numbers NRF-2019H1D3A2A01102248 and NRF-2020H1D3A2A02110965) through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT.

## 3.10 Appendix: Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134441.

# 4. GENERAL DISCUSSION

The results of the study indicate that *Lactobacillaceae* fermentation can effectively degrade CGs from flaxseed, which is consistent with an earlier study by Lei et al. (1999). In their research, Lei et al. (1999) reported that certain strains of *L. plantarum* were capable of breaking down CGs in laboratory media within 30 hours of fermentation. Similarly, the current study found a significant decrease in linustatin and neolinustatin levels in flaxseed after 24 hours of *Lactobacillaceae* fermentation.

Interestingly, this study's findings contradict those of a previous study by Yamashita et al. (2007), which suggested that freshly ground flaxseed was the most effective source of enzymes for decomposing cyanogen compounds. In contrast, the current study demonstrated that CGs can be removed from whole flaxseed through *Lactobacillaceae* fermentation without the need for grinding the seed. This is due to the fact that *Lactobacillaceae* produces β-glucosidase, an enzyme that breaks down CGs into hydrogen cyanide (HCN), while the acidic broth of Lactobacillaceae fermentation facilitates the volatilization of HCN.

The study monitored the reduction of major CGs, linustatin and neolinustatin, and total HCN, by taking samples at 24 hours, 48 hours, and 72 hours during fermentation. Results showed that CGs were removed from whole flaxseed after 72 hours of fermentation in both bench and large-scale experiments. To improve the study, collecting samples more frequently, such as every hour, could be done to create a curve that shows how CGs degrade over 72 hours. This curve would then estimate the optimum fermentation time.

The *Lactobacillaceae* identified in this study belonged to the genus *Lactobacillus*, *Limosilactobacillus*, *Levilactobacillus*, *Lentilactobacillus*, *Lacticaseibacillus*, and *Lactiplantibacillus.* This differs from the in-house consortium of *Lactobacillaceae* bacteria trained by Ratanapariyanuch et al. (2016, 2017) and upgraded by Tse et al. (2020), indicating that the consortium of bacteria can change and evolve in different food processing applications.

Further investigation into its use in other food fermentations, such as Korean kimchi, could provide a deeper insight into the utility of this process.

The study found that the treated flaxseed via *Lactobacillaceae* fermentation retains its nutritional components, such as oil and free fatty acids. This is consistent with previous research by Wu et al. (2012), which showed similar results using enzymatic fermentation to remove CGs from flaxseed meal. The study also observed an improvement in SDG concentration. This may be due to the consumption of the flaxseed hull, which contains lignans, by the *Lactobacillaceae*, leading to the release of SDG. Further research is needed to understand this mechanism.

The downside of the end product, dried fermented flaxseed, is the absence of flaxseed fibre (flax gum) since the flax gum was collected during the process to concentrate it further using ethanol as a byproduct. Additionally, the removal of the gum resulted in a yield of 626.3 g of dried fermented seed from processing 1 kg of untreated flaxseed. To achieve the dual objectives of preserving flaxseed fibre and increasing yield, modifications are required in the degumming and drying processes.

In this study, the fermented flaxseed was dried using a food dehydrator overnight at 60 °C, which was not sufficiently high to eliminate the bacteria that remained on the flaxseed. To enhance the drying process, a couple of steps can be taken. Firstly, the drying temperature can be raised to 74°C to pasteurize the flaxseed, ensuring that the dried flaxseed is safe for human consumption. Secondly, the moisture content of the wet flaxseed should be monitored throughout the process, enabling estimation of the optimal drying time.

Having discussed the pros and cons of this study, it is evident that *Lactobacillaceae* fermentation holds promise as a method to reduce CGs while preserving the nutritional components of whole flaxseed. These results present potential applications in the food industry.

# 5. SUMMARY CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Summary and Conclusions

The conclusion of the study is that using a consortium culture of *Lactobacillaceae* for fermenting whole flaxseed is a promising approach for removing cyanogenic glucosides (CGs) while maintaining its nutritional properties. Results showed that after 72 hours of fermentation at 30°C, CGs were significantly reduced while the concentration of flaxseed oil and SDG content improved. Furthermore, the fatty acid composition of the whole flaxseed remained unchanged during fermentation.

The use of whole flaxseed as opposed to flaxseed powder has several advantages. Firstly, using whole flaxseed ensures that all the nutrients, including oils and fatty acids, are preserved in their natural state. Additionally, the hull of the flaxseed, which contains lignan, can be consumed by *Lactobacillaceae* during fermentation, leading to an improvement in the SDG concentration.

Overall, the results of this study suggest that *Lactobacillaceae* fermentation of whole flaxseed is an effective method for removing harmful CGs while retaining its nutritional value. The CG-free flaxseed produced through this process can be utilized in various applications, including human food and animal feed, and can be exported to international markets with restrictions on CG levels in flaxseed-based foods.

## 5.2 Recommendations

### 5.2.1 Studying the Microbial Composition

To gain a better understanding of the microbial composition of the *Lactobacillaceae* consortium, it is recommended to conduct more in-depth microbiological analysis, including sequencing and microbial identification.

### 5.2.2 Exploring Other Temperature and Time Conditions

Different food products may require different fermentation conditions to achieve optimal results. It is recommended to explore other temperature and time conditions to determine the best conditions for removing CGs from flaxseed or other food products. Additionally, it is recommended to explore the duration and temperature required for drying wet flaxseed to enhance the efficiency of the drying process, ensuring that the final product is both pasteurized and consistent.

### 5.2.3 Evaluating Nutritional and Health Benefits

The fermented flaxseed has the potential to offer a range of nutritional and health benefits. It is advisable to carry out studies that assess the nutritional composition and potential health advantages of fermented flaxseed, while also determining the optimal dosage for consumption.

### 5.2.4 Investigating Commercial Applications

The results of this study suggest that the *Lactobacillaceae* fermentation of whole flaxseed is a promising technology for removing CGs from food products. It is recommended to conduct a feasibility study to investigate the commercial applications of this technology and determine the most economically viable way to scale up the process for industrial applications.

Furthermore, it is recommended to perform a techno-economic analysis to evaluate the economic viability of the process, as well as a life cycle assessment to assess the environmental impacts associated with the entire life cycle of both the product and the process.

### 5.2.5 Evaluating the Effect of Fermentation on Other Compounds

It is possible that the *Lactobacillaceae* fermentation could influence other compounds in the flaxseed or other food products. It is recommended to evaluate the effect of fermentation on the quality and safety of these products to ensure that they are safe for consumption.

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# APPENDIX: SUPPLEMENTARY FIGURES AND TABLE

(A (B

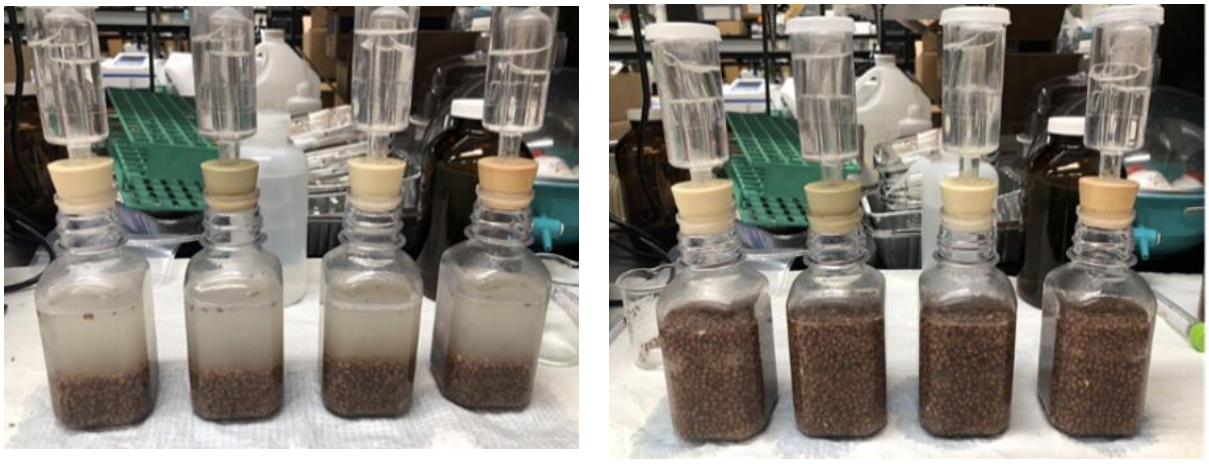

**Figure S1**. Bench-scale bacterial fermentation experiments: (A) 0 h fermentation; (B) after 72 h fermentation.

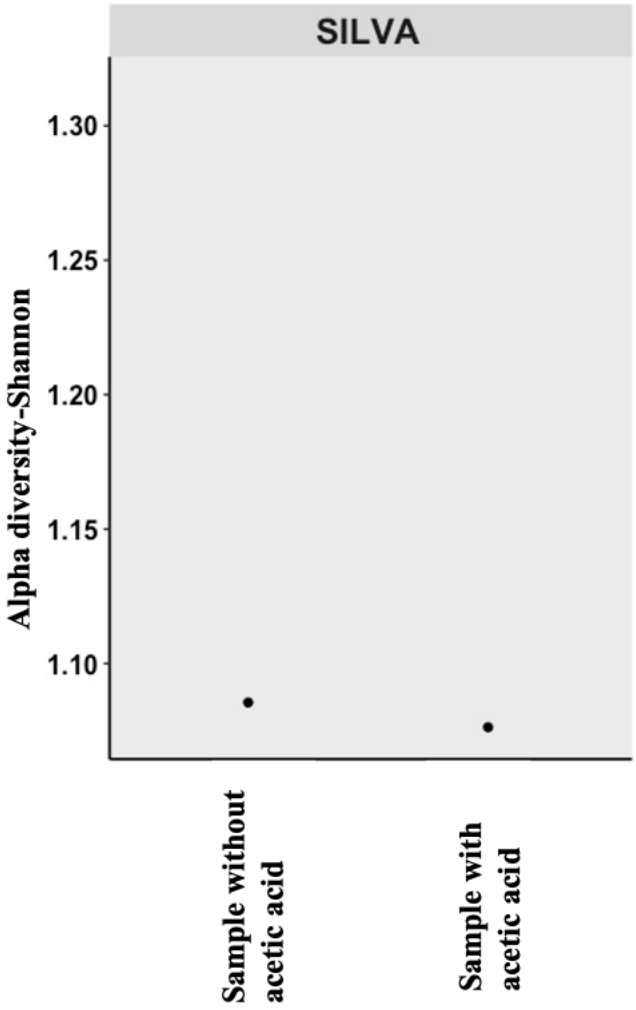


**Figure S2**. Alpha-diversity of bacterial culture used in flaxseed fermentation, without the presence of acid (left) and in the presence of acid (right).

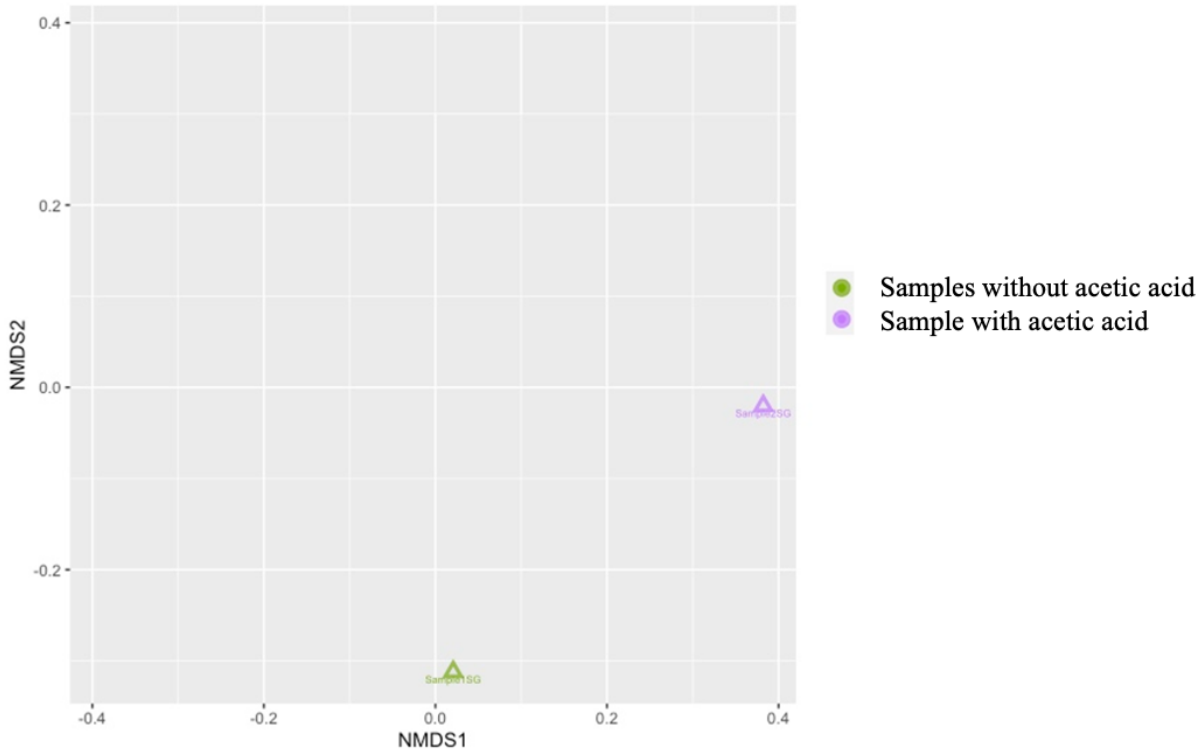


**Figure S3**. Beta-diversity of bacterial culture used in flaxseed fermentation, without the presence of acid (purple) and in the presence of acid (green).


**Figure S4.** Bench-scale fermentation at 30 °C using a Büchi water bath B-490 (Flawil, Switzerland) for 72 h.


**Figure S5.** 340 g flaxseed fermentation in a VWR 1390FM mechanical oven incubator (Radnor, PA, USA) at 30 ℃ for 72 h.

**Figure S6.** 1 kg flaxseed fermentation flaxseed fermentation in a VWR 1390FM mechanical oven incubator (Radnor, PA, USA) at 30 ℃ for 72 h.


**Figure S7**. Robot Coupe C80 automatic sieve (Moncton, NB, Canada)


**Figure S8**. A VWR® forced air oven (Radnor, PA, USA)


**Figure S9.** A Cabela's® 160 L commercial-grade food dehydrator (Sidney, Nebraska, USA)

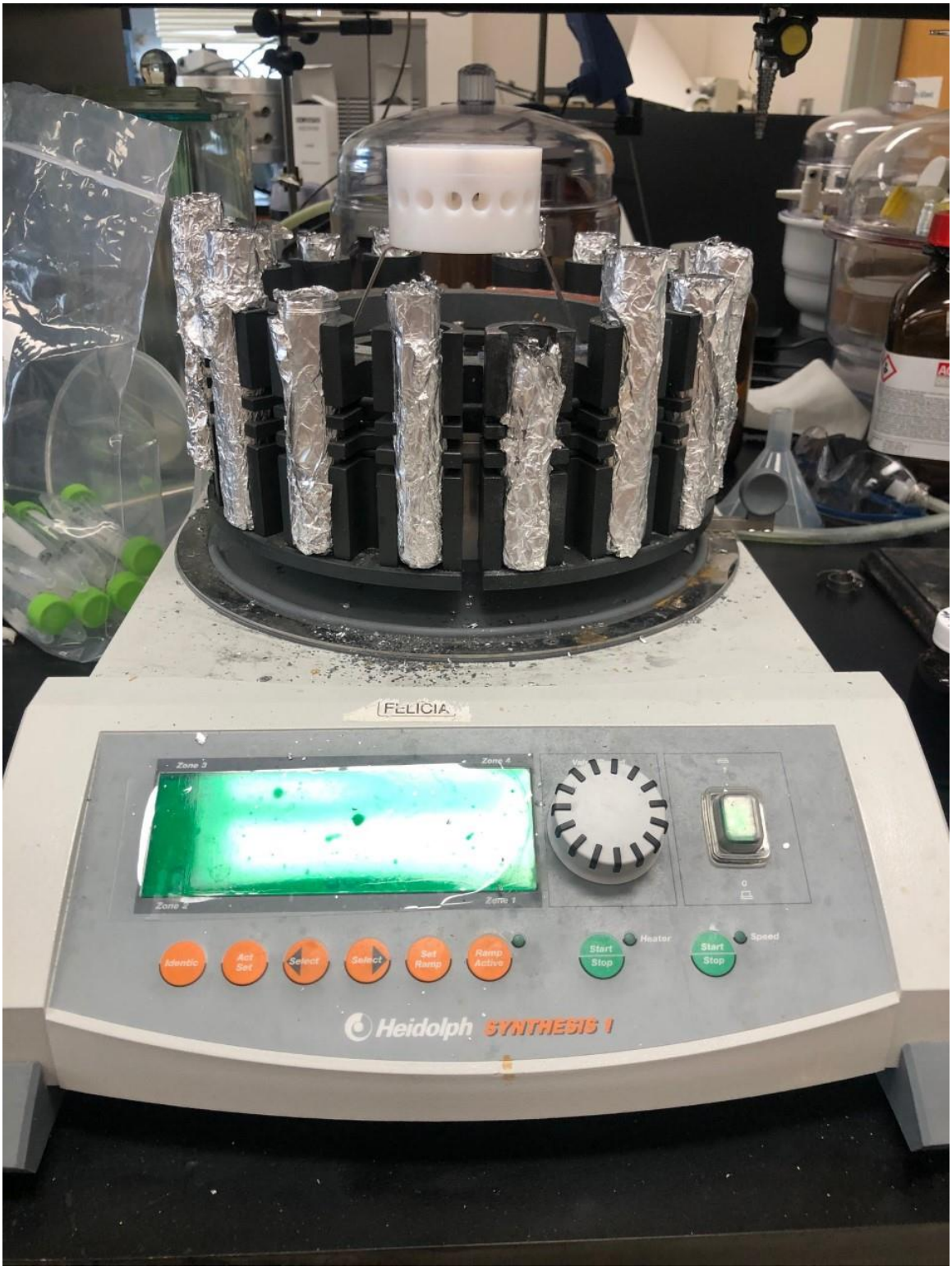


**Figure S10.** A Heidolph Synthesis 1 shaker (Schwabach, Germany)


**Figure S11.** A Beckman Coulter Allegra X-22K centrifuge (Brea, CA, USA)


**Figure S12.** A Büchi rotavapor R-210


**Figure S13.** Bruker Avance III HD 500 MHz spectrometer (Mississauga, ON, Canada)

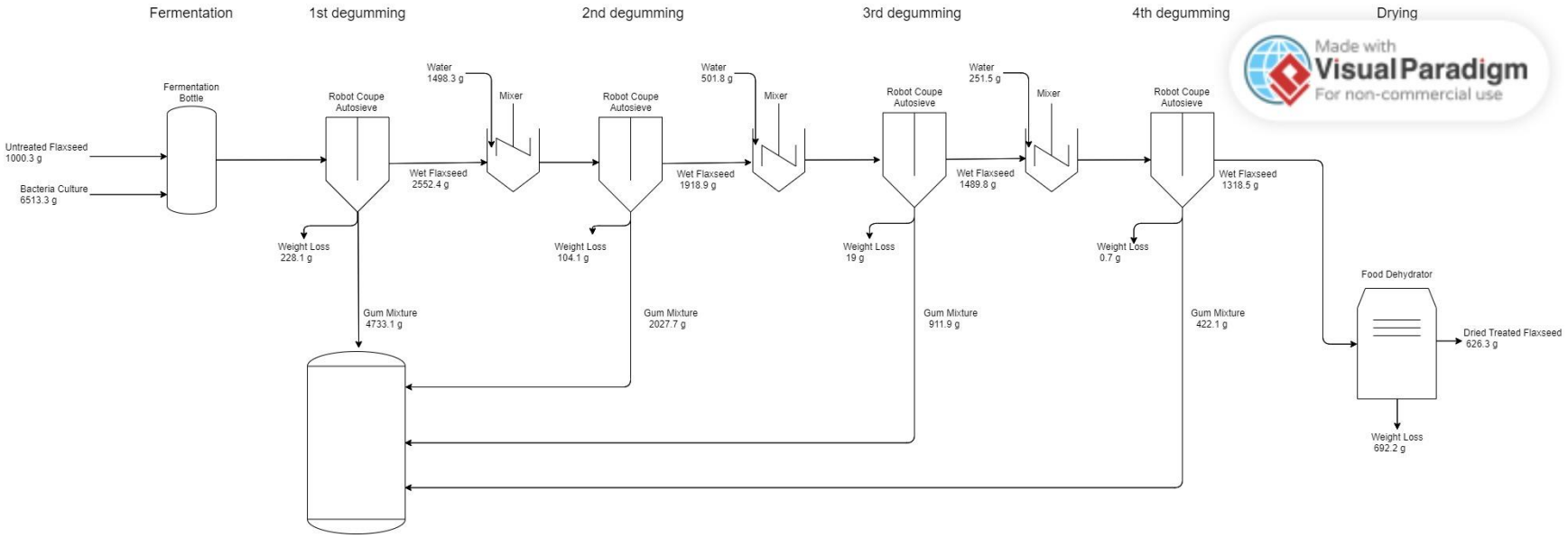


**Figure S14.** Flaxseed Fermentation Process Flowchart Created using Visual Paradigm (visual-paradigm.com)

**Table S1.** Mass Balance Data for Flaxseed Fermentation Process

| | Input (g) | Output(g) | Weight loss (g) |
|---|---|---|---|
| Fermentation | 7513.6 | 7513.6 | 0 |
| 1st degum | 7513.6 | 7285.5 | 228.1 |
| 2nd degum | 4050.7 | 3946.6 | 104.1 |
| 3rd degum | 2420.7 | 2401.7 | 19 |
| 4th degum | 1741.3 | 1740.6 | 0.7 |
| Drying | 1243.3 | 626.3 | 617 |

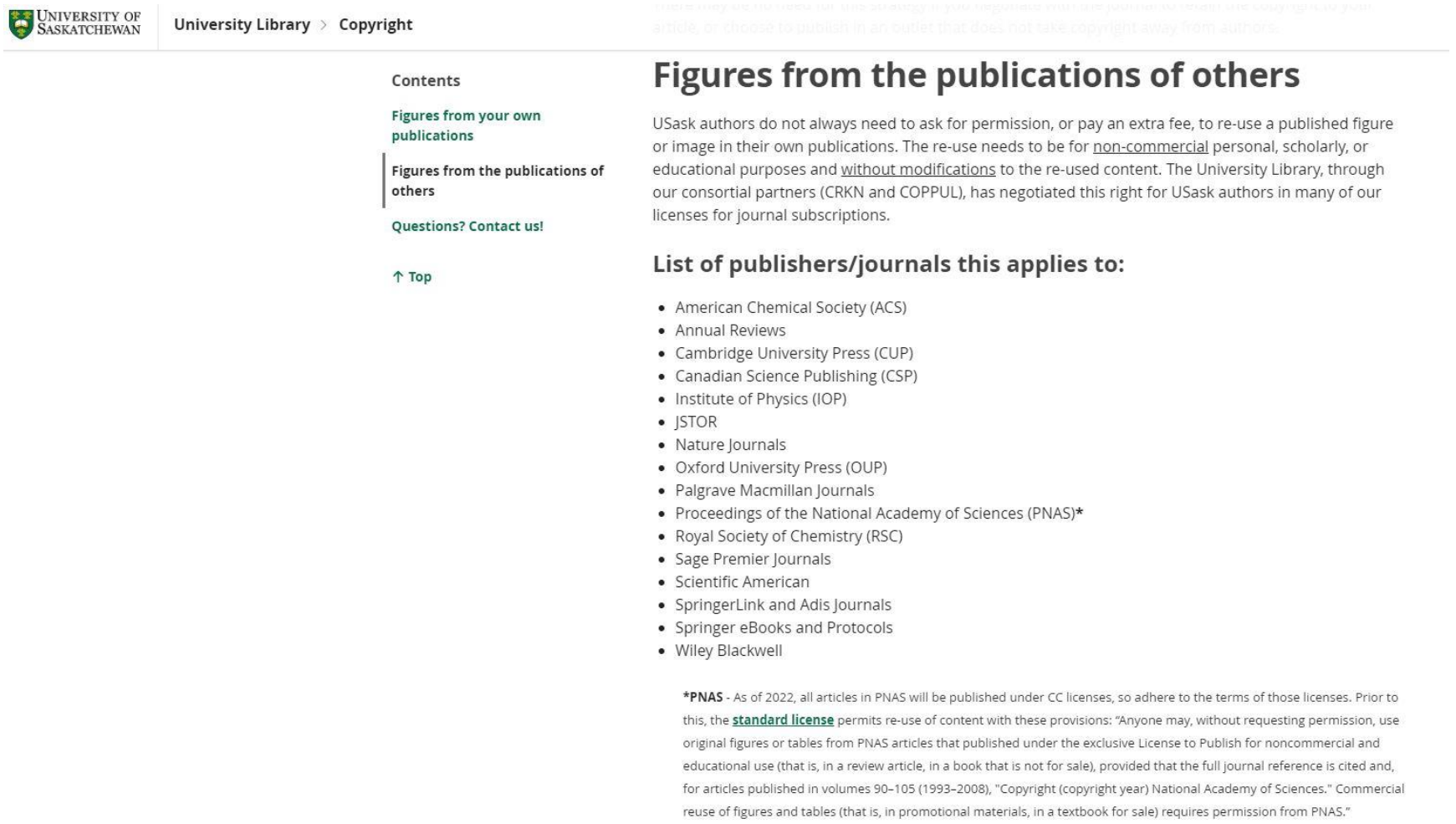


**Figure S15.** Copyright using Figures from the Publications of Others

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