ABSTRACT

Title of Thesis: FERMENTING KALE VEGETABLE (*Brassica oleracea Var Sabella*) IMPROVES ITS PROPERTIES AS A FUNCTIONAL FOOD

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The properties of kale as a functional food are well known. Fermentation is a process that has been shown to improve health impacts of foods. In this study we sought to determine how fermentation further improves or augments the functional food properties of kale. We tested six different fermentation methods which included traditional practices and inoculation with different bacterial species and compared outcomes to the unfermented control. After 16 days of fermentation, we quantified (i) selected bioactive components and (ii) anti-nutritional factors. We then determined (i) the antioxidant capacity of the whole vegetable, (ii) the microbiota composition of the vegetable and (iii) the anti-inflammation capacity of the ethanolic extract of the vegetable.

Fermentation significantly increased (i) the quantities of total polyphenols from 8.54 to 10.71 mg GAE/g (ii) sulforaphane from 960.8 ± 41.76 to 1777 ± 45.95 μg/g, and (iii) antioxidant capacity from 61.99 to 67.37 % respectively, and antinutritional factors

oxalate and tannin content significantly reduced by 49 % and 29.83 % respectively. Fermented kale extract exhibited potent anti-inflammatory effects in macrophages by reducing the iNOS expression by 84.3% and TNF-α, IL-1β, and IL-6 mRNA levels by 62, 68, and 85.5 %, respectively. Fermenting kale changed the surface microbiota by reducing the population of the inflammation-inducing Proteobacteria while increasing health-promoting Firmicutes; including Lactobacillus. All fermentation methods had a beneficial impact compared to the unfermented control, but the mixed culture of *L. lactis* and *L. acidophilus* was most effective.

In summary, fermenting enhanced the health benefits of kale by increasing concentration of total polyphenol, sulforaphane content, antioxidant capacity, anti-inflammation capacity and reducing the quantity of anti-nutritional factors. Furthermore, it promoted the prebiotic and/or probiotic vehicle properties of the vegetable by changing the proportion of beneficial bacteria and those associated with inflammation.

FERMENTING KALE VEGETABLE (*Brassica oleracea Var Sabella*) IMPROVES ITS PROPERTIES AS A FUNCTIONAL FOOD

by

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List of Abbreviations

DPPH: 2,2-diphenyl-1-picrylhydrazyl radical

ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

TPC: Total phenolic content

GAE: Gallic acid equivalent

TE: Trolox equivalent

QA: Quercetin equivalent

AC: Antioxidant capacity

LAB: Lactic acid bacteria

iNOS: Inducible Nitric Oxide Synthase

NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

LPS: Lipopolysaccharide

IL-1β: Interleukin-1 beta

IL-6: Interleukin-6

TNF-α: Tumor Necrosis Factor-alpha

Chapter 1: Introduction

1.1 Functional Foods

A functional food can be defined as any food or food components that provide health benefits beyond essential nutrition positively impacts health, physical performance, or state of mind. Any natural or processed foods that contain biologically active compounds, which, in defined, effective, and non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers for the prevention, management, or treatment of a chronic disease or its symptoms are considered functional food (Gur et al., 2018). The main component behind a functional food is bioactive compounds which are primary and secondary metabolites with nutritive and non-nutritive natural components which usually occur in small amounts in various food sources and exhibit beneficial biological activities like antioxidants, chemo- and cardio-preventive functions and can even reduce risk or prevent the onset of certain diseases (Gur et al., 2018). Foods that contain minerals, vitamins, dietary fibers, and fatty acids with additional biologically active components, like antioxidants and probiotics, are considered functional foods (Csapó et al., 2019).

1.2 Genus Brassica

The Brassicaceae family, or cruciferous vegetables, includes many economically important species, mainly edible oil plants, vegetable species, spice plants, and feed plants. They include cabbage, cauliflower, broccoli, brussels sprout, kale, spinach, and mustard which belong to the genus *Brassica* (family *Brassicaceae*), are cultivated and consumed globally (Hahn et al., 2016). Cruciferous vegetables are foods rich in nutritive composition and an excellent dietary fiber source. Besides,

cruciferous vegetables contain various bioactive glucosinolates and S-methyl cysteine sulfoxide, including sulfur-containing cancer-protective chemicals (Ağagündüz et al., 2022). The health benefits of cruciferous vegetables are associated with various phytonutrients including glucosinolates, polyphenols, carotenoids, or terpenoids groups (Šamec et al., 2019).

Green curly kale (*Brassica oleracea var. sabellica* L.) is a leafy vegetable popular in Southern America, Europe, and Northern Asia (Michalak et al., 2018). Kale originated from the eastern Mediterranean and Asia regions and later spread worldwide through immigrants, travelers, and merchants. It has been considered a food crop since 2000 B.C. (Satheesh and Workneh, 2020a). Kale is an annual crop whose size and nutritional variation depend on the variety and growing conditions. Varieties include green kale, dwarf kale, marrow-stem kale, tronchuda kale, curly leaf kale, scotch kale, tree kale, and bore kale (Satheesh and Workneh, 2020). Kale is listed as one of the powerhouse fruits and vegetables (PFV) as it is rich in flavonoids, polyphenols, vitamins, carotenoids, glucosinolates, prebiotic carbohydrates, and dietary fiber, which confer numerous health benefits and reduces the risk of chronic diseases (Migliozzi et al., 2015; Šalić and Šamec, 2022a).

The most popular cruciferous vegetable in the United States is broccoli, whose phytochemistry and biological activity were extensively studied and reviewed. However, in the last couple of years, the popularity of kale has increased, and there is even an event celebrated in the United States as National Kale Day (on the first Wednesday in October). The celebration casts light on kale's versatility and amazing health benefits and plays a crucial role in increasing awareness and encouraging the cultivation and inclusion of kale in diets (Šamec et al., 2019). Generally, kale

vegetable is consumed as raw salads or beverages but due to its health-promoting properties use of kale in novel bioactive food stuff is gaining interest over the last few years (Michalak et al., 2020).

1.3 Fermented Foods

Fermented foods are defined as "foods or beverages produced through controlled microbial growth, and the conversion of food components through enzymatic action" (Marco et al., 2017). Different foods including meat and fish, dairy, vegetables, soybeans, cereals, and fruits have been fermented and as a method of preservation through organic acids and ethanol produced. It also helps enhance sensory attributes (taste, flavor, color, texture) (Dimidi et al., 2019). Fermentation processes are classified based on primary metabolites produced and microorganisms involved during the fermentation process. For example, alcohol and carbon dioxide production (yeast), acetic acid production (*Acetobacter*), lactic acid production (lactic acid bacteria belonging to genera such as *Leuconostoc*, *Lactobacillus*, and *Streptococcus*) (Marco et al., 2017). In recent years, fermented foods like kefir, kombucha, sauerkraut, tempeh, natto, miso, kimchi, and sourdough bread are becoming more popular globally because of their health benefits from the probiotic effect of their constituent microorganisms, the fermentation-derived production of bioactive peptides, biogenic amines, and conversion of phenolic compounds to biologically active compounds, as well as the reduction of anti-nutrients (Dimidi et al., 2019).

Fermented cabbage products like sauerkraut and kimchi are globally popular fermented *Brassica* products (Michalak et al., 2018). Gundruk is a non-salted fermented green leafy vegetable prepared from the lactic acid fermentation of fresh

leaves of rayo (*Brassica rapa*), mustard (*Brassica juncea*), and cauliflower (*Brassica oleracea*). It is indigenous to the Nepali people and is popular in the Himalayan region of Nepal, India, and Bhutan (Tamang and Tamang, 2010).

1.4 Justification of the Study

Broccoli is the most widely studied and reviewed *Brassica* vegetable. Considering the undeniable nutritional and health-beneficial potential of kale and the advantages of fermented foods, only limited scientific data were available on how the fermentation process impacts on health-beneficial aspects of this vegetable. Different brassica vegetables are globally consumed as popular fermented vegetables. However, few scientific explorations have been made regarding using kale as a fermented vegetable and a potential value-added functional food. With better mineral compositions, kale contains a high concentration of oxalates which is a major anti-nutritional component, along with tannins, phytates, and nitrogen compounds (Šamec et al., 2018). Reduction of antinutritional factors present in this vegetable is significant for efficient nutrient uptake during its consumption but studies on the ways to reduce the antinutritional factors like tannins and oxalates were very limited.

Fewer studies have been reported regarding probiotic potential of fermented kale juice (Kim, 2017; Szutowska and Gwiazdowska, 2021) and the fermentation of kale leaves with *Lactobacillus* strains to develop functional food with antitumor potential (Michalak et al., 2018, 2020). Preliminary studies have concluded that kale improves metabolic health (Raychaudhuri et al., 2021) and attenuates inflammation and modulates gut microbial composition (Shahinozzaman et al., 2021). Thus, in this follow-up study, we studied the effect of the fermentation of kale on its role as a potential functional food. This research helped to gather scientific evidence on

whether fermented kale can provide health benefits well beyond unfermented kale based on parameters like change of microbiota composition, reduction of anti-nutritional factors, quantity of bioactive components (polyphenols, flavonoids, carotenoids, glucosinolates), antioxidant and anti-inflammatory capacities of the vegetable. Fermented kale is not yet commercially produced or consumed but lactic acid fermentation of kale has a great potential as a functional food (Michalak et al., 2020; Szutowska et al., 2021).

1.5 Objectives of the Study and Hypothesis Tested

Objective 1: Determine the impact of fermentation on the composition of bioactive components and anti-nutritional factors in kale.

Hypothesis: Fermentation enhances the availability of bioactive components and reduces anti-nutrition factors.

To test this hypothesis, bioactive components (total polyphenol, flavonoid, carotenoid, glucosinolates) and antinutritional factors (oxalate, phytate, tannin) in fermented and unfermented kale samples were quantified using spectrophotometric and high-pressure liquid chromatography techniques.

Objective 2: Determine the impact of fermentation on anti-inflammatory and antioxidant capacity of the kale.

Hypothesis: Fermentation enhances anti-inflammatory and antioxidant capacity of kale.

To test this hypothesis, we obtained kale ethanolic extract from fermented and unfermented kale samples for use in the DPPH and ABTS free radical scavenging

assays. *In vitro* tests for anti-inflammation effects were performed by qPCR in 264.7 RAW macrophages.

Objective 3: Determine the impact of fermentation on the bacterial composition of kale.

Hypothesis: Fermentation enhances the prebiotic/probiotic properties of kale by enhancing the abundance of health-associated bacteria.

To test this hypothesis, the microbiota composition of the vegetable was determined by 16S sequencing of bacterial DNA isolated from each sample. Comparisons were done between fermented and unfermented kale samples.

Chapter 2: Literature Review

2.1 Functional Foods

In 1984, the term "functional food" was first coined in Japan. The Japanese government established a new product category called Food for Specific Health Uses (FOSHU), which referred to a food that contained an ingredient with health benefits and was officially approved to claim its effects on the human body. This led to the creation of a dedicated legislative framework. The United States followed suit in the 1990s by developing the first health claim act; but did not provide a formal definition of ‘functional foods’ (Alongi and Anese, 2021).

The concept of "functional foods" is becoming acceptable or popular. Despite multiple definitions, they all revolve around the potential of consuming certain foods and nutrients to achieve health benefits and reduce disease risk. Fruits and vegetables and culinary herbs have been extensively studied for their health-promoting potential, and these foods possess exciting properties, mainly relating to the antioxidant and anti-inflammatory action of their components. which may impact the development of various diseases, including cardiovascular, neurodegenerative, or cancer. These positive effects are attributed to different bioactive compounds, such as phytochemicals, polyphenols, vitamins, minerals, or organic acids (Guerreiro et al., 2022; Fernandes et al., 2023). The physiological effects of functional food may vary, but their categories of action include physical performance, cognitive, behavioral, and psychological function, organ or system function, and combating chronic disease. Researchers are putting much effort into advancing our knowledge of functional foods (Aoi et al., 2004).

The future of functional foods could be impacted by biotechnology, leading to the development of crops that could improve public health worldwide. Golden rice and iron-enriched rice are examples of biotechnology-derived crops that can provide increased levels of beta-carotene and iron, with the potential to prevent iron deficiency anemia and vitamin A deficiency-related blindness globally. Future research can unravel other foods enriched with nutritive or non-nutritive substances to prevent chronic diseases like cancer, diabetes, osteoporosis, and other diet-related disorders (Falk et al., 2002; Shimizu and Hachimura, 2011).

Functional foods are becoming increasingly popular as people seek ways to support their overall well-being through food choices. There has been a significant rise in research focusing on functional foods globally. This surge has profoundly impacted the growth of the industry. The functional food market was around USD 162 billion in 2018 and is projected to reach USD 280 billion by 2025, with a consistent annual growth rate of around 8 % (Alongi and Anese, 2021).

2.2 Green Curly Kale

Green curly kale, classified as *Brassica oleracea L. convar. acephala var. sabellica* is a leafy vegetable popular in Southern America, Europe, and Northern Asia (Michalak et al., 2018). It is recognized by its ruffled leaves, fibrous stalks, and typically deep green color (Olsen et al., 2009a). Kale is listed as one of the powerhouse fruits and vegetables (PFV) as it is rich in flavonoids, polyphenols, vitamins, carotenoids, glucosinolates, prebiotic carbohydrates, and dietary fiber, which confer numerous health benefits and reduce the risk of chronic diseases (Michalak et al., 2020).

2.3 Nutritional value of kale

Kale is rich in minerals and nutrients. With about 3.6% dietary fiber and 8% total carbohydrates, it stands out among other cruciferous vegetables as the best source of triglycerides, folate, nicotinic acid, and vitamins C, K, A, B6, B2, and B1. Kale is also abundant in essential macro- and micronutrients such as sodium, K^{+}, Mg^{2+}, Ca^{2+}, and Fe2+. The remarkable vitamin content is impressive, with over 1,000 times the daily recommended amount of vitamin K, 98 times the amount of vitamin A, and 71 times the amount of vitamin C. As a result, the nutraceutical industry has recently taken notice of kale's health benefits (Khalid et al., 2023; Nayak et al., 2015; Thavarajah et al., 2016). Drinking a 250 ml glass of green curly kale juice can fulfill up to 63% of the recommended daily intake for Ca^{2+}, 38% for Mg^{2+}, 22% for K^{+}, 18% for Mn^{2+}, and 89% for $Na^{2+,}$ respectively and is also suitable for gut microbes as a source of prebiotic carbohydrates ranging from 0.4 to 6.7 g per 100 g vegetable (Joseph and Montefrio, 2020; Kim, 2012). The composition of kale according to the USDA Food Composition Databases is given in (Table 1).

Table 2.1. Proximates, minerals, vitamins, and fatty acids composition per 100 g of kale according to the USDA Food Composition Databases (Šamec et al., 2018)

Proximate	water (g)	84.04
	energy (kcal)	49
	protein (g)	4.28
	total lipids (g)	0.93
	Carbohydrate (g)	8.75
	Fibers (g)	3.6
	Sugars (g)	2.26
Minerals	Ca (mg)	150
	Fe (mg)	1.47
	Mg (mg)	47
	P (mg)	92
	K (mg)	491
	Na (mg)	38
	Zn (mg)	0.56

Vitamins	vitamin C (mg)	120
	thiamin (mg)	0.11
	riboflavin (mg)	0.13
	niacin (mg)	1
	B-6 (mg)	0.271
	folate (Âµg)	141
	vitamin A (Âµg)	500
	vitamin E (mg)	1.54
	vitamin K (Âµg)	704.8
Fatty acids	saturated (g)	0.091
	monounsaturated (g)	0.052
	polysaturated (g)	0.338

2.4 Bioactive components and potential health benefits of kale

Carotenoids, glucosinolates, polyphenols, and vitamins C and E are abundant in kale, making it an excellent source of nutrients. The high levels of sulforaphane in kale activate the Nrf2 gene, which increases insulin sensitivity and protects against oxidative damage caused by hyperglycemia such as nephropathy, retinopathy, and cardiomyopathy (Khalid et al., 2023). In addition to the essential nutritional compounds, kale contains antioxidants, such as polyphenols, carotenoids, glucosinolates, and vitamins C and E. Foods rich in antioxidants protect against free radicals and reactive oxygen species (ROS), thereby preventing human chronic diseases. A study of 38 commonly used vegetables reported that kale, spinach, broccoli, and rhubarb have the highest antioxidant activity (Zhou and Yu, 2006). In addition, Sikora et al. (2008); Sikora and Bodziarczyk (2012) reported that the antioxidant activity of kale at 33.2 µmol TE/g based on ABTS assay is much higher than that of broccoli, brussels sprouts, and green and white cauliflower and was kale extract also showed *in vitro* protection against the oxidation of low-density lipoproteins (LDL), which may indicate a potential protective effect against cardiovascular disease (Kural et al., 2011).

Kale's health benefits are associated with the polyphenols and glucosinolates (GSLs) which have free radical scavenging properties and also affect inflammatory properties (Choe et al., 2018). The GSLs' breakdown products are associated with anticarcinogenic properties (Verhoeven et al., 1997).

Glucosinolates

Glucosinolates are characterized by a core structure consisting of a sulfated isothiocyanate group linked to thioglucose. Their side chain modifications increase account for diversity of glucosinolates which are not biologically active until they are enzymatically hydrolyzed by the plant enzyme myrosinase, resulting in various bioactive breakdowns products such as nitriles, thiocyanates, and oxazolidinethiones (Figure 1). There are approximately 200 different glucosinolates, including aliphatic, aromatic, and indolic varieties, and their presence in different *Brassica* species isgenetically predetermined. Each cruciferous plant has a distinct glucosinolate profile that includes over ten different types, although only 3-4 are predominant (Šamec et al., 2019). The total glucosinolates content in kale from different growing locations is shown in (Table 2).

Table 2.2. Total glucosinolates content in kale from different growing locations (Šamec et al., 2018)

Location	**Content (µmol/g dW)**
Poland	2.25 ± 0.09
Poland	26.87 ± 0.23
Spain	23.04 - 93.90
USA	4.2 - 10.6
Turkey	34.52 - 60.94
Spain	11.00 -52.79

When cruciferous vegetables are fermented, most glucosinolates undergo hydrolysis and other transformations. In fermented products, these glucosinolates are either absent or present in minuscule amounts in their native form (Ciska et al., 2021). The extent to which glucosinolates are hydrolyzed during fermentation is determined by their chemical and microbiological stability. For instance, glucoiberverin is hydrolyzed quickly, while 4-methoxyglucobrassicin is considered the most stable (Ciska et al., 2021). Degradation products of glucosinolates are abundant in fermented cruciferous vegetables, and various examples are displayed (Figure 2.1).

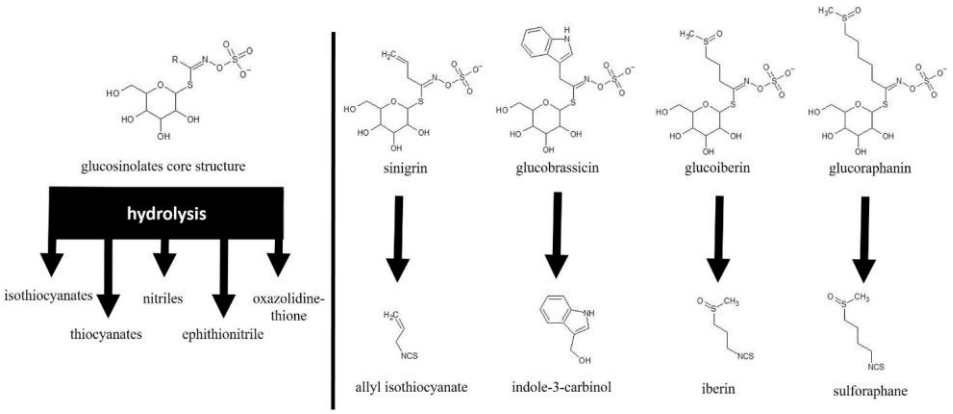


Figure 2.1. Glucosinolates core structure, representative of glucosinolates and their hydrolysis products (Šamec et al., 2019)

Polyphenols

Polyphenols have numerous health-related benefits (Mithul et al., 2021). Among the polyphenols found in cruciferous vegetables, flavonoids - predominantly flavonols - hydroxycinnamic acids, and anthocyanins are the most diverse and widespread (Cartea et al., 2011). According to Olsen et al. (2009b) polyphenols are not usually present in free form in fresh cruciferous vegetables. Instead, they are typically heavily glycosylated and acrylated, resulting in the formation of complex molecules. Various factors can cause fluctuations in the polyphenol content

throughout the fermentation process, particularly in complex forms (Šamec et al., 2021).

Carotenoids

Carotenoids are pigments consisting of around 850 tetraterpenes found in photosynthetic organisms. They play a crucial role in human health as they serve as precursors to vitamin A, protect against photodamage, act as antioxidants, boost immunity, and support reproduction (Maoka, 2020a). Kale is one of the best vegetable sources of carotenoids, with lutein and β-carotene comprising up to 40% and 34.8% of the total carotenoids, respectively (Szutowska and Gwiazdowska, 2021) . Decrease in carotenoids content by 17-31% during fermentation were reported by Szutowska et al. (2021) and. Odongo et al. (2017). The total carotenoid content decreased by 75% after fermentation where lutein had the most significant decrease of 98%. This decrease in carotenoid content during lactic-acid fermentation is due to the formation of volatile carotenoid cleavage derivatives, which contribute to the flavor and aroma of fermented products (Mapelli et al., 2020).

2.5 Antinutritional Factors in Kale

Some chemical compounds present in plant tissues are anti-nutritional factors with the potential to hinder the absorption of vital nutrients in humans. The effects of these factors can range from mild reactions to serious health concerns and even fatal consequences. Notable anti-nutritional factors such as nitrates, phytates, tannins, oxalates, and cyanogenic glycosides have been associated with various health risks. However, there are various processing techniques, such as cooking and blanching, that can help to decrease the levels of anti-nutritional factors in plant-based foods and increase their nutritional value for human consumption (Chilton et al., 2015; Natesh et

al., 2017). Many plant-based foods, including kale, contain oxalic acid and its salts, which can decrease the bioavailability of minerals like calcium. According to Satheesh and Workneh, (2020b) kale has a high oxalate content of 297 mg/100 g by fresh weight. Since it is a significant source of calcium, its oxalate content is a crucial factor to consider for mineral absorption. Phytate can negatively affect mineral absorption by forming insoluble complexes with calcium, iron, and zinc, leading to nutrient deficiencies (Satheesh and Workneh, 2020b).

2.6 Lactic acid fermentation of cruciferous vegetables

Fermentation is based on producing lactic acid from sugar under microbial activity. The fermentation of white cabbage, first used in Central and Eastern Europe, is known worldwide. Products made from shredded fermented cabbage are known as sauerkraut, but the whole heads can also be fermented. Similarly, *gundruk* is another example of a non-salted fermented green leafy vegetable prepared from the lactic acid fermentation of fresh leaves of rayo (*Brassica rapa*), mustard (*Brassica juncea*), and cauliflower (*Brassica oleracea*) indigenous to the Nepal and is popular in the Himalayan region of Nepal, India, and Bhutan (Ghimire et al., 2020; Tamang and Tamang, 2010). In other parts of the world, different cruciferous vegetables are fermented. In many cultures, fermented cruciferous products are considered functional foods or superfoods because they are a good source of vitamins, minerals, prebiotic fibers, polyphenols, active compounds derived from glucosinolates, phytoestrogens and bioactive (Ciska et al., 2021; Satheesh and Workneh, 2020a; Septembre et al., 2018).

Lactic acid fermentation is based on the production of lactic acid from sugar. A typical example of the lactic fermentation of cabbage is shown in Figure 2.2. In the

fermentation process, lactic acid production is crucial because it has a dual function: prevention from spoilage and enrichment of nutrients, sensory attributes in the product (Šalić and Šamec, 2022b).

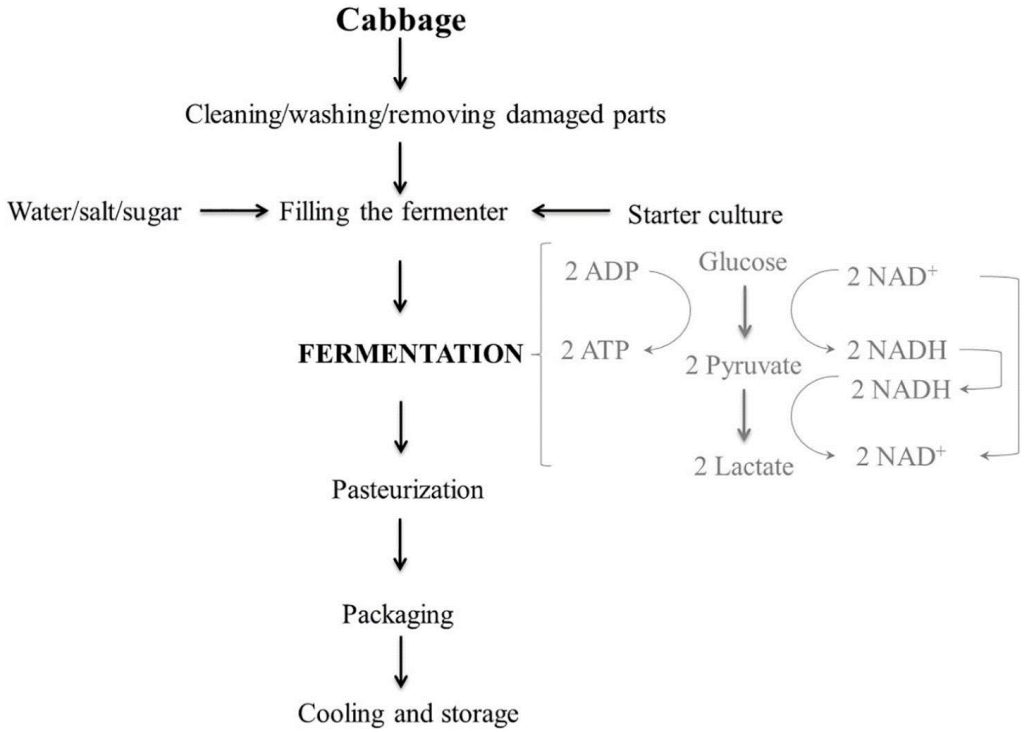


Figure 2.1. Schematic process of cabbage lactic fermentation (Šalić and Šamec, 2022b).

Generally lactic acid fermentation process can be done spontaneously by indigenous microbial cultures on the vegetables or by adding starter cultures (Li and Gänzle, 2020). The mixture of indigenous microorganisms responsible for fermentation processes on vegetables depends on the type of vegetable, place of growth, temperature and harvesting conditions. They include lactic acid bacteria (LAB) such as *Enterococcus*, *Streptococcus*, *Leuconostoc, Lactobacillus*, and *Pediococcus* and yeasts and molds *Debaryomyces*, *Kluyveromyces*, *Saccharomyces* (Sharma et al., 2020). When the fermentation process occurs under optimal/favorable conditions, leading role will have LAB belonging primarily to *Leuconostoc* (L.

mesenteroides), *bacillus* (*Lactiplantibacillus plantarum*, *Lactiplantibacillus pentosus*, *Levilactobacillus brevis, Limosilactobacillus fermentum, Lacticaseibacillus casei*), *Weissella, Enterococcus and Pediococcus* (*P. pentosaceus*) genera. *Lactiplantibacillus plantarum* is the most used fermentation species, LAB (Kimoto et al., 2019).

Chapter 3: Materials and Methods

3.1 Kale vegetable sample

The vegetable used in the study was from one farm (Spring Valley Farm in Romney, West Virginia), purchased on the same day at the Farmer's Market in Downtown Silver Spring, Maryland. According to the details provided by the farm the variety of the kale was Winterbor.

3.2 Kale fermentation

Fresh kale leaves from the market were cleaned and cut into small pieces (~ 2-3 cm). The samples were mixed up and then divided into 7 groups for the different fermentation treatments. Each group was divided into 3 to give 3 replicates of each treatment. The seven groups are shown in Table 3.1.

Six different fermentation methods and one unfermented control group were tested. The processes consisted of two groups that followed traditional household methods; spontaneous fermentation with naturally occurring bacteria, and fermentation with 2% salt (NaCl) based on vegetable weight. The other four groups used different bacteria species combinations as shown below in Table 3.1.

Table 3.1. Different sample groups and fermentation treatments used in the study

S.N.	**Sample groups**	
1	Control	Unfermented
2	F1	Spontaneous fermentation with naturally occurring bacteria
3	F2	Fermentation with 2% salt added per vegetable weight
4	F3	*Lactococcus lactis* (ATCC 11454)
5	F4	*Lactobacillus acidophilus* (ATCC 314)
6	F5	*Lactococcus lactis* and *Lactobacillus acidophilus*
7	F6	*Lactococcus lactis*, *Lactobacillus acidophilus*, and *Clostridium butyricum*

For group F1, about 400 g vegetable pieces were put into 1 litre mason jars (n = 3), pressed with the sterile pestle then sealed and left to ferment naturally. For group, F2 2% table salt w/w to the vegetable pieces was mixed with vegetable pieces before placing them into the jars. For groups F3-F6 specific bacteria cultures (Table 3.1) in a 10^7 CFU/g ratio were mixed into the vegetable before placing them in the jars as shown before by Ghimire et al., (2020) and Michalak et al., (2018). All fermentations were in triplicate at room temperature (18–20°C) for 16 days while the u unfermented control was frozen. All samples were then freeze-dried, homogenized into a fine powder using a food grinder, and stored in an airtight container at -20 °C freezer till further analysis.

3.3 Determination of pH

The pH value was analyzed using a digital pH meter. The pH meter was calibrated using standard buffer solutions of pH 4.0, pH 7.0, and pH 10.0. The pH electrode of the calibrated pH meter was inserted into the fermented kale in the fermentation jar, and stable pH values were recorded as recommended by Vatansever et al. (2017).

3.4 Determination of acidity % (as lactic acid)

According to the protocol described by Cai et al. (2019) we homogenized 2 g of kale sample, suspended it in 10 ml deionized (DI) water. and titrated it against 0.1 N sodium hydroxide (NaOH) solution using phenolphthalein indicator. The DI water blank was also tested. We used the formula below:

Titratable Acidity % (as lactic acid) = (V1 - V0) × Normality of NaOH × Equivalent weight of lactic acid × 100 / Sample weight (g)

Where V1 = the volume of NaOH solution consumed by the sample

V0 = the volume of NaOH solution consumed by the blank DI sample

3.5 Determination of total phenolic content

A slight modification to the method outlined by Chin et al. (2022) was employed to extract bioactive compounds. Each 2 g samples were blended with 20 ml of 80% (v/v) methanol, placed on a shaker plate for 30 minutes, vortexed for 1 minute and centrifuged at 4000 rpm and 30°C for 10. The resulting supernatant was filtered through Whatman No. 1 filter paper. The process was repeated, and the supernatants were combined and adjusted to a final volume of 50 ml using 80 % (v/v) methanol solvent. The filtrate was stored at -20°C to analyze total polyphenol content.

The total polyphenol content (TPC) was measured using a modified Folin-Ciocalteu spectrophotometric method. Exactly 200 µl sample extract, 1.5 ml $NaCO_3$ (75 g/l), and 1.5 ml freshly diluted (10-fold) Folin-Ciocalteu reagent was prepared. The solution was then incubated in darkness for 2 hours at room temperature, and its absorbance was measured at 725 nm using a spectrophotometer. Gallic acid was used to prepare a standard curve ranging from 0.1 to 0.8 mg/ml. The TPC was expressed in milligrams of gallic acid equivalents per 100 g sample on a dried weight basis (mg GAE/100 g db) using the standard calibration curve as described in Chin et al. (2022).

3.6 Determination of total flavonoid content

A slight modification to the method outlined by Chang et al (2020) was employed to extract and quantification of flavonoid compounds. Each 1 g sample was blended with 10 ml of 80 % (v/v) ethanol on a shaker plate for 30 minutes, vortexed for 1 minute and centrifuged at 4000 rpm at 30°C for 10 minutes. The supernatant was filtered through Whatman No. 1 filter paper. The process was repeated, and the

supernatants were combined and adjusted to a final volume of 25 ml using 80% (v/v) ethanol solvent and stored at -20°C.

The $AlCl_3$ colorimetric method based on the procedure outlined by Chang et al. (2020) was slightly modified for the total flavonoids assay. Quercetin (10mg) was utilized to construct the standard calibration curve by mixing with 10 ml of 80% ethanol and then making serial dilutions ranging from 25 to 100 µg/ml. Exactly 0.5ml of each sample, standard or blank (DI water) mixed with 1.5 ml of 95 % ethanol, 0.1 ml of 10 % $AlCl_3$ 0.1 ml of 1M potassium acetate, and 2.8 ml of deionized water.. After a 30-minute incubation at room temperature, the absorbance was measured at 415 nm using a UV-Vis spectrophotometer.

3.7 Determination of beta-carotene content

Beta-carotene was quantified according to the method described by Panwar et al. (2015). A β-carotene standard stock solution (10.9 mg/100 ml) was prepared in n-hexane and stored under refrigeration in an amber-colored bottle before use. Further dilutions from this stock solution were done with n-hexane to yield final concentrations between 1.9 to 10.9 mg/ml.

Kale samples (200–300 mg) were mixed with 8 ml ethanol containing 0.5 % (v/w) Butylated Hydroxytoluene (BHT) and vortexd for 1 min before adding 2 ml of hexane: acetone (2:1 ratio) solution. The mixture was incubated for 10 min in the dark at room temperature then centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and, an equal volume of 15 % alkaline (KOH) methanol containing 0.5 % BHT was added and incubated at 80°C for 15 min in a water bath before immediately chilling it on ice for 5–10 min for saponification. A solution of 4ml distilled water and 3ml petroleum ether containing 0.5 % BHT

wasre added to the saponified extract for better phase separation. The mixture centrifuged at 5,000 rpm for 10 min at 4 °C, followed by transfer of the upper colored organic phase to a fresh tube. The leftover residue was re-extracted twice with 4 ml of petroleum ether containing 0.5 % BHT, and the upper phases were collected and pooled and dried in a rotary vacuum evaporator. The residual carotenoid extract suspension was made in 2 ml of mobile phase solvent and filtered through a 0.45 µm syringe filter into an amber-colored HPLC sample vial and immediately analyzed by HPLC with a UV detector. Aliquots of 20 µl were injected into Agilent reverse-phase C18 (5 µ, 4.6 mm×250 mm) column at 30 °C. The mobile phase consisted of methanol: acetonitrile: chloroform (50:40:10) with 0.5% BHT. A flow rate of 0.7 ml/min was maintained, and elution of beta-carotene was monitored at 450 nm. Peaks were identified by their retention time and quantified using a standard curve generated from the standards.

3.8 Determination of glucoraphanin and sulforaphane content

Using the method described by Bello et al. (2018) and Sivakumar et al. (2007). Each 0.5 g of kale samples were extracted with 12.5 ml of 70 % methanol (sample-to-solvent ratio of 1:25 w/v) in a water bath at 70 °C for 30 minutes. Samples were then centrifuged at 4000 rpm for 30 minutes at 4°C. The supernatants was dried using a rotary vacuum evaporator at 40°C. Dried samples were dissolved in 3 ml HPLC-grade water tand filtered through 0.20 µm syringe filters.

Glucoraphanin and sulforaphane were quantified acording to the method described by Celik et al. (2014). The mobile phase for sulforaphane (acetonitrile: water, 30: 70) v/v and mobile phase for glucoraphanin (acetonitrile: water: formic acid, 1: 99: 0.1) v/v were filtered with 0.45 um syringe filter before use. Five mg of

standards of sulforaphane and glucoraphanin dissolved in 10 ml acetonitrile were used to prepare the stock solutions and then serially diluted in the respective mobile phase to make the standards with six different concentrations ranging from 50 to 500 ug/ml respectively. Exactly 10ul of the standards and samples were injected into ESA reverse-phase C18 (3um, 150 mm×3 mm) column with a flow rate of 0.3 ml/min flow rate under the UV detector in 230 nm at room temperature. Standard calibration graphs for glucoraphanin and sulforaphane were constructed by plotting the area under the chromatograms versus concentrations of the standards.

3.9 Determination of anti-nutritional factors

Oxalate content

Oxalate present in the samples was extracted with HCl and then reacted with $CaCl_2$ to convert it into calcium oxalate precipitate as described by Ranganna (2007). About 2g of sample was mixed with 25 ml of 1.5 % HCl solution, heated in the water bath at 95ºC for 10 min, cooled and filtered through Whatman No.1 filter paper. The filtrate was treated with 10ml of 0.025M CaCl2 mixed/stirred for 5 min and allowed for calcium oxalate to precipitate for 30 minutes. Using a pre-weighed dry filter paper and crucible, the sample was filtered, the filter paper was placed into the crucibles and dried in a hot air oven at 100-105 º C to constant weight. Oxalate content was calculated by the difference in the weight of filter paper and expressed as mg/g of sample dry weight.

Tannin content

The tannin content was determined by the Folin-Ciocalteu assay with slight modifications to the process described by Ranganna (2007). About 1 g of freeze-dried homogenized kale powder was extracted in 25 ml of 80 % ethanol with continuous

shaking for 2 hr and filtered through Whatman No.1 filter paper. A tannic acid standard stock solution of 10 mg/ml prepared by dissolving 100 mg tannic acid in 10 ml of 80 % ethanol serially diluted to prepare standards of 0.1, 0.2, 0.3, 0.3, 0.4, 0.5, and 1 mg/ml. In test tubes, 1ml of the filtered extracts was mixed with 5 ml of Folin-Ciocalteu reagent. After 5 min incubation, at room temperature, 4 ml of $NaCO_3$ (75 g/l) was added and incubated for 30 min at room temperature. Absorbance for standards and samples was measured at 725 nm using a UV-Vis spectrophotometer. From the standard curves of absorbance against the concentration of tannic acid, the amount of tannins present in the sample was calculated as mg TAE/g dw.

Phytate content

Phytate content was determined according to the method described by Ranganna (2007). Freeze-dried homogenized kale powder 0.5 g was extracted in 10 ml of 2% trichloroacetic acid solution with continuous shaking in a plate shaker for 30 minutes, then centrifuged at 3000 rpm for 5 minutes. Exactly 1 ml of the supernatants was mixed with 1ml of 0.3% $FeCl_3$ solution, and 8ml of DI water d and incubated at room temperature for 30 min. Absorbance was ere determined at 500 nm using a UV-Vis spectrophotometer. The amount of phytate was calculated using the formula:

$$(\text{Phytate (mg/g)} = (A \times V \times 1000)/(E \times W)$$

Where A is the absorbance of the sample, V is the final volume of the reaction mixture (in ml), E is the extinction coefficient of the phytate-Fe complex (1.43 x 10^4 M^-1 cm^-1 at 500 nm), and W is the weight of sample in gram.

3.10 Determination of antioxidant activity

Extraction of kale extracts

Extract of each sample was obtained using 80% ethanol following the method described by Hussain et al. (2016). About 2 g sample from each sample was blended with 20 ml of 80% (v/v) ethanol, placed on a shaker plate for 30 minutes, vortexed for 1 minute and centrifuged at 4000 rpm and 30°C for 10 minutes. The supernatant was filtered through Whatman No. 1 filter paper. The process was repeated, and the supernatants were combined and adjusted to a final volume of 50ml using 80 % (v/v) ethanol solvent. The filtrate was stored at -20°C.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The DPPH assay was performed according to the method described by Hussain et al. (2016). The sample extract (10 ml) was mixed with a 60 mM solution of DPPH in 2 ml ethanol, and kept in the dark at room temperature for 30 minutes before measuring the absorbance at 517nm using a UV-Vis spectrophotometer after setting it with a blank to zero using ethanol. The DPPH solution was freshly prepared, kept in flasks covered with aluminum foil, and stored in the dark at 4°C until use. The scavenging activity of the extract was expressed as the percentage of inhibition of the DPPH radical, calculated using the formula:

$$\text{Inhibition percentage (IP)} = [(\text{A control} - \text{A sample}) \times 100]/\text{A control}$$

Where, A control is the absorbance of the control (containing all reagents except the sample), and A sample is the absorbance of the sample, both measured at 517nm.

Determination of IC 50 for DPPH activity

To determine the IC 50 value, 20 ml of the extract obtained above was dried under nitrogen to obtain its precise weight. A 1 % solution of the dried extract was

prepared in methanol, to give a working concentration of 0.01 g/ml. Volumes ranging from 1 ml to 10 ml of the solution was transferred to different test tubes. To each, 2 ml of DPPH solution was added. The A control test tube contained only the DPPH solution.. The samples were vigorously shaken and kept in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm using a UV-Vis spectrophotometer. The IC 50 (DPPH) value, representing the concentration of the extract that caused a 50 % reduction in DPPH absorbance, was determined by analyzing the absorbance versus concentration graph as shown by Hussain et al. (2016).

2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) (ABTS) assay

The radical-scavenging capacities of kale extracts were determined using the method described by Yu et al. (2018). Antioxidant capacities of kale extracts were evaluated against ABTS free radicals. ABTS free radicals were generated by oxidizing ABTS with manganese dioxide at room temperature. The ABTS free radical stock solution was prepared by dissolving 0.2205 g of ABTS chromophore diammonium salt in 80 ml of DI water, stirred using a magnetic stir bar and then manganese dioxide powder was slowly mixed into the solution until the pale green color changed into deep blue-green color. The stock solution was filtered and diluted with phosphate buffer to prepare a working solution. The stock solution was adjusted to an absorbance of 0.700 ± 0.005 at 734 nm (UV-Vis) by diluting with phosphate buffer to obtain a working solution. Trolox was used as a standard. The standard curve was plotted between a concentration ranges of 1.25 to 75 ug/ml. A blank consisting of 2 ml phosphate buffer and 160 ul DI water was used. The the sample reaction mixture comprised 160 ul of standard/sample with 2ml of ABTS working solution. The

mixture was vortex mixed for 30 s, and absorbance was measured at 734 nm after 90 s reaction time using a UV-Vis spectrophotometer.

3.11 *In vitro* assessment of anti-inflammatory properties

For the *in vitro* anti-inflammatory assay only 3 treatments were evaluated. Unfermented kale was compared with traditional fermentation (2% salt w/w), and kale fermented with mixed culture of *L. lactis* and *L. acidophilus*). The samples were extracted on 80 % methanol as described before in an assay of total phenolic content. The methanolic extracts were dried using a rotary vacuum evaporator (at 40° C), and the dried extracts were further dissolved in Dimethyl sulfoxide (DMSO) to make a final concentration of 100 µg/ml. The sample groups used for this assay are presented in Table 3.2.

Table 3.2. Treatments used for *in vitro* anti-inflammatory assay.

Control	**LPS (100ng/ml)**	**Unfermented kale extract (100ug/ml)**	**Traditional fermentation (100ug/ml)**	**LAB fermentation (100ug/ml)**
Cell medium only	Cell medium + LPS	Cell medium + LPS (+ Unfermented kale extract	Cell medium + LPS + 2 % salt fermented kale extract	Cell medium + LPS + Lactic acid bacteria (*L. lactis* and *L. acidophilus*) fermented kale extract

Cell culture

Murine macrophages RAW 264.7 (ATCC, Manassas, VA, USA) were cultivated in high-glucose Dulbecco's Modified Eagle's Medium (DMEM)

supplemented with 10% fetal bovine serum (Cytiva Hyclone, USA), 100 units/mL penicillin (Gibco, NY, USA) at 37°C in a humidified atmosphere containing 5% CO2.

Cell treatment

The cell treatment method and dose selection (100 µg/ml) were based on previous studies by Hwang and Lim (2014) and Jeong et al. (2018). In a 6-well plate, 6.8×10^5 cells in 1 ml of cell culture medium were seeded and incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. After 2 days confluent cells were treated with 100 µg/ml of kale methanolic extract for 4 hours. Cells were then washed with cold phosphate-buffered saline (PBS) and stimulated with 1 µg/ml lipopolysaccharides from which *E. coli* (Sigma-Aldrich, MO, USA) for 20 hours. Followed by RNA extraction and cDNA synthesis. Two replicates were used for each of the sample extracts resulting in a total of 6 readings (n = 3×2) for each sample group.

RNA isolation and cDNA synthesis

Cells were washed with ice-cold Phosphate-buffered saline (PBS) before adding and scrapping them in 1 ml TRIzol reagent. Contents were homogenized in with 250 ul chloroform and centrifuged at 10,000 rpm for phase separation. The top supernatant layer was collected, mixed with 550 ul isopropanol, and centrifuged at 13,000 rpm for 20 min. The pellet was again washed with 1 ml of 75% ethanol, centrifuged at 9,500 rpm. The pellet was redissolved with 25ul nuclease-free water in 1.5 ml Eppendorf tube. RNA concentration was determined using Qubit RNA BR Assay Kits and Qubit 4 fluorometer (Thermofisher Scientific, MD, USA). About 2000 ng of the RNA from each sample was used for cDNA synthesis using the 2X RT master mix reagents and thermal cycler protocol as provided in High-Capacity cDNA

Reverse Transcription Kit (Thermofisher Scientific, MD, USA). The cDNA was stored at -80°C for further qPCR applications.

Analysis of target genes by qPCR

Genes encoding pro-inflammatory cytokines interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and Inducible Nitric Oxide Synthase (iNOs) determined by qPCR. Primer sets for IL-1β, IL-6, TNF-α, iNOs, and β-actin (all from IDT Technologies, Coralville, IA, USA) were used to quantify inflammation levels. RT-PCR cycling conditions on a CFX 96 (Bio-rad, CA, USA) were 2 min at 50 ºC and 2 min at 95ºC, followed by 40 cycles of two-step PCR denaturation at 95 ºC for 15 s and annealing extension at 60 ºC for 1 min. Duplicate assay samples contained 10 ng cDNA and 10uM primers in 2X PowerUp SYBR Green Master Mix (Thermofisher Scientific, MD, USA) in a final volume of 10 ul. The relative amount of target mRNA in each sample was normalized to beta-actin, the endogenous control gene. Data were analyzed according to the $2^{-\Delta\Delta CT}$ method, and fold difference was calculated in reference to LPS control.

Table 3.3. Primer sequence used for qPCR.

Gene	Forward	Reverse
iNOS	CAC CTT GGA GTT CAC CCA GT	ACC ACT CGT ACT TGG GAT GC
TNF-a	TAC TGA ACT TCG GGG TGA TTG GTC C	CAG CCT TGT CCC TTG AAG AGA ACC
IL-1ß	CCA GCT TCA AAT CTC ACA GCA G	CCA GCT TCA AAT CTC ACA GCA G
IL-6	TCC AGT TGC CTT CTT GGG AC	GTA CTC CAG AAG ACC AGA GG

3.12 Determination of microbiota composition in vegetable samples

The changes in microbiota composition of unfermented raw kale and fermented kale samples were analyzed by 16S sequencing technique as described by Shahinozzaman et al. (2021) using DNA extracted from each vegetable sample.

DNA extraction and amplification of the 16S hypervariable regions

To extract DNA, a bead beating method was used on 100mg of freeze-dried samples using a FastPrep-24 (MP Biomedical, OH, USA). The DNA extraction was done using the reagents and protocol provided in the DNeasy PowerLyzer PowerSoil kit (Qiagen, MD, USA). DNA concentration was measured on Qubit 4.0 Fluorometer using Qubit™ dsDNA BR assay kit (Thermofisher Scientific, MD, USA). The DNA samples were stored at -80°C for further analysis. Seven hypervariable regions i.e., V2, V3, V4, V6, V7, V8, and V9 were amplified by PCR using the reagents and manufacturer's protocol provided in the Ion 16S™ Metagenomics kit (Thermofisher Scientific, MD, USA). Amplification products were purified using Agencourt AMPure XP beads (Beckman Coulter, Nyon, Switzerland) on the DynaMag™-2 magnetic rack to remove primer dimers and small mispriming products, washed with fresh 70% ethanol, then eluted into 15 µl of Nuclease-free water. The purified PCR product was quantified by the Qubit™ dsDNA BR assay kit (Thermofisher Scientific, MD, USA) before calculating DNA for library preparation.

Library preparation, template preparation, and sequencing

Amplification products were purified using Agencourt AMPure XP beads (Beckman Coulter, Nyon, Switzerland) on the DynaMag™-2 magnetic rack to remove primer dimers and small mispriming products, washed with fresh 70% ethanol, then

eluted into 15 µl of Nuclease-free water. The purified PCR product was quantified by the Qubit™ dsDNA BR assay kit (Thermofisher Scientific, Rockville, MD, USA) before calculating DNA for library preparation. Libraries were prepared and bar-coded (ligation) using end repair buffer, enzymes, and manufacturer's protocol in the Ion Plus Fragment Library kit (and the Ion Xpress™ Barcode Adapters 33-53 (Thermofisher Scientific, Rockville, MD, USA). After ligating and barcoding, samples were purified using Agencourt AMPure XP beads (Beckman Coulter, Nyon, Switzerland) on the DynaMag™-2 magnetic rack, washed with fresh 70% ethanol, then eluted into 20 µl of low TE in 1.5 ml Eppendorf tubes. The library concentration was determined by qPCR based on reagents and protocol provided in Ion Universal Library Quantitation Kit (Thermofisher Scientific, MD, USA). Libraries were diluted to yield 10 pM inputs for template preparation and enrichment using an Ion OneTouch 2 System and the Ion PGM Hi-Q OT2 Kit following the instructions in the kit user guide (Pub. no. MAN0010902). After template enrichment on the Ion OneTouch2, template-positive Ion PGM Hi-Q Ion Sphere Particles (ISPs) with 400 base-pair average insert libraries were used for sequencing on a 530 Chip using the Ion GeneStudio S5 system (ThermoFisher Scientific, Grand Island, NY, USA). After quality control, 9,519,705 high-quality reads from V3 to V4 region of 16S rRNA gene sequences were obtained from 21 samples and the sample reads ranged from 68,281 to 892,702 reads.

3.13 Statistical analysis

All experiments were carried out in triplicate. Data are expressed as mean ± standard error of the mean. Statistical analysis was performed by a one-way analysis of variance (ANOVA), followed by a post hoc test (Tukey test) using GraphPad Prism

9 (San Diego, CA, USA). P values less than 0.05 were considered statistically significant.

Microbiome data diversity analysis

The Ion Torrent Suite software package was used to analyze sequencing data. Taxonomic classification was performed based on relative abundance and compared between each sample group using Microsoft Excel and GraphPad Prism software. Using the Galaxy workflow (https://huttenhower.sph.harvard.edu/galaxy), we determined the taxa most likely to explain differences between the seven groups using linear discriminant analysis (LDA) effect size (LEfSe) which utilizes a non-parametric Kruskal-Wallis rank sum test to assess differential features with significantly different abundances between assigned taxa and performs LDA to estimate the effect size of each sequence variant. LDA scores ranking differential taxa are displayed on a bar chart according to their effect size. A significant alpha level of 0.05 and an effect size threshold of a four times greater difference was used for displaying results.

Chapter 4: Results

4.1 Physicochemical analysis (pH and titratable acidity %)

The pH level and titratable acidity % were measured on day 1 (before fermentation) and day 16 (post-fermentation). The initial pH value of unfermented kale 5.72 ± 0.04 decreased during fermentation. A statistically significant difference ($p < 0.05$) was noted in pH values between the unfermented and fermented kale in all groups. Among the six different fermentation groups, pH values ranged from 4.52 ± 0.10 (natural fermentation) to 3.74 ± 0.05 (fermentation group with *L.lactis*, *L. acidophilus* and *C. butyricum*) (Figure 4.1).

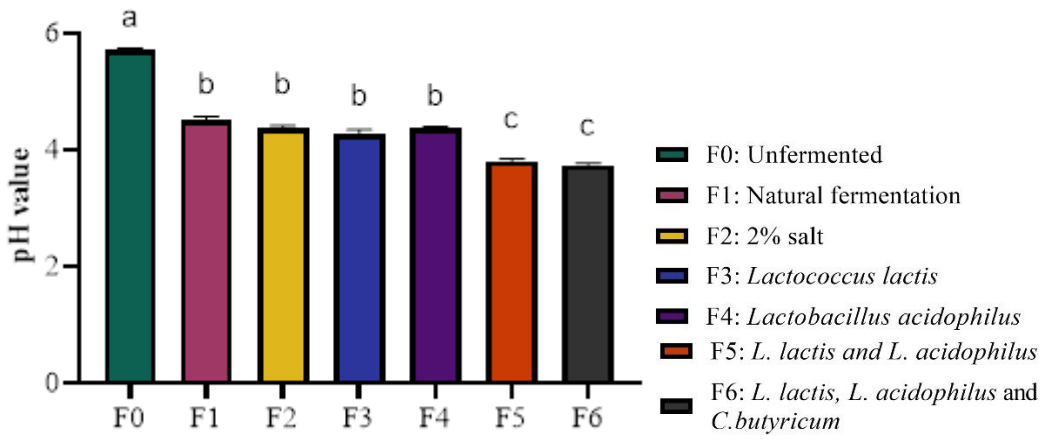


Figure 4.1. Effect of fermentation on pH value of kale. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

Titratable acidity (lactic acid equivalent) significantly increased following the fermentation in all groups compared to the unfermented group. The mixed cultures of *L.lactis* and *L. acidophilus* induced the highest increase in titratable acidity but the increase was not statistically different to other fermentation groups (Fig. 4.2).

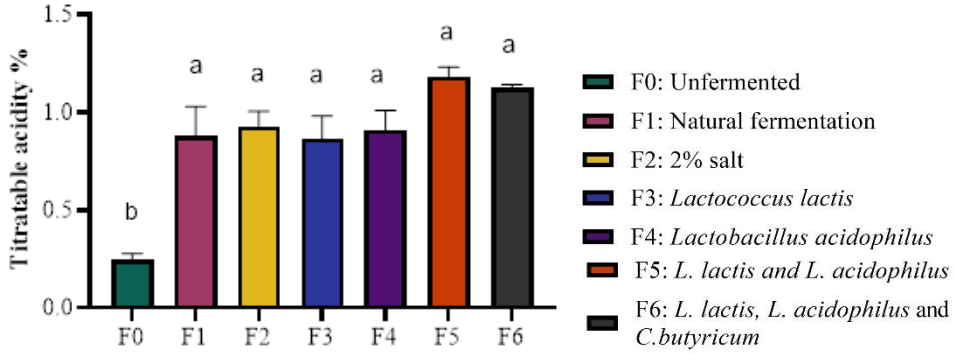


Figure 4.2. Effect of fermentation on titrable acidity % (as lactic acid). Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

4.2 Total polyphenol content

Total polyphenol content in terms of gallic acid equivalent ranged between 8.539 ± 0.16 to 10.71 ± 0.35 mg GAE/g as presented in Figure 4.3. The unfermented samples had the lowest concentration, while the highest concentration was observed in fermentation group with mixed cultures of *L.lactis* and *L. acidophilus*. All fermentation groups showed a slight increase in total polyphenols compared to unfermented kale, but significant increases ($p < 0.05$) were observed only in the groups with mixed bacterial cultures.

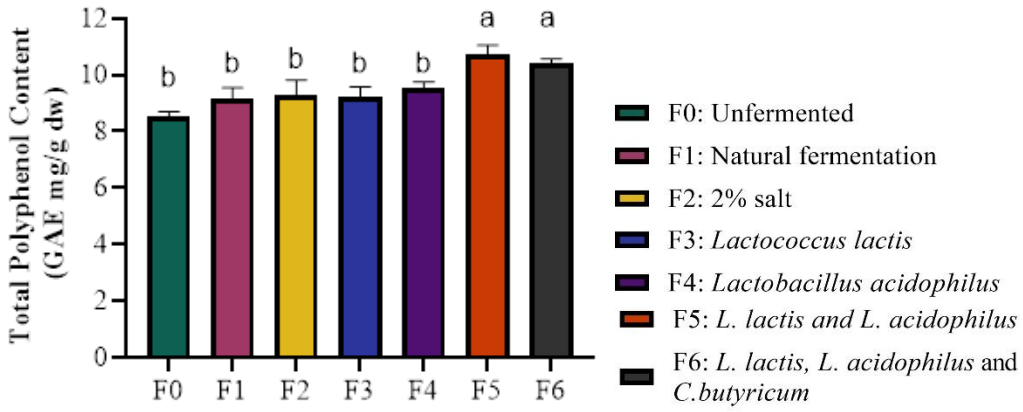


Figure 4.3. Effect of fermentation on total polyphenol content (mg GAE/g dw) of kale. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

4.3 Total flavonoids content

The total flavonoid content measured in terms of Quercetin equivalent varied from 5.35 ± 0.48 to 6.20 ± 0.19 mg QE/g dry sample weight (Figure 4.4). The mixed cultures of *L. lactis* and *L. acidophilus* recorded the highest flavonoid content (6.20 ± 0.19), followed by the unfermented kale (5.93±0.39), while fermentation with *L. lactis* had the lowest value (5.35 ± 0.48). However, there were no statistically significant differences ($p < 0.05$) between the unfermented and fermented kale in total flavonoid content.

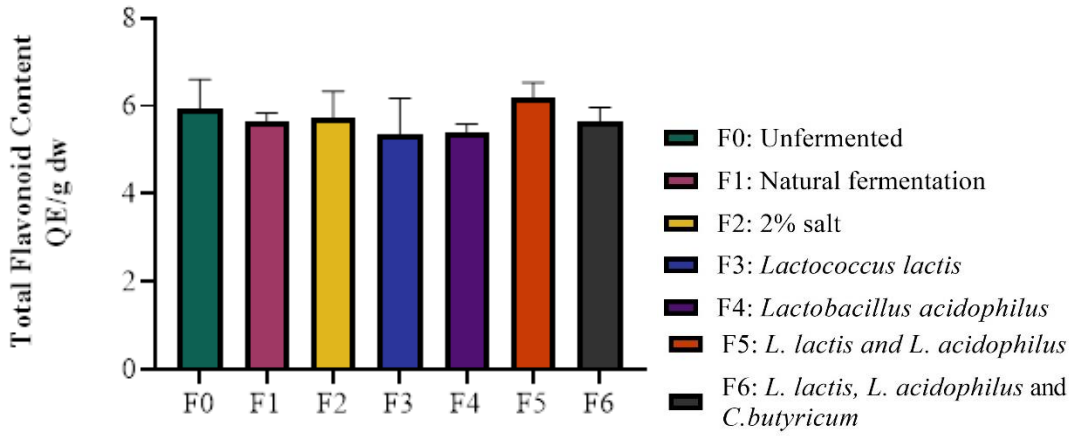


Figure 4.4. Effect of fermentation on total flavonoid content (mg QE/g dw) of kale. Results are shown as mean ± SEM. ($p<0.05$; n=3).

4.4 Beta-carotene content

All fermentation methods significantly lowered beta carotene levels. Beta-carotene levels ranged from 4.44 ± 0.78 to 15.86 ± 0.71 mg/100g dw (Figure 4.5). Unfermented kale had the highest beta-carotene content at 15.86 ± 0.71 mg/100g dw,

followed by fermentation group with mixed cultures of *L. lactis* and *L. acidophilus* at 9.42 ± 0.08 mg/100g dw, and the lowest was in group fermented with 2% salt at 4.44 ± 0.78 mg/100g dw. However, the decrease among all fermented groups was not different.

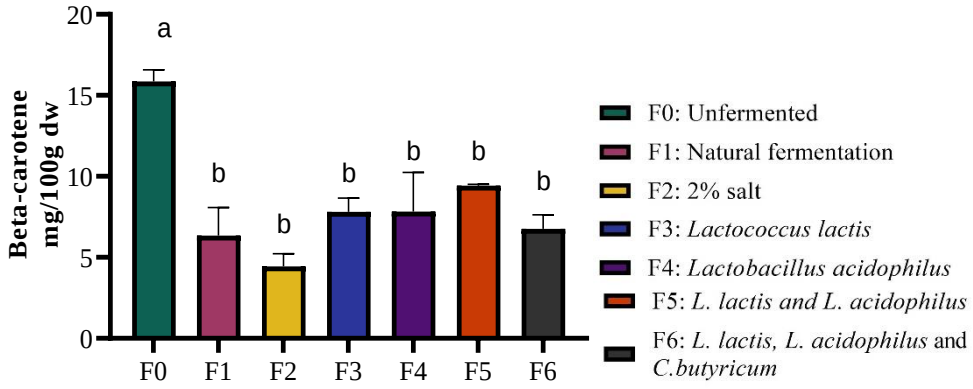


Figure 4.5. Effect of fermentation on beta-carotene content (mg/100g dw) of kale. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

4.5 Glucosinolates (glucoraphanin and sulforaphane content)

The glucoraphanin and sulforaphane contents ranged between 84.02 ± 15.79 to 229.4 ± 13.14 µg/g and 960.8 ± 41.76 to 1777 ± 45.95 µg/g, respectively. Glucoraphanin content was highest in the unfermented kale and significantly decreased ($p < 0.05$) in all fermented groups. Sulforaphane content was significantly higher ($p < 0.05$) in all fermented groups compared to unfermented kale. GroupF5 with mixed cultures of *L.lactis* and *L. acidophilus* had the lowest amount of glucoraphanin 84.02 ± 15.79 µg/g and highest amount of sulforaphane, 1777 ± 45.95 µg/g. The 2% salt fermentation group significantly differed from groups with mixed cultures of *L.lactis* and *L. acidophilus* regarding glucoraphanin and sulforaphane content (Figure 4.6 and 4.7). Overall, unfermented kale had a significantly higher

amount of glucoraphanin compared to fermented kale while fermented kale had a significantly higher amount of sulforaphane compared to unfermented kale.

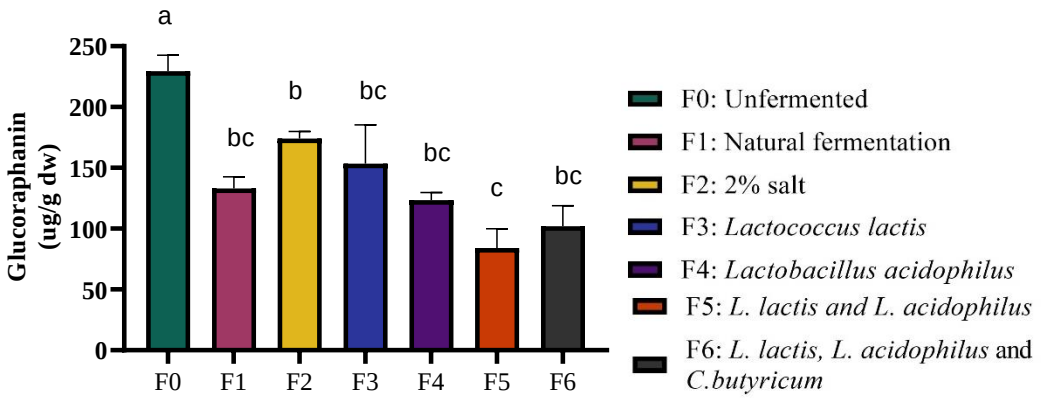


Figure 4.6. Effect of fermentation on glucoraphanin content (ug/g dw) of kale. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

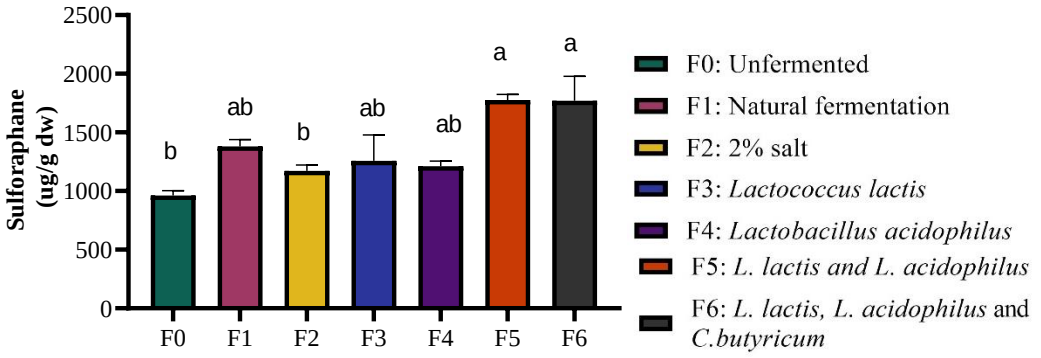


Figure 4.7. Effect of fermentation on sulforaphane content (ug/g dw) of kale. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

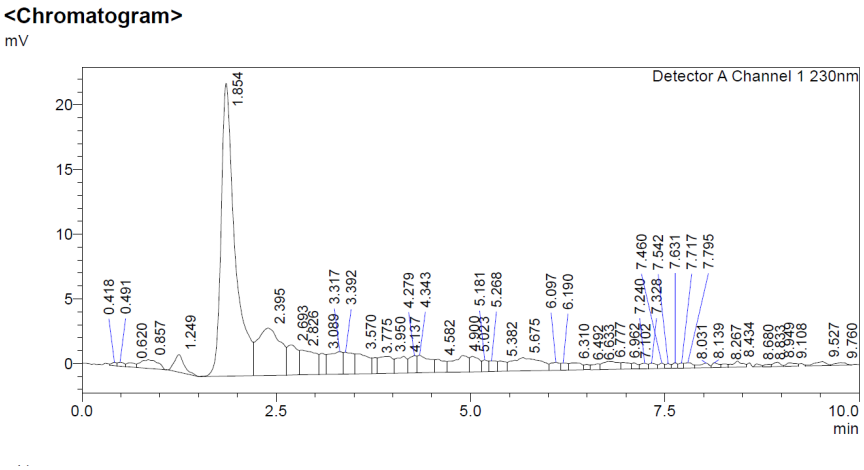


Figure 4.8. A representative chromatogram of fermented kale where glucoraphanin and sulforaphane peaks are at retention time 1.2 and 1.8 minutes, respectively.

4.6 Effect of fermentation on antinutritional factors in kale

Oxalate content

The amount of oxalate in unfermented kale was 0.75 ± 0.01 mg/g dw. All fermentation methods decreased oxalate content compared to the unfermented group ($p < 0.05$). Group (F5) with mixed cultures of *L.lactis* and *L. acidophilus* reduced oxalate content the most by 49 % to give a vlaue of oxalate content at 0.38 ± 0.01 mg/g dw, as shown in Figure 4.9. However, there was no significant difference in % of oxalate reduced among all fermentation methods.

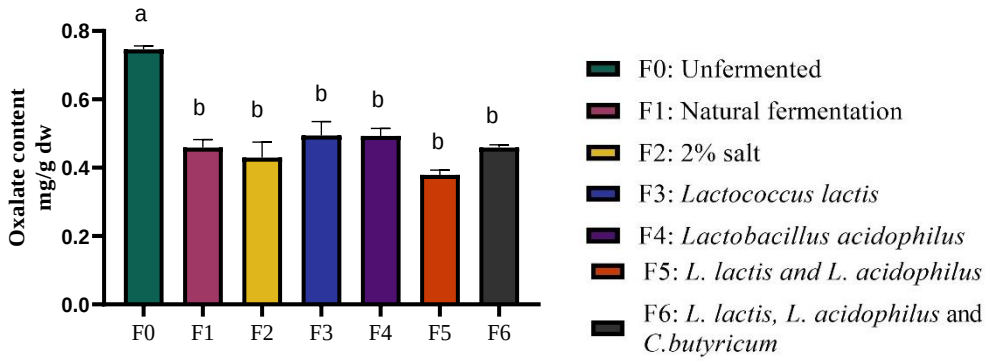


Figure 4.9. Effect of fermentation on oxalate content in kale. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

Phytate content

The quantity of phytate in unfermented kale was 2.345 ± 0.09 mg/g dw. All Fermentation methods slightly decreased the phytate content but not to a significant level statistically. Fermentation group with *Lactococcus lactis* had the lowest phytate content, measuring at 1.95 ± 0.11 mg/g dw, as demonstrated in Figure 4.10.

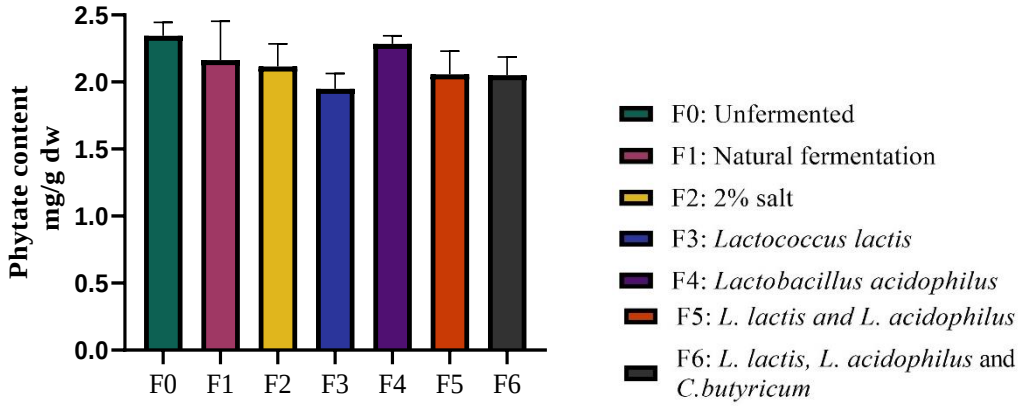


Figure 4.10. Effect of fermentation on phytate content in kale. Results are shown as mean ± SEM ($p<0.05$; n=3).

Tannin content

Unfermented kale contained 9.05 ± 0.61 mg/g dw of tannin. Although all fermentation groups slightly decreased tannin content compared to unfermented kale, significant decreases were observed only in F1 and F5 which had the lowest amount of Tannin at 6.35 ± 0.46 mg/g dw, Group (F5) with mixed cultures of *L.lactis* and *L. acidophilus* reduced tannin content the most by 29.83 % as shown in Figure 4.11.

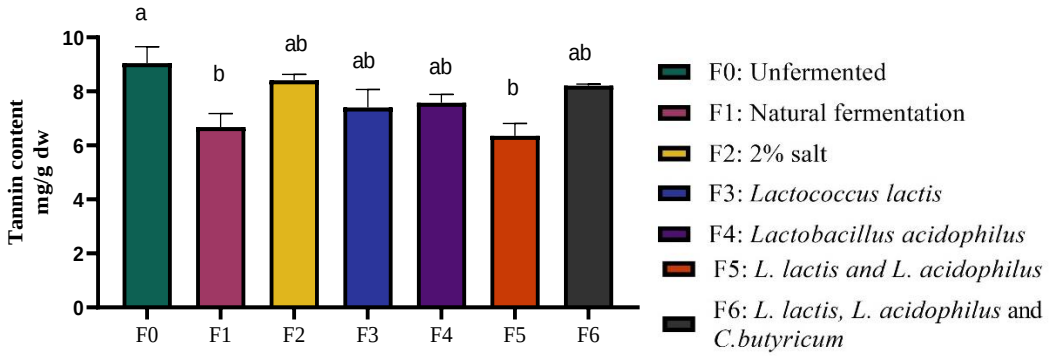


Figure 4.11. Effect of fermentation on tannin content in kale. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

4.7 Effect of fermentation on the antioxidant capacity of kale

DPPH free radical scavenging assay

Unfermented kale demonstrated the lowest antioxidant activity at 61.99 ± 1.48%. All fermentation methods significantly increased antioxidant activity ($P<0.05$; n=3). Group F6 mixed culture of *L.lactis*, *L. acidophilus*, and *C. butyricum* showed the highest antioxidant activity at 67.37 ± 0.58 % but the increase in anti-oxidant capacity due to fermentation was not different among all methods. (Figure 4.12).

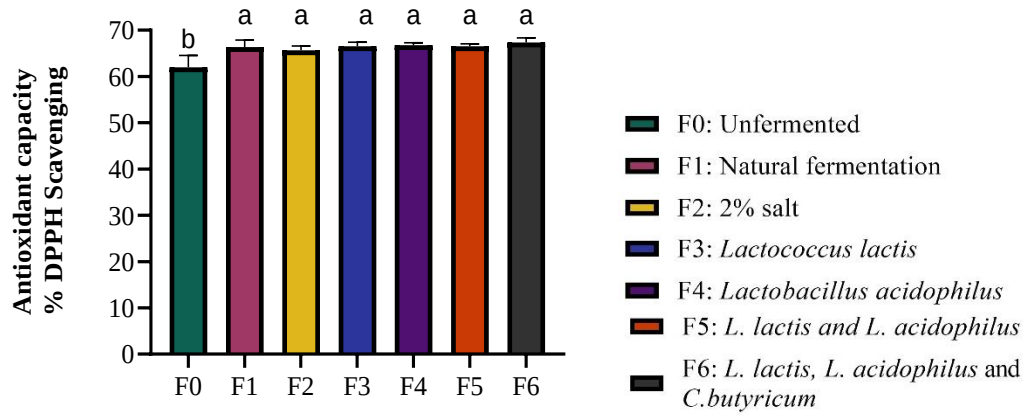


Figure 4.12. Effect of fermentation on antioxidant activity of kale. DPPH free radical scavenging capacity of unfermented and fermented kale extracts. Results are shown as mean ± SEM, 'a'- Different letters indicate

significant difference between treatments according to Tukey test ($p<0.05$; n=3).

Furthermore, the half-maximal inhibitory concentration (IC50) values of the ethanolic extract against DPPH analyzed using ascorbic acid as a reference standard. The IC50 values of kale extracts ranged between 186.7 ± 1.39 to 220.9 ± 3.41 ug/ml., IC 50 value of ascorbic acid was 75.56 ± 0.29 ug/ml, almost 3 times lower than unfermented kale. A highly significant difference ($p < 0.0001$) was observed between ascorbic acid, gallic acid standard and kale extracts. Fermentation by the mixed culture of *L. lactis* and *L. acidophilus* reduced IC 50 value compared to unfermented kale significantly as shown in Figure 4.13.

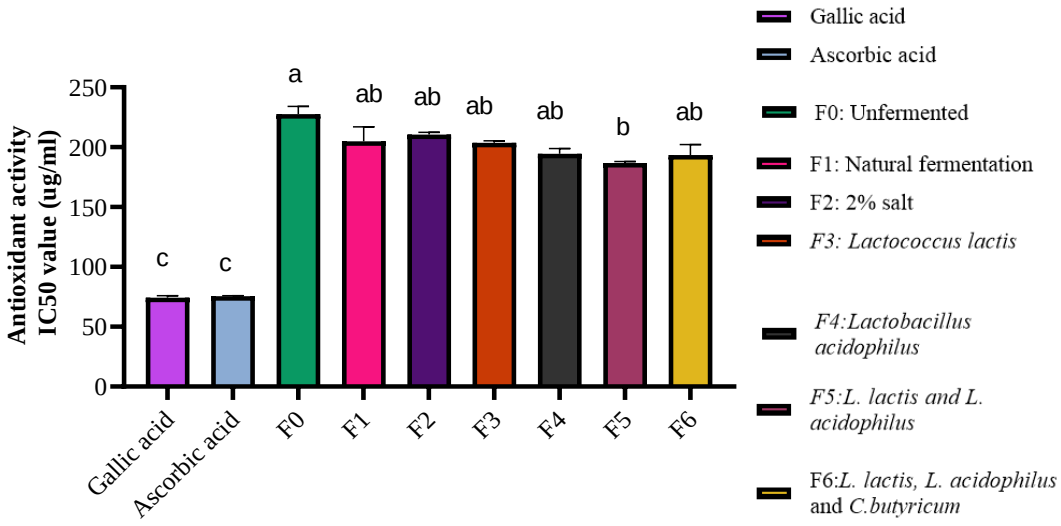


Figure 4.13. Effect of fermentation on antioxidant activity of kale. IC50 values of unfermented and fermented kale extracts. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

ABTS free radical scavenging capacity

The antioxidant capacity of kale extract evaluated against ABTS free radicals ranged between 1096 ± 32. 9 to 1354 ± 63.75 µM TE/g dw. A significantly higher

antioxidant capacity were observed in groups F5 and F6 fermented with mixed culture of LAB and LAB+ *C. butyricum*) (Figure 4.14).

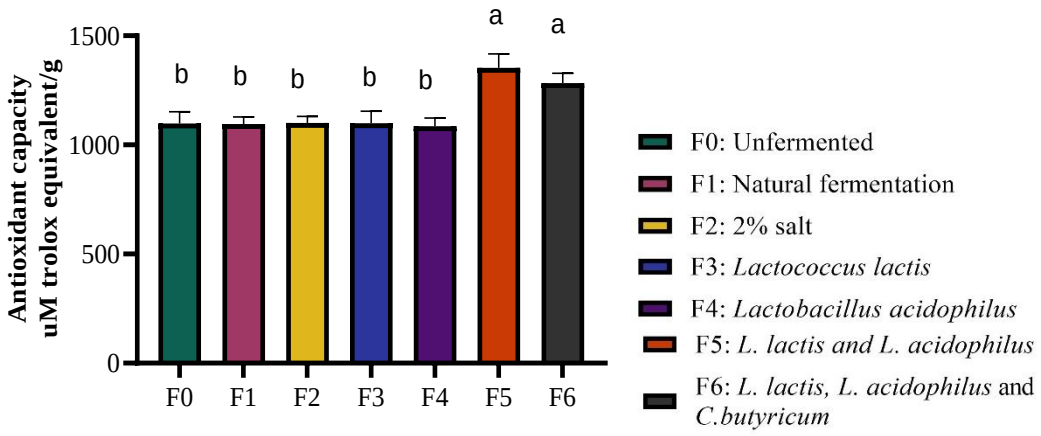


Figure 4.14. Effect of fermentation on antioxidant activity of kale expressed as ABTS free radical scavenging capacity µM TE/g dw. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

4.8 Effect of fermentation on anti-inflammatory properties of kale

Nitric oxide synthase (iNOs) gene expression

After stimulating cells with LPS, the inhibition of iNOs expression by kale extracts was expressed as a percentage of the control group (LPS only). The results showed a significant reduction ($p < 0.05$) in the gene expression of iNOs both in fermented and unfermented kale. The highest iNOs expression in the LPS control group (100%) was significantly reduced to 15.72 ± 7.07% by LAB-fermented kale, followed by unfermented kale (21.56 ± 4.87%) and traditionally fermented kale (24.44 ± 7.39%), as shown in Figure 4.15. All kale extracts significantly reduced inflammation, even though fermented kale reduced inflammation more than unfermented kale, but there was no statistically significant difference between fermented and unfermented kale ($p < 0.05$).

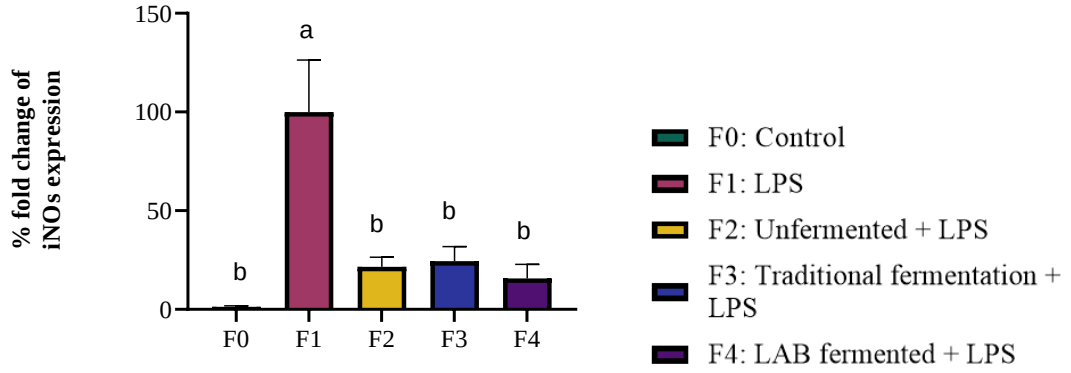


Figure 4.15. Effects of Kale on LPS induced inflammation. Percentage fold change (inhibition) of iNOs expression compared to LPS treatment. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

Interleukin-1 beta (IL-1β) gene expression

The inhibition of IL-1β expression by kale extracts was expressed as a percentage of the control LPS only group. Both fermented and unfermented kale extracts reduced IL-1β expression ($P<0.05$). The highest IL-1β expression in the LPS control group (100%) was significantly reduced to 31.8 ± 9.57% by LAB-fermented kale, followed by traditionally fermented kale (42.0 ± 18.1%) and unfermented kale (75.7 ± 16.2%), as shown in Figure 4.16. All kale extracts significantly reduced inflammation, even though fermented kale reduced inflammation more than unfermented kale, but there was no statistically significant difference between fermented and unfermented kale ($p < 0.05$).

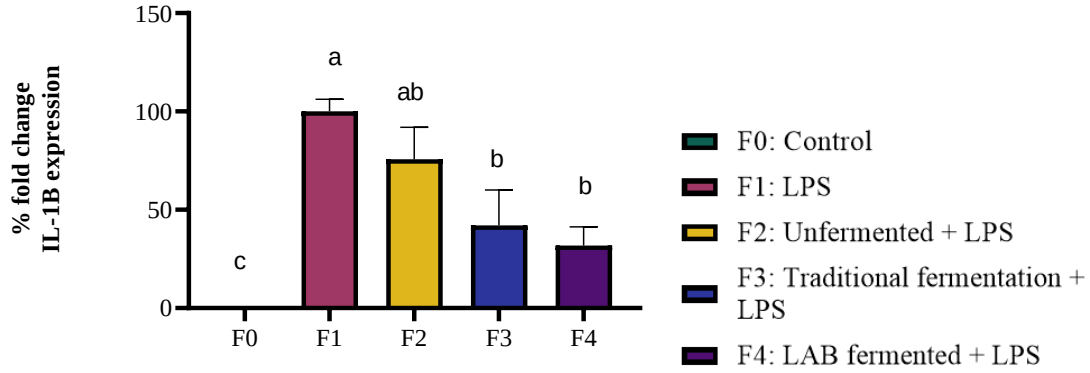


Figure 4.15. Effects of Kale on LPS induced inflammation. Percentage fold change (inhibition) of IL-1B expression compared to LPS treatment. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

Interleukin-6 (IL-6) gene expression

The inhibition of IL-6 expression by kale extracts was expressed as a percentage of the control group. The results showed a highly significant reduction ($p < 0.0001$) of IL-6 gene expression in both fermented and unfermented kale treatment groups. The highest IL-6 expression in the LPS control group (100%) was significantly reduced to 14.45 ± 7.75% by LAB-fermented kale, followed by traditionally fermented kale (16.72 ± 7.79%) and unfermented kale (18.16 ± 4.42%), as shown in Figure 4.17. All kale extracts significantly reduced inflammation, but there was no statistically significant difference between fermented and unfermented kale ($p < 0.05$).

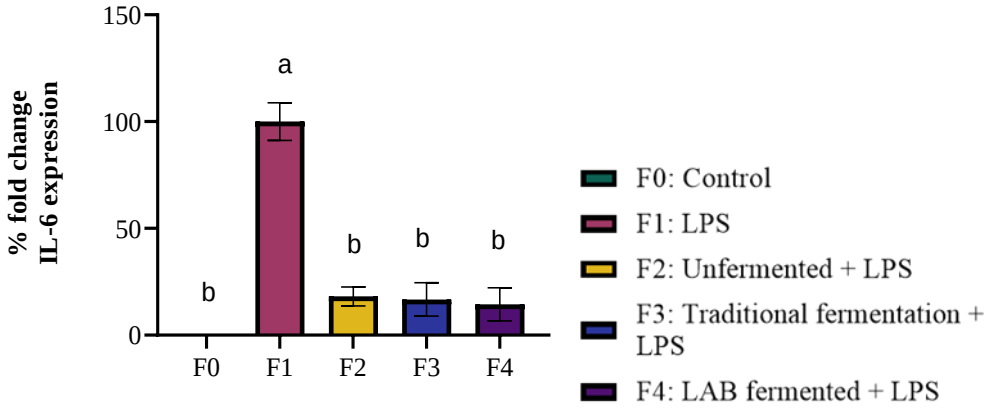


Figure 4.17. Effects of Kale on LPS induced inflammation. Percentage fold change (inhibition) of IL-6 expression compared to LPS. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; $n=3$).

Tumor necrosis factor-alpha (TNF-α) gene expression

The inhibition of TNF-α expression by kale extracts was expressed as a percentage of the control group. The results showed a highly significant reduction ($p < 0.05$) of TNF-α expressions in both fermented and unfermented kale treatment groups. The highest TNF-α expression in the LPS control group (100%) was significantly reduced to 38.41 ± 3.51% by LAB-fermented kale, followed by unfermented kale (48.12 ± 3.75%) and traditionally fermented kale (48.83 ± 12.88%), as shown in Figure 4.18. All kale extracts significantly reduced inflammation, even though fermented kale reduced inflammation more than unfermented kale, but there was no statistically significant difference between fermented and unfermented kale ($p < 0.05$).

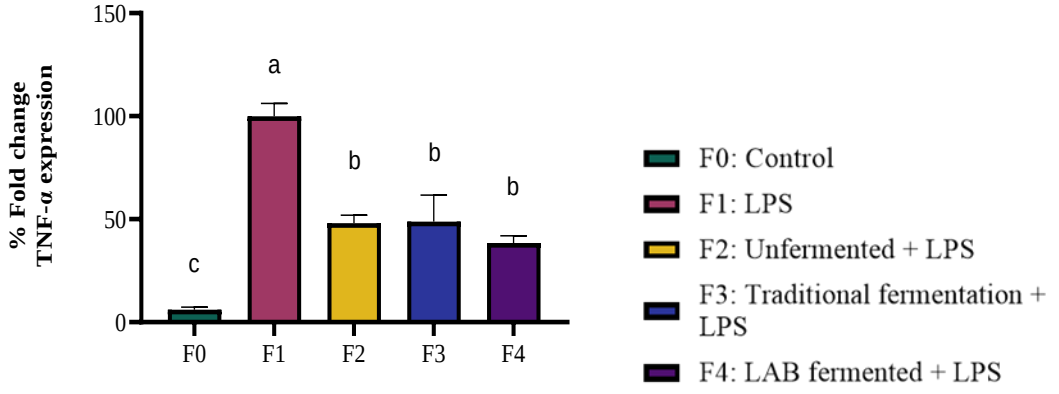


Figure 4.18. Effects of Kale on LPS induced inflammation. Percentage fold change (inhibition) of TNF-α expression compared to LPS. Results are shown as mean ± SEM, 'a'- Different letters indicate significant difference between treatments according to Tukey test ($p<0.05$; n=3).

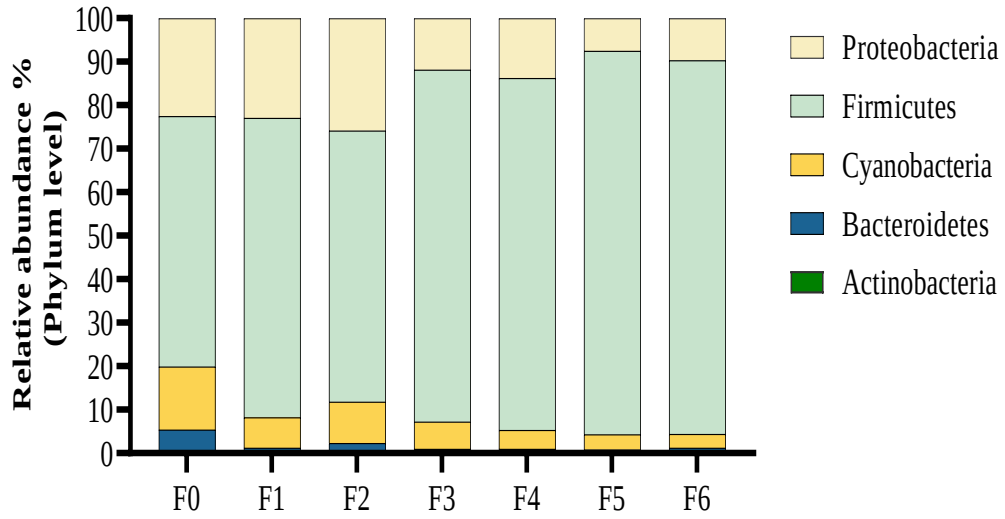
4.9 Effect of fermentation on the surface microbiota of kale

Fermentation 1 (natural fermentation) and fermentation 2 (2% salted fermentation) had higher abundance of Proteobacteria and with fermentation groups innoculated with mixture of *L.lactis* and *L. acidophilus* (fermentation 5) had significantly higher abundance of Firmicutes. The microbiota are further summarized as % relative abundance changes in phylum, class, order, and family level.

Relative abundance at phylum level

The microbiota of unfermented kale had 57.6% Firmicutes (22.5% Proteobacteria (14.57%, Cyanobacteria (and 4.7% Bacteroidetes. After fermentation Firmicutes increased to between 80.94 to 88.16 % in fermentation groups inoculated with lactic acid bacteria cultures (F3-F6) compared to unfermented kale. In traditional fermentation groups F1 (natural) and F2 (2% salted) a higher abundance of Proteobacteria were observed compared to other fermentation groups. Phylum

Bacteroidetes and Cyanobacteria decreased in all fermentation groups (Figure 4.19). All Lactic acid fermentation groups decreased Proteobactera.

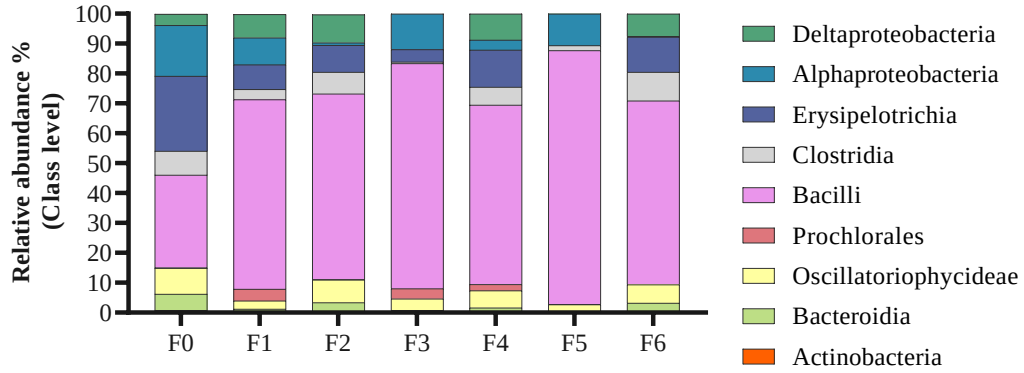


F0: Unfermented
F1: Natural fermentation
F2: 2% salt
F3: *Lactococcus lactis*
F4: *Lactobacillus acidophilus*
F5: *L. lactis and L. acidophilus*
F6: *L. lactis, L. acidophilus* and *C.butyricum*

Figure 4.19. Effect of fermentation on microbiota composition of kale at phylum level.

Relative abundance at class level

As presented in Figure 4.20 the relative abundance of class Bacilli increased 2 folds in the range of (60.2 to 85.1) % in fermented kale samples compared to the unfermented vegetable (31.4%). The highest abundance (85.06%) of Bacilli was observed in fermentation group F5 (fermented with mixed culture of *L. lactis* and *L. acidophilus*).

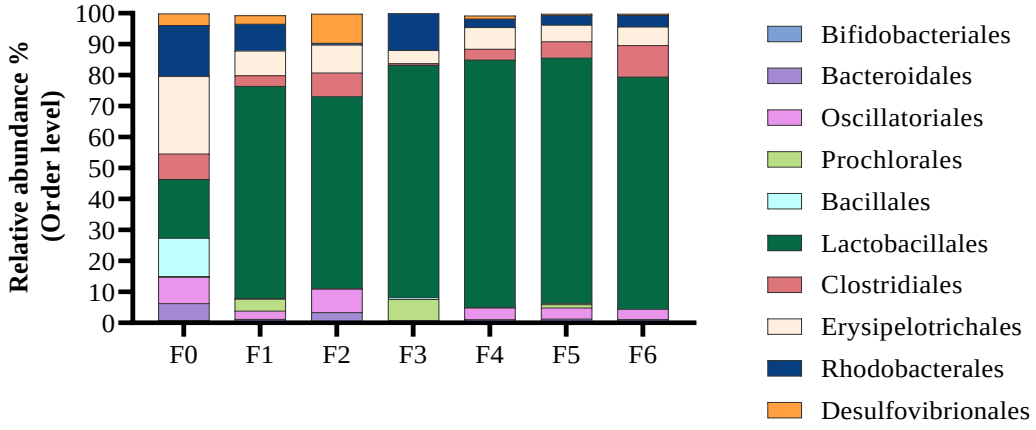


F0: Unfermented
F1: Natural fermentation
F2: 2% salt
F3: *Lactococcus lactis*
F4: *Lactobacillus acidophilus*
F5: *L. lactis and L. acidophilus*
F6: *L. lactis, L. acidophilus* and *C.butyricum*

Figure 4.20. Effect of fermentation on microbiota composition of kale at class.

Relative abundance at order level

Unfermented kale had higher abundance of Erysipelotrichales (25.17%) followed by Lactobacillales (19.19%), Rhodobacterales (16.99%), Bacillales (12.34 %), and Oscillatoriales (8.50%) respectively. In fermented kale samples Erysipelotrichales, Rhodobacterales, Oscillatoriales, and Bacteroidales decreased, while Lactobacillales increased up to 4 fold compared to unfermented kale as presented in Figure 4.21.



F0: Unfermented
F1: Natural fermentation
F2: 2% salt
F3: *Lactococcus lactis*
F4: *Lactobacillus acidophilus*
F5: *L. lactis and L. acidophilus*
F6: *L. lactis, L. acidophilus* and *C.butyricum*

Figure 4.21. Effect of fermentation on microbiota composition of kale at order level.

Relative abundance at family level

The microbiota of unfermented and fermented kale varied widely at family level. Erysipelotrichaceae, Streptococcaceae, Rhodobacteraceae, and unclassified Oscillatoriales had higher relative abundance in unfermented kale. In traditional fermentation (group natural and 2% salt) Leuconostocaceae, Erysipelotrichaceae, Streptococcaceae, Rhodobacteraceae, Desulfovibrionaceae, unclassified Oscillatoriales showed higher relative abundance as presented in Figure 4.22. While in fermentation groups inoculated with LAB cutures (group F3-6) Streptococcaceae and Lactobacillaceae had significant relative abundance.

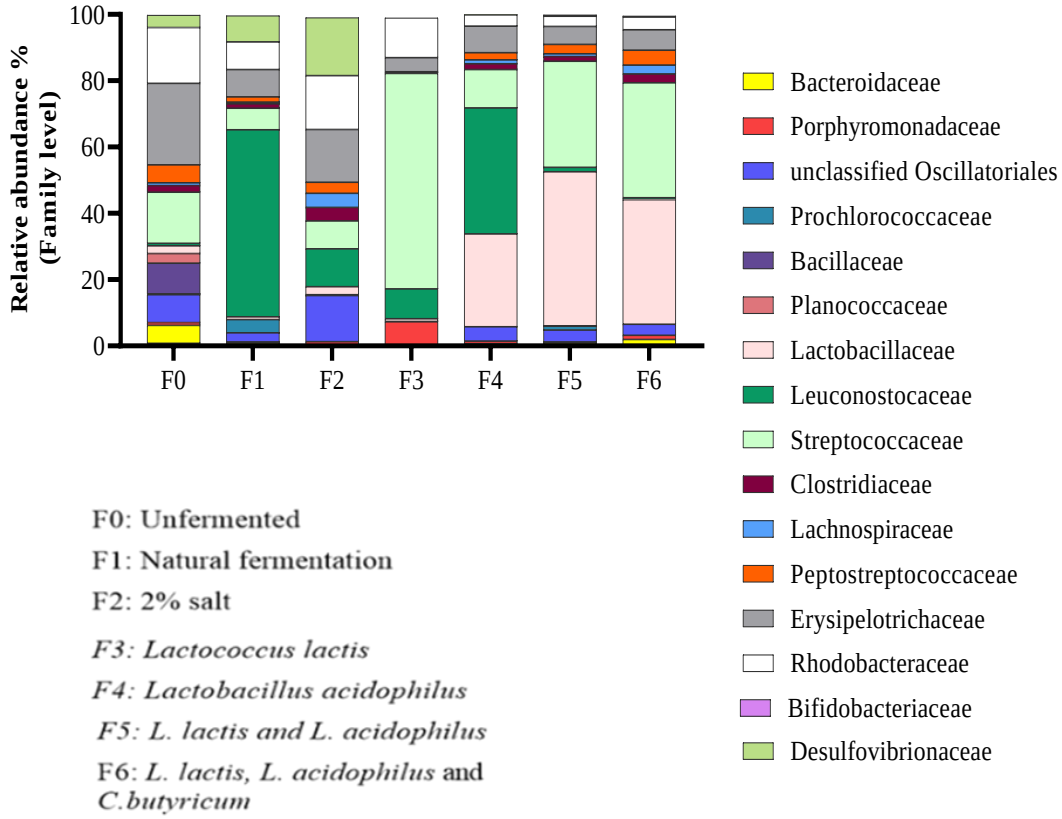


Figure 4.22. Effect of fermentation on microbiota composition of kale at family level.

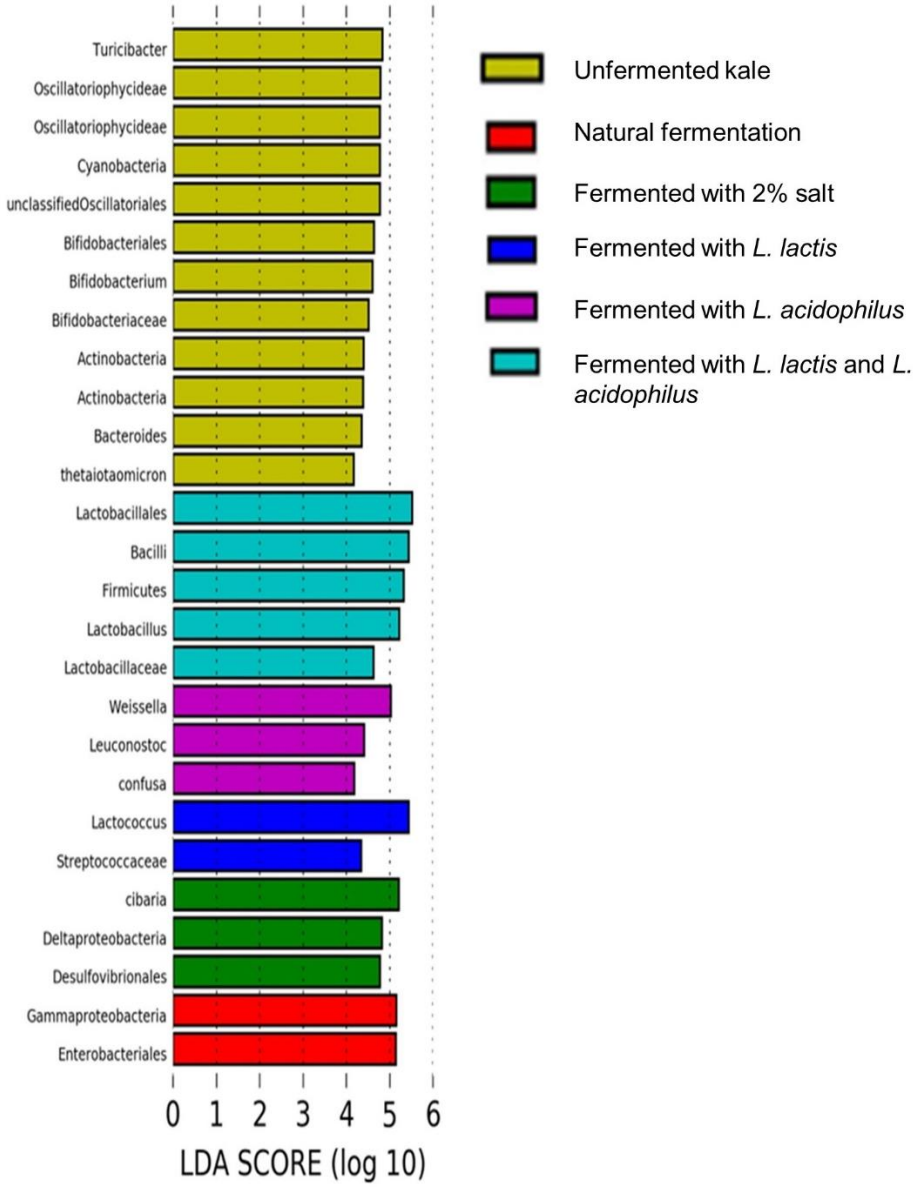


Figure 4.23. Linear discriminant analysis (LDA) effect size analysis between unfermented and fermented kale; LDA score > 4.0 among unfermented and fermented kale groups**.**

Chapter 5: Discussion

5.1 pH and titratable acidity

The pH value and acidity determine physicochemical characteristics. In this study, the pH of kale dropped 5.72 ± 0.04 to 3.74 ± 0.05 after 16 days of fermentation. Similar findings were reported by Vatansever et al. (2017) who fermented red cabbage fermented with 2% salt for 14 days; pH values decreased from 5.72 ± 0.11 to 3.76 ± 0.11. Hunaefi et al. (2013) also reported a drop of 4.3 and 3.8 in pH for red cabbage fermented with 1.5% salt and lactic acid bacteria respectively. Additionally, Ghimire et al. (2020) observed a decrease in pH from 6.59 to 3.71 during the spontaneous fermentation of *Brassica juncea* leaves for 15 days., Fermentation groups F5 and F6, which involved the combination of *L. lactis* and *L. acidophilus*, resulted in a the largest decrease in pH which can be attributed to more effective utilization and conversion of sugars present in kale into lactic acid by the mixed culture of lactic acid bacteria.

Similarly, before fermentation, the titratable acidity % (as lactic acid) % was relatively low; 0.243 ± 0.03 %. The increase in acidity by LAB fermentation was in agreement with work done in red cabbage Vatansever et al. (2017) where the acidity increased from 0.20 to 1.5 %. Similarly, Iga-Buitrón et al. (2023) reported a significant increase in titratable acidity after broccoli fermentation and found a notable difference between spontaneous fermentation and fermentation using mixed LAB (*Levilactobacillus brevis* and *Lactococcus lactis*) which were 1.18 and 1.33 % respectively.

The higher level of acidity and lower pH values in fermentation groups involving LAB compared to traditional fermentation groups without LAB inoculation

is due to the metabolic activities of the LAB used (Iga-Buitrón et al., 2023). During fermentation, microorganisms convert the natural sugars in kale into lactic acid, lowering its pH and creating an acidic environment that inhibits the growth of harmful bacteria, yeast, and molds while promoting beneficial bacteria (pH < 4.6). This also contributes to the tangy taste of fermented foods. Factors such as initial pH, bacterial strains, fermentation temperature, and duration all affect the extent of pH change (Vatansever et al., 2017).

Thus, among all fermentation groups tested, F5, which utilized a mixed culture of *L. lactis* and *L. acidophilus*, was most favorable in achieving superior lactic acid fermentation.

5.2 Total polyphenol content

Vegetables owe their overall antioxidant activity to polyphenols, as noted by Zhou and Yu (2006). In this study, the total polyphenol content increased slightly in all fermentation groups compared to unfermented kale with groups F5 and F6 with mixed LAB and *C. butyricum* showing the highest and statistically significant increases. The total polyphenols observed in this study were higher than those reported by Lučić et al. (2023), who found 4.0 mg GAE/g dw in hydroponically grown kale. Similarly, Yu et al. (2018) reported total polyphenols in the range of 1.59 to 2.33 mg GAE/g fresh kale in a study on home food preparation techniques' effects on kale bioactivity. However, the findings of Liu et al. (2023), reporting total phenolics of 17 mg/g, were higher than those of this study. Satheesh and Fanta, (2020a) reported a total polyphenol content in the range of 2.01 to 11.67 mg GAE/g in their review of bioactive compounds present in kale. The variation in total polyphenol content observed in different research findings might be due to kale cultivars'

variation, growing conditions, and the extraction process applied during the studies (Yu et al. 2018).

When vegetables undergo fermentation, their cell wall breakdown results in the release of various compounds, including polyphenols, which are naturally present but may be trapped within the cell structures. The enzymatic activities of microorganisms' during fermentation can help release these polyphenols, making them more accessible and contributing to a significant increase in total polyphenol content. Specifically, lactic acid bacteria lead to esterase enzymatic activity, catalyzing the hydrolysis of ester groups, which improves the bioavailability of phenolic acids. This could be the reason for the significant increase in total polyphenols in fermented kale (Salas et al. (2022). Some of the polyphenols in cruciferous vegetables exist as precursor compounds, such as glucosinolates and during fermentation, microorganisms' enzyme production converts these precursor compounds into active polyphenols increasing the total polyphenol content (Iga-Buitrón et al. 2023). The changes in polyphenol content during fermentation differ depending on the variety of vegetables, fermentation conditions, microbial strains used, and fermentation duration. (Olsen et al., 2010, 2012).

5.3 Total flavonoids content

Kale is a rich source of flavonoids, particularly quercetins which are antioxidants protective against free oxygen radicals and various illnesses, including cardiovascular disease, cancer, atherosclerosis, and chronic inflammation. Moreover, quercetin supports the production of enzymes that detoxify cancer-causing agents (Biondi et al., 2021). Previous studies by Sidsel et al. (2009), Susanne et al. (2012), and Olsen et al. (2009) have reported similar results for total flavonoid content in kale

vegetables, with flavonoid amounts of 6.61, 8.92, and 6.46 mg/g, respectively (Satheesh and Fanta, 2020a).

Study conducted by Marinova et al. (2005), found that the amount of flavonoids present in different fruits and vegetables varied from 0.2-1.9 mg/g. Similarly, Bahorun et al. (2004) discovered that Mauritian vegetables contained a total flavonoid content of 0.94-4.5 mg/g, with broccoli having the highest levels and carrot having the lowest levels (Satheesh and Fanta, 2020b). These results indicate that kale is a superior source of flavonoids when compared to other commonly consumed vegetables. Fermented kale showed a slight increase in total flavonoid content which could be due to enzymatic hydrolysis. During fermentation, lactic acid bacteria and other microbial species produce a range of enzymes, including glycosidases. These enzymes break the glycosidic bonds between sugars and flavonoid compounds, releasing aglycones or free flavonoids. Consequently, the total flavonoid content increases, and the bioavailability is higher due to the conversion of glycosylated flavonoids to their aglycone forms. Further study using ultrahigh-performance liquid chromatography photodiode array high-resolution multistage mass spectrometry (UHPLC-PDA−ESI/HRMS) could be used to understand more about the effect of fermentation on flavonoids (Dereje et al., 2023).

5.4 Beta carotene content

Approximately 850 tetraterpene pigments known as carotenoids are found in photosynthetic organisms. These pigments are vital for human health as they act as precursors for vitamin A, protect against photodamage, act as antioxidants, boost immunity, and aid reproduction (Maoka, 2020b). Kale is a good source of carotenoids, with lutein and β-carotene being the most prevalent types (Lee et al., 2020).

In this study beta-carotene content were similar to previous studies by Sikora and Bodziarczyk (2012), who have reported 3.88 to 44 mg/100 g beta-carotene content in kale. Fermented kale was found to have up to an 80% reduction in total carotenoids and a 72% reduction in beta-carotene. Szutowska et al (2021) also discovered a significant decrease in carotenoids and beta-carotene content during the fermentation of kale juice. The bacterial culture used in the fermentation process significantly impacted the reduction of beta-carotene, with *L. plantarum* causing a more significant decrease compared to *L. sakei*. The decrease in beta-carotene during fermentation was attributed to the production of various metabolites by lactic acid bacteria and the development of acidic conditions (Szutowska et al., 2021c).

According to a study by Odongo et al (2017) on Ethiopian kale, raw kale contained lutein, zeaxanthin, and β-carotene which decreased by 75% after fermentation. During lactic acid fermentation, volatile carotenoid cleavage derivatives are created, contributing to fermented flavor and aroma (Mapelli et al., 2020). Certain microorganisms like lactic acid bacteria can transform particular carotenoids during fermentation, such as beta-carotene being transformed into other carotenoids like zeaxanthin or lutein. This transformation can cause variations in the specific carotenoid composition of fermented kale. However, there have not been any comprehensive metabolomics studies reported that profile carotenoids and their derivatives before and after lactic acid fermentation of cruciferous vegetables and explain carotenoid chemistry and potential biodegradation (Szutowska et al., 2021c).

5.5 Glucosinolates content

Glucosinolates (GSLs) are another critical phytochemical that has excellent health benefits in human health. The most consumed and well-studied glucosinolates

within our diets are those derived from methionine, such as sulforaphane and allyl-glucosinolates, and aromatic glucosinolates, such as phenethyl- and benzyl-glucosinolate (Traka, 2016).

From the observations made in this study, glucoraphanin content was higher in the unfermented kale, significantly decreasing in the fermented kale. However, sulforaphane content was significantly higher in fermented kale than in unfermented kale. The fermentation groups with mixed LAB culture were found to have the lowest amount of glucoraphanin and the highest amount of sulforaphane.

According to a study conducted by (Liang et al., 2013), different *Brassica* vegetables such as broccoli, brussels sprouts, cabbage, and cauliflower contained glucoraphanin in the range of 0.25 to 19.22 mg/g and sulforaphane content ranged 60 to 1700 ug/g. The amount of glucoraphanin found in kale in our study was lower but sulforaphane content was higher than other cruciferous vegetables reported. In another study reported by Cai et al (2019) the unfermented broccoli had a glucoraphanin content of 3423.7 μmol/kg. However, this content drastically reduced to 712.4 μmol/kg after fermentation. A decrease in glucoraphanin content (40-65%) after the fermentation of curly kale was also reported by Szutowska et al (2020). A study by Hwang and Lim (2014) observed sulforaphane content in the range of 60.6 ± 5.1 to 620.2 ± 14.1 (μg/g) in broccoli samples extracted using different solvents. These values were very lower compared to the findings on kale in this study.

The lower glucoraphanin content in unfermented kale could be due to the conversion of glucoraphanin into sulforaphane by the action of the myrosinase enzyme released during chopping and grinding kale for freeze-drying Cai et al (2019). *Brassica* plants contain myrosinase, which hydrolyzes glucosinolates to produce

sulfur-containing isothiocyanate sulforaphane. Sulforaphane is a natural inducer of phase II enzymes in both human and animal bodies, aiding in detoxifying cancer-causing chemicals. Several factors directly influence sulforaphane formation, including plant species, genotype, pre-harvest conditions, and post-harvest processing. Glucoraphanin, the sulforaphane precursor, is also greatly affected by these factors (Gu et al., 2012). Bacteria displayed myrosinase-like activity while undergoing fermentation, facilitating glucosinolates hydrolysis. The myrosinase-like activity in LAB may result in different GLS breakdown products like sulforaphane in the fermented kale sample (Szutowska et al., 2020).

5.6 Effect of fermentation on the antinutritional factor present in kale

Oxalate content

Plant-based foods often contain oxalic acid and its salts, which may hinder the bioavailability and absorption of essential minerals such as calcium. Excessive oxalate in diet can also lead to the formation of kidney stones. To mitigate these risks, it's advisable to process these foods to reduce their oxalate levels if possible (Wadamori et al., 2014). Satheesh and Fanta (2020) have reported that kale has an oxalate content ranging from 0.08 to 23.02 mg/g by dry weight. The observations from this study (0.75 mg/g dw) were within the reported range. This difference could be due to variations in the analysis method, species, and agricultural conditions (Satheesh and Fanta, 2020). In this study, it was observed that the fermentation decreased the initial oxalate present in the kale by 50% similar to a study by Wadamori et al.(2014), who reported a 38.5 % reduction of oxalate content in *Kimichi* preparation using mixed vegetables. During lactic acid fermentation oxalate contents decrease because the lower pH causes the insoluble oxalate bound to calcium ions to

transform into soluble oxalate. Secondly soluble oxalate in the fermentation mixture which can then be utilized as an energy source by oxalotrophic bacteria such as *Lactobacillus acidophilus*, which has been shown to possess oxalate-degrading activity. This process ultimately reduces the oxalate contents of the final product. *Lactobacillus acidophillus* and *Lactobacillii plantarum* have beenreported t to degrade oxalate in fermented vegetable (Wadamori et al., 2014; Weese et al., 2004).

Phytate content

In the human body, phytate can have anti-nutritional effects by forming insoluble complexes with calcium, iron, and zinc through strong chelation leading to the deficiency of iron and zinc. Kale has lower phytate content of 0.12 mg/100 g compared to other green leafy vegetables with the range of 0.412–1.3 mg/100 g (Satheesh & Workneh Fanta, 2020a). But the amount of phytate observed in this study was higher than that range. This difference could be due to variations in the analysis method, variety, and agricultural conditions (Satheesh and Fanta, 2020). With fermentation, phytate content was observed to decline but not to a significant level compared to unfermented kale. Phytases are enzymes produced by *lactobacilli* and other bacteria during lactic acid fermentation. These enzymes can break down and decrease phytate concentration in fermented food and lead to an increase in the availability of minerals, which enhances the nutritional value of kale (Kim, 2017b; Samtiya et al., 2020).

Tannins content

Tannins are naturally occurring substances found in many plant-based foods and beverages, such as fruits, vegetables, nuts, and tea. While tannins have some beneficial health effects, such as antioxidant and antimicrobial properties, they can

also have negative effects on nutrient absorption and digestion. Tannins can bind to certain dietary proteins and carbohydrates, forming complexes that are difficult to digest thus reducing bioavailability of nutrients, making them less accessible for absorption in the gastrointestinal tract. Tannin content of 0.15 mg/100 g of had been reported in kale grown in Nigeria (Satheesh and Fanta, 2020). But the values observed in this study were higher compared to this value. Similarly, Fahmia et al (2019) reported 182 to 290 mg/100g tannin in cabbage samples. The variation in concentration of tannin among the *Brassica* vegetables could be due to extraction and analysis method, variety, and agricultural conditions (Satheesh and Fanta, 2020).

In this study a significant decrease ($p < 0.05$) in tannin content in kale was observed in kale fermented with mixed culture of *L.lactis* and *L. acidophilus.* Fahmia et al (2019) also reported a significant decrease in tannin content in cabbage during lactic acid fermentation using *L. plantarum* inoculum at variations of 5%, 10%, 15% and 20% (w/v). They also reported variation in tannin content according to the pH level of the fermentation biomass. So in this study the variation observed in tannin content among the different fermentation group can be correlated with the effect of variation of pH among the fermentation group as well as to the bacteria present as different bacteria have different tannase activity to break down the tannin (Fahmia et al., 2019).

5.7 Effect of fermentation on antioxidant capacity of kale

The increase of DPPH free radical scavenging properties in fermented kale can be positively correlated with high polyphenols and sulforaphane contents in fermented kale. In a study conducted by Rahman et al. (2022) on the antioxidant potential of different brassica vegetables (cabbage, cauliflower, and broccoli), IC50 values for

DPPH free radical scavenging were reported in the range of 90 ± 2.52 to 400 ± 10 ug/ml and the IC50 values observed in our study is similar to this observation. The IC50 value was higher than the results reported by Lan et al. (2005). Furthermore, the free radical scavenging potential also varies depending on the solvent used for sample extraction (Lan et al., 2005; Rahman et al., 2022).

The ABTS free radical scavenging value for unfermented kale obtained in this study corroborated with the value reported by Yu et al. (2018), where the ABTS scavenging values ranged from 1163.52 to 1262.57 μmol Trolox per g kale without microwave treatment, but the value for kale fermented with *L. lactis* and *L. acidophilus* were significantly higher.As per the results reported by Salas et al. (2022), the ABTS free radical reducing capacity ranged between 41.34 ± 2.85 to 194.27 ± 14.99 μmol Trolox per 100 g of broccoli which is lower than the values obtained for kale in this study. The enhanced ability of fermented kale to scavenge free radicals is due to the formation of new antioxidant compounds. During fermentation, microorganisms produce or release bioactive compounds like phenolic compounds, flavonoids, and other antioxidants. These compounds contribute to the overall antioxidant capacity of the fermented product and can potentially improve its health benefits (Salas-Millán et al., 2022). Several studies have evaluated the relationship between total phenolic content and antioxidant capacity and have found a strong correlation between total phenolic content and antioxidant capacity in selected vegetable products. (Zhang and Yu, 2021). Similarly, according to Figueiredo et al (2015), sulforaphane also contributes to free radical scavenging as it has antioxidant potential. This study indicated that lactic acid fermentation of kale with *L. lactis* and *L. acidophilus* would provide more health benefits than unfermented kale regarding its higher antioxidant properties.

5.8 Effect of fermentation on the anti-inflammatory capacity of kale

During an inflammatory response, proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are produced by macrophages. Previous studies have shown that LPS treatment triggers the production of TNF-α and IL-6 in RAW 264.7 cells, inducing their secretion (Jeong et al., 2018). The expression of in mammalian cells is a key factor in high nitric oxide output, responsible for many immune cell bactericidal and tumoricidal actions. The transcription factor NF-κB predominantly regulates the expression of host defense proteins, including iNOS. Activating NF-κB is necessary for cytokine production, a sign of inflammation. TNF-α, IL-1β, and IL-6 are the inflammatory cytokines whose expression is regulated by NF-κB (Hwang and Lim, 2014).

Overall, kale extracts, either fermented or unfermented, reduced inflammation significantly. Compared to unfermented and traditionally fermented kale, LAB-fermented kale extracts had higher anti-inflammation capacity. However, no significant difference was observed between fermented and unfermented kale extracts. Similar Inhibition of iNOS, TNF-α, IL-1β, and IL-6 gene and protein expression in LPS-induced RAW 264.7 cells by broccoli ethanolic extracts and *Artemisia montana* leaf extract were observed by Hwang and Lim (2014) and (S. H. Jeong et al., 2018) respectively.

Fernandes et al (2023), reported that Raw 264.7 cells inhibited proinflammatory cytokines significantly in non-encapsulated and encapsulated kale sprout samples. The maximum inhibition was observed with Sulphur and Selenium encapsulated kale samples in the corresponding IL-1β (28.5%, 29.6%), IL-6 (36.6%, 54.3%), and TNF-α (75.1%, 90.1%) cytokines, respectively, compared to controls stimulated with LPS.

Similarly, (Garcia-Ibañez et al., 2023) also reported inhibition of pro-inflammatory cytokine profile (TNF-α, IL-6, and IL-1β levels) released from human macrophage-like HL-60 cells treated with different cruciferous extracts.

The quercetin and kaempferol and sulforaphane confer the anti-inflammatory properties of kale. These compounds can interfere with the production of inflammatory substances like Nrf2 (Nuclear factor erythroid 2-related factor 2), which combat inflammation, ultimately reducing inflammation (Tilg, 2015). Furthermore, through fermentation, complex phytochemical substances are broken down into bioactive polyphenols of smaller sizes. Scientific studies indicate that these polyphenolic compounds, found in fermented products, can have a beneficial impact on microbiota metabolism and growth, as well as suppress the production of inflammatory cytokines and minimize inflammatory responses (Shahbazi et al., 2021).

It has been demonstrated that fermented cruciferous foodssuch as Kimchi and Sauerkraut possess anti-inflammatory properties aand attentate production of NO in LPS-induced murine macrophages RAW 264.7. The anti-inflammatory activity of sauerkraut may be partially attributed to allyl isothiocyanate and indol-3-carbinol. These compounds function through various mechanisms including the inhibition of pro-inflammatory cytokine production (such as IL-1β and TNF-α), pro-inflammatory enzyme expression (such as iNOS), and the activation of NF-κB pathway (Shahbazi et al., 2021). Additionally, sauerkraut reduces the levels of pro-inflammatory microRNA-155 in induced macrophages (Wagner et al., 2012). Similarly, a bioactive compound found in Kimchi, KIMCHI3-(40-Hydroxyl-30,50-dimethoxyphenyl) propionic acid, has been shown to alleviate inflammation in LPS-stimulated BV2 microglial cells. This is achieved by diminishing LPS-induced pro-inflammatory

cytokines secretion such as TNF-α and IL-1β. The compound works by inhibiting NF-κB, MAPKs, and PI3K signaling pathways (Jeong et al., 2015).

5.9 Effect of fermentation on microbiota of kale

The fermentation process was carried out for 16 days, and changes in the microbiota composition were evaluated to see how the fermentation process can increase the abundance of health-beneficial probiotic bacteria in the product. The high-throughput sequencing was applied to reveal the microbial community structure of samples. After quality control, 9,519,705 high-quality reads from V3 to V4 region of 16S rRNA gene sequences were obtained from 21 samples and the sample reads ranged from 68,281 to 892,702 reads.

Microbiota changes during different stages/time intervals were not studied on this research due to time limitation. In the unfermented kale bacterial phylum *Bacteroidetes*, *Cyanobacteria*, *Proteobacteria*, *Actinobacteria* were in higher abundance, along with *Firmicutes.* Eventually, after fermentation, the abundance of *Firmicutes* increased as expected. But in the samples fermented with traditional/household practices with spontaneous/natural fermentation and salting methods (groups Fermentation 1 and 2), *Proteobacteria* and *Cyanobacteria* had higher relative abundance than other fermentation groups. From the linear discriminant analysis, kale fermented with a mixed culture of *L. lactis* and *L. acidophilus* (Fermentation 3-5) produced significantly higher relative abundance of health-beneficial probiotics bacteria like Lactobacillus. While the spontaneous fermentation group had a significantly higher abundance of Gammaproteobacteria which are known to contain bacteria that can cause inflammation and are associated with disease. Phylum Firmicutes contains species that are capable of converting

sugars into lactic acid through fermentation. This lactic acid is essential in preserving, enhancing flavor, and improving the texture of fermented vegetables. Lactobacillus and other Firmicutes bacteria produce lactic acid that affects the taste, tanginess, and overall flavor of the fermented products. Some strains of Firmicutes phylum are probiotic bacteria that provide health benefits to the host when consumed in adequate amounts. The presence of certain Lactobacillus strains in fermented vegetables can promote gut health and digestion, giving potential probiotic effects. In the fermented kale abundance of Bacteroidetes decreased while Firmicutes increased which can be beneficial to prevent obesity (Shahbazi et al., 2021).

Chapter 6: Conclusions and recommendations

This study aimed to determine the impact of fermentation on the composition of bioactive components and anti-nutritional factors in kale, its anti-inflammatory and antioxidant capacity, and bacterial composition. The objectives and hypotheses were successfully tested, revealing significant observations and implications.

Objective 1 focused on the impact of fermentation on the composition of bioactive components and anti-nutritional factors in kale. The hypothesis that fermentation enhances bioactive components and reduces anti-nutritional factors was supported by the findings. Total polyphenol content increased in all fermentation groups, with significant increases observed in groups fermented with mixed lactic acid bacteria (LAB) cultures. Fermentation facilitated the release of trapped polyphenols and the conversion of precursor compounds into active polyphenols. Antinutritional factors like phytate, oxalate, and tannin were reduced during fermentation which can result in increased availability of minerals, proteins, and other nutrients present in kale. However, fermented kale showed a reduction in beta-carotene, a reduction attributed to the creation of carotenoid cleavage derivatives during lactic acid fermentation. Glucoraphanin content decreased in fermented kale, but sulforaphane content increased. The myrosinase-like activity of LAB during fermentation facilitated the conversion of glucoraphanin into sulforaphane.

Objective 2 aimed to determine the impact of fermentation on the anti-inflammatory and antioxidant capacity of kale. The hypothesis that fermentation enhances these properties was supported. Both fermented and unfermented kale extracts showed a significant reduction in inflammation, but LAB-fermented kale extracts exhibited higher anti-inflammatory effects. Fermentation also contributed to

the overall antioxidant capacity of kale through the production and release of bioactive compounds such as polyphenols, sulforaphane, and flavonoids. Kale fermented with *L. lactis* and *L. acidophilus* showed the highest antioxidant capacity compared to other test samples.

Objective 3 focused on comparing the changes in the bacterial composition of kale after fermentation. The hypothesis that fermentation enhances the prebiotic properties of kale by enhancing the abundance of health-associated bacteria was supported. All Lactic acid fermentation groups decreased *Proteobactera*. This Phylum is home to several bacterial taxa responsible for inducing inflammation and is generally associated with diseases. Therefore, fermentation improved the prebiotic properties of kale. Firmicutes phylum, particularly *Lactobacillus* strains, showed increased abundance in fermented kale, which can provide potential probiotic effects and promote gut health. Traditional/household fermentation groups had a significantly higher abundance of *Gammaproteobacteria* which might contain bacteria that can cause health problems so fermentation with LAB cultures resulting in a higher abundance of Firmicutes can provide healthy fermented kale.

The implications of the findings suggest that fermentation enhances the health benefits of kale by enhancing its increasing its antioxidant capacity and anti-nflammation capacity, improving the abundance of health-beneficial bacteria, and reducing antinutritional factors. The bioactive compounds whose concentration increased during fermentation contribute to these effects, making fermented kale a potentially valuable addition to a healthy diet. Nevertheless, the study had certain constraints. Time limitations prevented the detailed profiling of changes in various phenolic acids and individual flavonoids, glucosinolates, and the rate of microbiota

changes during different stages or time intervals. To gain a more comprehensive understanding of fermented kale, future research can delve into these aspects. Moreover, thoroughly examining the breakdown and conversion of beta-carotene to other carotenoids during fermentation and the sensory evaluation of fermented kale would prove valuable for exploring kale as a value-added product.

In summary, this study demonstrated the positive impact of fermentation on kale. Fermented kale can be considered a valuable functional food with potential health benefits. Further research in the mentioned areas would contribute to a deeper understanding of the effects of fermentation on kale and its applications in the food industry and human health.

Appendices

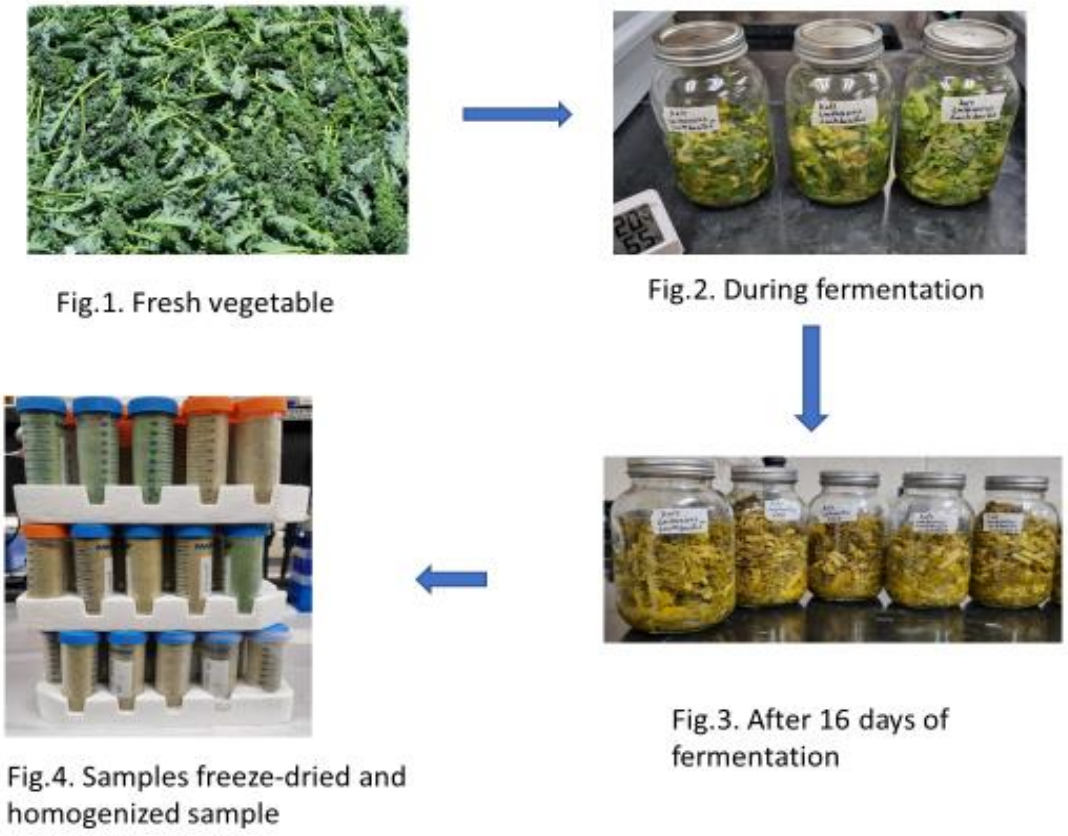


Figure S1. Representative images from the fermentation process

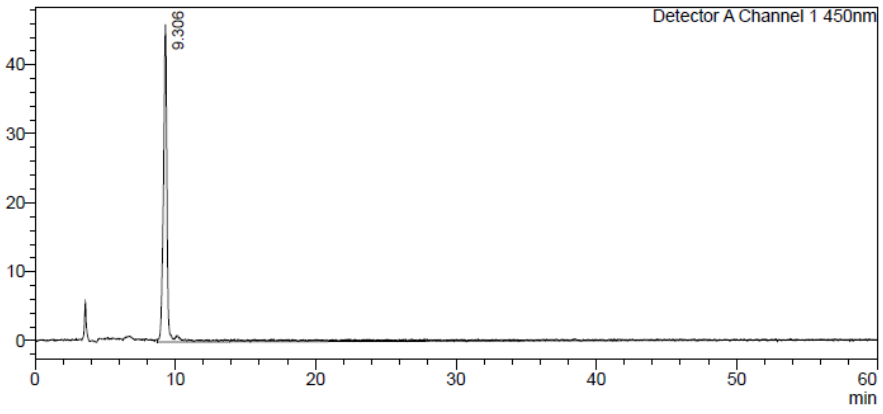


Figure S2. A representative HPLC chromatogram of beta-carotene standard at retention time 9.3 minutes

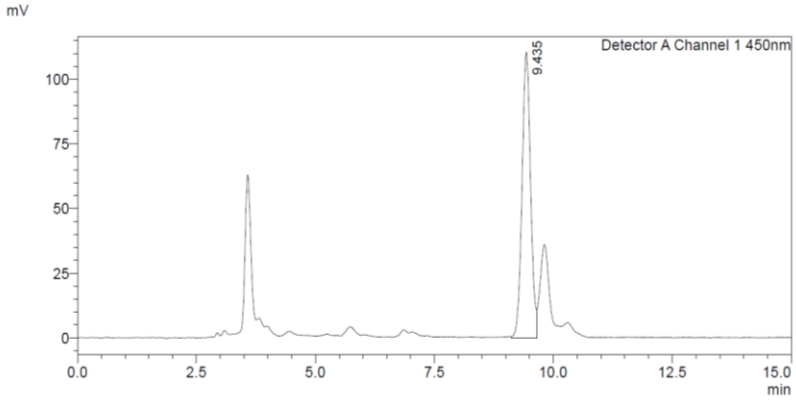


Figure S3. A representative HPLC chromatogram of beta-carotene in unfermented kale extract

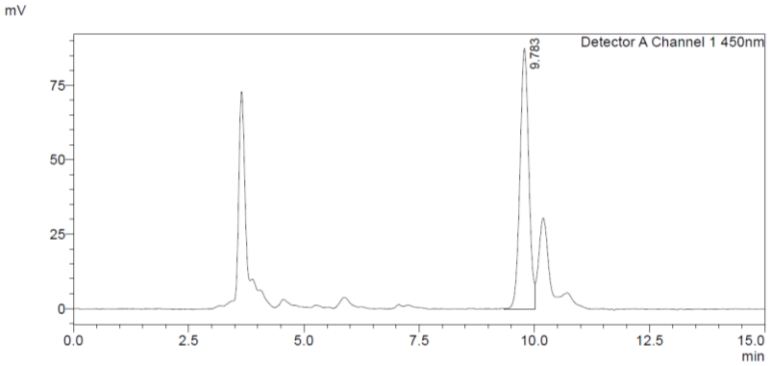


Figure S4. A representative HPLC chromatogram of beta-carotene in fermented kale extract

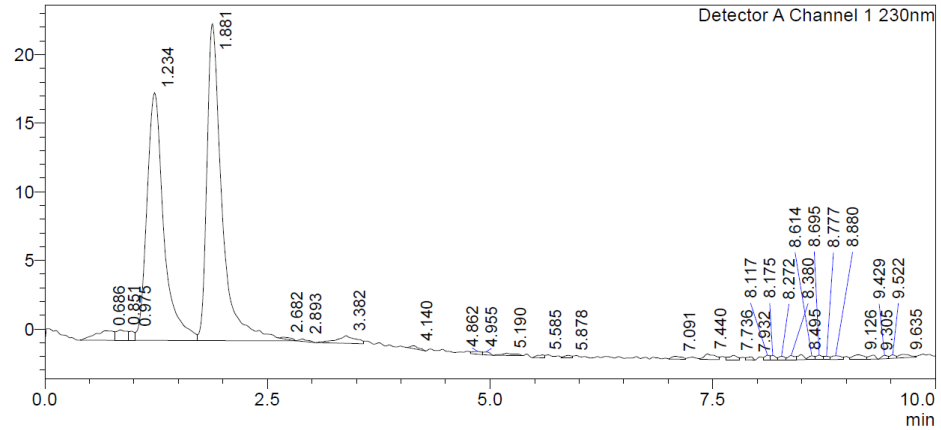


Figure S5. A representative chromatogram for Glucoraphanin and sulforaphane mixed standard at retention time 1.2 and 1.8 minutes respectively

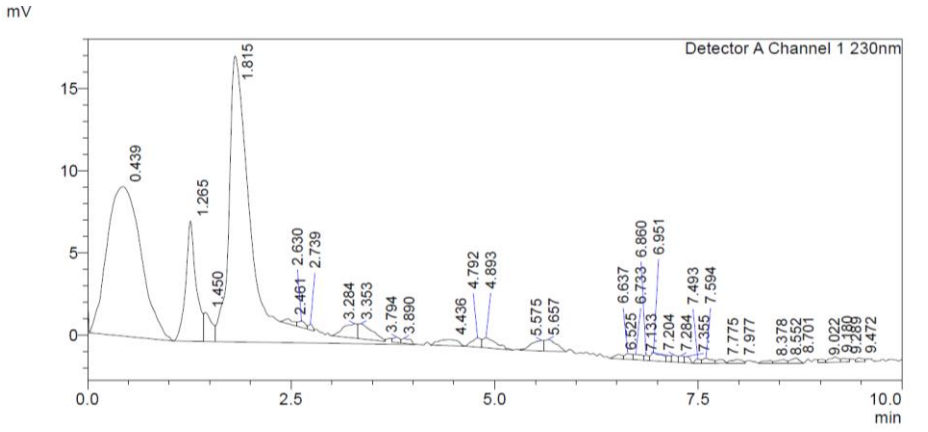


Figure S6. A representative chromatogram of unfermented kale, Glucoraphanin, and Sulforaphane peaks at retention time 1.2 and 1.8 minutes, respectively

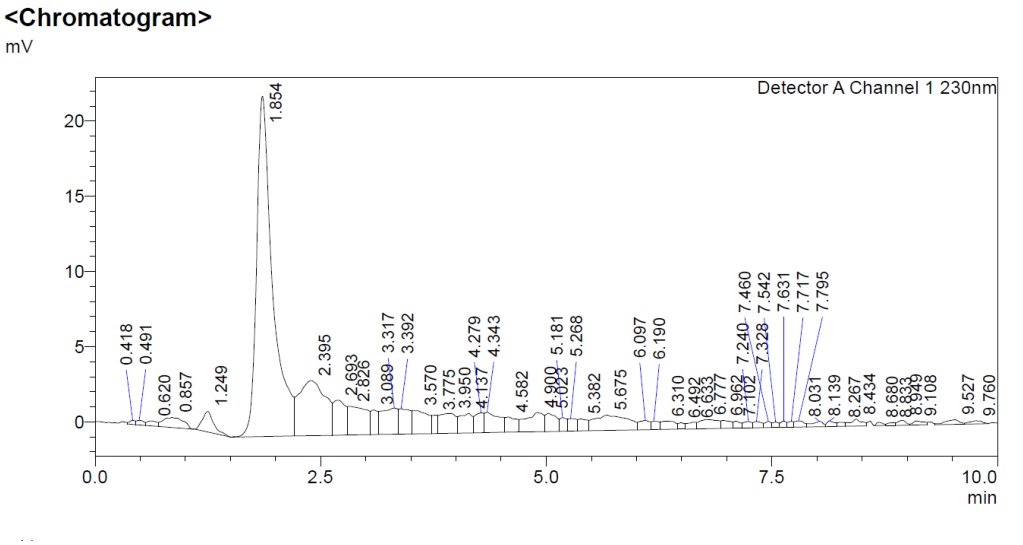


Figure S7. A representative chromatogram of fermented kale, Glucoraphanin, and Sulforaphane peaks at retention time 1.2 and 1.8 minutes, respectively

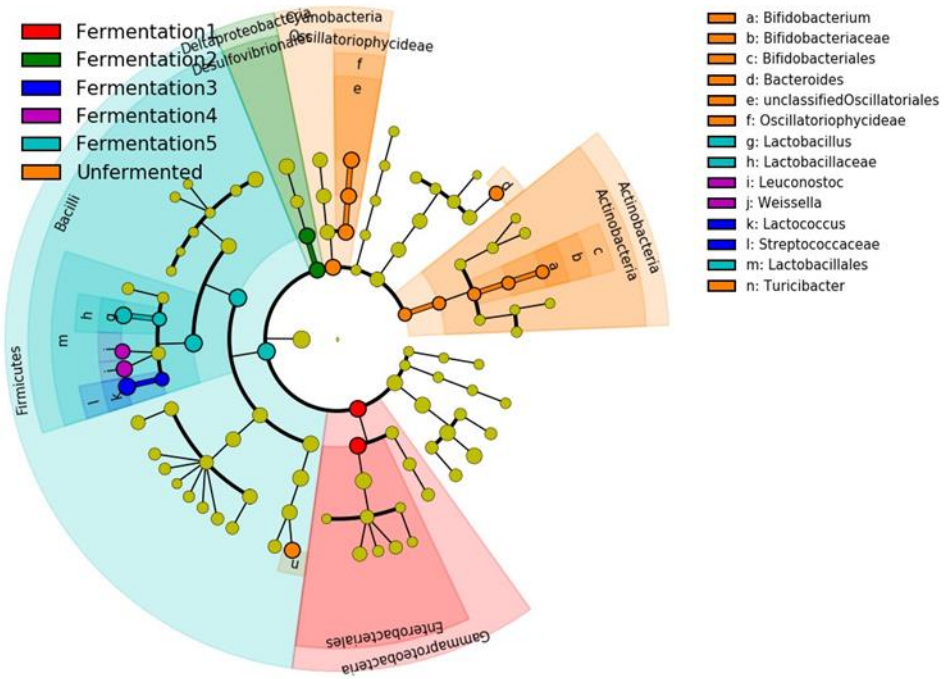


Figure S8. Linear discriminant analysis. Cladogram of differentially abundant taxonomic clades; LDA score > 4.0 among unfermented and fermented kale

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