Effect Of Natural Media Amendments on Crop Quality Under Controlled Environmental Conditions

By

Thilini K. Wickremasinghe

A Thesis Submitted to the School of Graduate Studies in
Partial Fulfillment of the Requirement of the Degree of

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Supervisor (Prof. Lakshman Galagedara)

Date

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Abstract

The effect of different natural media amendments on the physical, chemical, and biological properties of soil is well studied and documented. Although there is more information on changes in soil parameters, few studies have demonstrated effects on crop quality. Herein, a greenhouse study was set up to identify the effect of wood ash (WA), paper sludge (SL), biochar (BC), and its combinations on kale's nutritional profile grown in two different soil types (soil type 1, soil type 2) in Newfoundland, Canada. Pathway analysis revealed that although the same media amendments were used, the kale quality parameters were significantly altered by the grown soil type. It was shown that for soil type 2, most of the parameters were significantly enhanced compared to CTL (control) ($p<0.05$). For soil type 1, upon the addition of WA, SL, and BC there was a significant increase in the marketable yield; however, the crop's nutritional parameters were not significantly changed. The lipidomics results showed that for soil type 1, WSBC (wood ash+sludge+biochar) amendment appeared to be effective in enhancing the functional lipids of 1,2 DGs, 1,3 DGs, ω6 lipids, and phospholipids. Whereas in kale grown in soil type 2, most of the studied functional lipids were increased upon the addition of SL and WBC (wood ash+biochar) media amendment. This different impact of media amendments on soils was also suggested from the pathway analysis revealing significantly different ($p<0.05$) lipid pathways associated with each soil type. In conclusion, WA, SL, and BC and the combination amendments showed a positive effect on kale's nutritional profile, and this approach can be used to produce functional foods in controlled environmental conditions.

General Summary

To determine the effect of natural media amendments of wood ash, sludge, and biochar on the nutritional profile of kale, a greenhouse study with two different soil types (soil type 1 and soil type 2) was carried out. Kale grown in soil type 1 showed an increased marketable yield upon adding WA (wood ash), with no significant changes in the measured nutritional parameters. Comparatively, most of the parameters of kale grown in soil type 2 showed increased values, especially upon the addition of WBC (wood ash+ biochar) media amendment. The functional lipids of diglycerides (DG), phospholipids (PL), and omega-6 (ω6) lipids were enhanced in kale grown in soil type 1 when WSBC (wood ash+ sludge+ biochar) was used, while in soil type 2 most of the lipids were modulated upon the addition of SL (Sludge) and WBC. Overall, it was shown that WA, SL, and BC could be used as potential media amendments to modulate the nutritional parameters where their effects significantly vary on the soil type.

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List of Abbreviations and Symbols

CTL = Control

WA = Wood ash

WBC =Wood ash + Biochar

SL = Sludge

SBC = Sludge + Biochar

WASL = Wood ash + Sludge

WSBC = Wood ash + Sludge + Biochar

TAA = Total antioxidant activity

TPC = Total phenolic content

FSV = Fat-soluble vitamins

WSV = Water-soluble vitamins

CCE = Calcium carbonate equivalent

CBPPL = Corner Brook Pulp and Paper Ltd.

CEC = Cation Exchange Capacity

EC = Electrical Conductivity

Chapter 1: Effect of natural media amendments on crop quality

1.1: General introduction

1.1.1: Natural media amendments: their efficacy and concerns

Changes in soil chemical, physical and biological properties have been a major concern that has been mainly occurring through many anthropogenic activities (Larney & Angers, 2012). Increased urbanization and industrialization cause the generation of a higher number of waste products (Sharma et al., 2017). According to Ellis et al. (2010), a study on changes in biomes from 1700-2000 states that nearly 50% of change from wild to anthropogenic occurred by the early 20th century. The adoption of poor agricultural management practices has also been identified as the key reason for the loss of topsoil with less organic matter content; such reductions of organic matter content affect the replenishing ability of soil due to the changes in the soil available nutrient content and alterations in soil, water, and temperature regimes (Larney & Angers, 2012). Therefore, the successful restoration of land needs to be achieved by recreating the topsoil with the required amount of organic matter (Akala & Lal, 2000). The common practice of restoring the land is via importing the soil from elsewhere, but as per Larney and Angers (2010), this method is uneconomical, and the area from which soil is to fix the problem will eventually be degraded. Natural media amendments such as manure, biosolids, paper pulps, sludges, and waste from food processing industries can restore the lost soil properties (Sharma et al., 2017).

According to Vaish et al. (2016), nearly 4 billion Mg of municipal, industrial, and hazardous waste are generated globally, while 1.6-2 billion Mg are generated as municipal solid wastes (MSW). A report on Canada statistics (2008) estimated that around 181 Tg (wet weight assumed) of manure is produced from all livestock species in Canada, a 16% increase compared to 1981.

Cogger et al. (2006) reported that in Canada, nearly 0.5 Tg of the dry weight of biomass is produced per year. Recycling of wastes or organic matter such as livestock and human waste has been used to reclaim the soils from the early days, increasing both soil fertility and crop quality and reducing the amount of waste (Barrow, 2011).

The effect of different types of amendments in increasing the chemical, physical and biological properties of degraded soil is well studied (Pascual et al., 1999; Scullion & Malik, 2000). Although those amendments have been used for many decades, the negative impacts, such as the presence of higher concentrations of heavy metals, higher ratios of nitrogen (N) to phosphorous (P), toxins, pathogens, and bad odour, are concerns (Larney et al., 2011). Some may argue that using such amendments on soil is the sole method of dumping waste into the ground. Thus, Larney and Angers (2012) stated those soil amendments need to be used judiciously, as soils are resilient and benefit from increasing the soil organic matter content while imposing less burden on landfill sites. The authors also stated that the positive impact of using soil amendments is due to its ability to serve as fertilizers compared to common inorganic fertilizers in the market, which only add the nutrients such as N, P, and K (potassium) but no organic matter.

A study by Gardner et al. (2010) found that adding biosolids to a copper mine tailings site in British Columbia, Canada was an effective way to increase both soil quality and fertility compared to inorganic fertilizers. A similar study by Reid and Naeth (2005) stated that the addition of biosolids and composted paper sludge in the establishment of vegetation cover on tundra kimberlite mine tailings in Northwest Territories, Canada was showing a superior effect compared to the fertilizers. Although much scientific research claims the benefit of natural soil amendment usage, those are not widely available in the market as products due to less availability of published articles in the peer-reviewed literature (Abbott et al., 2018).

Furthermore, the data available were developed mostly on experimental field trials or participatory on-farm research activities (Schut et al., 2016). According to Abbott et al. (2018), before applying any soil amendment, it should be assessed whether it addresses the objectives of the farm production. According to the authors, the assessment should address:

i. Substituting or decreasing the usage of chemical fertilizers
ii. Helping in increasing the organic carbon content of the soil
iii. Usage of C and other nutrients from recycled waste products
iv. Addressing the need for alternative amendments
v. Addressing specialized markets via meeting farming and product process standards.

Apart from the above, the study also states that the selection of the amendments should be at least based on criteria such that it should predict the reduction or overcoming of the constraints associated with amendment application. Also, all applications of amendments should be based on the site specificity of soil and seasonal constraints associated with both plant and soil health. The current demand for natural media amendments has been identified due to the novel emphasis on soil health improvement and the increased demand for organic food production (Lockie et al., 2004). A survey carried out on cotton growers in Australia claimed that there is an improvement in soil structural stability, microbial biomass activities, soil organic matter content, and plant nutrient availability by using organic amendments (Quilty & Cattle, 2011). Many scientific studies claim the benefit of soil amendment usage, thus are not widely available in the market as products due to less availability of published articles in the peer-reviewed literature (Abbott et al., 2018). Furthermore, the data available are developed mostly on experimental field trials or participatory on-farm research activities (Schut et al., 2016).

Although many forms of soil amendments available, their cost-effectiveness, i.e., their efficacy and profitability in the agricultural industry, especially in terms of application and transportation costs towards farmers, has been a concern (Quilty & Cattle, 2011). Another major concern is the consistency of the crops, as there is a high uncertainty of the plants' responses to those soil amendments compared to conventional systems (Abbott et al., 2018). Castán et al. (2016) state that, since many soil amendments are available, selecting an amendment that addresses local soil constraints without adding any additional risks is more difficult. The difference in the cost between organic and inorganic fertilizers, and the possibility of the decline of finite inorganic sources such as phosphorous (Cordell et al., 2009) mainly due to growing energy demand, make natural media amendments with different functionalities more important in increasing both soil and crop quality (Dorian et al., 2006). In addition, as the current world is moving towards fewer carbon economies, recycling of waste matter from agriculture, municipal and industrial wastes is highly likely to increase (Cordell et al., 2009).

1.1.2: Newfoundland soils, paper mill wastes (wood ash and dewatered sludge) and biochar

1.1.2.1: Wood ash

The product that remains after the combustion of wood or unbleached wood fibre is defined as wood ash (WA) composted with many inorganic and organic compounds (Risse & Gaskin, 2002). Those ashes generated from wood have a good amount of most required plant nutrients except for less amount of N due to its low heat vaporization point (Brais et al., 2015). The constituents of the WA are highly dependent on the source as well as factors such as industrial combustion system, combustion temperature, the collection location, cleanliness of the wood material (Risse & Gaskin, 2002) boiler type (Pugliese et al. 2014), and contamination sources (Bottom ash, fly ash, etc) (Pitman, 2006). Due to such high variability of associated factors, it has been difficult to generalize WA properties (Demeyer et al., 2001).

In North America, it has been reported that pulp and paper industries generate a tremendous volume of waste products, for instance, Canada produces more than 1 Mg of wood ash and 4.7 Mg of sludge annually (Cherian & Siddiqua, 2019). A study on wood ash reports that it has shown the ability to increase the soil pH levels and dissolved organic carbon (DOC) levels which may contribute to soil nitrogen (N) availability through different mechanisms (Jokinen et al., 2006; Molina et al., 2007). Wood ash utilization in agriculture has been researched for more than eight years in the regions of Fennoscandia (Ludwig et al. 2002), but its usage in Canadian boreal forests has yet to be further investigated (Huotari et al., 2015). Most soils in western Newfoundland are highly acidic with a loamy structure due to persistent conditions such as high rainfall and humidity, the forest cover of coniferous trees making the accumulation of organic material (Page, 1971). These acidic soils require a large amount of lime per hectare to achieve the target pH level for crop growth (Anderson et al., 2013).

Since there is an added cost towards the purchase and transportation of lime, wood ash has been considered an economical alternative for lime (Arshad et al., 2012). There is a possibility of the presence of metallic and metalloid compounds in wood ash, such as cadmium (Cd), barium (Ba), and arsenic (As), which can be phytotoxic, making testing of ash important before usage as a soil amendment in agriculture (Dahl et al., 2010). Besides being identified as a plant nutrient material, wood ash has been widely used as an acid neutralizer due to the high pH and presence of calcium (Ca), magnesium (Mg), K hydroxides, and oxides (Saarsalmi et al., 2001). The characteristics of those oxides and hydroxides greatly varied on factors such as temperature, combustion, and the period of storage (Demeyer et al., 2001). There is less information on using wood ash to improve crop quality in Canada (Arshad et al., 2012). A study carried out in Alberta, Canada by Lupwayi et al. (2009) reports that via application of both wood ash and lime altered the functional bacterial composition and increase the biomass and carbon accumulations in acidic soil.

A similar study by Patterson et al. (2004) states that the application of wood ash with inorganic N fertilizers increased barley biomass and canola seeds yield at 25 Mg ha^{-1} application rate but the changes in soil properties have not been assessed.

1.1.2.2: Secondary Sludge/Dewatered sludge

Paper and pulp industries are rapidly growing, and concerns have been raised due to the accumulation of increasing waste (Simão et al., 2018). In 2013, Swedish Forest Industries Federation (n.d.) estimated that paper and pulp production reached 403 Mg and 179 Mg, respectively. According to World Wildlife Fund (WWF), there are around 400 million Mg of paper consumption annually which is responsible for nearly 5-16% of bio residual accumulation, and this amount gets highly varied according to the type of paper product that gets produced (Xu & Lancaster, 2008).

Most past analysed research has reported that sludge is generally consistent with high numbers in organic matter, nitrogen, and phosphorous making it more suitable as a natural amendment for agriculture (Ribeiro et al., 2010). Usually primary sludge, mainly consisted of cellulose fibre and different fillers such as calcium carbonate, kaolinitic, titanium oxides, and compounds such as potassium, and sodium (Cabral et al., 1998). The accumulation of sludge depends upon both the production process and the technology adopted in wastewater treatment (Fahim et al., 2019). Simão et al. (2018) state that such treatment differences impact the distinct features of primary and secondary sludges where sludge generated mainly constituent with more than 50% of water with a pH range of 6.6 to 8.2. Due to its high water content, it is essential before using it in any industry partial or whole dewatering process (Cabral et al., 1998).

Although many compounds are beneficial for plant growth, the high occurrence of toxic metals in both primary and secondary sludges can pose a significant impact on human health and environmental hazards. It has been recommended that before the application of sludge to land it needs to be accurately assessed for its parameters affecting overall quality (Abdullah et al., 2015). A study on the effect of paper mill sludge on potato yield, nitrogen deficiency, and disease incidence by N'Dayegamiye et al. (2013) reports that during the fall and springtime periods, it has shown a significant increase in the nitrogen uptake from the potatoes and its marketable yield was nearly eight times higher compared to unfertilized control. The research also demonstrated that the combination of inorganic nitrogen fertilizers significantly increased the yields, specific gravity, and N uptake by the potato plants compared to the control. Apart from the above a study on secondary sludge effects on volcanic soils in Chile by Gallardo et al. (2010) showed a significant increase ($p<0.05$) in microbial and enzymatic activities after the application of sludge and maximum biological activity was achieved after 15-30 days of application at a rate of 50 Mg ha^{-1}. A similar study by Gallardo et al. (2012) reported that the usage of secondary sludge improved soil physical and chemical properties, and its organic content.

1.1.2.3: Biochar

According to the international biochar initiative (IBI), biochar (BC) is defined as the production of a solid material after the thermochemical conversion of the biomasses such as agricultural residues (Wang et al., 2018) under limited oxygen conditions (Agegnehu et al., 2017). BC can be achieved through gasification, pyrolysis, flash carbonization, and hydrothermal carbonization (Wang et al., 2018). Since there are different feedstocks, production methods, and temperatures are associated, biochar's physicochemical properties can get highly varied (Sun et al., 2014; Weber & Quicker, 2018).

According to Anyika et al. (2016) state that there can be secondary contaminants present in the biochar that was made from toxic solid wastes; however, many researchers have figured out the potential benefit of biochar as a soil amendment (Ding et al., 2016; Xiao et al., 2018). Application of biochar to the soils has generally shown to be beneficial due to its stable or recalcitrant C content, which remains sequestered for a longer period compared to its natural form availability; This makes the usage of biochar as a product itself or as a combination of other ingredients with many applications, such as an agent for improving soil properties, absorbing particular environmental pollutants, mitigating of greenhouse gases, and improving the usage efficiency of natural resources (International Biochar Initiative, 2015).

A meta-analysis study carried out by Omondi et al. (2016), reported that soil amended with biochar significantly reduced the soil bulk density by 3-31% in 19 soil samples out of 22. Similarly, Blanco-Canqui (2017) reviewed 22 published articles and concluded that there was a 12% reduction in bulk density in all soil samples. Apart from increasing the soil's other physical properties, it has been shown that the application of biochar can increase soil pH levels reducing soil acidification (Yu et al., 2019). A correlation study by Yuan and Zhang (2011) reported that there was a strong positive correlation between increased pH levels in acidic soil of Ultisol with biochar alkalinity (r=0.95). Similarly, a study by Amin and Eissa (2017) on amended soil by using biochar derived from maize on a calcareous soil (pH of 7.9) has shown increased soil N efficiencies and organic content, while fresh weights of zucchini have been increased by 26.7, 55.0, and 195.0% for biochar application rates of 6.3, 12.6 and 25.5 g per pot, respectively. It has been observed that biochar can be used as a material of remediation for contaminated soils due to its ability to immobilization of heavy metals, reducing its bioavailability and enhancing plant growth; the biochar characteristics such as the presence of large surface areas, pores volumes, and specific functional groups are being identified as key elements in adsorbing the heavy metals (Ahmad et al., 2018; Inyang et al., 2016).

Lu et al. (2017) studied the effect of rice-based biochar at a 5% application rate on soil contaminated with Cd, Cu, Pb, and Zn and found that metal concentrations were reduced in the order of Cd<Cu<Pb< Zn expressed in terms of diethylene triamine peracetic acid (DTPA) while it also reduced acid extractable metals of Cd, Cu, Pb and Zn by 11%, 17%, 34%, and 6%, respectively.

1.2.3: The effect of natural media amendment on crop quality

Despite the well-addressed benefit of natural amendments to soil properties, there are fewer studies available addressing the bioactive compounds in edible plant materials (Selma et al., 2010). As per Bourn and Prescott (2002), although there were many attempts to investigate the differences in the nutritional qualities between conventional and amended soils, the variations in study designs and study approaches make it hard to compare the findings. Nevertheless, most studies agreed that the application of different fertilizers has positively affected the quality and yield of the crops (Rendig, 2015; Syltie et al., 1982).

Most of the studies have shown that the higher the N content, the higher the uptake of N in the forms of nitrate or ammonium ions in the crops (Brunsgaard et al., 1994; Schaller & Schnitzler, 2000; Sørensen et al., 1994). A similar study by Yun and Ro (2009) using isotopic N stated that there was an increase in the plant N- content with the application of compost. Past studies have figured that with higher rates of N applications, the levels of proteins and antioxidants have increased (Poiroux-Gonord et al., 2010; Yañez-Mansilla et al., 2015). The phenolic content and the ascorbic acid content have been increased in a study by Antonious et al. (2014) with the application of chicken manure and sewage sludge compared to no amendment. Thus, a similar study conducted by Zhao et al. (2007) reported that there is no significant difference in measured phenolic compounds in between the organic (compost + fish emulsion) and conventional (N-P-K + $CaNO_3$) media amendments.

Similarly, a study by Hargreaves et al. (2008) stated that the total antioxidants, yield, and vitamin C content of raspberries were not affected by adding composted tea as an amendment where the author suggests that such differences may occur due to changes in the precipitations throughout the year may have affected the nutrient availability in the soils.

Since there is an expansion of usage of different soil media amendments in agriculture with limited justified evidence when applied by farmers, the range of availability of soil amendments, its physio-chemical proprieties, and the potential of improving both yield and nutritional qualities need to be well addressed (Abbott et al., 2018).

1.2.4: Lipids: As bio-actives in functional foods and novel biomarkers

In the last decades, consumer demands in food production have changed considerably (Betoret et al., 2011) and consumers thoroughly believe that the foods that we consume directly contribute to health (Mollet & Rowland, 2002). Today foods are not only considered as media used to satisfy hunger and provide basic nutrients but also help prevent diseases which will improving physical and mental health (Nöthlings et al., 2007). The World Health Organization and the Food and Agriculture Organization (WHO/FAO) state that dietary patterns along with current lifestyle habits create major risk factors concerning the development of cancers, coronary heart disease, diabetes: type-2, obesity, osteoporosis, and periodontal disease (WHO, 2003). In this regard, Functional foods play an outstanding role in human health and well-being (Betoret et al., 2011).

According to Stuchlík and Zák, (2002), food can be defined as "functional" if it is shown to beneficially affect one or more target functions in the body, beyond providing adequate nutritional effects. Authors also state that functional foods can be natural food, where a beneficial component has been added, or food from which different technological and biotechnological approaches have removed a component.

The increasing demand for such foods can be mainly attributed to the increasing cost of healthcare, the increase in life expectancy, and the desire to improve the quality of life (Roberfroid, 2000). It has now widely accepted that the health of a population is influenced by diet (Betoret et al., 2011), especially by the high levels of phytochemicals present in plants (Reganold et al., 2010; Schreiner & Huyskens-Keil, 2006). Considering the bioactive compounds present in plants, plant lipids have gained popularity in developing functional foods (Stuchlík & Zák, 2002; Yang et al., 2018).

Chemically, lipids are defined as hydrocarbons of C6 to C32 long-chain, containing a functional headgroup / hydrophilic carboxyl group at one end and a methyl group at the terminal end (Patel et al., 2020). These hydrocarbons can be saturated, monounsaturated, or polyunsaturated, where there are 0, 1, and more than 1 double bonds present are, respectively (Cholewski et al., 2018). Considering the different fatty acid types, omega-3(ω3) and omega-6 (ω6) polyunsaturated acids which are considered essential fatty acids due to the lack of certain enzymes in humans that help in the desaturation process where; consequently, these fatty acids must be obtained from dietary sources (Patel et al., 2020). Epidemiological and experimental studies have shown that increasing ω-3 polyunsaturated fatty acids (PUFAs) in the diet has significant benefits against colon, breast, prostate, pancreatic cancers (Riediger et al., 2009), and cardiovascular diseases (Chen et al., 2013).

Glycolipids (GL) and phospholipids (PL) represent the major building blocks for biological membranes. In plants, phosphoglycerolipids are the predominating lipid class in the plastid membranes. The chloroplasts mainly consist of galactolipids which play an important role in the photosynthesis of higher plants, algae, and certain bacteria (Hölzl & Dörmann, 2007). Numerous studies have shown that galactolipids derived from plants exhibit various medicinal properties such as antitumor activity (Shirahashi et al., 1993; Murakami et al., 1995), and anti-inflammatory activity (Cateni et al., 2001; Kharazmi, 2008).

Dietary PLs have benefits of inhibiting cancer growth (Sakakima et al., 2007), significant reduction of total cholesterol (Simons et al., 1977; Wójcicki et al., 1995), and helping in restoring the immunological functions in the elderly population (MacZek et al., 1998). Diglycerides (DG) and phytosterols (St) are another new class of functional lipids common in vegetables which are in low concentrations (1–10% w/w) compared to triglycerides (TG) (Kovacs & Mela, 2006). Studies conducted by Kristensen et al. (2006) stated that compared to TG, the regular intake of 1,3-DG isomer helps in preventing obesity and diseases such as hypertension, gallbladder disease, and some types of can. Despite the well-documented soil benefits related to the use of different media amendments, few studies have investigated their influence on the phytochemical profile of edible plant materials (Sousa et al., 2005). A study by Vidal et al. (2018) stated that functional food development can be achieved via modulation of growth conditions such as the usage of vermicompost, potassium humate, and volcanic minerals to enhance the crops with targeted functional ingredients under controlled environmental conditions.

Plants that are subjected to environmental stresses develop different response mechanisms including changes in metabolisms (Bolton, 2009). Responses to biotic and abiotic stresses leading to modification of glycerolipid composition are well documented in plants (Böttcher et al., 2009; Li et al., 2008). Therefore, based on the research inconsistency on changes in crop quality upon the addition of natural media amendments, the following hypotheses were set up to conduct the research.

1.2: Hypothesis

1. There is a positive effect of wood ash (WA), paper sludge (SL), and biochar (BC) as natural growth media amendments to soil on the nutritional profile of vegetable crops.
2. There is a positive effect of natural media amendments on intact functional lipids content of plants which may contribute as a novel approach to the production of functional foods.

1.3: Objectives of the thesis

Based on the above hypotheses, research was set up to achieve the following objectives.

1. To determine the effects of paper mill wastes (WA, SL) and BC as natural growth media on the nutritional profile of kale grown under controlled environmental conditions.
2. To analyse analyse the above natural media amendments on the intact functional lipids profile of kale grown under controlled environmental conditions.

1.4: Thesis organization

This thesis is mainly organized into three main chapters and written in manuscript style.

Chapter 01: Describes the general background information on different natural media amendments, benefits, concerns of its usage, research problems, and overall study objectives. This chapter also emphasizes the need to address the nutritional quality of crops grown in natural media-amended soils.

Chapter 02: This chapter describes the effect of natural media amendments (wood ash, sludge, and biochar) on the nutritional profile of Kale grown under controlled environmental conditions.

Chapter 03: This chapter describes the effect of natural media amendments on the intact functional lipid profile of Kale grown under a controlled environment assessing using ultra-high performance liquid chromatography coupled to C30 reverse phase chromatography and heated electrospray ionization high-resolution tandem mass spectrometry (UHPLC-C30RP-HESI-HRMS/MS).

Chapter 04: Describes the overall discussion, conclusion, and future recommendations for the entire thesis.

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Chapter 2: The effect of wood ash, paper sludge, and biochar media amendments on the nutritional quality of kale grown under controlled environmental conditions.

Abstract

The effect of different types of natural media amendments in increasing chemical, physical and biological parameters of degraded soil is well studied. However, fewer studies are available addressing the modulation of plants' bioactive compounds upon adding those amendments. This study aimed to evaluate different growth media formulations (wood ash, paper sludge, and biochar) on the yield and nutritional profile of kale under controlled environmental conditions. A greenhouse study was conducted in two soil types (soil type 1 and soil type 2) with different growth media formulations of control (CTL), control + biochar (CBC), wood ash (WA), wood ash + biochar (WBC), sludge (SL), sludge + biochar (SBC), wood ash + sludge (WASL), and wood ash + sludge + biochar (WSBC). The nutritional parameters of total carbohydrates, total proteins, total fatty acids profile, antioxidant activities, phenolic content, total mineral content, and marketable yield were measured using standard protocols and expressed on a dry weight (DW) basis. The pathway analysis indicated that although the same media amendments were used, significantly different metabolic pathways were associated depending on the soil type ($p<0.05$). It was shown that upon the addition of WBC media amendment, most of the parameters such as total trace minerals (1.23 ± 0.23 mg g^{-1} of DW), ω3-lipids (i.e., lipid compounds containing omega-3 double bond) (14.73 ± 0.88 mg g^{-1} of DW), vitamin E (0.47 ± 0.04 mg g^{-1} of DW), vitamin B2 (0.123 ± 0.002 mg g^{-1} of DW), and B (0.01 ± 0.002 mg g^{-1} of DW) were significantly enhanced in soil type 2 compared to control. Comparatively, using natural media amendments of WA, SL, and BC in soil type 1 increased the marketable yield without altering the nutritional parameters. In conclusion, this study suggests that WBC could be used as a potential media amendment to modulate the nutritional parameters where its effects significantly vary on the used soil type.

Keywords: Wood ash, Paper sludge, Biochar, Nutritional parameters, Podzolic soil

2.1 Introduction

The wide applications of synthetic chemical fertilizers in crop production have become a major concern due to their negative impact on soil and ecosystems more broadly (Zhang et al., 2017). Intending to overcome such negative impacts, using natural growing media amendments has rapidly come to the agricultural scenario under both controlled environment and field conditions (Iheshiulo et al., 2017). The sole use of inorganic fertilizers has raised concerns such as high cost and has directed the farmers towards more organic sources of media amendments (Suge et al., 2011). The addition of natural media amendments to the soil can improve its physical, and chemical properties and biological activities (Clemente & Bernal, 2006; Agbede & Ojeniyi, 2008). Currently, many organic sources have been used to enhance crop production such as vermicompost, animal manure, green manure, crop residue, humate extracts, volcanic minerals, and different types of rock dust, etc. (Iheshiulo et al., 2017). Apart from the above, the usage of waste by-products of paper mill industries, such as primary /secondary sludge (SL) and wood ash (WA), have been identified and used as amendments in agriculture from the early days (Lafond, 2004). Primary sludges have shown a positive effect on soil quality due to their ability to increase soil parameters such as soil organic matter, water-holding capacity, soil aggregations, and infiltrations. In contrast, secondary sludge has been reported to improve crop production by enhancing the availability of N nitrogen(N) and phosphorous(P) (Camberato et al., 2006).

The expansion of bioenergy production across Canada has produced more wood ash as waste that usually ends up in landfills (Hannam et al., 2018). The reports by Elliott and Mahmood (2006) have shown a significant reduction in landfilling rates of wood ash by 2013, thus providing a great economic value by closing the nutrient cycle in forestry operations. Due to its associated alkaline properties, one of the main practical benefits of wood ash is its potential to use as a substitute agricultural lime.

Additionally, wood ash contains mineral nutrients such as P and potassium (K) and trace elements which are essential in plant growth; since there are many forms of concentrated compounds mainly present in produced wood ash, the probability of the presence of contaminants such as toxic organic compounds and heavy metals have reported low (Vance & Mitchell, 2000). A report on problematic soils by Lal (2015) mentioned that many soil management strategies have been implemented and proposed with the aim of improving soil quality, where amendments with biochar (BC) have shown great possibilities (Wu et al., 2017; Fang et al., 2018). Biochar is usually made by pyrolysis of carbon-rich waste material from agricultural residuals (Inyang et al., 2016) with unique phytochemical and heavy metal sorption properties (Wang et al., 2015; Wang et al., 2018). Recent research by Fang et al. (2018) states that biochar as a soil amendment or as a soil conditioner can improve soil properties, especially in problematic soils, which also helps in promoting plant growth conditions but Yu et al. (2019) state that many previous reviews were majorly focused only on interactions between the soil components and biochar, where research on plant growth in biochar amended soils are limited.

A study by Lazcano and Domínguez (2011) stated that most of the natural media amendments are comprised of humic and non-humic substances which supply plant growth-promoting compounds such as minerals, free amino acids, humic acids, fulvic acids, and phytohormones. Abbey et al. (2018) reported that the composition and availability of those compounds get highly varied in the natural media amendment type used. It may also significantly affect health-promoting (secondary metabolites) compounds in plants. The effect of different soil management techniques on the nutritional profile of different crops has been reported with inconsistent results (Heaton, 2002). Considering the past literature reviews, fewer studies have been conducted to compare the nutritional quality of crops grown conventionally and organically (Herencia et al., 2011).

A two-year greenhouse research study by Pitura and Michałojć (2012) reports that increasing N content in the growing medium reduced the vitamin C content of leafy celery and butterhead lettuce while the protein content of those plants increased with the increase of N rates. Application of sewage sludge and chicken manure as a soil amendment has been shown to increase both the physical and chemical properties of soil; the study also has demonstrated the increasing content of total phenolics and ascorbic acid content in kale and collard compared to non-amended soils (Antonious, 2016). Research by Zhao et al. (2007) reported that there was no significant effect on Chlorogenic acid and Quercetin glycosides in lettuce upon the addition of organic (compost + fish emulsion) and conventional (N-P-K + $CaNO_3$) fertilization. In a study on the effect of vermicast, potassium humate, and inorganic fertilizers (N_{20}-P_{20}-K_{20}) on *Plectranthus spp's* phytochemical parameters, it was shown that accumulation of carotenoids, carvacrol contents, and total phenolics was highest in organic media amendments compared to inorganic which was majorly promoting plant growth only (Zhang et al., 2017). Previous research findings demonstrated that varying media amendments could have a different impact on the phytochemical profiles of kale and lettuce (Abbey et al., 2018).

Based on past research, there is little to no information on the effect of paper mill wastes (i.e., wood ash, sludge) and biochar on crops' nutritional profiles. Therefore, I hypothesize that there is a positive effect of wood ash, sludge, and biochar on the nutritional profile of crops. The study's objective herein was to investigate the effect of the paper mill wastes and biochar on kale's nutritional profile grown in two soil types under greenhouse conditions. Two different soil types were selected to investigate the effects of amendments in low and high-pH soils due to the presence of highly acidic soils with a loamy structure in Western Newfoundland (Page, 1971). Kale was selected as the representative leafy vegetable for this study due to its high consumer demand and consumption. This study aims to investigate the usage of paper mill wastes with biochar in formulating soil amendments.

It also aims to identify suitable natural media amendments for crop quality enhancement with different soil types, potentially promoting agricultural self-sufficiency in this province.

2.2 Materials and methods

2.2.1 Experimental treatments and design

A greenhouse experiment was conducted at Wooddale, Grandfalls-Windsor, Newfoundland and Labrador (NL). The experimental treatments were wood ash (WA), dewatered paper sludge (SL), a combination of wood ash and dewatered sludge (WASL), and limestone (Control). One set of treatments was mixed with biochar (BC), where the amendments were lime+ biochar (CBC), wood ash+ biochar (WBC), sludge + biochar (SBC), and wood ash + sludge + biochar (WSBC). Lime is used as the control (CTL). WA and SL samples were collected from Corner Brook pulp and paper mill for 15 days, and 30 composite samples were made.

The composite samples were firstly air-dried and ground using Wiley mill (A.H. Thomas Co., Philadelphia, PA, USA). The ground samples of WA and SL were homogenized and sieved using a 2 mm mesh and were sent to the University of Guelph (Agriculture and Food Laboratory) for complete mineral nutrient analysis (Table 2.1). The composition of BC used in this study was analyzed by Gabilan laboratory, Salinas, California, USA, and results are presented in Table 2.2.

Two different soil types used in this experiment are referred to as soil type 1 and soil type 2 separately. Soils were collected from a newly cleared forest land from Wooddale provincial tree nursery-Grand Falls site, GrandFalls-Windsor (49.025 N, - 55.549 E), NL, Canada, in 2019. A composite subsample was sent to the Soil, Plant, and Feed Laboratory, Department of Fisheries, Forestry, and Agriculture, St. John’s, NL, to determine the basic soil properties.

Soil pH levels and soil textural analysis were determined by pH meter (Bluelab Combo Meter, Tauranga, New Zealand) and hydrometer. Soil 1 had a pH of 5.2 and a sandy loam texture, whereas soil 2 had a pH of 5.8 and a silt loam texture. The application rates of WA and PS were calculated based on CCE (Calcium Carbon Equivalencies) provided by the Soil, Plant, and Feed Laboratory, Department of Fisheries, Forestry, and Agriculture, St. John's, NL. The following Equation 1 was used to calculate the application rates of amendments.

$$\text{Application rate} = \frac{\text{Area} \times \text{Lime requirement (Mg/ha)}}{\text{CCE} \times (100 - \%\ \text{Moisture content})} \qquad \text{(Eq. 1)}$$

The target pH values of soil 1 and 2 were 6.3 and 7.1, respectively. For soil type 1, the application rates of WA, SL, and CTL were 17.25, 55 Mg ha^{-1}, and 7.1 Mg ha^{-1},, respectively. WASL treatment was formulated as a mixture of WA and SL rates at 13.8 and 11 Mg ha^{-1}, respectively. For soil type 2 CTL, WA, SL, and WASL application rates were 8.1 Mg ha^{-1}, 20 Mg ha^{-1}, 94 Mg ha^{-1}, and 13.8 Mg ha^{-1}, respectively. The addition and non-addition of BC were kept at a rate of 20 Mg ha^{-1}, and 0 Mg ha^{-1}, respectively, for both soils (Abedin & Unc, 2020).

Calculated above amendments were mixed into bulk with air-dried, sieved soils (4 mm) separately. A complete randomized block design with one plant per treatment and three replicates for each media amendment was set up separately for each soil type (n=48). For each pot, 5 kg of the above mixes were added, and the pots were maintained at 60% water-filled pore space (WFPS) using tap water.

All pots were left for 7 days for soil stabilization before seeding. However, due to the COVID-19 pandemic, the greenhouse facility was closed for three months, and the study was halted from March 2021 to June 2021. After this period, pots were re-sampled to measure the soil pH. In CTL and SL treatments, the soil pH was dropped below the target pH.

Additional limestone and sludge were added to get the target soil pH (6.3) and then watered to maintain 60% WFPS. After correcting for the mineral N, total P, and K in the amendments, all the pots were added with urea, triple super phosphate, and muriate of potash to supply the N:P: K rates of 180:52:128 and 150:87:149 per ha for soil type 1 and soil type 2, respectively. Fertilizers were applied as per soil test reports and crop requirements stated above.

Table 2.1: Basic physical and chemical properties of wood ash (WA), and paper sludge (SL) used in the current study.

Parameters	**Wood ash**	**Sludge**	**CCME limits for biosolids**
pH	12.6	8.2	
E.C. (Total Salts)	0.00952	0.00032	
Ammonium-N	0.746	876	
Nitrate-N	5.09	2	
Magnesium, Extractable	565	1170	
Potassium, Extractable	2420	1360	
Sodium, Extractable	265	321	
Calcium, Extractable	23600	5440	
Inorganic Carbon	16400	2020	
Organic Carbon	34500	399000	
Calcium, Total	168000	32500	
Phosphorus, Total	24000	10100	
Potassium, Total	23600	4390	
Arsenic (As)	2.5	2.1	41
Cadmium (Cd)	1.3	3.1	15
Chromium (Cr)	130	55	1000
Cobalt (Co)	14	5.1	150
Copper (Cu)	190	94	1500
Lead (Pb)	20	35	300
Calcium Carbonate Equivalent (CCE)	10.4	12.9	

Note: All units are in mg kg^{-1}, dSm^{-1} for EC (Electrical Conductivity), and percent (%) for organic carbon, organic matter, and CCE (Calcium Carbonate Equivalent). CCME = Canadian Council of Ministers of the Environment

Table 2.2: Physiochemical properties of biochar (BC) used in the current study

Properties	Dry weight basis
pH	9
EC (dS/m)	0.43
Moisture (%)	-
WHC (mL water per 100 g dry char)	74.9
Volatile matter (%)	8.5
Ash (%)	6.7
Fixed carbon (%)	84.5
H (%)	0.68
O (%)	7.84
N (%)	0.22
S (%)	0
H/C	0.1
O/C	0.07
Total ash (%)	7.1
Recalcitrant carbon (%)	76.2
Neutralizing value (% as $CaCO_3$)	4.9
Carbonate value (% as $CaCO_3$)	0.6
Bulk density (Mg/m^3)	0.19
Particle density (acetone)(g/cc)	1.57
Solid space (v/v%)	12.5
Void space (v/v%)	87.5

2.2.2 Greenhouse conditions

The seeds were purchased from Vesseys Seeds (Charlottetown, PEI, Canada), and seeding rates were calculated based on the manufacturer's recommendation. After seven days of soil stabilization in pots, 10 kale seeds were directly seeded into the pots at 1 cm depth (Ashenafi & Tewodros, 2018). The greenhouse temperature (Wooddale Agriculture and Forestry Development Center) was maintained approximately at 25°C for 16 h during the daytime and 16°C for 8 h at night with a mean relative humidity of 66% (Vidal et al., 2018). All the kale pots were maintained at 60% WFPS throughout the experimental period by adding 400-500 mL of water every 4 days. Pots were rearranged weekly to offset unpredictable occurrences due to variations in microclimate in the greenhouse (Abbey et al., 2018).

2.2.3 Harvesting

The kale leaves were harvested after 80 days of sowing the seeds per the manufacturer's recommendation. The harvested kale from all treatments was weighed to determine the fresh yield, and then samples were oven-dried at 65°C for three consecutive days or until a constant weight was achieved (Kim et al., 2017). Dried kale samples from each treatment were pulverized separately using a Cyromil (Reitch, Bonn, Germany), and ground samples were stored at room temperature for further nutritional analysis.

2.2.4 Nutritional analysis of kale

In all treatments, changes in the functional components such as total carbohydrates, total proteins, total fatty acids (FAMEs), total phenolic content (TPC), total antioxidant activity (TAA), macro minerals, trace minerals, antioxidants minerals, toxic minerals, water-soluble vitamins (WSV), fat-soluble vitamins (FSV), and marketable yield were measured.

2.2.4.1 Chemicals

Water-soluble vitamins Thiamine hydrochloride (vitamin B1), Riboflavin (vitamin B2), Nicotinamide (vitamin B3), Pantothenic acid (vitamin B5), Pyridoxine (vitamin B6), Folic acid (vitamin B9), and Cobalamin (vitamin B12) were purchased from Sigma Aldrich (St. Louis, USA). ICP-MS 43-element standard mix (IV-ICPMS-71A) was purchased from Inorganic Ventures (Christiansburg, Virginia, USA). Ferrous ammonium sulphate hexahydrate, *ortho*-dianisidine dihydrochloride, meta-Phosphoric acid, glacial acetic acid (HPLC grade), methanol (HPLC grade), acetone (HPLC grade), acetonitrile (HPLC grade), 2,4,6-tripyridyl-*s*-triazine (TPTZ), ferric chloride hexahydrate, anhydrous sodium sulphate, ethanol (95% v/v), hydrogen peroxide (30% v/v), sulfuric acid (98% v/v), sodium phosphate, hydrochloric acid (36% v/v), *ortho*-phosphoric acid (85% v/v), methylchroman-2-carboxylic acid (Trolox), sodium hydroxide, glycerol, sodium carbonate, and Folin-

Ciocalteu reagent were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Nitric acid (TraceMetal™ Grade), methanolic-HCl (Sigma-Aldrich, ON, Canada) chloroform (HPLC grade), Methanol (HPLC grade), hexane (HPLC grade). The following chemicals were purchased from Fisher Scientific (Ottawa, ON, Canada): LC-grade chloroform, methanol, acetonitrile, formic acid, acetic acid, and ammonium acetate. Deionized water (PURE LAB Purification System, ELGA Labwater, ON, Canada) and glucose standard (Oakville, Ontario, Canada) were used for solution preparation.

2.2.4.2 Total phenolic content (TPC)

Total phenolic content was measured using the method described by Thomas et al. (2010). Briefly, 100 mg of ground kale powder was suspended in 1 mL of HPLC grade 0.7% acidified ethanol, vortexed, and incubated at room temperature in the dark for 10 min. After the incubation, the samples were centrifuged at 5000 x g for 10 min, and the supernatant was decanted carefully into newly labelled vials. This supernatant was used to analyze the LPC (lipophilic phenolic content) and LAA (lipophilic antioxidant activity). The remaining pellets in the first tubes were then resuspended with 1mL of sodium phosphate buffer (pH 7.5) (made from a solution of mixture Na_2HPO_4 and NaH_2PO_4, 50 mM) and re-extracted as stated above to a new set of labelled vials. Those supernatants were used to determine HPC (hydrophilic phenolic content) and HAA (hydrophilic antioxidant activity).

Sample analysis for HPC and LPC was carried out by adding 25 µL of each standard and sample to a 96 wells microplate. For each well, 125 µL of 10-fold diluted Folin-Ciocalteu reagent was added, followed by 50 µL of either sodium phosphate buffer (pH 7.5, 50 mM) or acidified 0.7% ethanol depending on the measurement of HPC or LPC, respectively.

Then plates were incubated in the dark for 30 min, and absorbance was measured at 755 nm using a microplate reader (Cytation 3, BioTek, Vermont, USA). A standard curve (0.0–2.0 mM) was prepared using Quercetin, and the samples' phenolic content was expressed as mM Quercetin acid equivalents per g of kale dried weight.

2.2.4.3 Total antioxidant activity (TAA)

The TAA of the kale extracts was calorimetrically measured using the Ferric Reducing Antioxidant Power (FRAP) method by Thaipong et al. (2005) with minor modifications. Briefly, 20 µL of each standard and the extracted samples of HAA and LAA from above were reacted with 180 µL of freshly made FRAP solution, and the mixture was incubated in the dark for 30 min. Absorbance measurements were recorded at 593 nm on a Cytation3 imaging microplate reader. The TAA was determined by summing the LAA and HAA antioxidant activities. Reduced Trolox was used to develop the standard curve (0.0–0.5 mM), and the antioxidant power of the sample was expressed as µM Trolox equivalents per gram of dry sample.

2.2.4.4 Extraction of kale lipids and analysis of fatty acids (FA) using GC-FID

Total lipids were extracted using the method stated in Eggers and Schwudke (2016). Firstly, 500 mg of dried kale powder was mixed with MTBE/methanol (10:3; v/v) and incubated for 1 h at room temperature under continuous shaking. Then, 0.2 volume equivalents (compared to the total volume of MTBE/methanol) distilled water was added to the final volumetric ratio of 10:3:2.5 (MTBE, methanol, water). After mixing the samples thoroughly, the suspension was centrifuged for 10 min at 1000 g, and the upper lipid-containing phase was transferred into separate labelled collection vials. The remaining aqueous phase was re-extracted by 0.3 volume equivalents of the theoretical upper phase.

After diluting them to an appropriate concentration, the combined extracts were filtered and directly utilized for mass spectrometric analysis.

The FA profile of kale leaves samples was analyzed by hydrolyzing the ester linkages in the total lipid extract by 3N methanolic HCl to convert fatty acyls to free fatty acids, then methylate them to fatty acid methyl esters (FAMEs), which were then quantified using a GC flame ionization detector (FID) (Abbey et al., 2018). Briefly, aliquots of the extracted lipids (300 µL) from each kale sample were transferred to 2 mL vials along with 50 µl of nonadecanoic acid methyl ester (C19:0 FAME) (1 mg mL^{-1}) internal standard dissolved in 2:1 chloroform: methanol. The samples were then dried under a gentle stream of N_2, and after the drying process, samples were hydrolyzed and methylated by adding 100 µL of 3 N methanolic HCl. Samples were then vortexed, incubated at 80°C for 30 min, and cooled in a fume hood. Next, 0.8 mL of distilled water was added to each sample mixture, and the FAME mixtures were extracted three times using 500 µL of n-hexane. The extracted Hexane fractions were pooled (1.5 mL), dried under N_2, and the residues were re-suspended in 50 µL of n-hexane.

The analysis of FAMEs was carried out using Trace 1300 gas chromatography coupled to a Flame Ionization Detector (Thermo Fisher Scientific, Waltham, MA, USA). FAMEs were separated by a DB-23 column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies, Santa Clara, CA, USA) using helium as the carrier gas at a flow rate of 1 mL min^{-1}.

The sample was injected (1 µl) into the instrument using a tri-plus auto-sampler. The operation conditions maintained were as follows: The injection system was in spitless mode. The oven temperature was set up at 50°C (held for 1 min) and increased 20°C min^{-1} to 175°C (held for 1 min); afterward, it was increased again to 230°C at 4°C min^{-1} (held for 5 min).

FAMEs were identified and quantified by comparison of the retention times of the standards (Supelco PUFA No. 3 mix, Supelco 37 component mix, Supelco FAME mix (C8-C24; Sigma Aldrich, ON, Canada) and the FA amount of each sample was presented as mg g^{-1} of dried kale samples (Vidal et al., 2018).

2.2.4.5 Total carbohydrates

The carbohydrates were extracted from dried kale samples, as stated in Maness Niels (2010). Extracted carbohydrates were analyzed according to Sigma-Aldrich (USA-MA104) Kit standard procedure for the phenol-sulphuric method. Briefly, a 50 mg dry ground sample was accurately weighed into a round–bottom glass centrifugal tube (25 mL). Then, 5 mL 95% (v/v) of ethanol was added to each centrifuge tube and capped with a one-hole rubber stopper. Each tube was well mixed, placed into a water bath at 85 °C, and incubated in boiling ethanol for 20 min. Then, tubes were uncapped and centrifuged at 10,000×*g* for 10 min. The supernatant was decanted into new glass vials separately, and extraction was repeated three times. A standard glucose curve was prepared by adding directly to the microtiter plate (0, 2, 4, 6, 8, and 10 µL of the 2 mg mL^{-1}).

For each well, 20 µL of sample and 150 µL of the concentrated sulfuric acid were added and mixed well using the horizontal shaker. The plate was incubated at 90°C for 15 min in a dark condition. Then 30 µL of the developer was added and shaken again in the horizontal shaker at room temperature for 5 min. The colour change was measured at an absorbance of 490nm using a plate reader (BioTek, Vermont, USA). The results of total carbohydrates are expressed as mg g^{-1} of dried kale samples.

2.2.4.6 Total proteins

The extraction of proteins from dried kale samples was carried out as per Jones et al. (1989). 100 mg of dried samples were accurately measured into plastic vials and added with 1 mL of Na_3PO_4 buffer (pH: 7.5, 50 mM). The tubes were then centrifuged at 10000x g for 15 min, and the supernatant was analyzed by the coomassie plus (Bradford) assay kit (Thermo-Scientific (USA23236) for the microplate reader.

A standard curve is generated using the diluted albumin (BSA) standard, and proteins are expressed as mg g^{-1} of g of dried kale weight. Briefly, 10 µL of standard and samples were added to 96 microwell plates followed by 300 µL of Coomassie Plus Reagent, and each well was mixed well using a shaker for 1-2 min. Then the plate was kept for incubation at room temperature for 10 min. The absorbance was measured at 595 nm using the plate reader (BioTek, Vermont, USA).

2.2.4.7 Extraction of minerals and its analysis using inductively coupled plasma-mass spectrometry (ICP-MS)

100 mg of kale samples were digested in 10 mL of trace metal grade nitric acid (70% v/v) using a Multiwave Gomicrowave Digestion System (Anton Paar GmbH, Graz, Austria) operated under the following conditions: Initial temperature ramped to 180 °C for 10 min and then followed by a constant temperature of 180 °C for 40 min. A standards calibration curve ranging from 1 to 500 ppb was prepared using IV-ICPMS-71A standard mixture (Inorganic™ Ventures, Inc; Christiansburg, VA 24073, USA). Aliquots of the digests were diluted with 3% nitric acids and spiked with Rhodium103 (final concentration = 10 ppb) as the internal standard for ICP-MS analysis.

The ICP-MS analysis was conducted using the following parameters: an auxiliary gas flow of 0.79 mL min^{-1}, nebulizer gas flow of 1.01 mL min^{-1}, plasma gas flow of 14 mL min^{-1}, RF power of 1548 W, detector mode KED and a dwell time of 0.01s using Argon gas at a purity of 99.99 %. The results were expressed as mg g^{-1} of dried kale samples.

2.2.4.8 Determination of water-soluble vitamins (WSV)

The water-soluble vitamin extraction was carried out, as stated by Engel et al. (2010). Briefly, 8 mL of 0.1% (w/v) meta-phosphoric acid was added to 0.5 g of homogenized sample in amber flasks. After sonication in the dark for 18 min, the sample was centrifuged at 3500 rpm for 15 min. The supernatant was filtered through a 0.2µm regenerated cellulose membrane (Phenex™-RC syringe filters, Phenomenex, Torrance, CA), and 2% meta-phosphoric acid was added up to a volume of 8 mL

Stock solutions of individual vitamins (1 mg mL^{-1}) were prepared on ice and in low light as follows: Vitamin B1, B2, B3, B6, C, and B12 were dissolved in the mobile phase A (acetonitrile + 10 mM ammonium formate) solution; Vitamin B5, B7, and B9 were dissolved in 0.1 M NaOH solution. All solutions were placed at -80°C until use. A stock solution was made using aliquots of each vitamin to generate a calibration curve diluted using a 2% formic acid (0.2-50 ppm) calibration range. All solutions were prepared in amber glass vials. UHPLC completed the Analysis of targeted water-soluble vitamins- MS as stated in (Maurer et al., 2014) with modifications. A Luna C18 column (Dimensions: 2.0 × 100 mm^2, particle size: 3 µm, pore diameter: 100 Å; Phenomenex (CA, USA) was used. The solvent system used was as follows: Solvent A consisted of 10 mM of ammonium formate and acetonitrile, while solvent B consisted of 100% acetonitrile.

Chromatographic separation was performed at 35°C (column oven temperature) with a flow rate of 0.3 mL min^{-1}, and 10 µL of the extract was injected into the system. The solvent gradient separated the vitamins: ramp at 30 min, 55% solvent B; 22 min, 0% Solvent B, and 100 Solvent A.

The Orbitrap ESI source was run in positive ion mode. The following optimized parameters were used; sheath gas flow rate of 35; an auxiliary gas flow rate: 15; ion spray voltage: of 3.80 kV; capillary temperature: of 300 °C; Tube lens:100 V; capillary voltage: 35 V; mass range: 50-500 m/z; full scan mode at a resolution of 60,000 m/z; top-3 data-dependent MS/MS at a resolution of 30,000 m/z; and collision energy of 35 (arbitrary unit); injection time 22 min; isolation window: 2.0 m/z; automatic gain control target: 0.250; with dynamic exclusion setting of 30.0 s. Before usage, the mass spectrometer was externally calibrated to 1 ppm using ESI negative and positive calibration solutions (Thermo Scientific, MO, USA). The results were expressed as mg g^{-1} of dried kale samples.

2.2.4.9 Determination of fat-soluble vitamins (FSV)

The extraction of fat-soluble vitamins was carried out as per the method stated in Hrvolová et al. (2016). To 500 mg of the dried Kale sample, 4.5 mL of acetone: methanol (2:1, v/v), 1.5 mL of hexane, and 0.5% BHT (w/v), were added. Then all vials were subjected to sonication for 18 min, in ice water in a dark room condition. After the sonification for each vial 4 mL of cold 1M NaCl was added, and the samples were centrifuged at 1600 rpm for 10 min. After phase separation, the top layer of hexane was taken and filtered through a 0.45 µm Teflon membrane (Phenex™ Teflon® (PTFE); Phenomenex). Then, the filtrate was brought to volume with hexane (10 mL) and analyzed via UHPLC-MS, as stated by Hrvolová et al. (2016), with slight modifications. A polar acclaim C18 column (Dimension: 4.6 mm × 150 mm, particle size: 5 µm, pore diameter: 120 Å; Thermo Fisher Scientific, ON, Canada) was used for the analysis. The solvent system used was as follows. Solvent A consisted of methanol, 0.7 g L^{-1} of ammonium acetate, and 0.1% acetic acid, while Solvent B consisted of MTBE, methanol 80:20 (v/v), 0.7 g L^{-1} of ammonium acetate, and 0.1% acetic acid. The separation was carried out at 40°C (column compartment temperature) with a flow rate of 0.6 mL min^{-1}, and 20 µL of the extract was injected into the machine, and elution was carried out isocratically for 32 min. The Orbitrap MS was operated in the positive APCI ion mode to determine the fat-soluble vitamins.

The following optimized parameters were used for the Orbitrap Mass spectrometer: sheath gas flow rate: 25; ion spray voltage: 6 kV; an auxiliary gas flow rate: 5; Tube lens: 30 V; mass range: 60-900 m/z; capillary temperature: 300 °C; capillary voltage: 10.0 V; full scan mode at a resolution of 60,000 m/z; top-3 data-dependent MS/MS at a resolution of 30,000 m/z; and collision energy of 35 (arbitrary unit); injection time of 15 min; isolation window: 2.0 m/z; automatic gain control target: 0.250; with dynamic exclusion setting of 30.0 s. Before usage, the mass spectrometer system was externally calibrated to 1 ppm using APCI positive and negative calibration solutions (Thermo Scientific, MO, USA). The results were expressed as mg g^{-1} of dried kale samples.

2.2.5 Statistical analysis and data presentation

All the data for soil type1 and type 2 were analyzed by the multivariate approach of partial least squares discriminant analysis (PLS-DA) followed by a heatmap where the nutritional parameters that were accountable for the difference in the media amendments were selected based on Variable Importance in the Projection (VIP) ranking >1. One one-way ANOVA was carried out for each main cluster to determine the effect of natural media amendment on the nutritional properties.

Fisher's least significant difference (LSD) test at α = 0.05 was used to compare the treatment means, and significant parameters were presented in Demšar plots (Demšar, 2006). Pearson's correlation coefficients were used to determine the linear correlations between the nutritional parameters of kale samples. All the statistical analyses were performed using XLSTAT (Addinsoft, Paris, France) statistical software.

2.2.6 Pathway analysis

MetaboAnalyst 5.0 was used to perform pathway analysis (https://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtmL) (Chong et al., 2019) and the following parameters were set. Data normalization by None; Data transformation by log transformation; Data scaling by Pareto scaling; Pathway library: Arabidopsis thaliana (thale cress) in Kyoto Encyclopedia of Genes and Genomes data-base (KEGG). Pathways with impact values > 0 and –log (p) > 1.3 were considered the most significant ($p < 0.05$) pathways for the generation of functional lipids.

2.3 Results

This section explains the results for both soils separately based on the parameters that showed a significant difference (Table 2.2 and Table 2.3) among CTL, CBC, WA, WBC, SL, SBC, and WSBC amendments.

Table 2.3. Parameters for each cluster in the heatmap of soil type 1 and its significance

Cluster in heatmap	Parameters	R^2	F	Pr > F(Model)
G1	Total antioxidant minerlas	0.666	4.551	0.006
G1	Total PUFAs	0.518	2.453	0.065
G1	ω3-FA 18:3	0.523	2.510	0.060
G1	Total ω6 FAs	0.457	1.926	0.132
G1	ω6-FA 18:2	0.496	2.248	0.085
G1	Total ω3 FAs	0.487	2.174	0.094
G1	Total SFAs	0.543	2.711	0.047
G1	Cu	0.721	5.896	0.002
G1	FA:16:0	0.555	2.846	0.039
G1	Cr	0.439	1.790	0.158
G2	Fe	0.752	6.949	0.001
G2	FA:C22	0.576	3.102	0.029
G3	Mg	0.962	57.526	0.000
G3	Co	0.953	46.506	0.000
G3	Cd	0.970	74.772	0.000
G3	Total toxic minerals	0.921	26.774	0.000
G3	FA 18:0	0.652	4.289	0.008
G3	Pb	0.722	5.931	0.002
G3	As	0.469	2.019	0.116
G4	Total trace minerals	0.843	11.495	0.000
G4	FA 16:1	0.576	2.908	0.039
G4	Mn	0.905	20.301	0.000
G4	Total MUFA	0.326	1.038	0.446
G4	FA 24:0	0.590	3.084	0.032
G5	B	0.854	13.376	0.000
G5	Marketable yield	0.891	18.607	0.000
G5	K	0.472	2.041	0.112

Table 2.4. Parameters for each cluster in the heatmap of soil type 2 and its significance

Cluster in heat map	Parameters	R²	F	Pr >F(Model)
G1	Total trace minerals	0.754	7.006	0.001
G1	Marketable yield	0.778	7.999	0.000
G1	Na	0.778	8.021	0.000
G1	ω6-FA 18:2	0.563	2.941	0.035
G1	ω9-FA 20:0	0.463	1.969	0.124
G1	FA 16:0	0.596	3.375	0.021
G1	Total ω3 FAs	0.657	4.384	0.007
G1	ω3-FA 18:3	0.638	4.032	0.010
G2	ω6-FA 18:3	0.486	2.161	0.096
G2	B	0.794	8.783	0.000
G2	Total Proteins	0.809	9.684	<0.0001
G2	Vitamin:K1	0.587	3.253	0.024
G2	Total ω6 FAs	0.477	2.088	0.106
G2	ω3-FA 22:6	0.640	4.066	0.010
G3	Mg	0.945	39.482	<0.0001
G3	Retinol-A	0.803	9.331	0.000
G3	TAA	0.590	3.291	0.023
G3	Vitamin: C	0.538	2.658	0.050
G3	Total carbohydrates	0.748	6.789	0.001
G3	ω6:ω3	0.470	2.024	0.115
G4	β- Carotene	0.734	5.910	0.002
G4	Total FSV	0.700	5.007	0.004
G4	Vitamin:E(α-Tocopherol)	0.965	59.816	<0.0001
G4	ω9-FA 22:2	0.734	5.913	0.002
G4	K	0.394	1.394	0.278
G4	B	0.800	8.561	0.000
G4	HAA	0.636	3.739	0.015
G4	ω6-FA 20:4	0.572	2.863	0.041
G5	Cr	0.545	2.735	0.045
G5	Total antioxidant. minerals	0.595	3.364	0.021
G5	Fe	0.597	3.381	0.021

2.3.1 Effect of media formulations on nutritional parameters of Kale grown in soil type1

In the heatmap generated for soil type 1, the nutritional parameters have been clustered into five main groups (G1, G2, G3, G4, and G5), while the amendments are clustered into four main subgroups (SG1, SG2, SG3, and SG4) Figure (2.2). They are presented in demsar plots; for each parameter, the demsar plot shows the grouping of media amendment based on LSD mean values, while amendments that do not show any significant differences are connected in dots and lines. Considering the G1 (Figure 2.2 a) cluster the parameters of total antioxidant mineral content, total saturated fatty acid content (SFA), FA 16:0, and Cu (Copper) have shown significantly different ($p < 0.05$). The least amount is reported for all those parameters in G1 (Figure 2.2 a) via adding CBC amendment. The addition of CTL or no amendment has shown the highest values of 1.29 ± 0.11mg g^{-1} of DW; 0.0026 ± 0.0001 mg g^{-1} of DW; 1.68 ± 0.05 mg g^{-1} of DW for FA 16:0, Cu and SFA, respectively. The highest amount of total antioxidant mineral content has been shown upon adding WA amendment with a value of 0.335 ± 0.017 mg g^{-1} of DW.

Considering the G2 (Figure 2.2 b), the parameters of Fe and FA 22:0 have shown to be significant ($p < 0.05$). The highest amount of Fe has been shown for WA amendment (0.256 ± 0.010 mg g^{-1} of DW), while the values have been lowered upon the addition of SBC. The FA 22:0 also has shown the highest value (0.053 ± 0.012 mg g^{-1} of DW) when treated with WA amendment but upon addition of CBC the values have shown the least. It has also shown no significant difference ($p<0.05$) among the WA, WBC, WSBC, and CTL amendments. The parameters of Mg, Cd (Cadmium), Co (Cobalt), Pb (Lead), total toxic minerals, and fatty acid of FA 18:0 were shown to be significantly different ($p<0.05$) in cluster G3 (Figure 2.2 c). From all those parameters, amendment CTL showed the highest values. For example, the total toxic mineral content and FA 18:0 showed values of 0.0023 ± 0.0003 mg g^{-1} of DW and 0.245 ± 0.015 mg g^{-1} of DW, respectively.

Except for the Pb amount, it can also be seen that CTL is significantly different in all parameters from all other amendments ($p<0.05$). The least values for total toxic minerals (5.5E-04 ± 1.0E-05 mg g^{-1} of DW) and Cd (7.66E-06 ± 3.0E^{-07} mg g^{1} of DW) were shown upon the addition of WSBC amendment, while for the parameters of Mg, Co, Pb, and FA 18:0, the least values of 2.67 ± 0.186 mg g^{-1} of DW; 1.75E-05 ± 0.00001 mg g^{-1} of DW, 3.77E-04 ± 0.00001 mg g^{-1} of DW and 0.143 ± 0.009 mg g^{-1} of DW for amendments of SBC, WASL, WA, CBC respectively. In cluster G4 (Figure 2.2 d), the parameters of total trace minerals, Mn, and FA 16:1 and FA 24:0 had shown to be significantly affected ($p<0.05$) when natural media amendments were used. The highest amount of total trace minerals (0.036 ± 0.04 mg g^{-1} of DW) and Mn (0.14 ± 0.004 mg g^{-1} of DW) were shown upon the addition of the SL amendment. For the FA 16:1 and FA 24:0, the highest values of 1.289 ± 0.111 mg g^{-1} of DW and 0.072 ± 0.007 mg g^{-1} of DW were shown for CTL and SBC amendment, respectively.

Considering cluster G5 (Figure 2.2 e), the parameters of B and marketable yield were shown to be significantly different ($p<0.05$). The highest amount of B content (0.034± 0.004 mg g^{-1} of DW) was shown upon the addition of the SL amendment, where there are no significant differences among the WA, WBC, WSBC, SBC, and WASL amendments. The highest marketable yield (77.7± 1.726 mg g^{-1} of FW) was shown at WBC followed by WA amendment (71.6 ± 0.86 mg g^{-1} of FW). Pearson's correlation analysis (Figure 2.3 a) manifested a strong positive correlation between marketable yield and trace mineral content (r= 0.680; p=0). Apart from the above, a positive correlation was shown for PUFAs (r= 0.414; p=0.05) with plant K (Figure 2.3 b) amount, while a negative correlation between MUFAs and Mg was shown (r=-0.502; p=0.015) (Figure 2.3 c).

2.3.2 Effect of media formulations on the nutritional quality of kale grown in soil type2

The heatmap clusters the nutritional parameters into five main groups (G1, G2, G3, G4, and G5), while the media amendments have been clustered into three main groups (SG1, SG2, SG3, and SG4) (Figure 2.4). The parameters of total trace minerals, FAs of ω6-18:2, ω3-18:3, 16:0, total ω3 FAs, Na (Sodium), and marketable yield have shown a significant difference among amendments for G1) (Figure 2.5 a). Apart from the above, all the FAs showed the highest values for WBC treatment, while the lowest values were for the WSBC treatment. For example, in the fatty acid of ω3-18:3, two clear groups of mean values can be seen where the highest value of 13.23 ± 0.277 mg g^{-1} of DW is shown for WBC amendment. It can also be seen that WBC and CBC amendments are not significantly different. A similar trend can be seen for the parameter of total trace elements, where the highest value of 1.24 ± 0.234 mg g^{-1} of DW at WBC shows a significant difference ($p<0.05$) compared to CTL amendment. It can also be seen in the G1 cluster the marketable yield and Na shows a similar trend where the highest values were shown at WA treatment with the values of 162.73 ± 6.51 g of DW; 1.01 ± 0.121 mg g^{-1} of DW, respectively. The lowest values for marketable yield and Na were shown upon the addition of SL; the values were 72.11 ± 2.43 g of DW; 0.36 ± 0.032 mg g^{-1} of DW, respectively. Considering the G2 cluster (Figure 2.5 b), the parameters of vitamin B2, K1, ω3-FA 22:6, and total proteins are significant. In agreement with G1, the ω3-FA 22:6 appearing in G2 also shows a similar trend where the WBC amendment (0.391 ± 0.087 mg g^{-1} of DW) shows the highest while the lowest value was shown at WSBC (0.09 ± 0.016 mg g^{-1} of DW). Vitamin B2 has shown the highest values upon the addition of WBC amendment (0.01 ± 0.001 mg g^{-1} of DW), but there is no significant difference compared to WA ($p<0.05$). Similarly, for vitamin K1, the highest value of 0.03 ± 0.009 mg g^{-1} of DW value can be seen for WBC treatment, followed by WA treatment, while the lowest value was shown for CBC (0.003 ± 6.9E-05 mg g^{-1} of DW).

Considering the total proteins, it can be seen that values were significantly altered upon the addition of media amendments, where the highest values were shown at CTL treatment (1.26 ± 0.06 mg g^{-1} of DW).

Parameters of Mg (magnesium), vitamin A: retinol, vitamin C, total carbohydrates, and TAA clustered in G3) (Figure 2.5 c) are shown to be significant ($p<0.05$). In this cluster, parameters of Mg, vitamin C, and total carbohydrates show 8.2 3 ± 0.188 mg g^{-1} of DW, 8.30 ± 0.526 mg g^{-1} of DW, and 338.45 ± 29.0 mg g^{-1} of DW, highest at CBC amendment respectively. Regarding the TAA, it has been shown that upon the addition of CBC treatment, the antioxidant activity has reduced to 23.89 ± 0.771 µM of Trolox equivalent of DW. At the same time, it has also shown that there is no significant difference among CBC, CTL, and WA treatments. The highest amount of TAA was shown at WASL amendment with a value of 30.16 ± 1.456 µM of Trolox equivalent of DW.

In the G4 cluster (Figure 2.5 d), the parameters of β-Carotene, total fat soluble, vitamin E (α-Tocopherol), B , ω9- FA 22:2, and hydrophilic antioxidant activity have shown significant. In this cluster, it is clear that parameters such as total fat-soluble vitamins (FSV), β-Carotene and Vitamin E (α-Tocopherol) show the highest values of 1.85 ± 0.189 mg g^{-1} of DW, 0.72 ± 0.157 mg g^{-1} of DW, and 0.47 ± 0.044 mg g^{-1} of DW for WBC amendment respectively. A similar trend can be seen for the B parameter with the highest value of 0.012 ± 0.001 mg g^{-1} of DW shown upon the addition of WBC where there was no significant difference between WBC and WA amendment. The least amount of B was shown upon the addition of WASL amendment with a value of 0.004 ± 0.0005 mg g^{-1} of DW.

Considering the HAA, it can be seen that the SL amendment has helped increase the hydrophilic antioxidant activities compared to other amendments. At the same time, the lowest value was shown for the CBC amendment (18.25 ± 0.933 μM of Trolox equivalent of DW). A similar trend can be seen for the ω9- FA 22:2, where SL showed the highest value of 0.15 ± 0.008 mg g^{-1} of DW, while the lowest was shown for CTL (0.043 ± 0.007 mg g^{-1} of DW). In cluster G5) (Figure 2.5 e), the parameters of Cr (chromium), total antioxidant minerals, and Fe (iron) have shown significant differences among amendments. In Cr, upon the addition of WASL, the amount has shown increased values (0.001 ± 0.0002 mg g^{-1} of DW) where there is no significant difference among WA, WSBC, CBC, WBC, and SBC amendments. The parameters of total antioxidant minerals and Fe show the same trend where the highest amounts are reported at 0.426 ± 0.042 mg g^{-1} of DW and 0.421 ± 0.042 mg g^{-1} of DW for SBC amendment, respectively.

Pearson's correlation analysis (Figure 2.6 a) manifested a strong positive correlation between marketable yield and total trace mineral content (r= 0.729; p<0.0001). Apart from the above a strong positive correlation was shown for total proteins (r=0.861; p=0.0001) and retinol content (r=0.622; p<0.0001) with plant Mg amount (Figure 2.6 b and Figure 2.6 c). Interestingly a negative correlation between HAA and vitamin C was shown (r=-0.622; p=0.0001) (Figure 2.6 d) while TAA also showed a strong negative correlation with Mg present in the plant (r=-0.661; p=0) (Figure 2.6 e).

Table 2.5. Toxic minerals (µg g^{-1}) present in kale grown in soil type 1

Toxic minerals	**(WHO/FAO, 2007)**	**CTL**	**CBC**	**WA**	**WBC**	**SL**	**SBC**	**WASL**	**WSBC**
Cadmium	0.2	1.35	0.12	0.08	0.07	0.11	0.07	0.04	0.01
Lead	5	0.55	0.39	0.41	0.38	0.45	0.41	0.44	0.4
Arsenic	0.5	0.41	0.14	0.17	0.18	0.12	0.1	0.13	0.14

Table 2.6. Toxic minerals (µg g^{-1}) present in kale grown in soil type 2

Toxic minerals	**(WHO/FAO, 2007)**	**CTL**	**CBC**	**WA**	**WBC**	**SL**	**SBC**	**WASL**	**WSBC**
Cadmium	0.2	0.3	0.28	0.25	0.27	0.19	0.2	0.23	0.27
Lead	5	0.31	0.38	0.45	0.44	0.37	0.49	0.46	0.45
Arsenic	0.5	0	0.4	0.39	0.32	0.32	0.42	0.49	0.5

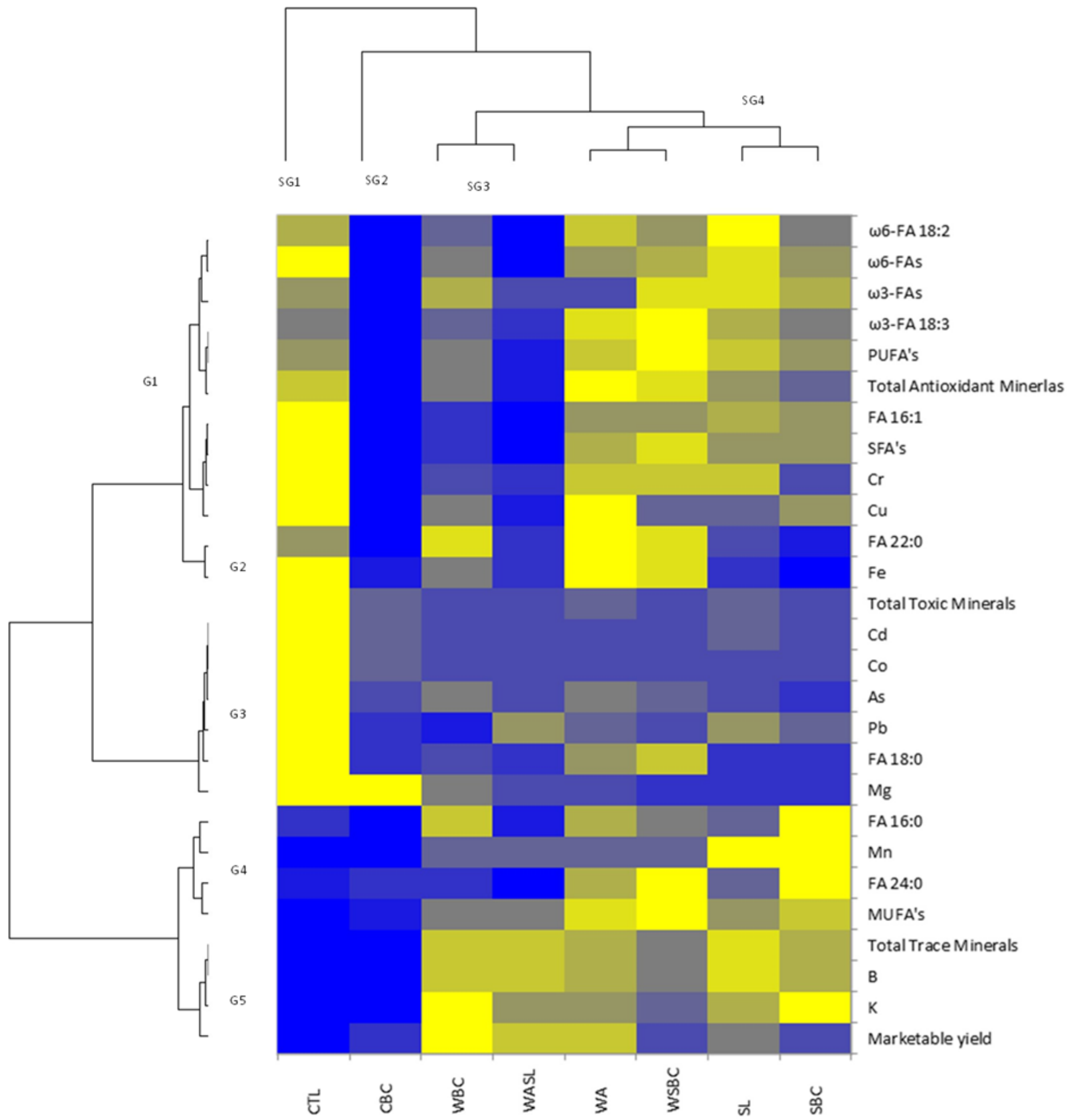
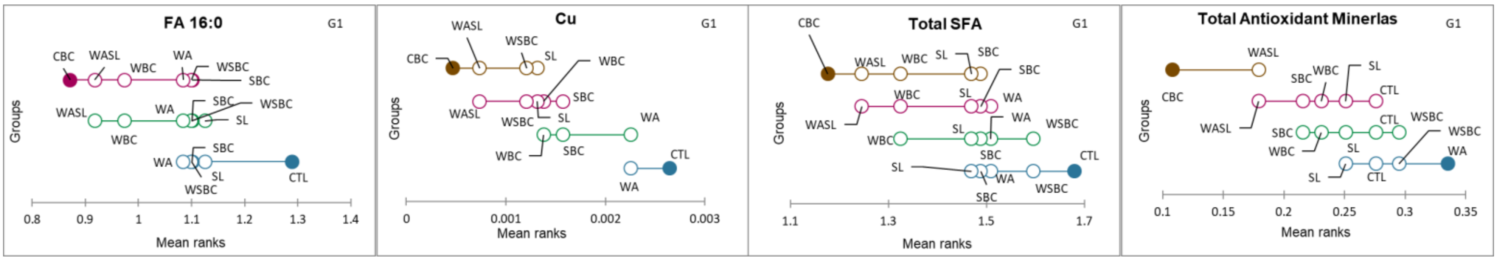


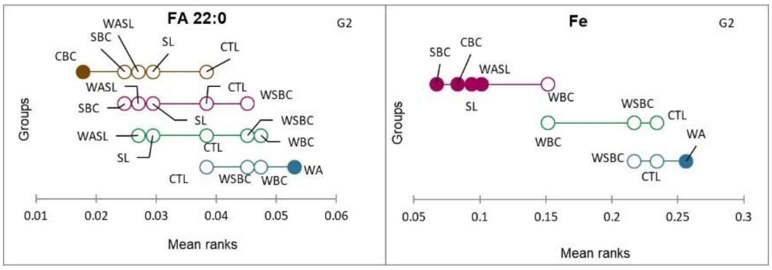
Figure 2.1. The heatmap for nutritional parameters of kale grown in with media amendments for soil type 1. A colour scale of blue to yellow is being used with colour calibration ranging from -1 to +1 respectively). CTL = control, CBC = control + biochar, WA = wood ash, WBC = wood ash + biochar, SL = sludge, SBC = sludge + biochar, WASL = wood ash + sludge, and WSBC = wood ash + sludge + biochar are amendments.

a



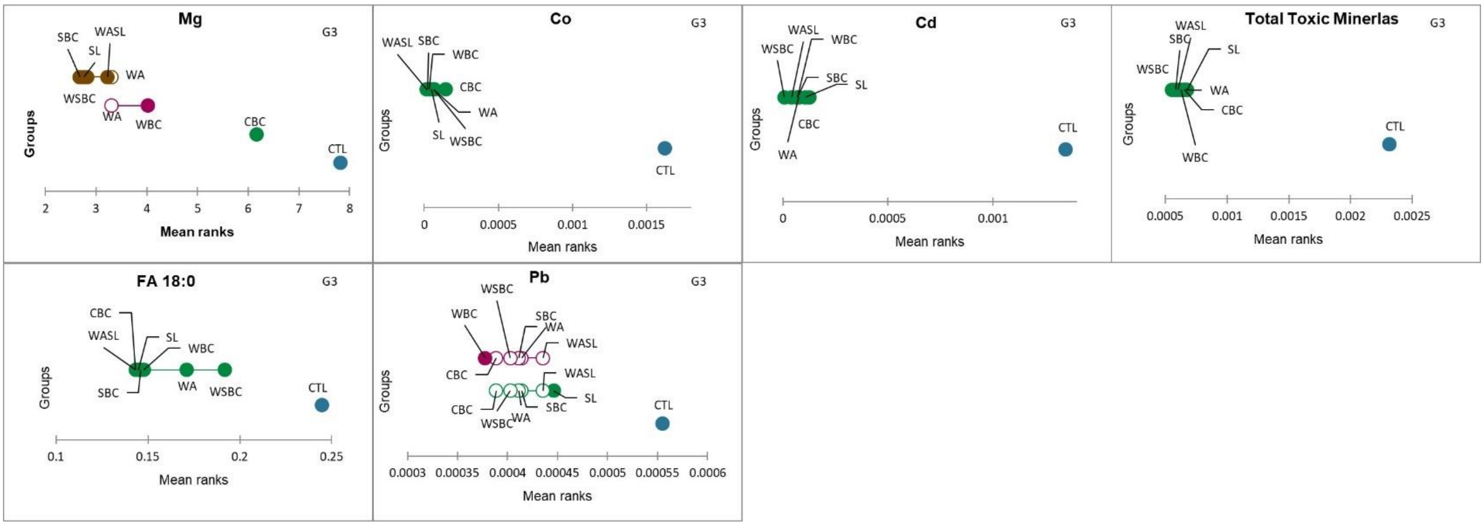
FA 16:0
G1
Groups
0.8 0.9 1 1.1 1.2 1.3 1.4
Mean ranks
Cu
G1
0 0.001 0.002 0.003
Mean ranks
Total SFA
G1
1.1 1.3 1.5 1.7
Mean ranks
Total Antioxidant Minerlas
G1
0.1 0.15 0.2 0.25 0.3 0.35
Mean ranks
CBC WASL WBC WA WSBC SBC SL CTL

b



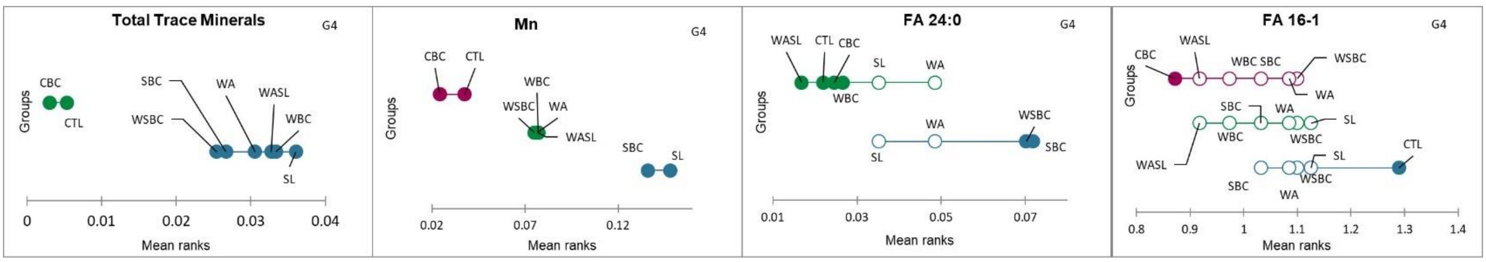
FA 22:0
G2
Groups
0.01 0.02 0.03 0.04 0.05 0.06
Mean ranks
Fe
G2
0.05 0.1 0.15 0.2 0.25 0.3
Mean ranks
CBC WASL SBC SL CTL WSBC WBC WA

c



Mg
G3
Groups
2 3 4 5 6 7 8
Mean ranks
Co
G3
0 0.0005 0.001 0.0015
Mean ranks
Cd
G3
0 0.0005 0.001
Mean ranks
Total Toxic Minerlas
G3
0.0005 0.001 0.0015 0.002 0.0025
Mean ranks
FA 18:0
G3
0.1 0.15 0.2 0.25
Mean ranks
Pb
G3
0.0003 0.00035 0.0004 0.00045 0.0005 0.00055 0.0006
Mean ranks
CBC WASL WBC WA WSBC SBC SL CTL

d



Total Trace Minerals
G4
Groups
0 0.01 0.02 0.03 0.04
Mean ranks
Mn
G4
0.02 0.07 0.12
Mean ranks
FA 24:0
G4
0.01 0.03 0.05 0.07
Mean ranks
FA 16-1
G4
0.8 0.9 1 1.1 1.2 1.3 1.4
Mean ranks
CBC WASL WBC WA WSBC SBC SL CTL

e

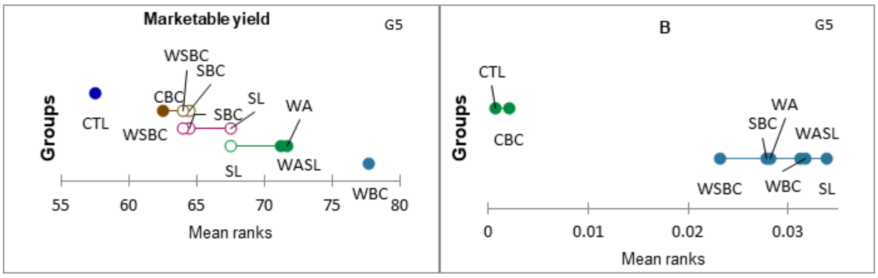


Figure 2.2. Demsar plots of a, b, c, d and e for the nutritional parameters shown in G1, G2, G3, G4 and G5 clusters of heat map of soil type 1 respectively. Only the significant ones(p<0.05) are shown. Groups of amendments that are significantly different (p <0.05) are not connected by dots. All the parameters are shown in mg g^{-1} of DW. CTL = control, CBC = control + biochar, WA = wood ash, WBC = wood ash + biochar, SL = sludge, SBC = sludge + biochar, WASL = woodash + sludge, and WSBC = wood ash + sludge+ biochar are amendments.

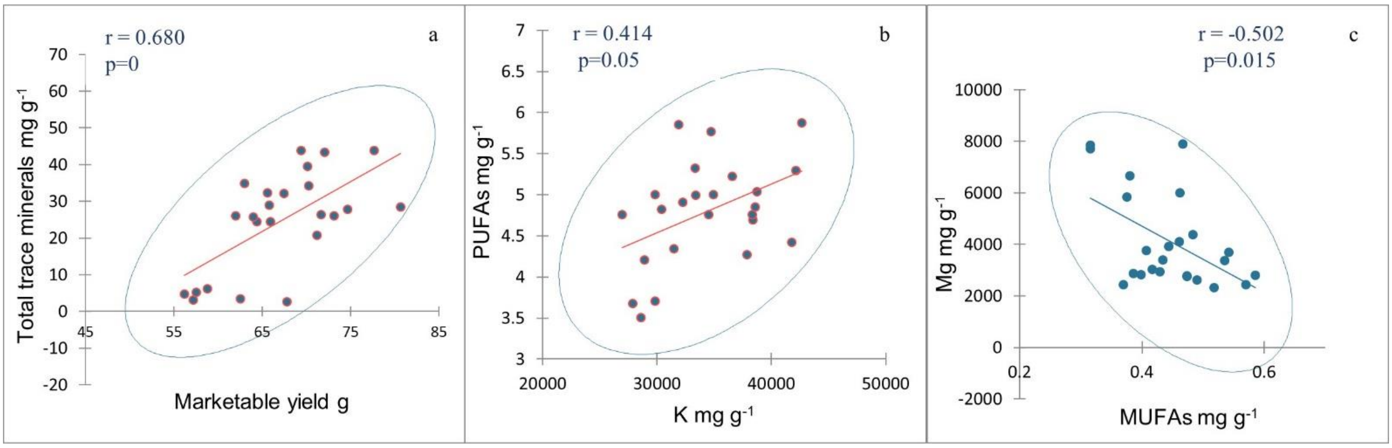


Figure 2.3. The Pearson correlation graphs of total mineral content and marketable yield (a), PUFAs and K (potassium) (b), and, MUFAs and Mg (magnesium) (c) for kale grown in soil type1.

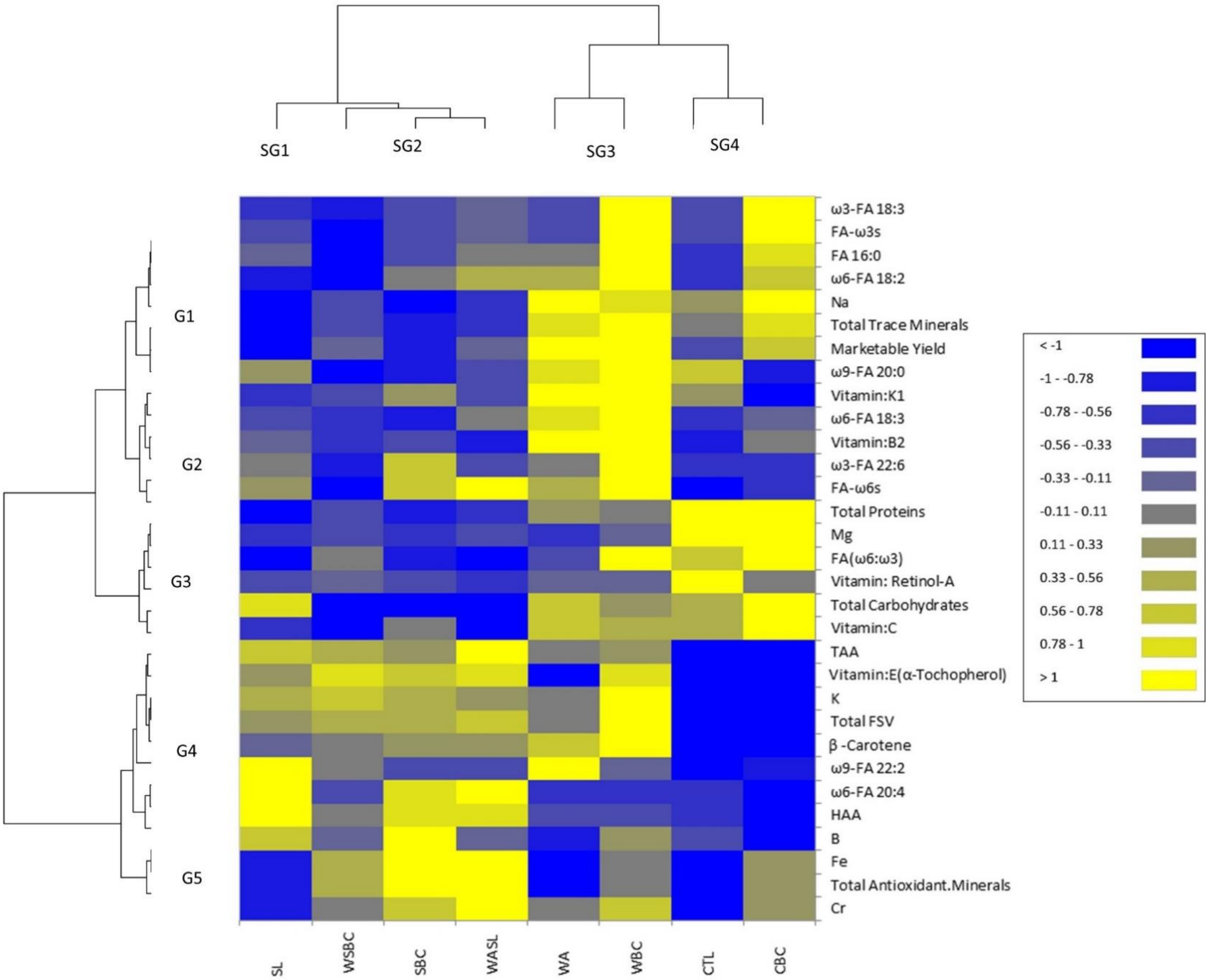
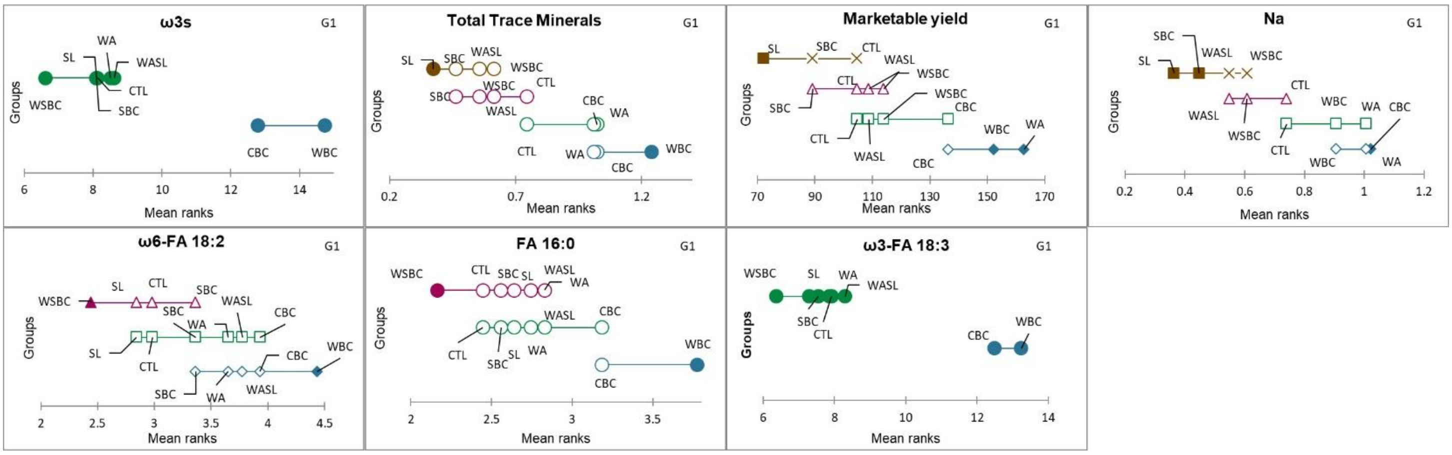
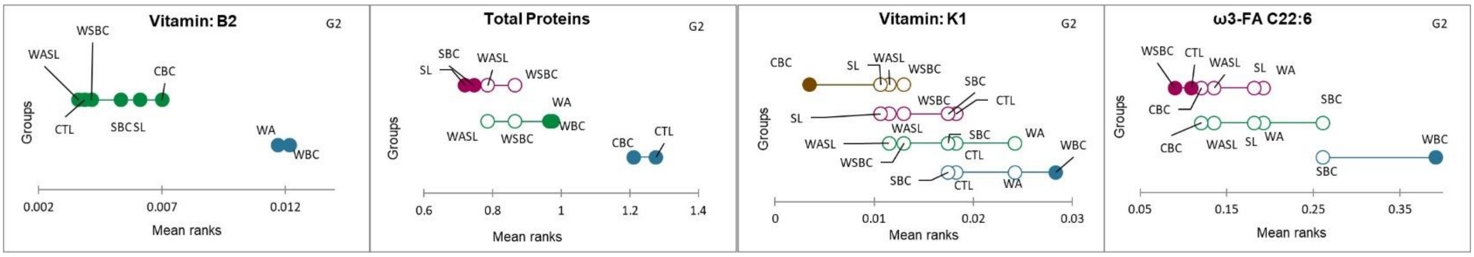


Figure 2.4. The heatmap for nutritional parameters of kale grown with media amendments for soil type 2. A colour scale of blue to yellow is being used with colour calibration ranging from -1 to +1 respectively). CTL = control, CBC = control + biochar, WA = woodash, WBC = woodash + biochar, SL = sludge, SBC = sludge + biochar, WASL = woodash + sludge, and WSBC = woodash + sludge+ biochar are amendments.

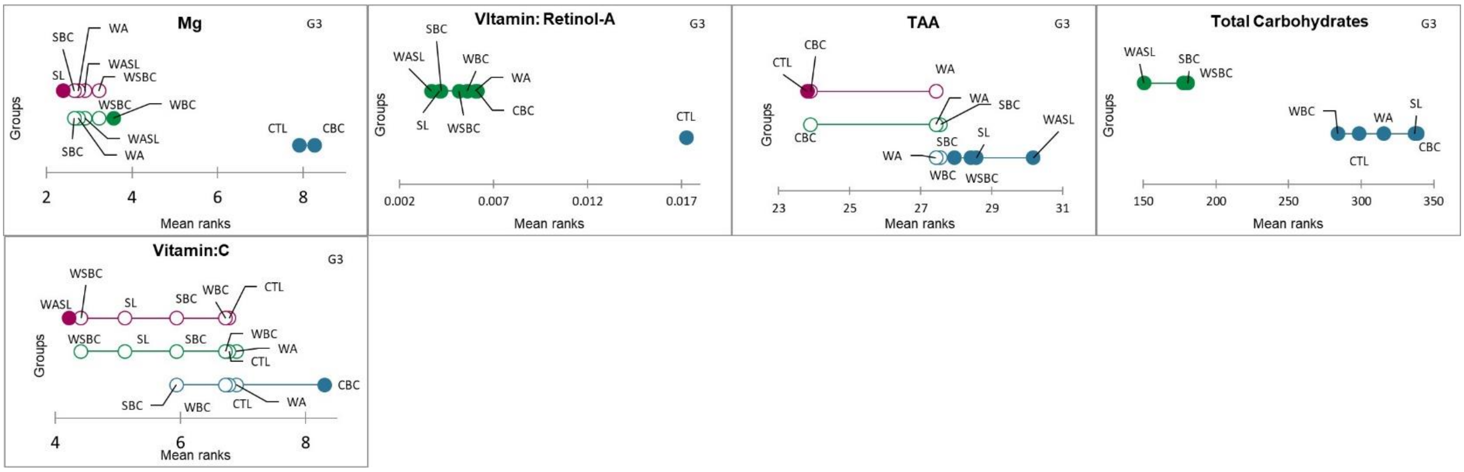
a


ω3s
G1
Total Trace Minerals
Marketable yield
Na
ω6-FA 18:2
FA 16:0
ω3-FA 18:3
Groups
Mean ranks

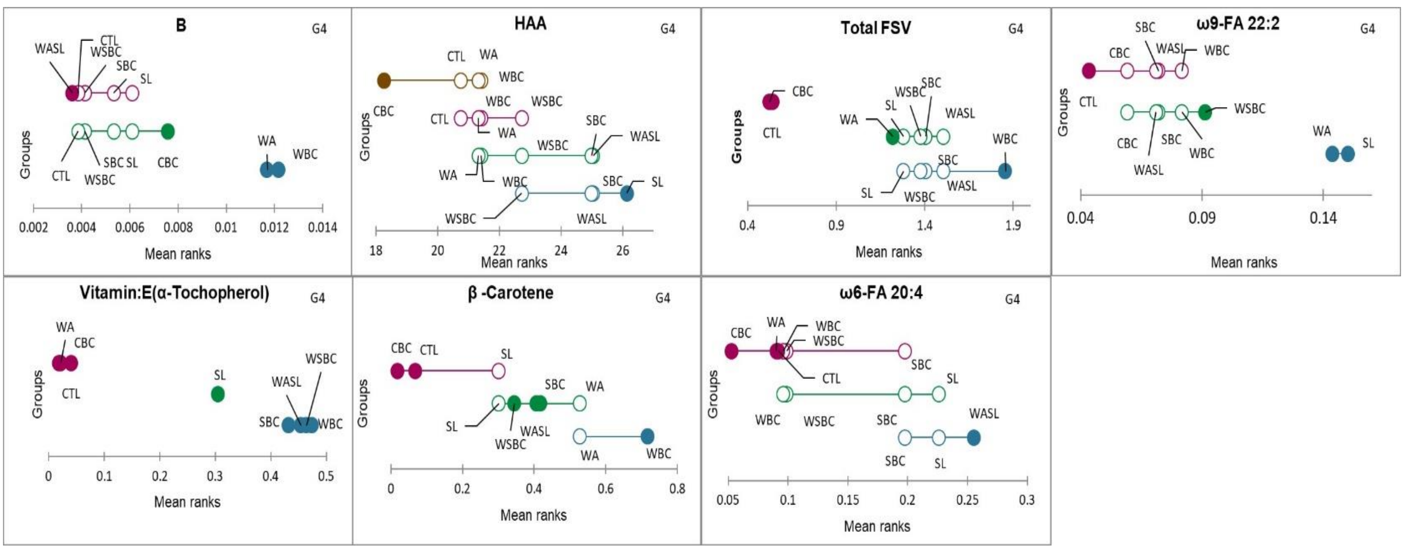
b


Vitamin: B2
G2
Total Proteins
Vitamin: K1
ω3-FA C22:6
Groups
Mean ranks

c


Mg
G3
VItamin: Retinol-A
TAA
Total Carbohydrates
Vitamin:C
Groups
Mean ranks

d


B
G4
HAA
Total FSV
ω9-FA 22:2
Vitamin:E(α-Tochopherol)
β -Carotene
ω6-FA 20:4
Groups
Mean ranks

e

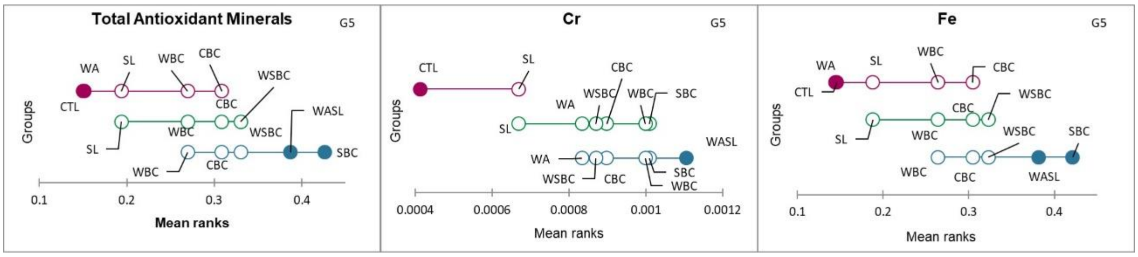


Figure 2.5. Demsar plots of a, b, c, and d for the nutitonal parameters shown in G1, G2, G3, G4 and G5 clusters of heat map of soil type 2 respectively. Only the significant ones($p<0.05$) are shown. Groups of amendments that are significantly different ($p <0.5$) are not connected by dots. The parameters of marketable yield, TAA /HAA are in g, µmol g^{-1} TROLOX equivalent respectively. All other parameters are in mg g^{-1} of DW. CTL = control, CBC = control + biochar, WA = wood ash, WBC = wood ash + biochar, SL = sludge, SBC = sludge + biochar, WASL = woodash + sludge, and WSBC = wood ash + sludge+ biochar are amendments.

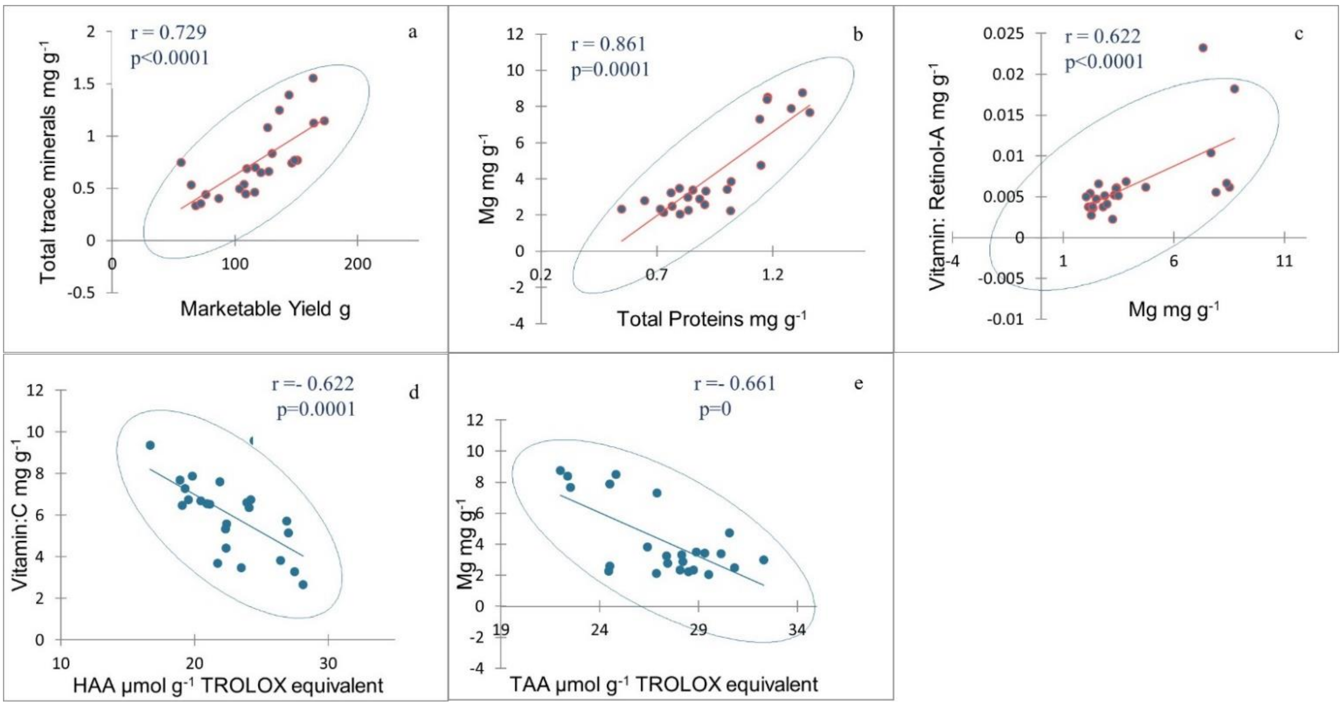


Figure 2.6 The Pearson correlation graphs of total mineral content and marketable yield, total PUFAs and K (potassium), and, total MUFAs and Mg (magnesium) for kale grown in soil type 2.

2.5.3 Pathway analysis of significant nutritional parameters for soil type 1 and soil type 2

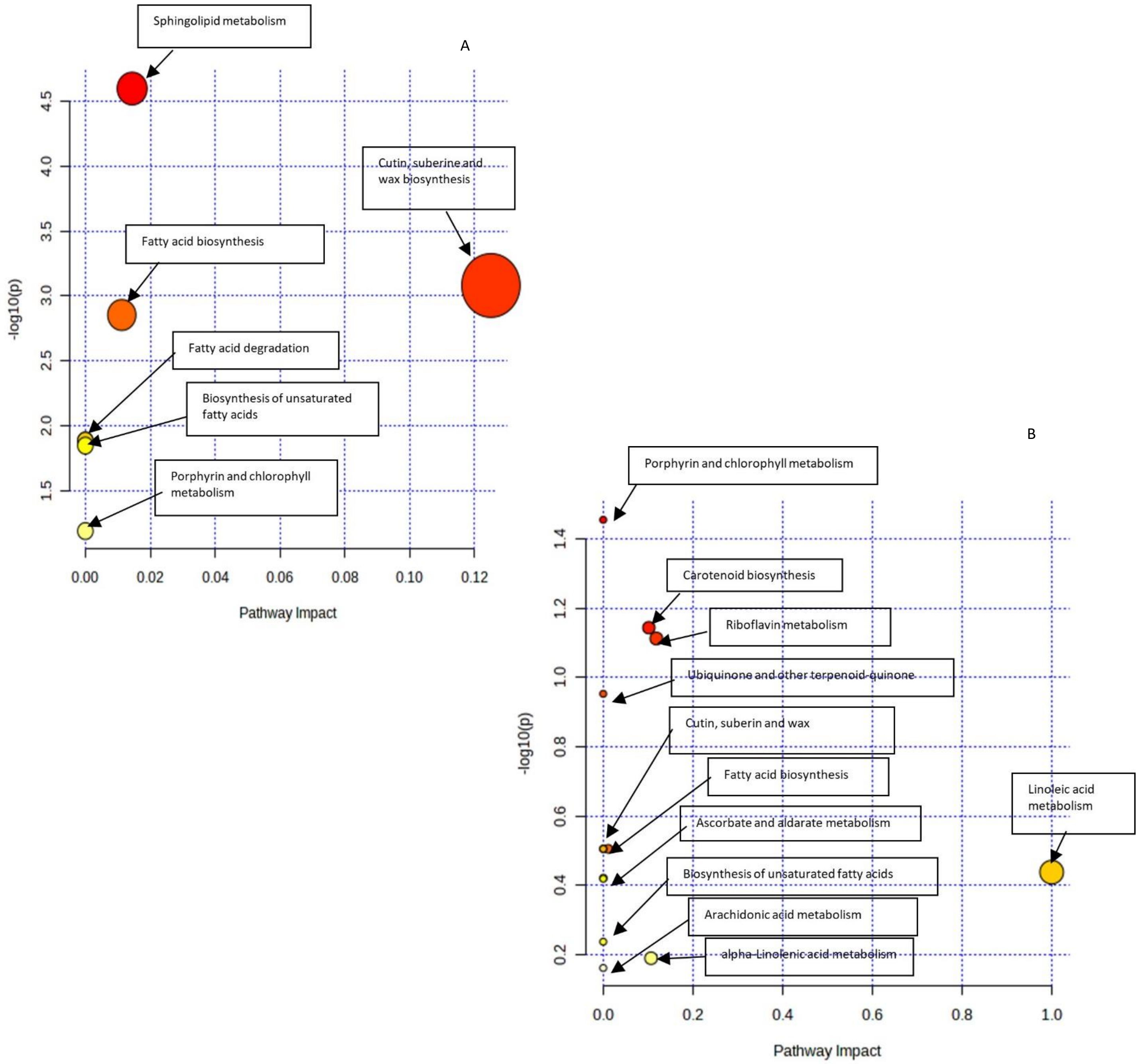


Figure 2.7 Overview of pathway analysis using Metaboanalyst 5.0. (A) Pathway analysis of soil type 1 for significant nutritional parameters (B) Pathway analysis of soil type 2 for significant nutritional parameters. The x-axis represents the pathway impact, and the y-axis represents log10 (p-value). Large sizes and dark colors represent major pathway enrichment and high pathway impact values, respectively.

Pathway analysis showed the significantly altered plant metabolic pathways ($p<0.05$ and Impact factor>0) when using natural media amendment (Figure 2.7). For soil type 1 sphingolipid metabolism, cutin, suberine, and wax biosynthesis and FA biosynthesis pathways were shown to be significantly affected (Table 2.7). Considering soil type 2 (Table 2.8), although many pathways were identified, none of them were significantly affected by natural media amendments.

Table 2.7. Metabolic pathways associated upon addition of natural media amendments for kale grown in soil type 1.

Pathway Name	p	-log(p)	Impact
Sphingolipid metabolism	2.5229E-5	4.5981	0.01442
Cutin, suberine and wax biosynthesis	8.3068E-4	3.0806	0.125
Fatty acid biosynthesis	0.0014026	2.8531	0.01123
Fatty acid elongation	0.013041	1.8847	0.0
Fatty acid degradation	0.013041	1.8847	0.0
Biosynthesis of unsaturated fatty acids	0.014247	1.8463	0.0
Porphyrin and chlorophyll metabolism	0.064909	1.1877	0.0

Table 2.8. Metabolic pathways associated upon addition of natural media amendments for kale grown in soil type 2.

Pathway Name	**p**	**-log(p)**	**Impact**
Porphyrin and chlorophyll metabolism	0.035061	1.4552	0.0
Carotenoid biosynthesis	0.071854	1.1435	0.10175
Riboflavin metabolism	0.077226	1.1122	0.11852
Ubiquinone and other terpenoid-quinone biosynthesis	0.11152	0.95265	0.0
Fatty acid biosynthesis	0.3127	0.50488	0.01123
Fatty acid elongation	0.3127	0.50488	0.0
Fatty acid degradation	0.3127	0.50488	0.0
Cutin, suberine and wax biosynthesis	0.3127	0.50488	0.0
Linoleic acid metabolism	0.3651	0.43759	1.0
Glycolysis / Gluconeogenesis	0.37948	0.42081	3.8E-4
Ascorbate and aldarate metabolism	0.38149	0.41851	0.0
Biosynthesis of unsaturated fatty acids	0.58	0.23657	0.0
alpha-Linolenic acid metabolism	0.64717	0.18898	0.10665
Arachidonic acid metabolism	0.69056	0.1608	0.0

2.4 Discussion

In this study, it was shown that kale grown in soil type 1 tends to show SFAs enhancement when no amendment (CTL) was used while the addition of other media amendment decreased the fatty acids. Several past studies have focused on reducing the intake of saturated fatty acids to mainly decrease the risk of cardiovascular diseases (CVD) (Briggs et al., 2017) where it contributes to increasing low-density lipoprotein (LDL) cholesterol, a strong risk factor for CVD (Mensink, 2016). Kale is considered to be rich in omega-3 fatty acids which are confirmed in this study for soil type 2; The linolenic acid (ω3-FA 18:3) has been prominent and shown the highest values upon the addition of the WBC amendment compared to other fatty acids. Therefore, using such amendments in soil type 2 can be considered an approach to produce kale with less saturated fatty acid content. Natural media amendments containing higher amounts of N content and EC appeared to stimulate monounsaturated and ω3 fatty acids (Abbey et al., 2018). Those findings confirm the current study for soil type 2 where it can be seen higher total nitrogen amounts are present in the initial WA stocks (Table 2.1) and the analysis report of soil type 2 has shown a high EC value too (Data not shown).

Studies by Ayaz et al. (2006) report that total unsaturated fatty acid content ranged from 129 μg g^{1} where among all, ω3-FA18:3 has reported as 85.3 μg g^{-1} per dry kale weight confirming the range of ω3- FA values in this study for soil type 2. A study by Calder (2015) states that ω3 PUFA carries health properties like anti-inflammatory and immunomodulatory while reports by Lee et al. (2014) state that consumption of ω3 rich food such as kale can help prevent diabetes and cardiovascular diseases.

A similar study by Abbey et al. (2018) using the media of vermicompost, K-humate, and volcanic minerals on kale found an increase in oleic acid content rather than linolenic acid in kale grown in vermicompost compared to other amendments. Apart from the above Vidal et al. (2018) reported an increase of a total of ω9-FA 18:1 and ω3-FA 16:3 FA when kale was grown in dry vermicast while a total of ω6-FA 18:2 FA accumulation has enhanced by the usage of volcanic minerals. Similarly, in the present study for soil type 2, kale that was grown in WBC amendment showed an increase in ω6-FA 18:2. Overall, it was clear that most of the FA levels were significantly modulated in amended soil treatments compared to the control (CTL) for soil type 2.

Considering the mineral content of kale grown in this study, the macro minerals of Mg was shown to be significantly affected by media amendments in both soil types. Compared to the past studies, the Mg amount in the current study shows significantly higher values, which may be due to a high extractable amount of Mg in soil type 2 (5600 mg kg^{-1}) (Data not shown). Apart from the above media of WA (565 mg kg^{-1}) and SL (1170 mg kg^{-1}), both do carry a higher amount of extractable Mg levels, which may also contribute to the increased levels. A study by Grace et al. (2000) where kale has grown in a pH 7.65 soil with neither fertilizer nor pesticide application has shown a higher amount of Mg levels (0.21%), confirming that factors such as the method of cultivation, cultivar, or species type and region of the production influence the mineral composition of kale leaves (Acikgoz & Deveci, 2011).

A similar effect for Na (Sodium) for soil type 2 was shown where adding amendments reduced the values. Many early studies have reported that plants' macro elements of P, Ca, K, and Mg contents are remarkably affected by wood ash applications (Clapham & Zibilske, 1992; Etiégni & Campbell, 1991; Vance, 1996). Although the current study did not manifest an increase in Mg, many past studies have shown and agreed that Ca and K contents of plants increase noticeably with the application of wood ash (Erich, 1991; Susan Erich & Ohno, 1992).

The increase of K content upon adding WBC was seen in the present study for both soil types, but the parameter does not show any significant difference ($p<0.05$). The inconsistencies in the contents and the exports of Mg, following wood ash application is probably the result of the interaction with Ca and/or K (Demeyer et al., 2001). This can also be confirmed in the current study where there is a negative correlation between K and Mg can be seen for both soil types (soil type 1: $r = -0.448$; $p = 0.028$; soil type 2: $r = -0.466$; $p= 0.022$) (Figure 2.6 and Figure 2.7). Early reports by Tucker et al. (1999) state that the amount of K and Mg in fruits and vegetables plays a potential role in managing bone mineral density, where the approach of using media amendments such as WA, BC, and SL needs to be carefully modulated.

Trace metals are essential for plant growth but are required in very small amounts, which involve metabolic functions, such as regulation of genes, cell protection, primary and secondary metabolism, hormone perception, signal transduction, and reproduction (Hänsch & Mendel, 2009; Mozaffari & Hays, 2019). Reports by Yang et al. (2003) state that adding organic fertilizers significantly increases the amounts of trace metals that accumulate in plants. Considering the total trace mineral content (B, Cr, and Co) in this study, significantly the highest amounts were shown for kale grown in SL (0.036 ± 0.004 mg g^{-1} of DW) and WBC (1.23 ± 0.23 mg g^{-1} of DW) amendments for soil type 1 and soil type 2, respectively.

A significant amount of B was increased upon the addition of SL amendment for soil type 1 where except for CTL and CBC, there was no significant difference ($p<0.05$) was shown with other amendments. Interestingly a similar effect was manifested in soil type 2, where the application of WBC increased the B values in kale. Early reports by Ferm et al. (1992) state that WA is an excellent source of B where it can be used in soils deficient in B. However, studies by Kukier et al. (1994) state that there can be induced toxicities and reduced plant growth when applying a higher amount of WA. Singh et al. (2016) state that plants utilize efficient and highly specific mechanisms for obtaining the essential plant micronutrients from the soil, even at very low concentrations. Plants can solubilize and absorb micronutrients at very low concentrations in the soil, even from nearly insoluble precipitates (Shrestha et al., 2019) due to plant roots which are assisted by chelating agents, changes in pH, and other redox reactions (Tangahu et al., 2011).

In this study, overall, the highest amount of total antioxidants mineral content (Mn, Fe, Cu, Zn, and Se) was shown in kale grown in WA amendment and SBC for soil type 1 and 2, respectively. A study carried out by Adanlawo and Ajibade (2006) to determine the effect of soaking two varieties of Roselle (*Hibiscus sabdariffa*) red and green in WA were also shown a significant increase in Zn and Mn content. A study by Saravanan et al. (2016) has shown that the cowpea plants grown in paper sludge-amended soils have shown a higher amount of iron as compared to the other minerals where the excess level of iron content in the plants and the soil is due to kind of the sludge applied for the plant to grow as they possess iron in the higher range.

A similar study by Sabia et al. (2021) states that the usage treatment 75% sewage sludge + 25% NPK showed the highest iron content in both kale (6.99 ppm) and spinach (6.64 ppm), respectively. Early reports by Bhattacharya et al. (2016) state that kale is a leafy vegetable rich in Fe compared to spinach (2.71 mg per 100 mg) while studies by Satheesh and Workneh (2020) suggest it as one of the best sources of iron fortifications. The current study has shown increased values of Fe content in kale when WA and SBC amendments were used for soil type 1 and 2, respectively. Therefore, the usage of such amendments creates possibilities for creating functional foods enhanced with Fe.

Some toxic minerals (As, Cd, and Pb) were present in the initial WA and SL stocks. Although no specific limits have been developed by the CCME and the Canadian food inspection agency (CFIA) on using organic waste material as growth media amendment, CCME has developed A and B compost categories and the biosolids limit for waste application in the agriculture industry. Both WA and SL used in this study were shown to lower concentrations of toxic minerals than the above compost categories for both soil types (Table 2.1). It was shown that upon adding media amendments, total toxic element levels have reduced for soil type 1 where Co and Cd elements were significant, while in soil type 2 there were no significant ones. The accumulation of salt and heavy metal content has been correlated with the unnecessary application of sewage treatment to the soil, causing plant toxicity (Hao & Chang, 2003).

Moreover, consuming foods containing higher toxic minerals leads to a nutrient-deficient body causing a weak immune system, changes in psycho-social performance, impedance in intrauterine growth, malnutrition, and a higher risk for gastrointestinal cancer and other related problems (Hao & Chang, 2003). It was shown that for both soil types, the level of selected toxic minerals almost showed low values compared to the standards by WHO/FAO (Table 2.5 and Table 2.6).

A similar study by Zubair et al. (2019) on using sewage sludge and aquatic weed compost found that heavy metal concentrations of kale exposed to those media were significantly higher than those in the untreated plants. This contradicts the current study for soil type 1, where except for no amendment (CTL), no significant difference was shown in total toxic minerals for all other media amendments. A study by Merino et al. (2006) states that there were no changes in heavy metal concentrations in the leaves or fruits of kiwi grown in an acidic soil amended with paper mill-generated wood ash. It also manifested a reduction of toxic minerals upon the addition of BC for WA and SL in the present study.

Biochar used in this study was shown high CEC, high carbon, and large groves produced at 550°C, (Table 2.3) might have impacted the heavy metal sorption capacity in the soil system by making organic complexes (Uchimiya et al., 2011) and reduced plant uptake. An increase in soil pH might help in the adsorption of heavy metal to the surface area of the BC and eventually may have reduced plant uptake. Functional groups such as -COO-, -COH, and -OH (Yuan et al., 2011) are present in BC which aids in the adsorption of heavy metals, and decreased bioavailability (Banik et al., 2018; Shaaban et al., 2013; Yuan et al., 2011). The results of the present study for soil type 1 are consistent with the previous study by Zhang et al. (2016) who observed a significant reduction in Cd mobility and prevention of Cd accumulation in rice when sewage sludge was amended with BC.

The present study has shown a significant difference in marketable yield, where the highest yield was seen at WBC and WA applications for soil types 1 and 2, respectively. In soil type 2, the lowest yield was manifested on SL; in soil type 1, no amendment (CTL) has shown the least. A significant amount of K and other trace elements in WA helps improve plant metabolism and increases plant growth and yield (Füzesi et al., 2015). A recent study by Mercl et al. (2018) reported that using WA as a sole amendment for maize plants increased biomass yield and improved nutritional status.

A similar study by Demeyer et al. (2001) states that the most yield increase of crops can be attributed to the increased availability of minerals such as K, P, and B in soils after adding WA. However, the lower plant yield of Kale crops in SL treatment might be due to the high soil C/N (data not shown) that may have led to N immobilization by soil microorganisms resulting in low mineralization and decreased plant growth (O'Brien et al., 2007).

The TAA and HAA of cruciferous vegetables like kale have shown both antioxidant and anticarcinogenic properties (Satheesh & Workneh, 2020). In this study for soil type 2, for TAA the highest value of 30.2 ±1.4 µmol g^{-1} Trolox equivalent of DW was shown after adding WASL media amendment. In contrast, the lowest values were shown for CBC and CTL, with no significant difference ($p<0.05$). A study Singh et al. (2004) using sludge as an amendment on sunflower fields has shown an increase in proline and ascorbic acid content at all exposure periods compared to CTL treatment confirming increased antioxidant activities. A similar study by Shakeel et al. (2020) also reports an increase in proline content, and the total antioxidant activity of beetroot increased significantly with increasing fly ash levels. The authors also suggest that elevated levels of antioxidant activity might be due to scavenging the oxidative stress caused by the higher concentration of fly ash in the soil. Moreover, the presence of antioxidant vitamins such as carotenoids, polyphenols, and glucosinolates have been mainly identified as compounds that possess antioxidant activities in kale-like greeny vegetables, while Plumb et al. (1996) report that compounds such as glucosinolates can be shown a rather low antioxidant activity but the hydrolysis products of glucosinolates have shown anticancer properties (Keum et al., 2004).

Plant carbohydrates form the main structural components and products of energy in two forms, cellulose, and hemicelluloses, which are polymers of glucose and 5-carbon sugars and other compounds, respectively. Starch is the principal form of a glucose polymer, which gets stored in cells as granules when produced than required (Sabia et al., 2021). This study shows that for soil type 2, the total carbohydrate amounts ranged from 150 mg g^{-1} to 349 mg g^{-1} of DW where amendment CBC showed the highest followed by the SL (Figure 2.2). The least amount of carbohydrates in kale grown in WASL media has shown a significant difference ($p<0.05$) compared to CTL.

A study by Zubair et al. (2019) reports that the enhanced levels of carbohydrates in sewage-sludge-supplemented plants are due to the occurrence of some important mineral ions such as Mn and Cu elements which are known to stimulate the two photosystems. For PS II, an O2 evolving system, Mn^{2+} is required, and the interaction between copper and ferredoxin on the reducing side of PS I is also considered important (Marschner's Mineral Nutrition of Higher Plants 2002). This agreed with the present study for soil type 2 where a positive correlation between total carbohydrates and total trace minerals can be seen (r =0.282; p =0.270) (Data not shown). A study by Pradhan et al. (2009) on urine and urine + ash fertilizer treatments showed a significant effect on total sugar content ($p<0.05$). Zeid et al. (2007) also reported that in plants irrigated with sewage water, the polysaccharide and total carbohydrate contents were higher than those grown on the control amendment.

Plant proteins play an important role in the development and form structural constituents, antibodies, and most hormones. Almost 90% of the proteins are reported to function as enzymes, which are key players in many fundamental cellular reactions required for proper development in the plant system (Sabia et al., 2021). In this study for soil type 2, it can be seen that the usage of amendments has reduced the protein content of kale. The highest total protein content was observed in the CTL treatment (1.27 ± 0.06 mg g^{-1} of DW) followed by the CBC treatment. The results are in line and in the range that was reported by Emebu and Anyika (2011) while a study by NA El-Tayeh et al. (2011) states that soluble proteins in shoots of cotton plants decreased remarkably by increasing sewage sludge levels at 30% which adheres with SL amendment in this study. A similar study by El-Samad et al. (2020) mentions a significant reduction in the protein content of some bean varieties (cv. Giza 843) grown in sewage sludge. However, the studies by Pradhan et al. (2010) manifest that beetroot grown in urea + wood ash media has shown a significant increase in protein content. Furthermore, protein contents in red beetroots were positively correlated with Mg^{2+} (r = 0.699; p = 0.0001).

Similarly, in our study for soil type 2 Mg is positively correlated with total protein content ($r = 0.861$; $p < 0.0001$) (Figure 2.6) where the amount of Mg was shown to be significantly low upon the addition of all media types, which may have led to the decrement of protein levelsThe fat-soluble vitamins present in kale grown in soil type 2 were significantly prominent upon the addition of WBC amendment (1.85 ± 0.18 mg g^{-1} of DW). Similarly, the highest amount of β -carotene was shown upon the addition of WBC (0.71 ± 0.15 mg g^{-1} of DW) where there was a significant difference in the β- Carotene content of kale grown in no media amendment (CTL). β -carotene is the precursor for retinol and vitamin A, and in kale, many studies have reported the presence in the range of 3.887– 44 mg per 100 g FW (Satheesh & Workneh Fanta, 2020), which is in agreement with the results from our study.

In the present study for soil type 2, a weak positive correlation ($r = 0.251$; $p = 0.013$) was observed between β -carotene and total antioxidant amount (Data not shown). Studies by Perera and Yen (2007) reported that consumption of carotenoid-rich foods may help reduce the occurrence of several diseases such as cataracts, cancers, cardiovascular diseases, age-related macular degeneration, diseases related to low immune functions, and other degenerative diseases. Pradhan et al. (2009) found that tomatoes grown in urea + wood ash did not show any significant difference in β- Carotene content compared to the no treatment. According to WHO the RDA (Recommended dietary allowance) of vitamin A is 700 - 900 μg day^{-1} (Trumbo et al., 2001) which can be seen in the present study as achievable through such usage of media amendments. Similarly, a study by Antonious et al. (2011) found that sweet potatoes grown in municipal sewage sludge (MSS) have shown an increased amount of β-carotene concentration (157.5 μg g^{-1} of FW) compared to MSS + yard waste treatments (99.9 μg g^{-1} of FW).

In the current study, the content of water-soluble vitamins, vitamin C (ascorbic acid) and vitamin B2 in kale were significantly affected by adding media amendments for soil type 2. Vitamin C is the major water-soluble antioxidant in plant cells and plays a major role in protecting cells against free radicals and oxidative damage (Wang et al., 2003). Past research has shown that ascorbic acid derivatives tested on cancer cells revealed promising anticancer activity (Naidu, 2003). In addition, ascorbic acid in most fruits and vegetables protects against cardiovascular diseases, high blood cholesterol, and high blood pressure (Ruxton et al., 2006). In plants, ascorbic acid is involved in xenobiotic detoxification, the cell cycle, and cell wall growth acts as a coenzyme in metabolic changes involved in photosynthesis and respiration processes (Franceschi & Tarlyn, 2002). According to Acikgoz (2011) humans require vitamin C as an essential component of their diet and since vitamin C is ubiquitous in all green plants, makes an easy source to take an adequate amount for humans in their daily diet. Kale is reported to have a high concentration of vitamin C than all other salad vegetables and vegetables of the *Brassicaceae* family (Edelman & Colt, 2016).

In the present study, the highest amount of vitamin C (8.3 ± 0.52 mg g^{-1} of DW) was shown when CBC amendment was used, which did not show a significant difference compared to CTL. This study's values were 62.27–969 mg per 100 g of FW, satisfying the RDA for both males and females (Acikgoz, 2011a; Sikora et al., 2012). An early three-year study by Wang et al. (2003) found similar results where there was no significant difference in the vitamin C content of corn and potatoes grown in composted manure compared to conventional treatment.

The highest amount of vitamin B2 was shown when WBC amendment (0.012 ± 0.0002 mg g^{-1} of DW) was used where there was no significant difference ($p<0.05$) with WA. However, a significant value reduction can be seen upon adding other amendments. In recent studies by Šamec et al. (2018), the concentration of riboflavin reported in kale is considered reasonably good which varies between 0.13–0.9 mg per 100 g of FW, which agrees with the current study.

Apart from that, a study by Litoriya et al. (2018) on determining the effect of organic and chemical sources on the nutritional quality of durum wheat found that riboflavin had a nonsignificant difference and was higher in organically grown wheat.

In this study, it was demonstrated that soil type 1 (pH 5.2) and soil type 2 pH 5.8) act differently upon the addition of different media amendments. This was also confirmed through pathway analysis results showing different significant metabolic pathways associated. Such variation might have occurred ue to the differences in soil pH and its organic constituents.

2.5 Conclusion

We investigated the effect of wood ash (WA), paper sludge (SL), and biochar (BC) on the nutritional profile of kale grown under controlled environmental conditions. It was clearly shown that depending on the soil type, the nutritional profile of kale got significantly varied. This was also confirmed through pathway analysis, where different metabolic pathways were shown to be significant for both soil types. Overall, it has been demonstrated that WA amendment in soil type 1 increased the yield thus, there was no significant effect of media amendments on most of the nutritional parameters of kale. Unlike soil type 1, the nutritional profile of kale grown in soil type 2 was significantly impacted by the media amendments compared to CTL. The WBC amendment has shown increased values of most of the nutritional parameters, while the addition of the WA has also led to an increase in yield. Although there is an increase in many nutritional parameters, we recommend routine tests of WA and SL for heavy metals before usage, which can be accumulated in the food chain. We may conclude that WA alone or amended with BC may increase the kale yield and nutritional parameters in high soil pH.

Findings from this study may contribute to optimizing the use of paper mill waste to fertilize crops, specially grown in podzolic soils, which will help to maintain or enhance the crop quality paving pathways to produce functional foods. However, this study was carried out for a single crop cycle in a controlled environmental condition; further studies need to be carried out on field experiments with many crop cycles.

2.6 References

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Chapter 3: The effect of natural media amendments on the intact functional lipids profile of Kale grown under controlled environmental conditions.

Abstract:

It is widely known and accepted that diets rich in vegetables/ plants significantly impact people's health. Vegetables contain higher levels of different phytochemicals that have shown positive correlations in reducing lifestyle-related diseases such as cardiovascular disease, cancers, type 2 diabetes, and other non-communicable diseases. The objective of this study was to investigate the effect of natural media amendments, i.e., wood ash (WA), paper sludge (SL), biochar (BC), and combinations (such as WSBC) on the intact functional lipids content of Kale grown under controlled environmental conditions. A greenhouse study was conducted with two soil types, soil type 1 and soil type 2, which were the variations of podzolic soils in Newfoundland. Experimental treatments that were used were: CTL (control), CBC (control + biochar), WA (wood ash), WBC (wood ash + biochar), SL (sludge), SBC (sludge + biochar), WASL (wood ash + sludge), WSBC (wood ash + sludge + biochar). In this study, intact functional lipids containing ω3 and ω6 fatty acids, including glycolipids, phospholipids, diglycerides, triglycerides, and sterols, were assessed using ultra-high-performance liquid chromatography-high resolution tandem mass spectrometry (UHPLC-HRMS/MS). The results revealed that for soil type 1, WSBC amendment showed higher functional lipids of 1,2-DGs, 1,3-DGs, ω6-lipids, and phospholipids. In contrast, functional lipids content was increased mostly upon adding SL and WBC media amendment for soil type 2. This different act of media amendment on soils was manifested from the pathway analysis revealing associated significant lipid pathways ($p<0.05$) for each soil type. In conclusion, the greenhouse study suggests using natural growing media amendments, particularly, SL, WBC, and WSBC could be a useful approach to producing kale with enhanced levels of functional lipids. Using such natural media

amendments could be a novel approach for developing functional foods enhanced with functional lipids in control environmental systems.

Keywords: Wood ash, Paper sludge, Biochar, Lipids, Podzolic soil

3.1 Introduction

Functional foods are defined as foods that have the capability of promoting health and disease prevention in addition to their main nutritional and processing values. The primary role of any main diet is to provide nutrients that will help in the metabolic requirements of an individual while it also provides the satisfaction and well-being through hedonistic attributes such as taste, colour, overall acceptance, etc. In fact, diet does not only help to achieve optimal health conditions in the human body, but it also plays an important role in reducing the risk of diseases. In the present society, there is a trend towards these nutritional changes mainly due to increase of aging population, desire for improved quality life and rapid increase in health care costs (Stuchlík & Zák, 2002).

The World Health Organization and Food and Agriculture Organization (WHO/FAO) stated that there is a relationship between several dietary patterns along with lifestyle patterns that contribute to developing disease conditions such as cancer, diabetes (type 2), obesity, cardiovascular diseases, and periodontal diseases (WHO, 2003). In this regard, it is now widely accepted that the health of a population is influenced by diet (Betoret et al., 2011), especially by the high levels of phytochemicals present in the plants/ vegetables (Reganold et al., 2010; Schreiner & Huyskens-Keil, 2006). Plant lipids have gained popularity in developing functional foods due to the bioactive compounds present in plants. (Stuchlík & Zák, 2002; Yang et al., 2018).

Fatty acids (FA) or lipids serve in diverse metabolic functions related to the growth and maintenance of cells and tissues and act as a caloric energy molecule that participates in cellular signalling events accompanied by different physiological processes (Zárate et al., 2017). Chemically FAs are hydrocarbons of C6 to C32 long-chain, containing a hydrophilic carboxyl group at one end and a methyl group at the terminal end (Patel et al., 2020). These FAs can be saturated (SFA), monounsaturated (MUFA), or polyunsaturated fatty acids (PUFA) where their unsaturation degree or the number of double bonds present are 0, 1, and more than 1, respectively (Cholewski et al., 2018). Considering the different fatty acid types of omega-3 (ω3) and omega-6 (ω6) belong to a category of PUFAs where the first double bond is located counting from the methyl end group is located between the 3^{rd} and 4^{th} carbon atom of the fatty carbon chain in acids. Similarly, the first double bond is situated between the 6^{th} and 7^{th} carbon counting from the methyl end group omega-6 fatty acids PUFAs (Patel et al., 2020). The fatty acids of ω3-FA16:3, ω3-FA18:3, and ω6-FA 18:2 are predominant in plants (Stuchlík & Zák, 2002).

These ω3 and ω6 fatty acids are considered essential fatty acids due to the lack of certain human enzymes that help in the desaturation process, which are consequently required to obtain from dietary sources (Patel et al., 2020). Epidemiological and experimental studies have shown that increasing ω3-PUFAs in the diet has significant benefits against colon, breast, prostate, and pancreatic cancers (Riediger et al., 2009), cardiovascular diseases (Chen et al., 2013), stress, anxiety, cognitive impairment, mood disorders, diabetic nephropathy, inflammatory bowel disease, and alzheimer's disease (Huang, 2010; Shapiro et al., 2010; Turner et al., 2011). Although the role of ω6- PUFAs on human health is not stated very clearly, the American heart association and many scientists state that the consumption of at least 5% to 10% of energy as ω6- PUFA will help in improving cardiovascular health (Harris et al., 2009; Czernichow et al., 2010). Like SFAs, MUFAs can be synthesized by the body and therefore are not considered essential dietary lipids.

The dietary MUFAs are considered to be biologically active and have been claimed to have various health effects based on epidemiological studies (Chen et al., 2013). Early reports by Keys (1997) state that Oleic acid (OA; 18:1, ω9), one of the important MUFA, was associated with reduced cardiovascular disease (CVD) in individuals consuming a Mediterranean diet, while studies by Paniagua et al. (2007) state that MUFAs have also been shown to lower blood glucose and triglycerides in type II diabetics patients. Apart from the above, Simopoulos (2002) reports that a lower ratio of omega-6/omega-3 fatty acids is more desirable in reducing the risk of many chronic diseases of high prevalence in Western societies and developing countries.

Glycolipids and phospholipids represent the major building blocks for biological membranes. In plants, phosphoglycerolipids are the predominating lipid class in the plastid membranes; The chloroplasts mainly consist of galactolipids which play an important role in the photosynthesis of higher plants, algae, and certain bacteria (Hölzl & Dörmann, 2007). The thylakoid membrane consists mainly of galactolipids, composed of 50% of monogalactosyldiacylglycerol (MGDG) and 26% of digalactosyldiacylglycerol (DGDG), phosphatidylglycerol and sulfoquinovosyldiacylglycerol (SQDG) (Spicher et al., 2016). Numerous studies have shown that galactolipids derived from plants, cyanobacteria, and green algae exhibit various medicinal properties such as antitumor activity (Shirahashi et al., 1993; Murakami et al., 1995), anti-inflammatory activity (Cateni et al., 2001; Kharazmi, 2008) and antiviral activity (Reshef et al., 1997). Apart from glycolipids (GLs), phospholipids (PLs) are amphiphilic lipids found in all plant and animal cell membranes, arranged as lipid bilayers; Basically, the PLs found in most cell membranes are glycerophospholipids which consist of fatty acid(s) esterified to a glycerol backbone, a phosphate group and a hydrophilic headgroup

(Küllenberg et al., 2012). Dietary PLs are the polar lipids extracted from the food products such as eggs, soybeans, milk, and marine organisms (Cohn et al., 2010).

Naturally occurring PLs of plant or animal origin, predominantly contain an unsaturated FA in the sn-2 position, such as oleic, linoleic, or linolenic acid, or the pro-inflammatory arachidonic acid (usually from animal origin) or the anti-inflammatory eicosapentaenoic acid (usually from marine origin). In contrast, the sn-1 position more offently carries a saturated FA, such as stearic acid or palmitic acid (Küllenberg et al., 2012). Many studies have shown that dietary PLs have benefits of inhibiting cancer growth (Sakakima et al., 2007), significant reduction of total cholesterol (Simons et al., 1977; Wójcicki et al., 1995), and helping to restore the immunological functions in the elderly population (MacZek et al., 1998).

Diglycerides (DG) are another new class of functional lipids common in vegetables which are in low concentrations (1–10% w/w) compared to triglycerides (TG) (Kovacs & Mela, 2006). In DGs the esterification occurs at either C1 and C2 or C1 and C3, leading to isoforms of 1,2-DG and 1,3-DG with metabolic characteristics distinct from triglycerides (Christensen, 2012). Studies by Kristensen et al. (2006) state that compared to TG the regular intake of 1,3-DG isomer helps prevent obesity and diseases such as hypertension, gallbladder disease, and some types of cancers. Triglycerides are lipid molecules with three fatty acids attached to the glycerol backbone (Shah & Limketkai, 2017).

The term medium-chain triacylglycerols (MCTs) refers to mixed triacylglycerols of saturated fatty acids consisting of a chain length of 6–10 carbons, and it has been found that MCTs have the potential of using as an energy source, especially in a variety of clinical nutrition settings such as fat malabsorption, pancreatic insufficiency, impaired lymphatic chylomicron transport, severe hyperchylomicronemia, and total parenteral nutrition (Marten et al., 2006).

The long-chain triglycerides (LCTs), which have fatty acids of > 12 carbons, are considered to be transported via chylomicrons into the lymphatic system, allowing for extensive uptake into adipose tissue (St-Onge & Jones, 2002). Many single-day experiments have revealed that replacing LCT with MCT in the diet may help lose weight after prolonged consumption (Dulloo et al., 1996; Scalfi et al., 1991).

Apart from the above, phytosterols have gained interest as functional lipids. The common dietary sources of phytosterols are vegetables, wood (e.g., tall oil), and vegetable oils (Chen et al., 2013). Common phytosterols are stigmasterol, β-sitosterol, and campesterol, which are members of the triterpene family (Jones & Abumweis, 2009). Reports by Verleyen et al. (2002) state that concentrations of phytosterols in vegetable oils range from 0.1% to 1.0%, and the typical consumption is 200–400 mg daily. The production of phytosterol-fortified foods has become popular because of its ability to decrease total and low-density lipoprotein (LDL) cholesterol in humans by mainly inhibiting the absorption of dietary cholesterol (Chen et al., 2013).

Various stabilized organic wastes, such as animal manure, vegetable waste, urban solid waste, and sewage sludge, can be used to amend soils (Casado-Vela et al., 2007). Additionally, adding organic wastes/natural media amendments to soil can reduce the rapid degradation of soil and improve physiochemical properties to achieve high productivity levels (Selma et al., 2010). Despite the well-documented soil benefits related to using different natural media amendments, few studies have investigated their influence on the phytochemicals of edible plant materials (Sousa et al., 2005). Vidal et al. (2018) state that functional food development can be achieved via modulation of growth conditions, such as using natural media amendments to enhance the crops with targeted functional ingredients under controlled environmental conditions. Plants subject to environmental stresses mostly lead the evolution of different response mechanisms, including changes in metabolisms (Bolton, 2009).

Responses to biotic and abiotic stresses leading to modification of glycerolipid composition are well documented in plants (Böttcher et al., 2009; Li et al., 2008). For example, studies by Torres-Franklin et al. (2007) found that a comparison of two cowpea cultivars, drought tolerant and drought susceptible submitted to moderate drought stress, revealed an increase in DGDG leaf content, thus contributing to plant tolerance to arid environments. Similarly, when the steady state gets altered by the compositional changes in the growing medium, it has been reported that there is a significant change in crop lipid content (Moellering & Benning, 2011).

The pulp and paper industry has a fundamental role in the worldwide economy, where global manufacturing was estimated to increase by around 400 million Mg annually. A report by the world wildlife fund (WWF) states that there is a 5-16% accumulation of biosolids (WA and SL) during the production process (Fahim et al., 2019). Pulp and paper mill by-products have been used for amending agricultural soils and producing crops such as corn (Ribeiro et al., 2010). The nutrient concentrations of paper mill bio-solids vary according to factors such as pulping method and the microbial decomposition level in secondary sludge treatment (Nunes et al., 2007). This variation in those amendments could change the accumulation levels of functional lipids and other phytochemicals in the cultivated crops (Vidal et al., 2018). Since there is limited information present on changes in the functional lipid profile of crops upon the addition of wood ash, sludge, and biochar, we hypothesized that the use of those different media amendments under controlled environmental conditions to produce plants could add values to the waste from paper mills while enhancing formulations may enhance the functional lipids content of the crop of kale grown under controlled environmental conditions. This research aims to study the effect of amendments on the functional lipids of kale grown under controlled environmental conditions.

Kale was used as the crop in this study due to its short biological cycle, consumer demand, and perceived health benefits, where the reports by Ayaz et al. (2006) state that kale is an excellent dietary source of $\omega 3$ fatty acids. Two different soil types with different pH values were used in this study as the agriculture is carried out on lands converted from forested podzols in boreal regions of Newfoundland and Labrador (NL) (Canadian Agricultural Services Coordinating Committee. Soil Classification Working Group. National Research Council Canada, 1998). The lipids profile of the kale produced from 2 types of soils, 1 control condition and 8 types of amendments were then analyzed to investigate the impact of using amendments from paper mill by-products.

3.2 Materials and Methods

For the detailed experimental design, treatment and growing greenhouse conditions, and different media amendments, please refer to section **2.2.1** in **Chapter 2**.

3.2.1 Membrane lipid extraction and lipidomic analysis

3.2.1.1 Chemicals

The HPLC grade acetonitrile, chloroform, and methanol (Fisher Scientific (Mississauga, ON, Canada); deionized water (Thermo Scientific, ON, Canada); HPLC grade acetic acid, formic acid, ammonium formate, and ammonium acetate (Sigma-Aldrich (Oakville, ON, Canada) were used.

3.2.1.2 Sample preparation and lipid extraction

All harvested kale samples were oven-dried at 65°C for three consecutive days until a constant weight was achieved (Kim et al., 2017). Dried samples of each treatment were pulverized separately using a Cryomil (Reitch, Bonn, Germany), and ground samples were stored at room temperature for further nutritional analysis.

The lipid extraction was carried out as per the method stated by Matyash et al. (2008). Briefly, 100 mg of dried kale samples grown on separate amendments were mixed with MTBE/ methanol (10:3; v/v), 4 replicates from each treatment were placed in separate glass centrifuge tubes and vortexed. Then all samples were kept in a continuous shaker and incubated for 1 h at room temperature. After the incubation, 0.2 volume equivalents (compared to the total volume of MTBE/methanol) water was added to the final volumetric ratio of 10:3:2.5 (MTBE, methanol, water). After thoroughly mixing the samples, the suspension was centrifuged at 1000 x g for 10 min, and the upper phase containing lipids was transferred into a collection vial which was directly analyzed by analyzed UHPLC-C30-RPLC.

3.2.1.3 Analysis of kale intact functional lipids using UHPLC-C30RP-HESI-HRMS/MS

The functional lipids classes of kale, as well as their molecular species, were separated through ultra-high performance liquid chromatography coupled to C30 reverse phase chromatography and heated electrospray ionization high-resolution tandem mass spectrometry (UHPLC-C30RP-HESI- HRMS/MS). Lipids in dried kale leaves were separated on an Accucore C30 reverse phase column (150 mm × 2 mm I.D., particle size: 2.6 µm, pore diameter: 150 Å). The mobile phase system was prepared using solvent A and solvent B consisting of acetonitrile: H_2O (40:60 v/v) and Isopropanol: acetonitrile: H_2O (90:10:1, v/v/v), respectively, and both solvent A and B were added 0.1% formic acid and 10 mM ammonium formate.

The separation was carried out at 30°C (column temperature) with a flow rate of 0.2 $mLmin^{-1}$, and in each run, 10 µL of the samples were suspended in MTBE; methanol and water were injected into the machine. The following system gradient for lipid classes and molecular species separation was employed according to the methods stated at (Pham et al., 2019). Briefly, 30% solvent B was first applied for 3 min; then solvent B increased to 43% over 5 min. Next, the gradient was increased in 1 min to 50% B, then to 90% B over 9 min followed by an increase to 99% B over 8 min, and finally kept at 99% B for 4 min. Before each new injection, the column was re-equilibrated to 70% solvent A (initial conditions) for 5 min. A Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an automated Dionex UltiMate 3000 UHPLC system controlled by both X-Calibur 4.0 and Chromeleon software, respectively was used for lipid analyses in this study.

The parameters for the Q-Exactive Orbitrap mass spectrometer were set as follows: sheath gas: 40, auxiliary gas: 2, ion spray voltage: 3.2 kV, capillary temperature: 300 °C; S-lens RF: 30 V; mass range: 200–2000 m/z; full scan mode at a resolution of 70,000 m/z; top-20data dependent MS/MS at a resolution of 35,000 m/z and collision energy set at 35 (arbitrary unit); injection time: 50 min; isolation window: 1 m/z; automatic gain control target: 1e5. Both ESI negative and positive calibration solutions were used for the instrument's external calibration to 1 ppm.

3.2.1.4 Data processing (UHPLC-C30RP-HESI-HRMS/MS).

Data were acquired and processed using X-Calibur 4.0. (ThermoScientific, MO, USA) and LipidSearch version 4.1 (Mitsui Knowledge Industry, Tokyo, Japan) software packages. For the identification and semi-quantification of the lipid classes and their molecular species present in the complex lipid samples, LipidSearch 4.2 software was used. The parameters used for identification were as follows: Target database: Q-Exactive; precursor tolerance: 5 ppm; product tolerance: 5 ppm; product ion threshold: 5%; m-score threshold: 2; Quan m/z tolerance: ±5 ppm; Quan RT (retention time) range: ± 1 min; use of all isomer filter and ID quality filters A, B, and C; Adduct ions: $[M + H]^+$ and $[M + NH4]^+$ for positive ion mode, and $[M\text{-}H]^-$ and [M + HCOO]−, for negative ion mode.

Following identification, the observed lipid classes and molecular species were aligned and merged using the following alignment parameters: search type: product; experiment type: LC-MS; alignment method: mean; RT tolerance: 0.15; calculate unassigned peak area: on; filter type: new filter; top rank filter: on; main node filter: all isomer peaks, M-score threshold: 5; ID quality filter: Fatty acyl chains position identification present in the molecular species found in the lipid classes of each sample was based on the fragmentation patterns of the MS/MS spectra, and manually confirmed using X-Calibur 4.0 according to the rules established for tandem mass spectrometry (Pham et al., 2019).

The functional lipid classes selected from the search were as follows:

Glycolipids of MGMG, MGDG, DGMG and SQDG; Phospholipids of phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG);Neutral lipids including DG, TG, phytosterols (Sts) consisting of β-sitosterol (SiE), stigmasterol ester (StE), campesterol ester (CmE), acylated hexosyl stigmasterol ester (AcHexStE), acylated hexosyl betasitosterol ester (AcHexSiE) and acylated hexosyl campesterol ester (AcHexCmE). All identified lipid classes are presented in nmol% values and for each lipid class, the data were re-categorized based on ω3 fatty acids containing, ω6 fatty acids containing, Saturated Lipids, Monounsaturated Lipids, Polyunsaturated lipids, Ratio of ω6:ω3, LCTs, SCTs(Short chain TGs), MCTs, 1,2-DGs and 1,3-DGs for Glycolipids, PLs, DGs, TGs, and Sts separately (Molecular level data are not shown).

3.2.2 Statistical analysis and data presentation

All the data for soil type 1 and type 2 were analyzed by the multivariate approach of Partial Least Squares Discriminant Analysis (PLS-DA) followed by a Heatmap where the functional lipids that were accountable for the difference in the media amendments were selected based on VIP ranking >1. To determine the effect of natural media amendment on the functional lipids, one-way ANOVA was carried out for each main cluster. Fisher's Least Significant Difference (LSD) test at $\alpha = 0.05$ was used in determining the significance of mean differences for each nutritional parameter and significant parameters ($p<0.05$) were presented in Demšar plots (Demšar, 2006). All the statistical analyses were performed using XLSTAT (Addinsoft, Paris, France) statistical software.

3.2.3 Pathway Analysis

MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml) (Chong et al., 2019) and the following parameters were set. Data normalization by None; Data transformation by log transformation; Data scaling by Pareto scaling; Pathway library: Arabidopsis thaliana (thale cress) in Kyoto Encyclopedia of Genes and Genomes database (KEGG). Pathways with impact values > 0 and –log (p) > 1.3 ($p< 0.05$) were considered the most significant pathways for the modulation of functional lipids.

3.3 Results

This section explains the results for both soils separately based on the parameters showing a significant difference (Table 3.1 and Table 3.2 in supplementary). For each type of soil, one control (CTL) and seven treatments using amendments of CBC (control + biochar), WA (wood ash), WBC (wood ash + biochar), SL (sludge), SBC (sludge + biochar), WASL (wood ash + sludge), and WSBC (wood ash+ sludge +biochar) amendments were investigated.

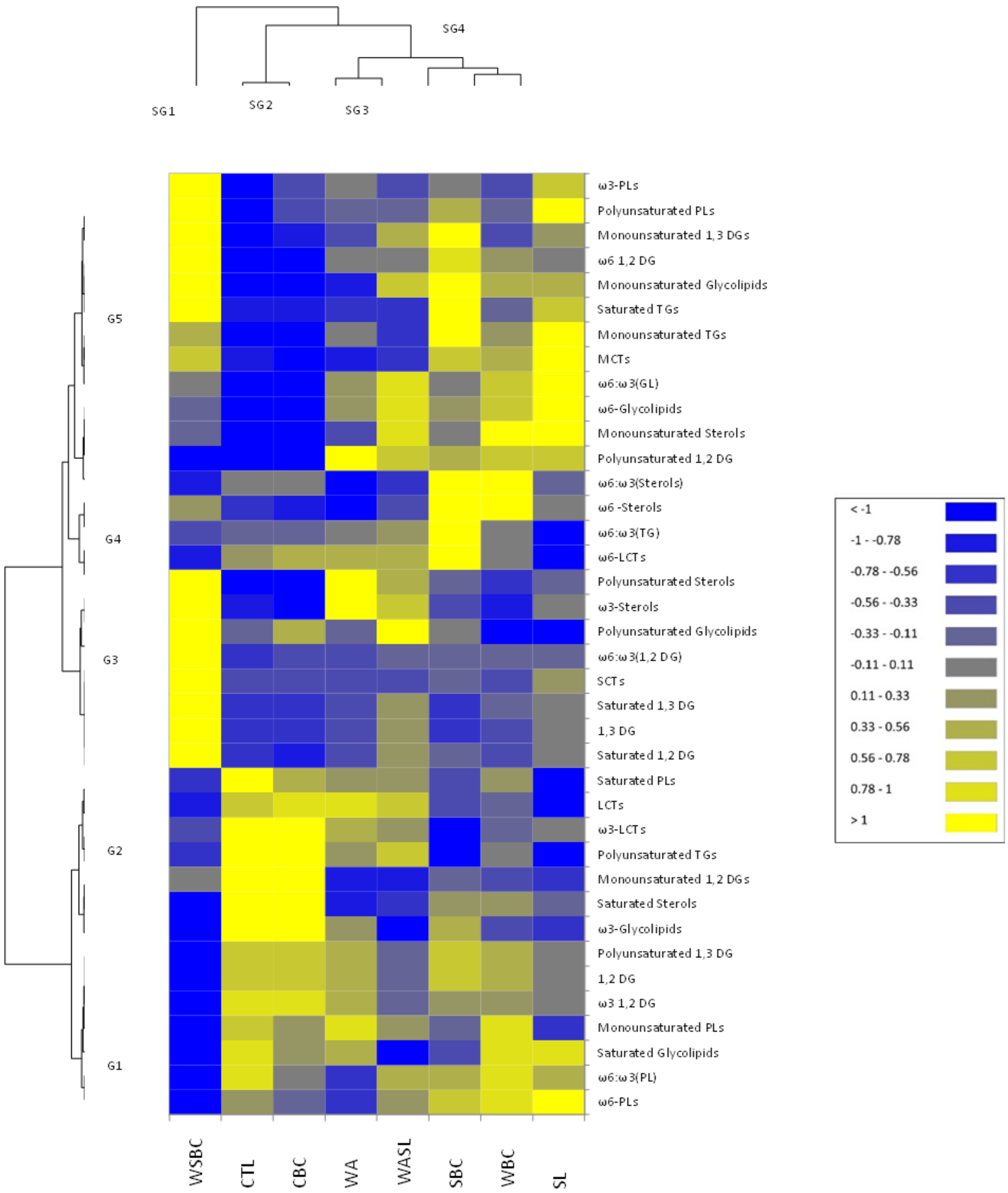
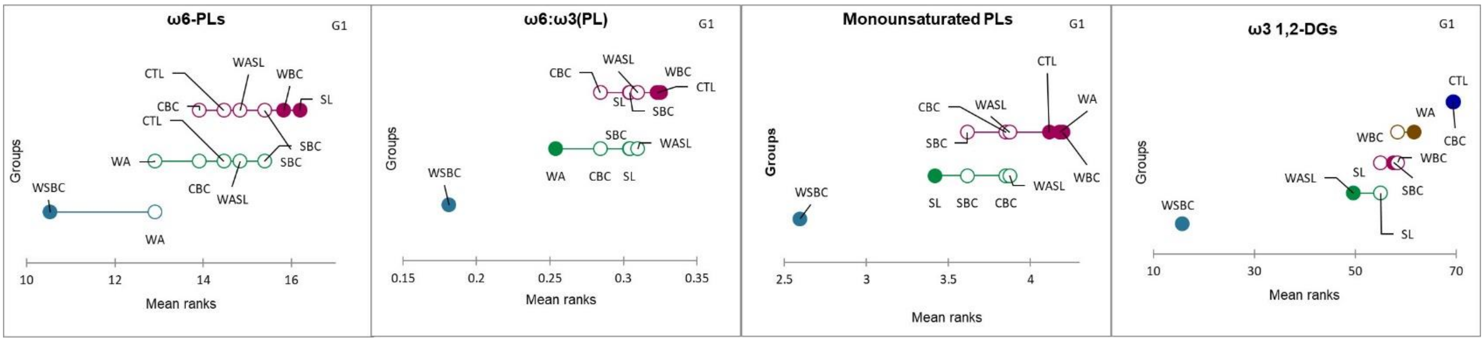
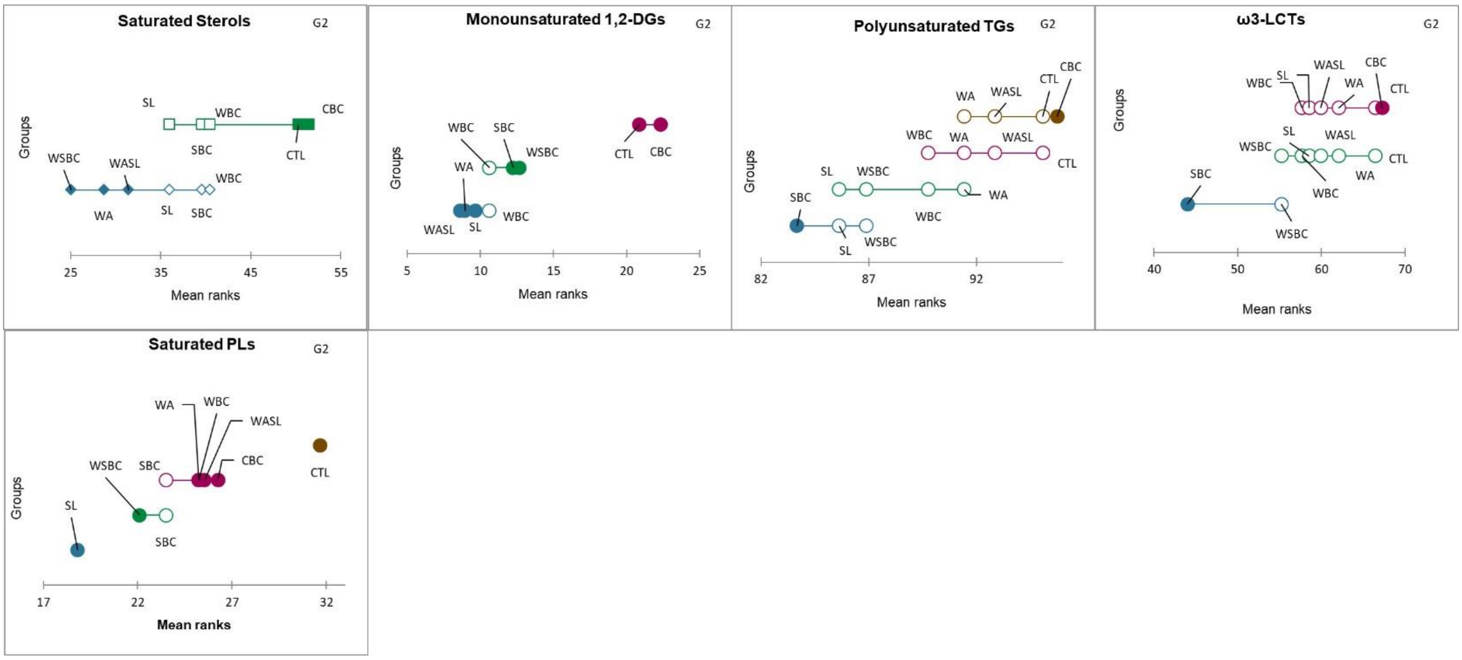


Figure 3.1. The heatmap for lipid classes of kale grown with media amendments for soil type 1. A colour scale of blue to yellow is being used with colour calibration ranging from -1 to +1 respectively. CTL = control, CBC = control + biochar, WA = wood ash, WBC = wood ash + biochar, SL = sludge, SBC = sludge + biochar, WASL = wood ash + sludge, and WSBC = wood ash + sludge + biochar are amendments.

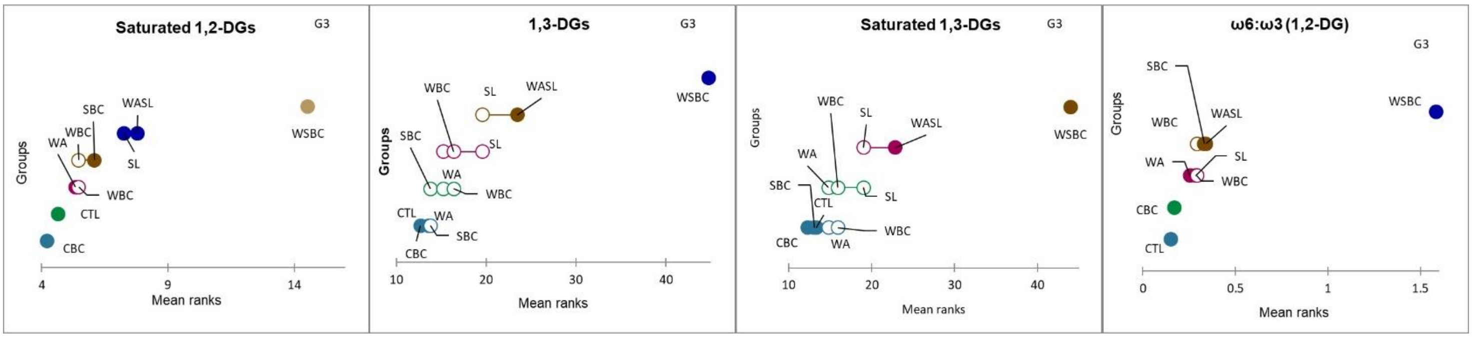
a


ω6-PLs
G1
CTL
WASL
WBC
SL
CBC
SBC
WA
WSBC
Groups
10
12
14
16
Mean ranks
ω6:ω3(PL)
WSBC
0.15
0.2
0.25
0.3
0.35
Monounsaturated PLs
2.5
3
3.5
4
ω3 1,2-DGs
30
50
70

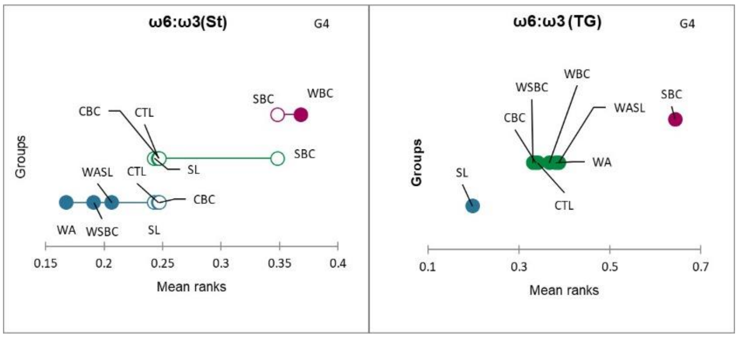
b


Saturated Sterols
G2
SL
WBC
CBC
SBC
CTL
WSBC
WASL
WA
25
35
45
55
Mean ranks
Groups
Monounsaturated 1,2-DGs
5
10
15
20
Polyunsaturated TGs
82
87
92
ω3-LCTs
40
50
60
70
Saturated PLs
17
22
27
32

c


Saturated 1,2-DGs
G3
4
9
14
Mean ranks
Groups
1,3-DGs
10
20
30
40
Saturated 1,3-DGs
ω6:ω3 (1,2-DG)
0
0.5
1
1.5

d



e

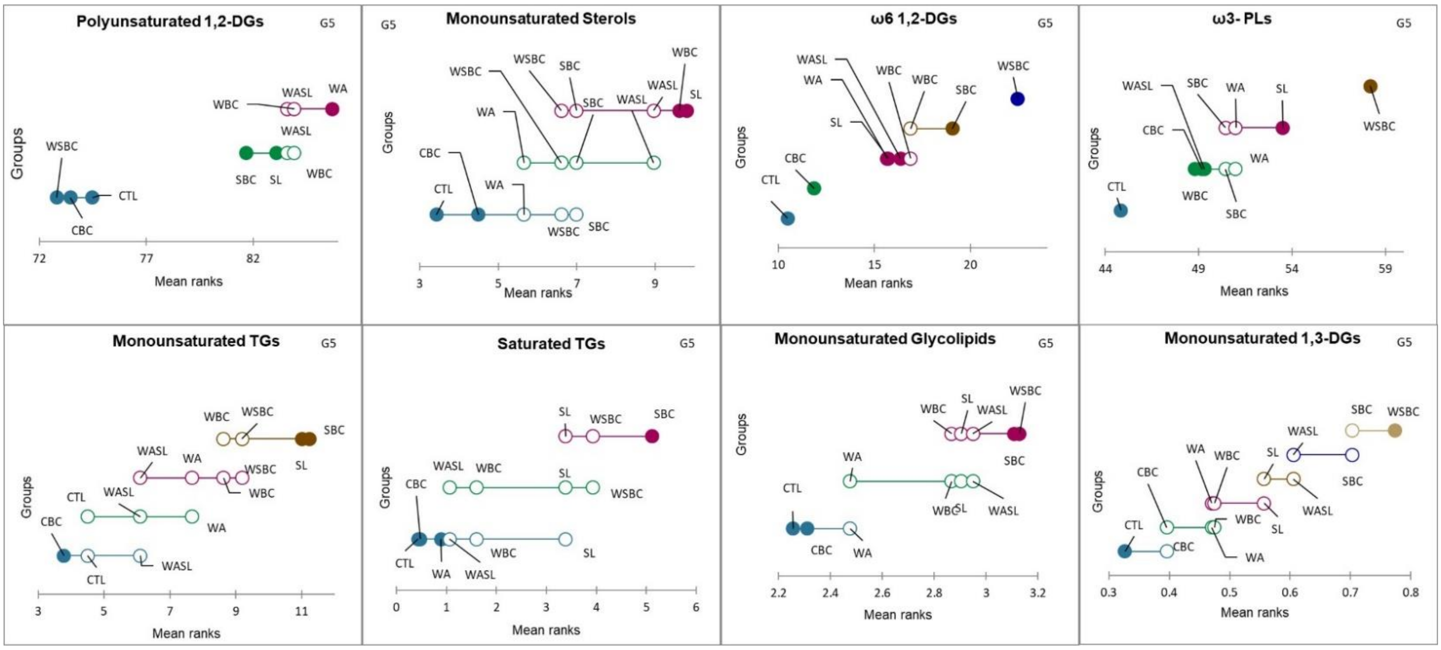


Figure 3.2. Demsar plots of a, b, c, d and e for G1, G2, G3, G4 and G5 clusters of heat map of lipid classes in kale grown with media amendments for soil type 1 respectively. Only the significant ones($p<0.05$) are shown. Groups of amendments that are significantly different ($p <0.5$) are not connected by dots. All other parameters are in nmol%. CTL = control, CBC = control + biochar, WA = wood ash, WBC = wood ash + biochar, SL = sludge, SBC = sludge + biochar, WASL = wood ash + sludge, and WSBC = wood ash + sludge + biochar are amendments.

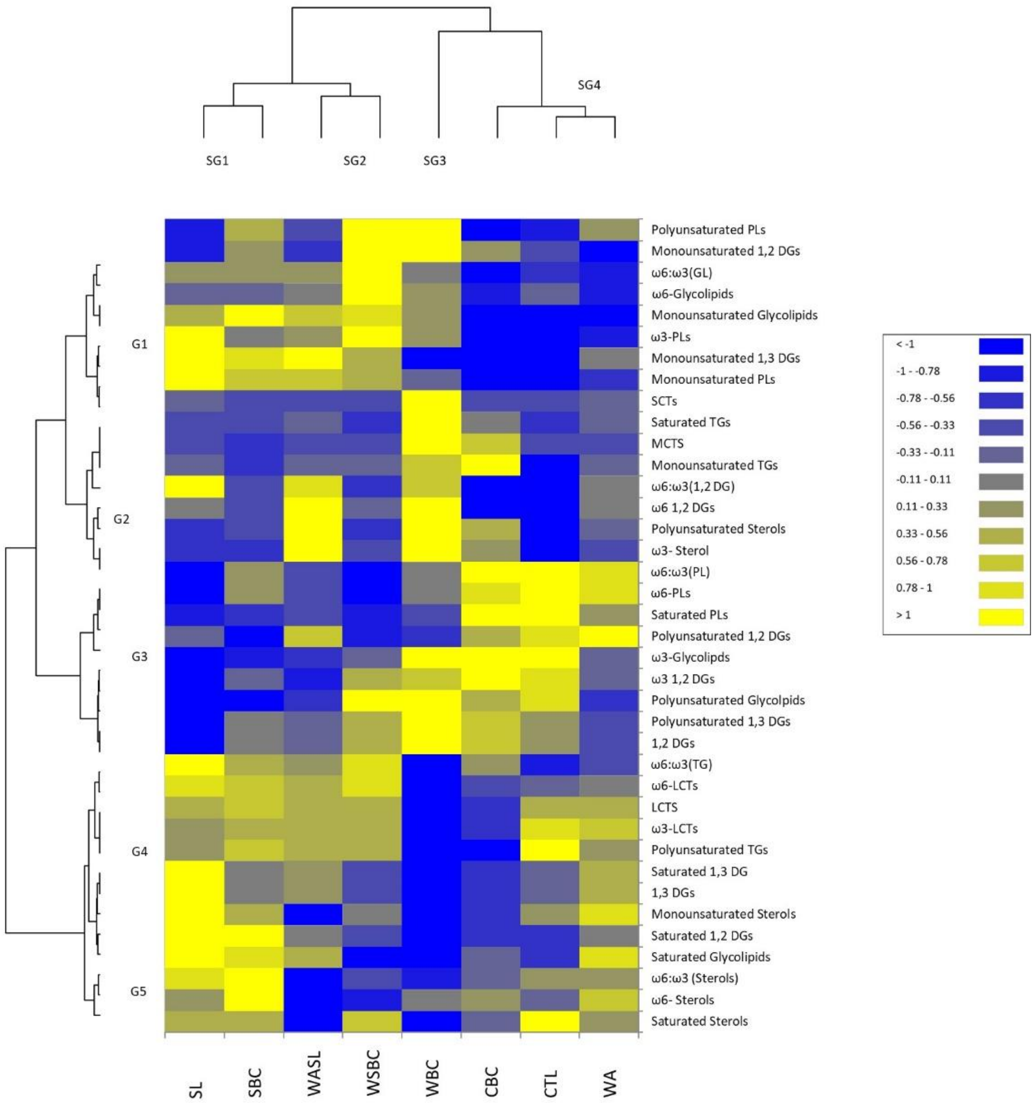
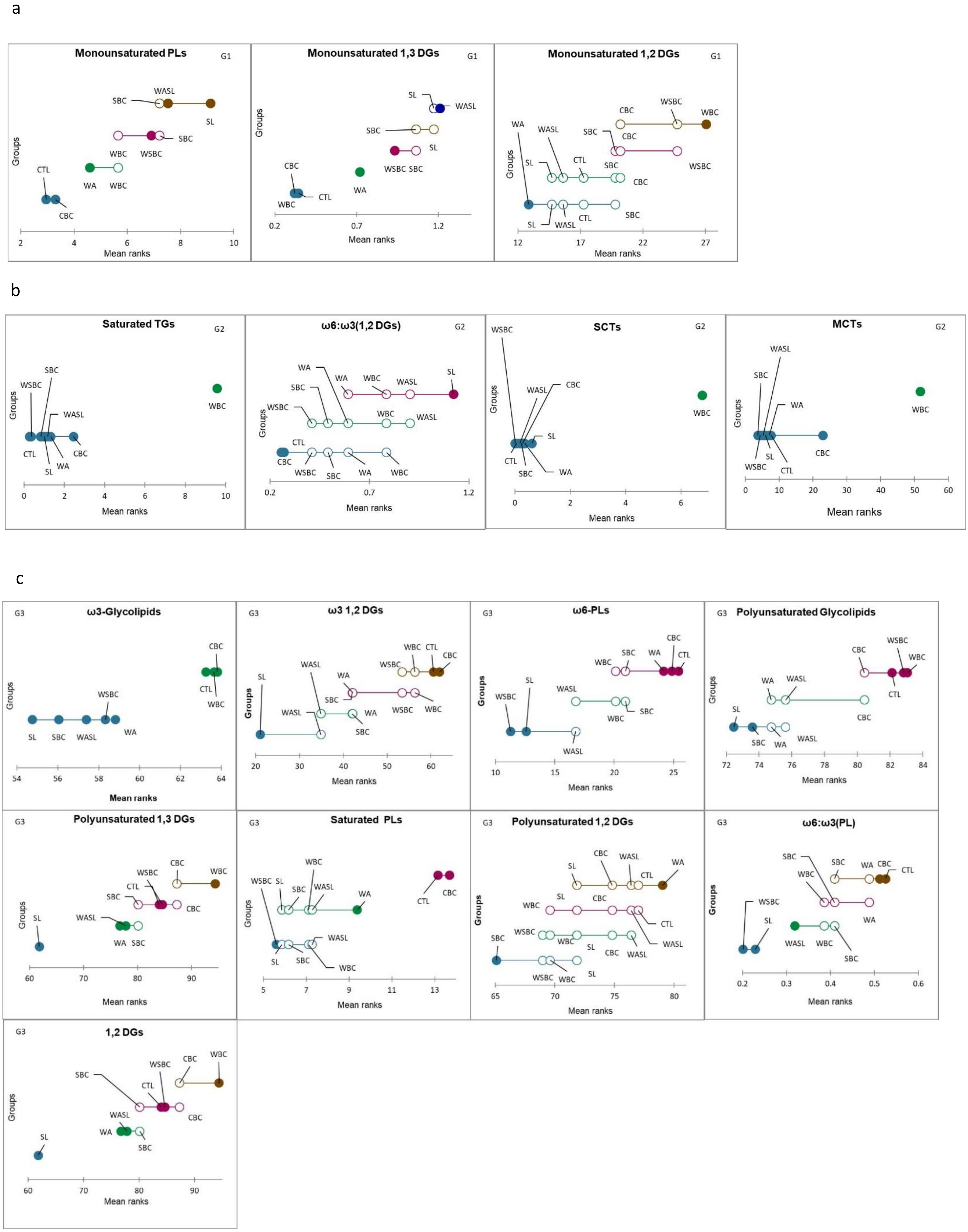


Figure 3.3. The heatmap for lipid classes of kale grown with media amendments for soil type 2. A colour scale of blue to yellow is being used with colour calibration ranging from -1 to +1 respectively. CTL = control, CBC = control + biochar, WA = wood ash, WBC = wood ash + biochar, SL = sludge, SBC = sludge + biochar, WASL = wood ash + sludge, and WSBC = wood ash + sludge + biochar are amendments.


a
Monounsaturated PLs
G1
Groups
Mean ranks
Monounsaturated 1,3 DGs
G1
Groups
Mean ranks
Monounsaturated 1,2 DGs
G1
Groups
Mean ranks
b
Saturated TGs
G2
Groups
Mean ranks
ω6:ω3(1,2 DGs)
G2
Groups
Mean ranks
SCTs
G2
Groups
Mean ranks
MCTs
G2
Groups
Mean ranks
c
G3
ω3-Glycolipids
Groups
Mean ranks
G3
ω3 1,2 DGs
Groups
Mean ranks
G3
ω6-PLs
Groups
Mean ranks
G3
Polyunsaturated Glycolipids
Groups
Mean ranks
G3
Polyunsaturated 1,3 DGs
Groups
Mean ranks
G3
Saturated PLs
Groups
Mean ranks
G3
Polyunsaturated 1,2 DGs
Groups
Mean ranks
G3
ω6:ω3(PL)
Groups
Mean ranks
G3
1,2 DGs
Groups
Mean ranks
CTL
CBC
SBC
WBC
WSBC
WA
SL
WASL

d

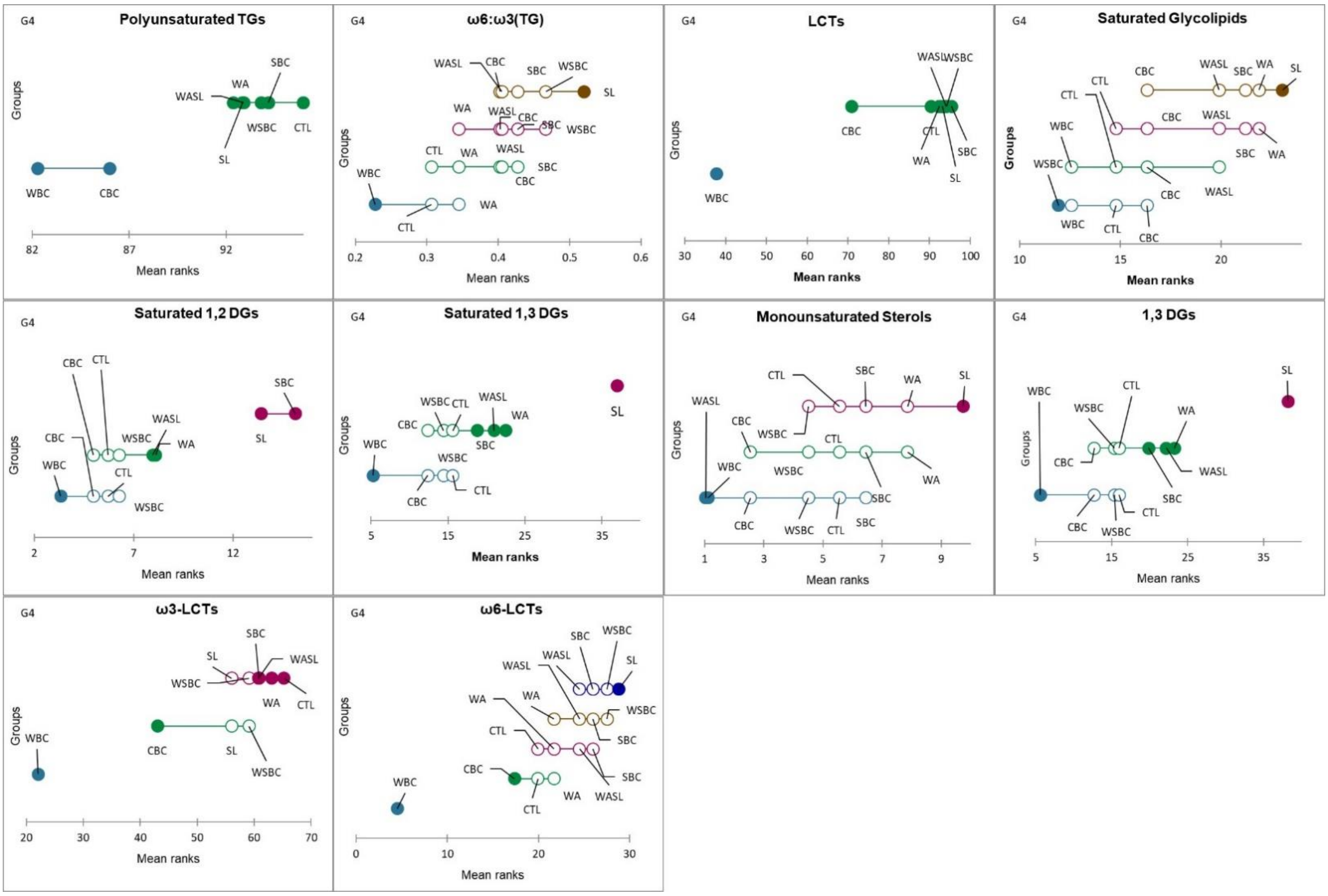


Figure 3.4. Demsar plots of a, b, c, and d for G1, G2, G3, G4 and G5 clusters of heat map of lipid classes in kale grown with media amendments for soil type 2 respectively. Only the significant ones(p<0.05) are shown. Groups of amendments that are significantly different (p <0.5) are not connected by dots. All other parameters are in nmol%. CTL = control, CBC = control + biochar, WA = wood ash, WBC = wood ash + biochar, SL = sludge, SBC = sludge + biochar, WASL = wood ash + sludge, and WSBC = wood ash + sludge +biochar are amendments.

3.3.1 Effect of media amendments on modulation of functional lipids of kale grown in soil type1

In the heatmap generated for soil type 1, the functional lipid parameters have been clustered into five main groups (G1, G2, G3, G4, and G5), while the amendments are clustered into four main subgroups (SG1, SG2, SG3, and SG4) (Figure 3.1). Considering the G1 cluster (Figure 3.2 a), the parameters of ω6-PLs, monounsaturated PLs, ω6: ω3(PL), and ω6 1,2-DGs were shown to be affected upon the addition of natural media amendments ($p<0.05$). In the G1cluster, when WSBC media amendment was added, there was a significant reduction in functional lipids. Considering the parameters of ω6-PLs and monounsaturated PLs, the highest values of 16.18± 1.02 nmol% and 4.19 ± 0.10 nmol% were manifested upon the addition of SL and WBC amendments, respectively. The overall highest amount of functional lipid was shown from the parameter of ω6 1,2 (69.47 ± 1.07 nmol%) during the usage of CTL amendment.

Considering the G2 cluster (Figure 3.2 b), the parameters of saturated sterols, monounsaturated 1,2-DGs, polyunsaturated TG's, ω3-LCTs, and saturated PLs were shown to be significantly affected by the media amendments (Except for the parameter of saturated PLs, all other parameters show a significantly high amount of functional lipids when CBC amendment is applied. Considering the saturated sterols and saturated PLs, the highest amounts of 51.05 ± 2.47 nmol% and 31.68 ± 0.66 nmol% were shown when CBC and CTL were added, respectively. Parameters of polyunsaturated TGs and ω3-LCTs show a similar trend where the highest values of 95.74 ± 0.52 nmol% and 67.25 ± 0.66 nmol% upon the addition of CBC respectively, while the lowest values of 83.65 ± 5.44 nmol% and 44.01 ± 6.93 nmol% were shown for SBC amendment respectively.

In cluster G3 (Figure 3.2 c), the parameters of saturated 1,2-DGs, 1,3-DGs, ω6: ω3 (1,2-DG), and Saturated 1,3-DGs were shown to be significantly affected by the usage of amendments ($p<0.05$). This cluster showed the highest amount of functional lipids when the WSBC amendment was used. For instance, in the parameters of saturated 1,2-DGs and 1,3-DGs the highest values of 14.52 ± 0.56 nmol% and 44.74 ± 1.95 nmol% were shown upon the addition of WSBC. In both parameters, it was also shown that the WSBC amendment was significantly different from all amendments ($p<0.05$). Considering the parameter of ω6: ω3 (1,2-DG), it followed the same trend where the highest (1.58 ± 0.39) monounsaturated lipids were shown from 1,2 DGs (27.06 ± 5.21 nmol%) upon using of WBC. The highest amount of monounsaturated PLs and 1,3 DGs were shown at SL and WASL amendment with values of 9.12 ± 0.89 nmol% and 1.21 ± 0.01 nmol%, respectively. The lowest (0.15± 0.01 nmol%) was shown upon the addition of CTL.

In cluster G4 (Figure 3.2 d), only two parameters of ω6: ω3-TG and ω6: ω3-sterol were shown to be significantly affected by different media amendments ($p<0.05$). There was a significant increase in the ω6: ω3-Sterol parameter when WBC amendment was used (0.36 ± 0.02) while the lowest was shown (0.16 ± 0.06) upon the addition of WA. The parameter of ω6: ω3-TG was shown the highest value of 0.64 ± 0.13 upon the addition of SL when SBC amendment was used where there was no significant difference between SBC and SL amendments.

The parameters of polyunsaturated 1,2-DGs, monounsaturated sterols, monounsaturated TGs, saturated TGs, monounsaturated glycolipids, ω6 1,2-DGs, monounsaturated 1,3-DGs, and ω3-PLs were shown significantly affected by the media amendments in G5 cluster (Figure 3.2 e) ($p<0.05$). The parameters of monounsaturated 1,3-DGs, ω6 1,2-DGs, ω3-PLs, and monounsaturated glycolipids follow the same trend where the highest values were shown upon the addition of WSBC amendment. In contrast, the lowest values were shown when CTL was used. For example, the parameter of ω3-PLs manifested the highest amount with a value of

58.20 ± 1.02 nmol% when WSBC was added, where there was no significant difference compared to all other amendments ($p<0.05$). The parameters of monounsaturated TGs and saturated TGs highest values of 11.22 ±3.39 nmol% and 5.11 ± 2.24 nmol% were shown for SBC amendment, respectively. The highest functional lipid amount of 85.71 ± 0.07 nmol% was shown for the parameter of polyunsaturated 1,2-DGs upon the addition of WA amendment is significantly different from SL ($p<0.05$). A similar trend can be seen on polyunsaturated glycolipids and 1,2 DGs except for polyunsaturated 1,2 DGs where the highest and lowest values of 79.03 ± 1.74 nmol% per DW; 65.08 ± 2.95 nmol% per DW were shown for WA and SBC amendments, respectively.

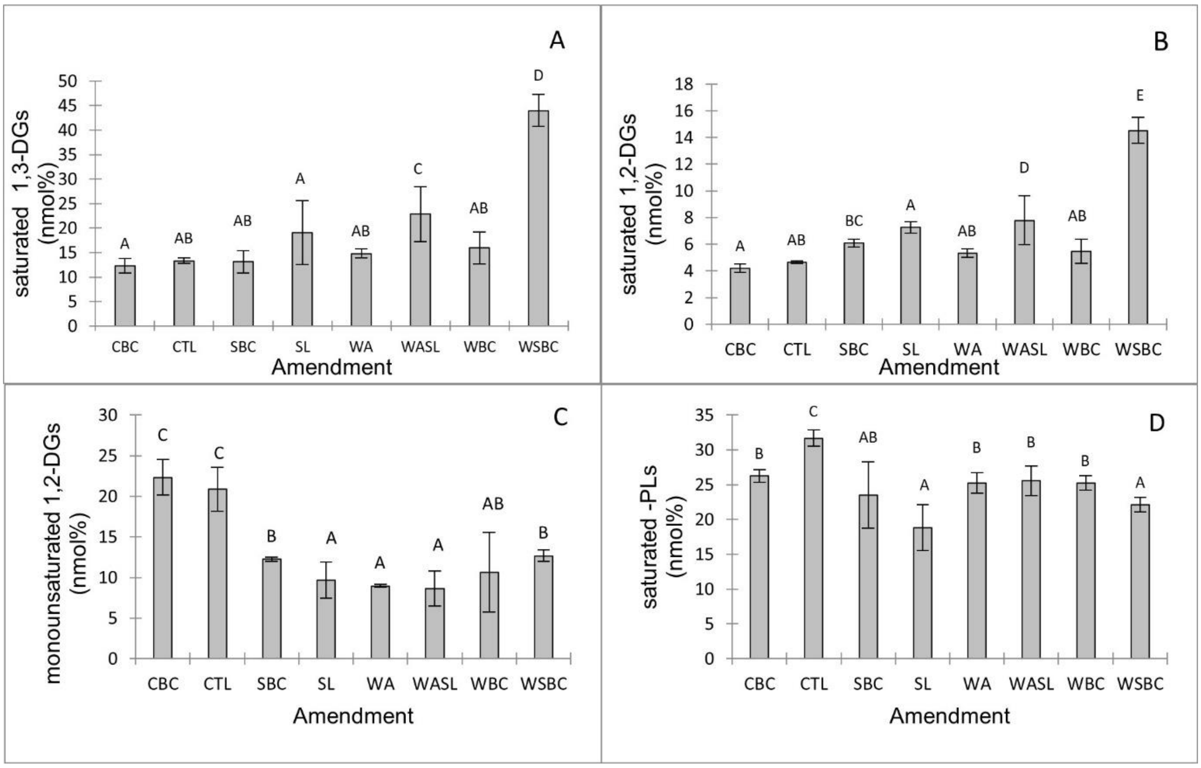


Figure 3.5. The natural media amendment effect on SFA-DG-1,3(A), SFA-DG-1,2(B), MUFA DG-1,2(C), and SFA-PL(D) in soil type 1. Each vertical bar represents the average of replicates ± SE (n=3). Different letters indicate significant differences among treatments at $p \leq 0.05$ according to Fisher's Least Significant test. CTL = control, CBC = control + biochar, WA = wood ash, WBC = wood ash + biochar, SL = sludge, SBC = sludge + biochar, WASL = wood ash + sludge, and WSBC = wood ash + sludge + biochar are amendments.

3.3.2 Effect of media amendments on modulation of functional lipids of Kale grown in soil type 2

The heatmap clusters the functional lipid classes into five main groups (G1, G2, G3, G4, and G5), while the media amendments have been clustered into four main groups (SG1, SG2, SG3, and SG4) (Figure 3.3). The parameters of monounsaturated PLs, monounsaturated 1,3-DGs, and monounsaturated 1,2-DGs were shown to be significantly affected among amendments for G1 ($p<0.05$). They are presented in Demsar plots; for each parameter, Demsar plot shows the grouping of media amendment based on LSD mean values. The amendments that do not show significant differences are connected in dots and lines. In this cluster (Figure 3.4 a), the highest amount of monounsaturated lipids was shown from 1,2-DGs (27.06 ± 5.21 nmol%) upon using WBC. The highest amount of monounsaturated PLs and 1,3 DGs were shown at SL and WASL amendment with values of 9.12 ± 0.89 nmol% and 1.21 ± 0.01 nmol%, respectively.

Considering the G2 cluster (Figure 3.4 b), the parameters of Saturated TGs, ω6: ω3(1,2-DG), SCTs, and MCTs were shown to be affected by the usage of natural media amendments ($p<0.05$). The parameters of saturated TGs and SCTs follow a similar trend where the highest was reported when WBC amendment was added with values of 9.58 ± 0.74 nmol% and 6.75± 2.27 nmol%, respectively. It can also be seen that in both cases, the WBC amendment is significantly different from other amendments ($p<0.05$). The parameter of MCTs does follow the same trend as above, where the highest amount (51.74 ± 4.63 nmol% was shown upon the addition of WBC treatment while the lowest was manifested on SBC usage (3.69 ± 0.70 nmol% Considering the parameter of ω6: ω3(1,2-DG) the highest value of 1.12 ± 0.03 was shown upon the addition of SL amendment, and there was no significant difference shown at WASL, WBC, and WA amendments ($p>0.05$).

The parameters of ω3-glycolipids, ω3-1,2 DG, ω6-PLs, ω6: ω3(PL), 1,2-DGs, saturated PLs, and polyunsaturated glycolipids, 1,3-DGs and 1,2-DGs have shown to be significantly affected by natural media amendments at G3 cluster (Figure 3.4 c). The parameters of ω3-glycolipids and ω3- 1,2-DG have shown a similar trend where the highest values of 63.80 ± 1.25 nmol% and 61.98 ± 3.94 nmol% were shown for CBC amendment, respectively. In both parameters, the least values of 54.76 ± 1.89 nmol% and 21.02 ± 1.97 nmol% were shown upon the addition of SL. Interestingly the variation of ω6-PLs was the highest (25.44 ± 1.97 nmol%) upon using CTL amendment. Considering the effect of media amendments on polyunsaturated lipids in this cluster, overall polyunsaturated 1,2-DGs had shown the highest value of 94.40 ± 1.97 nmol% upon adding WBC. In contrast, the lowest value was shown when SL was used (61.81 ± 3.31 nmol%). It can also be seen that the WBC amendment is significantly different from SL($p<0.05$). A similar trend can be seen on polyunsaturated glycolipids and 1,2-DGs except for polyunsaturated 1,2 DGs where the highest and lowest values of 79.03 ± 1.74 nmol%; 65.08 ± 2.95 nmol% were shown for WA and SBC amendments, respectively.

Considering the G4 cluster (Figure 3.4 d) in soil type 2, the parameters of ω3-LCTs, ω6-LCTs, polyunsaturated-TGs, ω6:ω3(TG), LCTs, saturated glycolipids, saturated 1,2-DGs, saturated 1,3-DGs, monounsaturated sterols and 1,3-DG have shown to be significantly affected by the changes of natural media amendments ($p<0.05$). In this cluster, the least amount of lipids was present upon the addition of WBC amendment except for the parameters of monounsaturated sterols and saturated glycolipids. Except for the parameters of ω3-LCTs, polyunsaturated TGs, saturated 1,2-DGs, and LCTs the highest amount of lipid content was shown at the usage of SL amendment where it was shown a significant difference compared to WBC amendment ($p<0.05$).

There is a similar trend in parameters of ω3-LCTs and polyunsaturated TGs where the highest values were shown upon CTL amendments usage with the values of 65.24 ± 2.20 nmol% and

95.97 ± 0.4 nmol% respectively. Considering the saturated significant lipids in the G4 cluster, it was shown that saturated 1,3-DGs were the highest (37.01 ± 3.34 nmol%) when SL amendment was used. Considering the ω6: ω3(TG) it follows the same trend where the highest value was shown upon the addition of SL (0.51 ± 0.06 nmol%) while the lowest was shown when WBC (0.22 ± 0.04 nmol%) amendment was used.

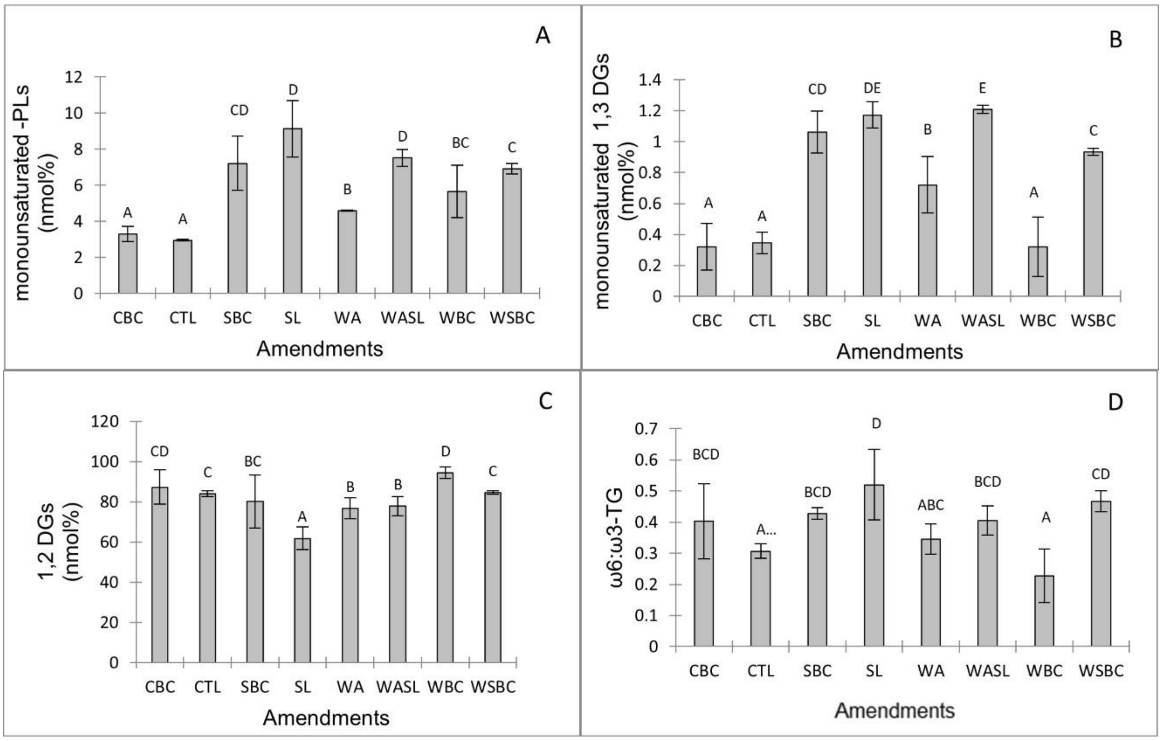


Figure 3.6. The natural media amendment effect on MUFA-PL(A), MUFA-DG-1,3(B), DG-1,2(C), and ω6:ω3-TG(D) in soil type 2. Each vertical bar represents the average of replicates ± SE (n=3). Different letters indicate significant differences among treatments at p≤0.05 according to Fisher's Least Significant test. CTL = control, CBC = control + biochar, WA = wood ash, WBC = wood ash + biochar, SL = sludge, SBC = sludge + biochar, WASL = wood ash + sludge, and WSBC = wood ash + sludge + biochar are amendments.

3.3.3 Pathway analysis of significant functional lipids in soil type 1 and soil type 2.

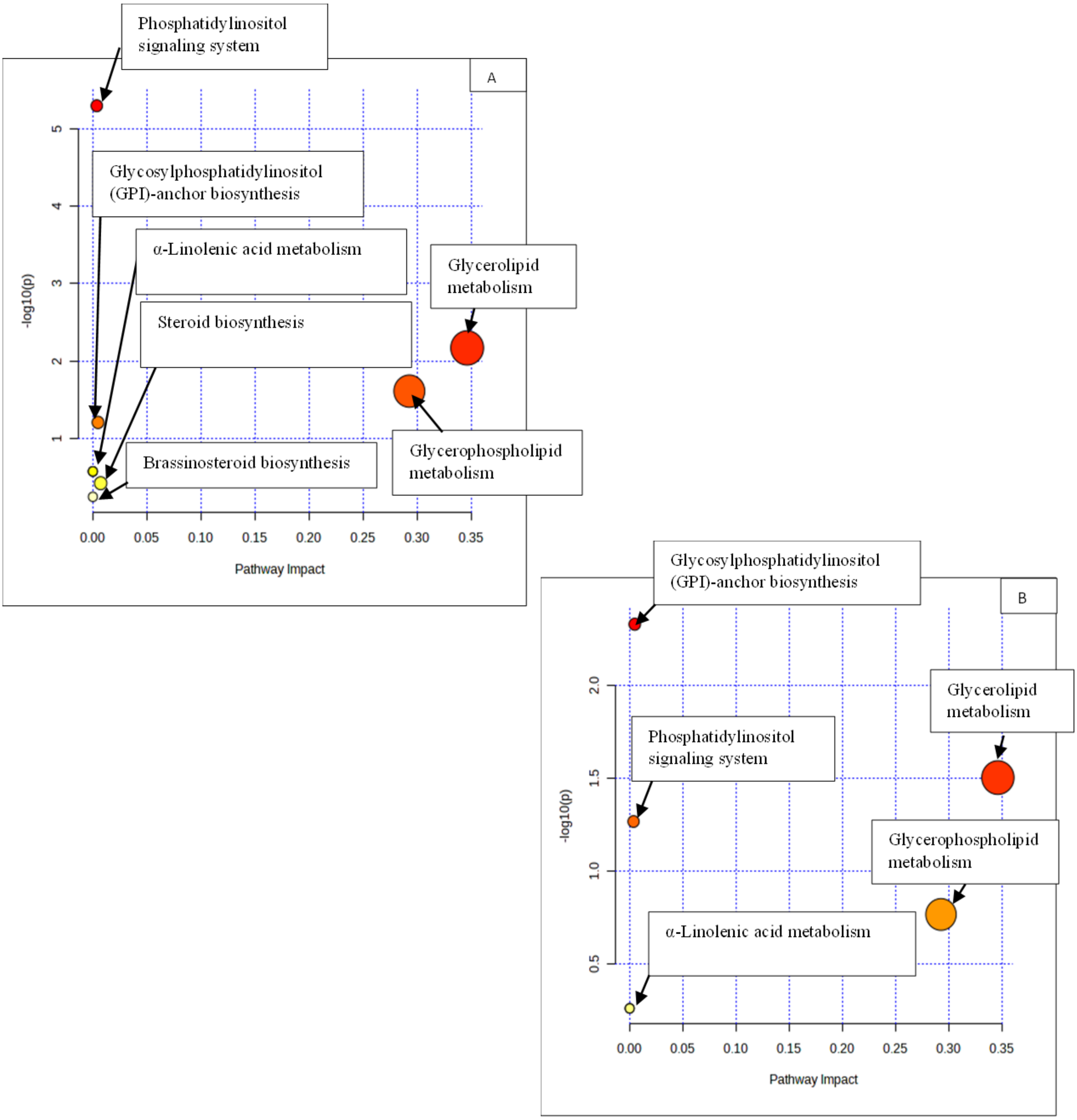


Figure 3.7. Overview of pathway analysis using Metaboanalyst 5.0. Pathway analysis showing significant functional lipids of soil 1 (A) and soil 2 (B). The x-axis represents the pathway impact, and the y-axis represents log10(p-value). Large sizes and dark colors represent major pathway enrichment and high pathway impact values, respectively.

Pathway analysis manifested significantly altered lipid metabolic pathways in each soil type when adding natural media amendments. Seven different pathways of Glycosylphosphatidlyinositol (GPI) anchor biosynthesis, phosphatidylinositol signalling, glycerophospholipid metabolism, arachidonic acid metabolism, α-linolenic acid metabolism, linoleic acid metabolism, phosphatidylinositol signalling system and, glycerolipid metabolism were found for both soil types (Figure 3.7). There was a significant difference in identified metabolic pathways ($p<0.05$ and Impact factor>0) of glycosylphosphatidylinositol (GPI)-anchor biosynthesis and glycerolipid metabolism for soil type 1 (Table 3.1). In contrast, for soil type 2 pathways of phosphatidylinositol signalling system, glycerolipid metabolism and glycerophospholipid metabolism were shown to be significantly altered upon the addition of amendments (Table 3.2).

Table 3.1. Different functional lipid pathways associated with soil type 1

Pathway Name	**p**	**-log(p)**	**Impact**
Phosphatidylinositol signaling system	5.1085E-6	5.2917	0.00362
Glycerolipid metabolism	0.0067399	2.1713	0.3461
Glycerophospholipid metabolism	0.024303	1.6143	0.29262
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	0.061514	1.211	0.00476
Arachidonic acid metabolism	0.2632	0.57971	0.0
Linoleic acid metabolism	0.2632	0.57971	0.0
α-Linolenic acid metabolism	0.2632	0.57971	0.0
Steroid biosynthesis	0.37358	0.42762	0.00721
Brassinosteroid biosynthesis	0.55939	0.25229	0.0

Table 3.2. Different functional lipid pathways associated with soil type 2

Pathway Name	p	-log(p)	Impact
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	0.0046893	2.3289	0.00476
Glycerolipid metabolism	0.031392	1.5032	0.3461
Phosphatidylinositol signaling system	0.054114	1.2667	0.00362
Arachidonic acid metabolism	0.54881	0.26058	0.0
Linoleic acid metabolism	0.54881	0.26058	0.0
α-Linolenic acid metabolism	0.54881	0.26058	0.0

3.4 Discussion

In this study, the monounsaturated lipids, polyunsaturated lipids (ω3s and ω6s), diglycerides, triglycerides, phospholipids, and plant sterols were shown to be modulated when natural media amendments were applied for both soil types. Monounsaturated lipids or monounsaturated fatty acids containing lipids have been identified as beneficial due to the potential benefit in the primary and secondary prevention of cardiovascular diseases (Schwingshackl & Hoffmann, 2014). Oleic acid (FA 18:1) is the most abundant fatty acid in plants (Kehelpannala et al., 2021). In plant immunity, it acts as a signalling agent and involves in the crosstalk between salicylic acid and jasmonic acid signalling pathways during pathogen infection (Kachroo et al., 2001). Li-Beisson et al. (2010) report that oleic acid is also a common component of membrane glycerolipids, including PC, PE, PI, PG, MGDG, DGDG, and SQDG. Interestingly in this study, the highest amount of monounsaturated lipids was shown from 1,2-DGs when CBC (22.33 ± 1.27 nmol%) and WBC (27.06 ± 5.21 nmol%) amendments were used for soil type 1 and soil type 2, respectively.

A study by Vidal et al. (2018) where the effect of potassium humate, dry vermicast, and volcanic minerals on the functional lipid profile of kale has studied states that all the above media amendments helped in increasing the oleic acid content in kale. Authors also report that such an increment of monounsaturated fatty acid (MUFA) may be due to a significant delta 9 desaturase enzyme increase. A similar study by Abbey et al. (2018) reports that natural media containing higher amounts of N content and electric conductivity (EC) appeared to be stimulating the production of monounsaturated and ω3 fatty acids. Those findings confirm the current study for soil type 2 where it can be seen higher total nitrogen amounts are present in the initial WA stocks (Table 2:1), and the analysis report of soil type 2 has shown a high EC value too (Data not shown).

A similar study by Sharma et al. (2011) found that using poultry manure and sugarcane mud as amendments to grow rape grains significantly increased the oleic and eurecic acid (FA 22:1) compared to the control agreeing with the current study. It was also seen that amendments with biochar were prominent in modulating the monounsaturated lipids content for both soil types. Lu et al. (2020) state that biochar enhanced the protection of the plant- or microbe-derived molecules while a study by Chen et al. (2021) states that the usage of rice biochar contributed to Organic matter stability and diversity enhancement in soil.

It is well established that SFA /SFA-containing lipids increase the low-density lipoprotein (LDL) cholesterol, a major risk in developing cardiovascular diseases (Mensink, 2016). In guideline on lifestyle management to reduce cardiovascular risk by the American heart association and American College of Cardiology (AHA/ACC) reports strong evidence (level A) for reducing SFA intake by 5% ~ 6% of calories. In this study for soil type 1, the highest amount of SFA-containing lipids was observed from plant sterol in kale in CBC amendment. However, there was no significant difference between CBC and CTL amendments. Overall, it can be seen that upon adding media amendments, there was a significant reduction in SFA-containing lipids (figure 3.2). This could be a promising approach to decreasing the accumulation of SFA-containing lipids in Kale. In soil type 2, except for saturated TGs, all other saturated lipids were enhanced when SL amendment was used. In contrast, overall, the lowest amount of SFA lipids was shown when WBC amendment was used. It was also shown that there was no significant difference between WBC, CTL, WASL, and WSBC amendments.

A study by Sharma et al. (2011) also found a similar effect where there was no significant difference in both palmiticpalmitic acid (16:0) and stearicstearic acid (18:0) contents in rape seeds grown in a seleniferous region when organic amendments were used. In our study for both soil types, polyunsaturated lipids, especially those containing ω3 and ω6 fatty acids, modulated significantly ($p<0.05$) when media amendments were used.

ω3 and ω6 fatty acids are essential to humans and must be obtained from different dietary sources (Dörmann & Benning, 2002). Kale is considered one of the vegetables rich in ω3 fatty acids, where Total unsaturated fatty acids reported in dry kale leaves as 129 $\mu g\ g^{-1}$ (Ayaz et al., 2006).

The authors also state that among unsaturated fatty acids, the highest value of 85.3 $\mu g\ g^{-1}$was shown from FA 18:3 (α-linolenic Acid). The findings of this work demonstrated that for soil type 1, the highest amount of ω3 was from long-chain triglycerides in CBC (67.2 5± 5.21 nmol%) where there was no significant difference ($p<0.05$) among media amendments except for SBC. A similar trend was seen from soil type 2 where the highest amount of ω3 lipids (65.24 ± 2.20 nmol%) was demonstrated from LCTs upon CTL amendment usage. The values shown in both soil types for ω3 lipids agree with the study by (Ayaz et al., 2006). Compared to CTL for both soil types, a reduction of ω3 lipids can be seen when most amendments were used. A similar trend of no significant difference in the accumulation of total ω3-FA 18:3 fatty acids was observed between the CTL and kale grown in the other amendments (Vermicast, K-humate, and Volcanic minerals) by Vidal et al. (2018). Such differences can occur due to the changes in the level or activity of delta 13 desaturase enzyme that catalyzes the biosynthesis of ω3-FA 16:3 from ω6-FA 16:2 (Stuchlík & Zák, 2002).

Considering the ω6 containing lipids, in this study, the highest amount was shown from 1,2 DGs lipids when CTL was used in soil type 1 while for soil type 2, the highest from LCTs upon addition of SL amendment. A similar study by Abbey et al. (2018), who observed the application of K-humate and volcanic minerals amendments seen suppressed Omega-3 fatty acids under the low pH of the K-humate and the high pH of the volcanic minerals, while Omega-6 fatty acid content was unaffected by media compositions.

It has been figured out that almost all the dietary PUFAs are plant-derived, and these are derived from SFAs; SFAs are progressively desaturated to form MUFAs, oleic acid ω9-FA 18:1, and the PUFAs of linoleic acid (LA) and α-linolenic acid (ALA) ω6-FA 18:2 and ω3-FA 18:3, respectively (Ursin, 2003). LA compromises up to 89% of total PUFA energy intake, while ALA typically accounts for only about 10% of total PUFA energy in adult diets (Kris-Etherton et al., 2000). This study demonstrated that the highest PUFAs containing lipids were shown from TGs when CBC amendment and CTL amendment were used for soil type 1 and soil type 2, respectively.

Previous research conducted by Simopoulos (2002) suggests that early human beings evolved on a diet with a ratio of ω6 to ω3 essential fatty acids (EFA) of ~ 1 whereas, in current Western diets, the ratio varies from 15/1–16.7/1; The presence of excessive amounts of ω6 polyunsaturated fatty acids and a very high ω6: ω3 ratio, as is found in today's Western diets, promote many diseases such as inflammatory and autoimmune diseases, cardiovascular disease, and, cancer, whereas increased amounts of ω3 PUFAs or a low ω6: ω3 ratio exert suppressive effects (Simopoulos, 2002).

In the present study, the lowest ratio of ω6: ω3(1,2-DGs) was shown when CTL was used for soil type 1, and soil type 2, it was shown upon the addition of CBC amendment where there was no significant difference ($p<0.05$) shown with CTL. Those results agree with the study by Sharma et al. (2011) where adding chicken manure, sugar cane press mud, and farmyard manure increased the ω6: ω3 ratio of sugar cane while the lowest was achieved from the control treatment.

Glycolipids are plant-derived lipids that have been identified as functional lipids, especially due to their known human health benefit, such as intestinal impairments; in literature, it has been found that the majority of ω3- PUFAs are from glycoglycerolipids which have strong stability against oxidations (Yamashita et al., 2022). Galactolipids are a subclass of glycolipids, the major membrane lipids in the chloroplast (Christensen, 2012). Past studies have demonstrated that the galactolipids from vegetable act as nutraceuticals with effects on the treatment or prevention of cancer (Hou et al., 2007) and anti-inflammatory diseases such as arthritis (Ulivi et al., 2011). Therefore, the production of vegetables enhanced with such bioactive compounds can be achieved through plant growth media modulations.

The current study for soil type 1 showed the highest glycolipids when WSBC amendment was used. However, those values are very low as the significant parameter was monounsaturated glycolipids. Considering soil type 2, it was shown that the polyunsaturated glycolipids content increased upon the addition of WBC amendment, but usage of SL showed a decrease in nmol% values. The reduction of polyunsaturated glycolipids in soil type 2 can be associated with N-deficiency due to the high C:N of SL amendment (Bellamy et al., 1995) while studies by Gaude et al. (2007) state that N deficiencies in higher plants such as *Arabidopsis* result in a co-ordinated breakdown of galactolipids and chlorophyll with deposition of specific fatty acid phytyl esters in thylakoids and plastoglobules of chloroplasts.

Due to the identified potential use of the health-promotive properties of galactolipids, several recent patents being granted to produce medicinal and functional food products containing galactolipids for the prevention or treatment of anti-inflammatory illnesses and cancers (Bagger et al., 2014; Shyur et al., 2019).

In the past decade, many studies have shown that despite diglyceride and triglycerides having similar energy values (~9 kcal g^{-1}) and absorption rates, the intake of diglycerides, in particular the 1,3-diglyceride (1,3 DG) isomer, has a positive effect in preventing body weight gain and related diseases (Kristensen et al., 2006; Maki et al., 2002). The benefits of 1,3-DG consumption are known to be related to its distinct metabolic pathway in the small intestine compared to TGs or 1,2-DG where in normal conditions, the TG and 1,2-DG are hydrolyzed to 2-monoglycerides (2-MG) and free fatty acids (FFA); these are readily resynthesized back to TG with the consequent fat deposition in the body tissues; the metabolic pathway of the 1,3-DG isomer occurs through the formation of the 1(or 3)-MG intermediate and FFA, thereby avoiding the 2-MG pathway to TG (Maki et al., 2002; Murase & Kimura, 2004; Nagao et al., 2000; Sakakima et al., 2007).

This study showed that kale grown in WA (soil type 1) and WBC (soil type 2) amendments have significantly increased the 1,2-DGs amounts compared to the 1,3-DGs. Nevertheless, the 1,3-DGs were shown to be significantly highest when kale was grown in the WSBC amendment (44.74 ± 1.95 nmol%) for soil type 1 while for soil type 2, the highest amount was shown when WBC amendment was used (38.18 ± 3.31 nmol%). In plants, DGs are synthesized in both the Endoplasmic reticulum (ER) and the chloroplast, where glycerol-3-phosphate (G3P) is esterified by lysophosphatidic acid acyltransferase (ATS) enzyme to form lysophosphatidic acid (LPA), which is transformed into phosphatidic acid (PA); PA is then de-phosphorylated by phosphatidate phosphatase (PAP) to form DGs (Bates & Browse, 2012). Therefore, it can be seen that WA and WSBC amendments appear to stimulate molecular species in PA and/or the subsequent removal of phosphate (de-phosphorylation) from these PA molecular species leading to the formation of DG molecular species (Vidal et al., 2018). Apart from the above, Dosso et al. (2020) state that most current vegetable oils are not a significant DG source; therefore, diglyceride-enriched food products must be obtained from other sources.

Recent studies by Ferretti et al. (2018) have been able to synthesize edible oils enriched with 1,3-DGs by glycerolysis of vegetable oils promoted by solid bases where usage of natural media amendments can also be a promising way to modulate especially 1,3-DG amounts in plants.

Medium-chain triglycerides (MCT) are triglycerides with fatty acids having a chain length of 6–12 carbons (Kovacs & Mela, 2006) and due to their physiological as well as functional characteristics, which have helped in treating various health disorders. Zhang et al. (2015) reported that a diet rich in MCT could increase fat oxidation and energy expenditure in healthy adults fed for 3 months. In the human body, medium-chain triglycerides are readily hydrolyzed by lingual and gastric lipases, and they differ from long-chain triglycerides (LCT, having fatty acids of >12 carbons), where the fatty acids of MCT are absorbed directly into the portal circulation and transported to the liver for rapid oxidation (Babayan, 1987). Early reports by Bremer (1983) report that, unlike long-chain fatty acids, the intramitochondrial transport of medium-chain fatty acids does not require the enzyme carnitine palmitoyltransferase, while Kovacs and Mela (2006) state that not requiring the above enzyme probably accelerates lipid oxidation and limits storage of MCT within tissues.Therefore, MCTs are used as a functional or nutraceutical oil in various food and pharmaceutical formulations (Jadhav & Annapure, 2022). Interestingly in our study, MCTs were only shown in soil type 2, when WBC amendment was used where it was significantly different ($p<0.05$) from other media amendments used, providing insights to produce functional foods enriched with MCTs.

Phospholipids are amphiphilic lipids found in all plant and animal cell membranes, arranged as lipid bilayers; They are mostly known as glycerophospholipids (GPLs), which consist of fatty acids esterified to the glycerol backbone, a phosphate group and a hydrophilic residue (e.g.choline, resulting in PC (phosphatidylcholine or lecithin) (Küllenberg et al., 2012).

Hartmann et al. (2009) studied the effect of PC on experimental arthritis (chemically induced using carrageen) in rats and found a significant reduction of arthritis after PC supplementation. Apart from the above several studies have investigated PLs for the inhibition of cancer growth; For example, an *in vitro* study with hepatic cancer cell lines showed a dose-dependent growth restraint when the cancer cells were cultured in the presence of soy and egg yolk PC (96.5% pure PC from soybeans and 99% pure PC from egg yolk) and menaquinone-4 (vitamin K2) (Sakakima et al., 2007).

Our study demonstrated that the highest amount of PLs containing ω3 fatty acids was shown upon the usage of WSBC amendment for soil type 1. Reports by Küllenberg et al. (2012) state that further functional product value comes from the health benefits of the Phospholipids being combined with the benefits of the selected nutrient(s) where one prime combination would be phospholipids with ω3 fatty acids. In soil type 2, the highest amount of ω6-PLs was shown when CTL was used whereas compared to soil type 1, an inverse effect was shown via the addition of WSBC amendment. The reduction of PLs for soil type 2 can be caused by P deficiency, where Gaude et al. (2008) state that membrane lipid remodelling during phosphate starvation in plants leads to decreases in phospholipids and increases in galactolipids contents. The presence of many past clinical studies and experimental studies have established that phospholipids such as PS, PC, GPC (glycerophosphocholine), and mixed phospholipids as first-rate nutraceuticals due to the characteristics such as perfect safety record, unique physicochemical characteristics, and a well-documented array of health benefits make them premier functional food constituents (Kidd, 2002). Phytosterols are natural components of human diets whereas, in the Western diet, an average of ~250 mg per day of phytosterols are consumed through vegetable oils, cereals, fruits and vegetables (Hicks & Moreau, 2001).

Recently, phytosterols have attracted greater attention due to its known hypocholesterolemic effect by lowering plasma total and LDL cholesterol levels without affecting plasma high-density lipoprotein (HDL) cholesterol concentrations (Zheng et al., 2014). The prospect of lowering cholesterol levels by consuming ''functional'' foods fortified with natural phytonutrients would seem more attractive to many than drugs or dietary restrictions (Moreau et al., 2002). In our study, the phytosterols of campersterol, β-stigmasterol, and sitosterol were found in both soil types. Interestingly in our study for soil type 1 the highest amount of sterols consisted of saturated fatty acids when CBC amendment was used where there was no significant difference between other media amendments except for WSBC. Compared to soil type 1, in soil type 2, the parameter of monounsaturated sterols was shown significantly low values, and the highest values were shown when SL media amendment was used.

In this study, it was demonstrated that soil type 1(pH 5.2) and soil type 2 (pH 5.8) act differently upon the addition of different media amendments. For soil type 1, the phosphatidylinositol signaling system pathways, glycerolipid metabolism and glycerophospholipid metabolism were shown to be significantly affected by media amendments. Soil type 2 also shows the glycerolipid metabolism pathway being significantly affected. In addition, glycosylphosphatidylinositol (GPI)-anchor biosynthesis seems to be altered in soil type 2 when natural media amendments were used.

3.5 Conclusion

The results obtained from this study demonstrate that natural growth media amendments are very effective in modulating most of the functional lipids in kale cultivated under controlled environmental conditions. Apart from the above pathway analysis, the functional lipids in kale get significantly vary according to the soil type. Overall, for soil type 1, WSBC amendment appeared to be most effective in enhancing the functional lipids of 1,2-DGs, 1,3-DGs, ω6 lipids, and phospholipids. Considering soil type 2, most of the studied functional lipids were increased after SL and WBC media amendments were added. From those findings, it can be clear that using natural media amendments can be a strategy for producing functional foods in control environments and production systems, such as greenhouses or environmental growth rooms. It is universally accepted that a strong relationship exists between diets high in vegetables and reduced risks of developing common lifestyle-related illnesses such as coronary diseases, cancer, type 2 diabetes, obesity, and osteoporosis. The greenhouse study suggests using natural growing media amendments, particularly, SL, WBC, and WSBC could be a useful approach to producing kale plants with enhanced levels of functional lipids. Increased access to vegetables such as kale with enhanced functional lipids could aid in increased consumption with potential implications in reducing common lifestyle-related illnesses. The study also showed that pathway analysis of functional lipids of kale could be an innovative and promising approach to elucidate the derivation pathways and provide new perspectives for further analysis of functional lipids of vegetables grown in various media amendments. To evaluate its disease resistance potential upon land application, more studies are needed to explore untargeted functional lipids under varying amendments.

3.6 References

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3.7 Supplementary materials

Table 3.3. Parameters for each cluster in the heatmap of soil type 1 and its significance

Cluster in Heatmap	Parameter (Functional Lipid)	R^2	F	Pr > F(Model)
G1	ω6-PL	0.519	3.703	0.007
G1	ω6:ω3-PL	0.553	4.240	0.004
G1	Saturated Glycolipids	0.376	2.066	0.088
G1	Monounsaturated PLs	0.503	3.468	0.010
G1	ω3 1,2 DGs	0.957	76.809	0.000
G1	ω3-Glycolipids	0.273	1.289	0.297
G2	Saturated Sterols	0.427	2.550	0.041
G2	Monounsaturated 1,2 DGs	0.896	29.643	0.000
G2	Polyunsaturated-TGs	0.579	4.711	0.002
G2	ω3-LCTs	0.506	3.511	0.010
G2	LCTs	0.245	1.112	0.388
G2	Saturated-PLs	0.810	14.630	0.000
G3	Saturated 1,2 DGs	0.964	92.862	0.000
G3	1,3 DGs	0.937	50.800	0.000
G3	Saturated 1,3 DGs	0.937	51.104	0.000
G3	SCTs	0.331	1.698	0.157
G3	ω6:ω3 (1,2 DG)	0.865	21.997	0.000
G3	Polyunsaturated Glycolipids	0.248	1.129	0.378
G3	ω3-Sterols	0.399	2.279	0.063
G3	Polyunsaturated Sterols	0.311	1.551	0.198
G4	ω6- lCTs	0.244	1.109	0.390
G4	ω6:ω3(TG)	0.644	6.198	0.000
G4	ω6-Sterols	0.244	1.109	0.390
G4	ω6:ω3(Sterols)	0.532	3.894	0.006
G5	Polyunsaturated 1,2 DGs	0.881	25.481	0.000
G5	Monounsaturated Sterols	0.502	3.457	0.011
G5	ω6-Glycolipids	0.397	2.260	0.065
G5	ω6:ω3(Glycolpids)	0.376	2.069	0.087
G5	MCTs	0.268	1.257	0.313
G5	Monounsaturated TGs	0.644	6.209	0.000
G5	Saturated TGs	0.476	3.110	0.018
G5	Monounsaturated Glycolipids	0.503	3.465	0.010
G5	ω6 1,2 DG	0.872	23.437	0.000
G5	Monounsaturated 1,2 DGs	0.804	14.094	0.000
G5	ω3-PLs	0.791	12.957	0.000

Table 3.4. Parameters for each cluster in the heatmap of soil type 2 and its significance

Cluster in heatmap	Parameter (Functional Lipid)	R²	F	Pr > F(Model)
G1	ω6-Glycolipids	0.229	0.679	0.688
G1	Monounsaturated Glycolipids	0.481	2.121	0.101
G1	Monounsaturated -PLs	0.872	15.512	0.000
G1	Monounsaturated 1,3 DGs	0.927	28.875	0.000
G1	Monounsaturated 1,2 DGs	0.656	4.361	0.007
G1	Polyunsaturated PLs	0.403	1.545	0.222
G1	ω6:ω3(Glycolipids)	0.261	0.807	0.594
G2	ω3-Sterols	0.529	2.569	0.056
G2	ω6 1,2 DGs	0.487	2.172	0.094
G2	Saturated TGs	0.872	15.586	0.000
G2	Monounsaturated TGs	0.414	1.618	0.201
G2	Polyunsaturated Sterols	0.451	1.877	0.141
G2	ω6:ω3(1,2 DGs)	0.570	3.030	0.031
G2	SCTs	0.778	7.994	0.000
G2	MCTs	0.732	6.259	0.001
G3	ω3-Glycolipds	0.724	5.997	0.001
G3	ω3 1,2 DGs	0.721	5.905	0.002
G3	ω6-PLs	0.734	6.304	0.001
G3	Polyunsaturated Glycolipids	0.676	4.766	0.005
G3	Polyunsaturated 1,3 DGs	0.739	6.472	0.001
G3	Polyunsaturated 1,2 DGs	0.588	3.263	0.024
G3	ω6:ω3(PL)	0.734	6.313	0.001
G3	1,2 DGs	0.739	6.472	0.001
G3	Saturated PLs	0.751	6.889	0.001
G4	ω3-LCTs	0.736	6.374	0.001
G4	ω6-LCTs	0.827	10.916	0.000
G4	Polyunsaturated TGs	0.708	5.543	0.002
G4	ω6:ω3(TG)	0.685	4.978	0.004
G4	LCTs	0.707	5.526	0.002
G4	Saturated Glycolpids	0.553	2.830	0.040
G4	Saturated 1,2 DGs	0.861	14.203	0.000
G4	Saturated 1,3 DGs	0.728	6.124	0.001
G4	Monounsaturated Sterols	0.563	2.950	0.035
G4	1,3 DGs	0.739	6.472	0.001
G5	ω6-Sterols	0.499	2.279	0.082
G5	Saturated Sterols	0.450	1.871	0.142
G5	ω6:ω3(Sterols)	0.486	2.164	0.095

Chapter 4: General conclusions and future recommendations

4.1. General conclusion

The results obtained from this research manifested that media amendments in wood ash (WA), sludge (SL), and biochar (BC) are effective in modulating the nutritional parameters of kale. In Chapter 2, it was shown that for soil type 2 (pH 5.8), upon the addition of WBC (wood ash + biochar) media amendments, most of the parameters such as total trace minerals (1.23 ± 0.23 mg g^{-1} of DW), ω3-FA (14.73 ± 0.88 mg g^{-1} of DW), vitamin E (0.47 ± 0.04 mg g^{-1} of DW), vitamin B2 (0.123 ± 0.002 mg g^{-1} of DW), and Boron (0.010 ± 0.002 mg g^{-1} of DW) were significantly enhanced compared to control. In contrast, kale grown in soil type 1 (pH 5.2) increased the marketable yield upon the addition of WA; thus, it did not manifest a significantly different ($p<0.05$) effect on the nutritional parameters. Pathway analysis results confirmed that although the same media amendments were used in both soil types, there were different significant ($p<0.05$) metabolic pathways associated with each soil type. Although there are fewer to no past research reports on the effects of such media amendments on crop quality, our results mostly agreed with similar studies by Ayaz et al. (2006) and Pradhan et al. (2009).

The results of Chapter 3 reveal that the complex lipids profile of kale was significantly altered upon the addition of the formulated natural media amendments. For soil type, 1 WSBC (wood ash +sludge +biochar) amendment appeared to be the most effective in enhancing the functional lipids of 1,2 diglycerides, 1,3 diglycerides, omega-6 lipids (i.e., lipids compounds containing ω6 double bond), and phospholipids. For kale grown in soil type 2, the major functional lipids content was primarily increased upon the addition of SL and WBC media amendments.

It is universally accepted that there is a strong relationship between diets high in vegetables, and reduced risks of developing common lifestyle-related illnesses such as coronary diseases, cancer, type 2 diabetes, obesity, and osteoporosis (Vidal et al., 2018). From the research findings, it suggests that the usage of natural media amendments (wood ash, sludge, and its combinations with biochar) can be a strategy for the production of vegetables/fruits with enhanced nutritional quality in controlled environmental production systems

4.2. Future recommendations

In this study it was manifested that for kale grown in soil type 1 (pH 5.2) showed increased values in total antioxidant mineral content, FA 22:0 and total trace minerals upon addition of WA and SL respectively (Table 4.1). Most of the measured parameters did not show any significant effect upon addition of media amendments. Comparatively for soil type 2 (pH 5.8) most of the measured parameters were significantly shown increased values upon usage of WBC amendment (Table 4.2). Although there was mostly a positive effect on the nutritional parameters of kale when WA, SL, and BC media amendments were used, it is highly recommended to carry out the routine test for the presence of heavy metals, especially for SL and WA. Such routine tests will prevent the bioaccumulation of heavy metals in the food chain. This study was conducted in controlled environmental conditions with a single crop cycle. We recommend conducting more controlled environmental research with different crops and repeated crop cycles grown in different soil types. We also recommend measuring each cycle's nutrient parameters before conducting any field experiments. Worthington (2001) stated that over the last 75 years, the question of whether inorganic chemicals and other agricultural methods, including organic farming/ natural media amendments, affect nutrient content is still unresolved; the author also stated that such inconsistencies of the results could be due to the variability in agricultural data resulting from uncontrollable factors such as rainfall and sunlight, which may also influence the plant nutrient content.

Therefore, such variability must be considered along with using any natural media amendments. Further studies need to be investigated on the plant's physiology changes and specific metabolic pathways alterations upon adding media amendments which will give the insight to produce more tailored nutritional properties in vegetables and fruits.

Table 4.1. Effect of natural media amendments based on the highest nutritional parameter values of kale grown in soil type 1 (pH 5.2)

Parameter	CTL	CBC	WA	WBC	SL	SBC	WASL	WSBC
Total antioxidant minerals			✓					
Saturated Fatty Acid Contents	✓							
Cu	✓							
FA 16:0	✓							
Fe			✓					
FA 22:0			✓					
Mg	✓							
Co	✓							
Cd	✓							
Total Toxic mineral content	✓							
FA 18:0	✓							
Pb	✓							
Total trace minerals					✓			
FA 16:1	✓							
Mn					✓			
FA 24:0						✓		
B						✓		
Marketable Yield				✓				

Table 4.2. Effect of natural media amendments based on the highest nutritional parameter values of kale grown in soil type 2 (pH 5.8)

Parameter	**CTL**	**CBC**	**WA**	**WBC**	**SL**	**SBC**	**WASL**	**WSBC**
Total trace minerals				✓				
Marketable yield			✓					
Na			✓					
ω6- FA 18:2				✓				
FA 16:0				✓				
ω3's				✓				
ω3-FA 18:3				✓				
Vitamin B2				✓				
Total Proteins	✓							
Vitamin K1				✓				
ω3-FA 22:6				✓				
Mg		✓						
Retionol-A	✓							
Total Antioxidant Activity (TAA)							✓	
Vitamin C		✓						
Total Carbohydrates		✓						
B				✓				
Hydrophilic Antioxidant activity (HAA)					✓			
Beta- Carotene				✓				
ω6: FA 20:4							✓	
Total Fat soluble vitamins				✓				
Vitamin E				✓				
ω9-FA 22:9					✓			
Cr							✓	
Total Antioxidant minerals						✓		
Fe						✓		

4:3 References

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