MODULATION OF BIOACTIVE COMPOUNDS IN UNDERUTILIZED LEGUME HORSE GRAM (*Macrotyloma uniflorum)* BY GERMINATION AND FERMENTATION

A Thesis

by

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ABSTRACT

Horse gram (*Macrotyloma uniflorum* Lam. Verdc.), although an underutilized pulse it is an excellent source of protein, dietary fiber, variety of micronutrients and is also traditionally used in various herbal therapeutic formulations. The present study advances the knowledge on impact of soaking, germination, cooking and fermentation on major bioactive compounds in horse gram seeds. Changes occurring in phenolic compounds, protein content, free amino acids, free radical scavenging activity, α-amylase and α-glucosidase enzyme inhibition activities as well as volatile compounds were determined in horse gram seeds as well as sprouts. It was also observed that germination of horse gram seeds for 72 h significantly increased DPPH free radical scavenging activity, most of the essential amino acids content except threonine, α-amylase and α-glucosidase inhibition activity. Cooking significantly increased DPPH free radical scavenging activity of horse gram sprouts while free amino acid content, α-amylase and α-glucosidase inhibition activity was reduced compared to fresh horse gram sprouts. Fermentation of horse gram sprouts by *Lb. plantarum* NRRL-B-4496 and *Lb. plantarum* NCDO-1193 for 48h also further increased the DPPH free radical scavenging activity, free amino acid content, α-amylase and α-glucosidase inhibition activity. HPLC-DAD-ESI/MS analysis of all the samples indicates changes in phenolic compounds during processes such as germination, cooking and fermentation. Such result can be attributed to conversion of glycosylated phenolic compounds to their more bioavailable forms i.e. aglycones such as kaempferol. Differences in free radical scavenging activity and total phenolic content indicate the

presence of certain metabolites other than phenolic compounds with potent antioxidant activity. Microbiological analysis determined that both lactic acid bacterial strains were able to utilize horse gram sprouts for optimum growth during 48h fermentation period. A total of 40 volatile compounds were detected using head-space solid phase micro extraction combined with GC-MS. Qualitative as well as quantitative changes in volatile compounds was determined during germination and fermentation of horse gram sprouts for 24h (day 1), 48h (day 2), 72h (day 3), 96h (day 4), 120h (day 5) by *Lb. plantarum* NRRL-B-4496 and *Lb. plantarum* NCDO-1193 as well as during natural fermentation. A remarkable increase in the amount of some organic acids such as acetic acid, alcohols and some volatile phenols such as eugenol was observed with increasing fermentation time. The results suggest that germination and lactic acid bacterial fermentation of horse gram seeds and sprouts respectively influences the levels of majority of the investigated bioactive compounds. Therefore, the present study supports the rationale that conventional processes facilitates release of bound compounds, conversion of complex metabolites to its more bioavailable form and biosynthesis of certain compounds with sensory as well as potential health benefits in addition of delivering probiotic characteristics to the consumers.

DEDICATION

TO MY LOVING HUSBAND

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NOMENCLATURE

LAB	Lactic acid bacteria
GAE	Gallic acid equivalent
BSA	Bovine serum albumin
TPC	Total Phenolic Content
MRS	de Man, Rogosa and Sharpe

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1. INTRODUCTION

The understanding of nutritional and health promoting properties of plant foods including legumes has insights from Hippocrates, the father of modern medicine, who more than 2000 years ago quoted "*Let food be thy medicine, and medicine be thy food*". Scientific evidences of such protective roles for plant based foods in health promotion are substantial[1]. Also the strength of these emerging scientific insights further guides worldwide policymaking in diet and health issues that informs and facilitates community and local programs to address dietary goals and to increase consumption of fruits, vegetables and legumes. These disease preventive food solutions are now integrated with traditional nutritional challenges such as hunger particularly hidden hunger and malnutrition. Hidden hunger is listed currently among the most serious global problems and it is no longer limited to the developing countries. In hidden hunger, high amount of calories from refined food is readily available but has micronutrient deficiencies and this is afflicting more than 2 billion individuals or one in three individuals, globally[2]. An important solution to these micronutrient deficiencies can be nutritionally enhanced edible seeds like legumes (used interchangeably as pulses). Edible legumes offer important and affordable source of micronutrients in human diet, as they are rich in proteins, energy, dietary fiber, phytochemicals, vitamins and minerals. Overall legumes have been recognized as the second most important group of crops after cereals [3, 4]. Pulses are seeds of annual legumes that include plants such as bambara beans, dry beans, horse gram beans, dry chickpeas, cow peas, dry lentils, lupins, dry peas, pigeon peas and vetches commonly

used for feeding humans and cattle. Besides basic nutritional benefits pulses provide agronomic benefits to the producers, as the rotational cultivation of pulses increase the amount of nitrogen in soil, thereby reducing the requirement of additional chemical fertilizers. Over last decade global pulses production has been increased from 61.5 million ton to 73 million ton with India accounting for a quarter of world production, which makes it the world's largest producer of pulses. India, with the largest vegetarian population depends on pulses as an important protein source, also represents more than one-fourth of the world total pulse imports followed by European Union, China, Pakistan and Egypt [5]. However, the overall global consumption rate of pulses has experienced a slow but steady decline when compared to the consumption rate of dairy and meat products and this is predicted to continue to rise considerably. However in light of global public health challenges, nutritional improvements and environmental challenges of reducing carbon foot print and improving soil biology for resilience of food production, development of pulse crop technologies, provides important opportunities to improve the consumption of plant based-foods.[6]. Although several common proteinaceous edible legumes like soybean, beans, peas, chickpea, cowpea and some others are available globally, traditional and localized underutilized pulses that are more resilient to climate change challenges and with excellent nutrient profiles offer new opportunities to contribute to global food security, health and environmental challenges.

One of such underutilized pulse is commonly known in India as Horse gram (*Macrotyloma uniflorum* (Lam.) Verdcourt (Syn., *Dolichos uniflorus* Lam., *Dolichos biflorus* auct. non L.). It is a pulse and fodder crop native to Southeast Asia and tropical

Africa, but its center of origin is India. The name *Macrotyloma* is derived from Greek word *makros* which means large, *tylos* meaning knob and *loma* means margin referring to its knobby statures in the pods [7]. It is an adverse climate tolerant pulse well known for its drought resistance and favorable agronomic characteristics for cultivation on less fertile dry soils. Some studies have shown its ability to improve soil fertility, resistance to abiotic stress factors like salinity and pH[8, 9]. Horse gram seeds are low in fat and are commonly consumed among the low income community. It has been reported to have lot of medicinal value and known to have relevance and use in traditional herbal compositions for treatment of kidney stones, menstrual complexes, diabetes, obesity, hypertension, and throat infection, cold and fever [10-13]. Due to its potential nutritional and medicinal potential and its capability to grow in wide range of adverse climatic conditions, the US National Academy of Sciences had considered Horse gram as a "Potential food source of for the future" [14]. Horse gram seeds have been extensively consumed in the past (2000 BC) and holds potential to be used as a "Food for millennia" [12]. Despite of being a potential source of protein and other nutrients, horse gram also contains certain phytochemicals such as phytic acid, tannins, saponins and flatulence causing oligosaccharides with anti-nutrient effects that limits the overall nutritional value of this pulse. However, such anti-nutrients or non-nutrient bioactive compounds have been recently considered to possess several health benefits especially when biologically processed and modified through germination, cooking and fermentation prior to consumption. Several scientific reports have supported processing mediated improvement in common legume based food products[15-18], however very little information is available in relation to effect of processing on major bioactive

compounds of horse gram seeds. Therefore, further scientific validation of these processes to enhance value of horse gram seeds has great potential not only to increase global awareness about such underutilized pulse for possible health benefits but also encourage cultivation around the world as a locally grown and environmental-friendly crop.

2. REVIEW OF LITERATURE

Horse gram seeds can potentially become integral part of common as well as nutrient rich diet. Since they exhibit strong free radical scavenging capacities which makes them useful for food supplements, natural antioxidant and therapeutic purposes for human health benefits[12]. Horse gram seeds are commonly popular for high protein comprises higher lysine content than pigeon pea and chickpea making it a good complement to a cereal based diet[19]. Nevertheless, they are also rich in tannins and polyphenols compared to the other legumes[13]. Such polyphenols and tannins are synthesized by plants for protection against predators and also in response to various stress conditions and accumulate phenolic compounds as part of their metabolism[13]. Such bioactive compounds undergo dormant stage as the soft seeds from the pods are subjected to drying for long term storage to produce hard though edible seeds. However, several factors like variety, environmental as well as agricultural condition and location of cultivation, affect overall nutritional profile of these seeds. For example horse gram black seeds contain relatively high levels of total phenolics and tannins compared to brown seeds, since they are different varieties evolved from different ecological adaptations[20]. Similarly, processing conditions also critically influences the levels of bioactive compounds in these seeds. Dried horse gram seeds have been reported by several researcher to be excellent source of dietary antioxidants as well as possess good antimicrobial activity against pathogens[21]. Raw seeds have also been studied to possess bioactive compounds for treatment of diabetes, hypercholesterolemia and obesity[10, 11, 22, 23]. Despite the numerous health benefits of raw

seeds, the major drawback in using the raw grain is that they are not easily rehydrated and difficult to cook, as the seed coat is hard and requires longer cooking time. In order to render dry horse gram seeds edible and palatable, soaking, sprouting and cooking is generally practiced. Therefore, certain conventional processing methods like soaking, germination and cooking is proposed in the present study to evaluate the changes in the nutritional quality of this poor man's pulse food.

2.1 Soaking

Soaking of grain legume is generally practiced before they are germinated or cooked, in order to make them consumable and ensure sensory quality. It is one of the important steps in production of legume-derived food products such as tempeh since improper soaking affects body and texture defects in the final product[24]. Optimum presoaking conditions facilitates cooking, germination and fermentation processes in the legumes. Soak water may or may not be discarded in traditional practices, however some studies showed decrease in polyphenol content of horse gram and some other lentils during soaking which is attributed to leaching of polyphenols in the in the external medium, hence it is suggested to reuse the soaked water instead of draining [25-28].

As discussed before, since dried horse gram seeds has high amount of antioxidants that are concentrated in the seed coat, consumption of foods prepared with unprocessed raw horse gram seeds have showed hyperglycemic properties[29]. Moreover, during conventional processing methods like soaking, germination and cooking dry seeds undergo important chemical changes resulting in better texture, improved digestibility and

palatability[26, 30]. Such changes during germination positively affect the bioavailability mainly due to extensive metabolic modification. Some reports have suggested both horse gram seeds and sprouts to be excellent examples of 'functional foods' as it has the potential to lower the risk of various diseases and exert health promoting benefits in addition to basic nutrition[31]. Since longtime, traditional processing methods like soaking, cooking, germination and fermentation have provided wide range of opportunities to exploit the actual potential of legumes.

2.2 Germination

Germination is one of the most simple and effective process to improve the overall nutritional value of pulses. During germination, the seed dormant reserve nutrients present in endosperms like starch, proteins and lipids are metabolized in the cotyledons and the products formed are translocated to the developing axis that nourishes sprouts to grow into a seedling and subsequently into a new plant. Therefore, the sprouted seeds or pulse sprouts have gained importance not only due to it higher nutritional value but also exerting value added health benefits [32]. In a previous study[31], horse gram sprouts showed higher total phenolic content as well as *in vitro* antioxidant activity compared to raw seeds. Such an increase was suggested to be due to the metabolic changes in dormant raw seeds as it undergoes the process of hydration followed by sprouting which led to synthesis or release of several bound bioactive compounds with good antioxidant activity. Germination also dramatically increased total phenolic compounds and total flavonoids in mung bean sprouts with increasing germination period, up to 4.5 and 6.8 times higher than original

concentration of mung bean dry seeds, respectively. The total antioxidant activity of mung bean sprouts was increased by 6 fold higher than that of raw mung bean seeds. Apart from antioxidant activity, germination of lentils also brings about an increase in the levels of amino acids, vitamins like ascorbic acid, riboflavin, thiamin and niacin [28, 33]. Several other studies demonstrated the effect of germination on dry legume seeds[17, 33, 34], however very few studies have reported constructive effect of germination on horse gram dry seeds[25, 26]. Therefore, the present study further adds to the rationale towards proposing that the germination process significantly increases phytochemical content and antioxidant activities in legumes or pulses particularly horse gram.[17, 35].

2.3 Cooking

Cooking quality of seeds is one of the important parameters of legumes, its commonly practiced to improve seed texture, digestibility and palatability. However, cooking quality varies to very high extent among all the legumes and it is critical to determine the acceptability of the seeds for human consumption[30]. Depending on the cooking quality of the legumes, cooking time differs widely among genotypes and is affected by the permeability of seed coat and the cotyledons to hot water [30]. Thermal processes like cooking not only structurally modifies the legume seeds but also modifies functional properties which in turn increases digestibility of seeds. Several reports showed significant decrease in certain heat-labile nutrients during cooking of [36]seeds or sprouts[25, 37-39], while some studies suggested thermal processes to be responsible for remarkable increase in the phytochemicals in sprouts[18]. In a previous study[25] it was observed that

cooking decreased polyphenol content in moth bean and horse gram which could be attributed to either binding of polyphenols with other organic substances like protein or structural alterations of polyphenols that render them incapable of giving the chemical color reaction measured by the analytical method. However interestingly, in the same study it was also observed that the loss of polyphenols was much less in the moth bean or horse gram sprouts compared to raw seeds cooking, suggesting cooking sprouts could be a beneficial choice over cooking legume seeds.

2.4 Fermentation

Fermentation is one of the oldest techniques used traditionally and commercially for food preservation. It is also considered as a simplest way to naturally derive nutritionally improved food products. It improves nutritional, organoleptic, technological and shelf life attributes in diverse range of fermented foods and beverages. There are approximately 5000 varieties of fermented foods consumed worldwide [40]. Such fermented food products comprise delicacies made from milk (dairy products), fruits (wine), vegetables (sauerkraut), fish (bagoong, colombo cure), meats (salami) and legumes (soy natto, soy miso). Similar to germination, fermentation also have been practiced to enhance the nutritional importance of legumes and legume based products for human consumption[28, 34, 41]. This crucial metabolic process also decreases the levels of certain anti-nutritional compounds like flatulence causing oligosaccharides and phytic acid [42] as the same time significantly increase the levels of bioavailable phenolic compounds[43]. However, selection of microorganism to be used as starter culture for fermentation of

legumes is an important constraint to its success, since legumes are considered to be a hostile environment for typical fermentation bacteria like lactic acid bacteria (LAB). Majority of dairy and food industries use lactic acid bacteria (LAB) as starter culture for fermentation. Such lactic acid fermentation primarily focusses on acidification in the raw ingredients through production of organic acids from carbohydrates with simultaneous breakdown of secondary metabolites to develop essential biomolecules which are referred to as bioactive compounds[44]. These bioavailable form of compounds are readily accessible by the consumer's body as well as possess several health benefits from prevention of cancer, chronic diseases like diabetes, CVD and obesity to improvement of general well-being of the consumer[15, 45, 46]. This is one of the main reason certain lactic acid bacteria have a huge market as probiotics, live microorganisms in products, which when administrated in adequate amounts confer a health benefits to the host"[47]. The effect of natural and LAB mediated fermentation on phenolic contents and antioxidant activity have been reported in several previous studies[28, 48]. Some studies reported fermentation increased total phenolic content (TPC) [15, 49] during fermentation while some studies found a slight decrease in TPC in naturally and LAB-fermented lentils [15]. However, the latter study mainly investigated TPC in the soluble fraction of fermented legumes, while LAB can also produce enzymes that can metabolize or transform soluble polyphenols to free form or other compounds better than naturally fermenting bacteria. Therefore, the present study is designed to simultaneously analyze changes occurring in bioactive compounds in naturally fermentation and LAB-mediated fermentation.

2.4.1 Lactobacillus plantarum

The genus *Lactobacillus* comprises variety of different species that display large degree of diversity. Among these groups, *Lactobacillus plantarum* is the most flexible and versatile species found in diverse environment like dairy, meat, plants or vegetables [50]. However, it is most frequently found as natural inhabitant of human gastrointestinal tract[51], therefore strains like *Lb. plantarum* 299v, *Lb. plantarum* PS128, *Lb. plantarum* NRRL-B 4496 are popularly recognized as probiotic bacteria that may confer numerous health benefits to the consumers[47, 52]. *Lactobacillus plantarum* is a gram positive facultative heterofermentative lactic acid bacteria. A complete genome sequencing study [53] for *Lactobacillus plantarum* WCFS 1 revealed that it contains 3052 protein-encoding genes and the genome encodes all enzymes required for the glycolysis and phosphoketolase pathways. It also encodes a large pyruvate-dissipating potential, leading to various end products of fermentation. It was demonstrated that this microbe focuses mainly on carbon catabolism, which is explained by its ability to import and utilize various sources of carbon. It also corresponds to the presence of potentially highly expressed genes encoding for enzymes required for central carbon metabolism, and exceptionally high number of sugar import systems including phosphoenolpyruvate (PEP) dependent sugar phosphotransferase systems. The presence of large group of surface-anchored proteins also indicates that *Lb. plantarum* has the potential to adhere to a large variety of surfaces and substrates for growth. Such ability might depict the higher scope of *Lb. plantarum* spp. for commercial utilization as starter culture to develop non-dairy probiotic products. During anaerobic fermentation, it utilizes various carbon

sources (carbohydrates) to convert into chiral configuration of lactate and alcohols due to genes *ldhL* and *ldhD* genes encoding lactate dehydrogenase. However, unlike some other lactobacillus spp. *Lb. plantarum* is also able to degrade lactate into acetate, ethanol, acetoin, formate and 2,3-butanediol under aerobic conditions, due to number of pyruvate dissipating enzymes. In addition, it also can produce hydrogen peroxide and carbon dioxide as byproducts, which potentially kills or inhibits undesirable bacterial growth, especially acid tolerant spoilage yeast and mold during fermentation. Hence, preliminary study involved two *Lactobacillus plantarum* spp. probiotic strain along with *Lactobacillus helveticus* spp.

2.5 Proposed Metabolic pathways

2.5.1 Deglycosylation

Fermentation hydrolyzes complex polyphenols into simpler and biologically more active compounds due to the ability of fermenting bacteria of deglycosylation[54]. It has been reported that conjugate glycosides are not readily absorbed intact across the intestine of healthy adults and they need to be hydrolyzed, releasing aglycones which are more bioactive forms of polyphenols that can be easily absorbed by the intestinal lining to confer proposed health benefits to the consumers[55]. Most of the *Lactobacillus* strains possess enzymes associated with the metabolism of phenolic compounds, while some of these enzymes are primarily described for *Lactobacillus plantarum*[54]. It has been reported that *Lactobacillus plantarum* spp. are able to decarboxylate the hydroxycinnamic acids, *p*-

coumaric and caffeic acids[56]. Molecular characterization studies on *L. plantarum* revealed that this bacterium species possess inducible gene encoding *p*-coumarate decarboxylase (PadA) having *p*-coumaric acid decarboxylase (PAD) activity, but interestingly these enzymes were found to be different in structure, specificity of substrate, expression and N-terminal amino acid sequence compared to other characterized decarboxylases from *Saccharomyces cerevisiae*, *Bacillus pumilus* and *Psuedomonas fluorescens*. Such results promoted use of lactic acid bacteria for fermentation of legumes over other microorganism in order to develop functional foods. A study observed an increase in the bioactive compounds of soybean (*Glycine max* cv. Merit) and mung beans (*Vigna radiate* [L]) due to fermentation by *Lactobacillus plantarum* CECT 748 T, this strain was able to efficiently convert glycosylated isoflavones into bioactive aglycones [44]. Another study [43] investigated the effect of *L. plantarum* fermentation on the content of phenolic compounds in *Vigna sinensis* flours and suggested fermentation as an appropriate and effective process for increasing nutritional and biological quality due to not only their bioavailability but also improvement in the concentration of phenolic compounds.

2.5.2 Proteolysis

It is strongly believed that during germination, proteases released are responsible for inactivation of proteinaceous anti-nutritional factors like lectin, amylase and trypsin inhibitors in seeds and also for breakdown of complex storage proteins into simpler peptides and free amino acids[57]. According to an earlier study, there are three crucial phases of proteolysis during seed germination[58]. First stage of hydrolysis involves release

of amino acids that can be further used for the synthesis of enzymes catalyzing the conversion of complex reserve nutrients into simpler form for transport, secondly bulk hydrolysis occurs when protein complexes hydrolyze into amino acids required for growing sprouts, at the end of sprouting, cellular proteins as well as enzyme proteins are further broken down into additional amino acids that are used by the seedling before the onset of photosynthetic growth[58]. Germination is known to have very little effect on crude protein content. A study reported 1.4% increase in protein content of green gram during germination, whereas another study[59] reported an increase of 2.5% in mung beans. Such an increase was attributed to biosynthesis of enzymes and protein during germination and compositional changes following the degradation of other constituents[35]. However, such an increase in crude protein content during seeds germination may not be necessarily due to an increase in true protein as most of analysis measure total nitrogen content of the samples that can be either due to presence of nucleic acid, free amino acids and polypeptides in legumes. Therefore, in the present study, chromatographic analysis to evaluate the changes in amino acid composition during germination, cooking and fermentation is of greater interest. Similar results were reported for significant increase in the amount of amino acids during germination of mung beans[59], while such rise corresponds with increasing germination time[35]. It was observed that leucine, phenylalanine and threonine were significantly higher in sprouts, although the amount of cysteine, methionine and lysine was found to be very low in mung sprouts.

Lactic acid fermentation has also been known to affect the amounts of amino acids in cereals and legumes. Bacterial fermentation usually involves optimum proteolytic

activity, while yeast mainly degrades carbohydrates. Hence the changes in amino acids composition during bacterial and yeast fermentation differ significantly; therefore, bacterial fermentation must be given more importance to control nutritional improvement of legumes over yeasts. Lactic acid bacteria particularly are equipped with protein-degradation system, although some strains of *Lb. plantarum* lack genes encoding extracellular proteases required for primary breakdown of protein, but interestingly they possess 19 highly expressed genes encoding intracellular peptidases and uptake systems for peptides, which aids in further degradation of peptides formed during germination into simpler amino acids in the course of fermentation. Despite of protein breakdown machinery, some genomic sequencing studies[52, 53] also revealed that *Lb. plantarum* possess genes that encodes for comprehensive pathways for *de novo* biosynthesis of amino acids.

2.6 Volatile Compounds

The consumer inclination towards products and ingredients of natural origin over synthetic has driven attention towards natural production of flavor and aromatic compounds. Lactic acid bacteria are known to produce a wide range of volatile organic compounds including several flavor and aromatic compounds[60]. As discussed before, *Lb. plantarum* is highly heterogeneous and highly adaptable species to different habitats, as well as possess pyruvate dissipating potential that leads to production of wide range of fermentation products. Such versatile nature of this bacteria employs it to be part of several non-dairy based fermented products that aids in production of various desired flavor and

aromatic compounds in different substrates. Volatiles are believed to be products or by-products of metabolic pathways such as generation of hydrocarbons, aliphatic alcohols and ketones from fatty acid biosynthesis, flavors formation from amino acid transformation. Keto acids most commonly formed due to conversion of amino acids by various enzymes, like aminotransferases, amino acid oxidases and dehydrogenases. Phenylalanine is known to be converted to phenyl pyruvic acid (PPA) by aminotransferases cell extract of *Lb. plantarum* and it is further transformed to benzaldehyde which holds a large market in the flavor industry[61]. In a study, it was observed that *Lb. plantarum* was able to produce high amount of lactate along with diacetyl, acetate, ethanol, acetaldehyde, acetone and acetoin in Obushera, a fermented sorghum beverage, while yeast produced high amounts of acetaldehyde and methyl alcohols which indicates *Lb. plantarum* spp. can be healthier alternative to yeasts for plant-based food fermentation[62].

2.6.1 Headspace solid-phase micro extraction (HS-SPME) with Gas Chromatrography-Mass Spectrometry (GC-MS)

Quantitative as well as qualitative analysis of those volatile compounds is also a challenge in regards to obtaining reproducible results for each fermentation batch, as large discrepancies in results can occur due to less sensitive volatile compounds extraction methods. Hence solid-phase micro extraction (SPME) method coupled with gas chromatography mass spectrometry can be a highly sensitive analytical method for analyzing volatile compounds of complex products like fermenting foods and require

small amount of sample for analysis[63]. An improved simple, rapid, precise and sensitive method proposed for semi-quantitative determination of wheat bread volatile compounds was based on headspace solid phase micro extraction-gas chromatography-mass spectroscopy[64]. In addition to extraction method and bacterial metabolism differing metabolite profile among similar food products, to some extent SPME fiber type also influences detection of bacterial volatile compounds. SPME fibers are available in variety of coating with different selectivity strength. Two SPME fiber type carbowax-divinyl benzene and carboxen-polydimethylsiloxane are popular choices for analyzing bacterial volatile compounds, although choice of GC column has less effect than SPME fiber type and fermenting medium on the volatile profile detected for a particular product[65].

Overall environmental factors act as crucial external factors for germination of seeds, therefore water intake for weakening the seed coat, metabolism and seed growth, light and air supply for respiration and temperature are considered as important factors for optimum metabolism during germination. Also modification in quantitative and qualitative phenolic and amino acid composition of legumes would considerably depend on the type of legume. Similarly, starter culture selection is necessary to ensure reproducibility of any fermented food product and compatibility of the growth requirements of bacteria in the fermenting environment for optimum survivability until consumption. Hence processing conditions and probiotic strain selection are important parameters in order to evaluate changes in the bioactive compounds during germination and fermentation. This led to some preliminary experiments prior to the final study.

Several researchers have reported the effect of soaking, germination, cooking and fermentation on bioactive compounds of common legumes[42, 48, 66] however there is very little information available on underutilized pulse horse gram (*Macrotyloma uniflorum*) and impact of processing on the nutritional value of this crop[25]. Therefore, the present study was undertaken to advance the knowledge of impact of some common processes such as soaking, germination and fermentation on major bioactive compounds of horse gram seeds and sprouts. The primary goal of this study is to exploit the nutritional potential of the poor man's pulse in order to obtain a position in the list of common pulses around the world along with being an efficient medium for probiotic bacteria to the general population as well as celiac and lactose intolerant consumers.

3. GERMINATION AND LACTIC ACID BACTERIAL FERMENTATION ENHANCES BIOACTIVE COMPOUNDS IN AN UNDERUTILIZED HORSE GRAM- *Macrotyloma uniflorum*

3.1. Abstract

Horse gram (*Macrotyloma uniflorum* Lam. Verdc.), an underutilized pulse and fodder crop, is native to Southeast Asia and tropical Africa. Impact of germination, cooking and lactic acid bacterial fermentation on health promoting bioactive compounds were determined through analyzing changes in phenolic compounds, protein content, amino acids, free radical scavenging activity, hyperglycemia relevant α-amylase and α-glucosidase enzyme inhibitory activities of both horse gram seeds and sprout extracts. Germination of horse gram seeds for 72 h significantly increased DPPH scavenging activity, essential amino acids content except methionine, tryptophan and threonine, α-amylase and α-glucosidase inhibitory activity compared to raw seeds. Furthermore, cooking also significantly increased DPPH scavenging activity; however, free amino acid content, α-amylase and α-glucosidase inhibition activity in sprouts were lower compared to fresh horse gram sprouts. Fermentation of horse gram sprouts using *Lb. plantarum* NRRL-B-4496 and *Lb. plantarum* NCDO-1193 for 48h further significantly increased the total phenolic content, DPPH scavenging activity, free amino acid content, α-amylase and α-glucosidase inhibitory activity. Results also indicated that fermented sprouts contain certain metabolites, other than phenolic compounds, with potent antioxidant activity. The fermented horse gram sprouts maintained optimum lactic acid bacterial population after

48h fermentation period. Overall germination and lactic acid bacterial fermentation of horse gram seeds and sprouts enhanced targeted health promoting bioactive compounds and has potential to deliver probiotic characteristics to the consumers. Possible mechanisms for underlying nutritional improvement could be due to release of bound compounds, conversion of complex metabolites to its more bioavailable form and *de novo* biosynthesis of certain compounds with health promoting properties.

3.2. Introduction

Hidden hunger is listed currently among the most serious global problem and it is no longer limited to the developing countries. Globally, it is afflicting more than 2 billion individuals, who obtain high calorie from refined food but have micronutrient deficiencies[2]. An affordable solution to these micronutrient deficiencies could be nutritionally enhanced edible dried seeds using pulses. Generally, legume family offer an important and inexpensive source of micronutrients in human diet, as they are rich in proteins, energy, dietary fiber, health promoting bioactives, vitamins and minerals. Legumes have been recognized as the second most important group of crops after cereals[3, 4]. In addition to basic nutritional benefits, pulses provide climate change resilience benefits to the producers in response environmental stress and the rotational cultivation of pulses increases the amount of nitrogen in soil, thereby reducing the requirement of additional fertilizers while improving soil health. Over last decade global pulses production has been increased from 61.5 billion kilograms to 73 billion kilograms with India accounting for a quarter of world production. While several common proteinaceous edible legumes like

soybean, beans, peas, chickpea, cowpea are available globally, traditional and localized underutilized pulses that are more resilient to climate change challenges, exhibits excellent nutrient profiles and demonstrate the potential to become "Food for millennia". We strongly believe pluses can offer new opportunities to contribute to global food and nutritional security, health and environmental challenges[12]. Therefore, Horse gram (*Macrotyloma uniflorum* (Lam.) Verdcourt (Syn., *Dolichos uniflorus* Lam., *Dolichos biflorus* auct. non L.), a pulse with an excellent source of protein, dietary fiber and bioactives was investigated[20]. It is traditionally being used in various herbal therapeutic formulations for treatment of kidney stones, menstrual complexes, diabetes, obesity, hypertension, throat infection, cold and fever[10-12]. Horse gram is also known as an adverse climate tolerant pulse well known for its drought resistance and favorable agronomic characteristics[8]. Despite being a potential source of protein and health promoting properties,, horse gram seeds also contain certain non-nutrient compounds such as phytic acid, tannins, saponins and flatulence causing oligosaccharides that limits the overall nutritional value of this pulse[13]. However, such non-nutrient compounds have been recently investigated to provide several health benefits especially when processed and modified through germination, cooking and fermentation prior to consumption[48, 67, 68]. Therefore, further scientific validation of such processes and their metabolic elucidation has merit for improving the nutritional value of this underexplored pulse for potential health benefits while contributing to advancing climate change resilient crop systems around the world. Germination of legume seeds is expected to improve nutritional value by activation of certain metabolic pathways and their related enzymes for conversion of

bioactive compounds to their simpler or bioavailable forms[28]. To further enhance health promoting properties of plant-based foods, incorporation of lactic acid bacteria (LAB) or probiotics is increasingly gaining importance. Additionally, challenges of allergies to dairy products, cholesterol intake and consumer's vegan preferences seem to divert the interests of food scientists towards non-dairy based probiotic products. Legumes, being good substrate for proliferation of probiotic lactic acid bacteria, legume fermentation using LAB has been extensively studied focusing on development of symbiotic functional foods with superior nutritional benefits[44, 69]. One such LAB namely, *Lactobacillus plantarum* spp. is widely accepted as an ideal starter culture for fermentation of plant materials, due to its ability of decarboxylation, deglycosylation and peculiar probiotic characteristics[70, 71]. Accumulative evidences suggest that soaking, germination, cooking and fermentation enhances bioactive compounds of most common pulses; however, very little information is available in relation to processing effect on the bioactive compounds of horse gram seeds[25, 26]. This expedition was undertaken with goal to make this poor man's pulse to be a one of the household legumes worldwide and demonstrate its potential for conventional as well as industrial applications, thereby highlighting the effect of soaking, germination, cooking and lactic acid bacterial fermentation on major bioactive compounds by determining changes occurring in phenolic compounds, protein content, amino acids, free radical scavenging activity, hyperglycemia relevant α-amylase and α-glucosidase enzyme inhibitory activities, amino acids composition of horse gram seeds, sprouts and fermented sprouts.

3.3. Materials and Methods

3.3.1. Raw Seeds

Dry horse gram (*Macrotyloma uniflorum*) seeds were procured from a local store but originally grown in the regions of Tamil Nadu, India. Raw seeds were cleaned and ground using coffee bean grinder to obtain fine flour that can pass through a 60 mesh sieve. Fine raw seed flour was packed in air tight container and stored at room temperature until analysis.

3.3.2. Chemicals

Folin-Ciocalteu, 3,5-dinitrosalicylic acid, Flavonoids- kaempferol, luteolin, apigenin, quercetin. Phenolic acids- gallic acid, p-coumaric acid, chlorogenic acid standards were purchased from Sigma Chemical Co. (San Diego, USA). o-pthaladehyde (OPA) and amino acids- aspartic acid, glutamic acid, asparagine, glutamine, serine, histidine, citruline, glycine, threonine, arginine, alanine, β-alanine, tyrosine, methionine, valine, tryptophan, phenylalanine, isoleucine, leucine, ornithine, lysine were purchased from Sigma Chemicals Co., (San Diego, USA). Solvents used for HPLC analysis were of HPLC grade.

3.3.3. Processing Methods

3.3.3.1. Germination and Cooking

Cleaned and weighed horse gram seeds were washed using 70% ethanol and with sterile water (three time), prior to soaking in sterile water (1:5) for 8 h at ambient

temperature. After soaking, seeds were placed over moist filter paper in a petri dishes (150 mm) and allowed germination under dark condition for 72 h in a closed incubator set at 25-28 °C with sterile water spray (twice/24 h). Duration of germination was selected to be 72 h based on the preliminary study attempted until 96 h, 72 h germination showed better total phenolic content, DPPH scavenging activity as well as absence of leaves. Samples for raw, soaked seeds and sprouts for each day were collected in triplicates. All the samples were crushed in clean mortar pestle and stored in dark at -20°C until analysis. A portion of horse gram sprouts were subjected to conventional cooking method, by boiling in sterile water at 95±5°C for 30 min until they appeared tender edible sprouts and allowed to cool down to room temperature before adding starter inoculation[25].

3.3.3.2. Inoculum preparation and fermentation

Lactic acid bacterial (LAB) strains *Lb. plantarum* NRRL-B-4496 and *Lb. plantarum* NCDO-1193 were obtained from Dept. of Plant Sciences, North Dakota State University. USA. The frozen cell culture was sub cultured thrice using MRS broth (Sigma, USA) by incubating at 37°C for 48 h under micro-aerobic conditions in order to be reactivated. The active cells were harvested and re-suspended in sterilized water in order to obtain 8 $\log_{10}$ cfu/ml. This cell suspension was used as starter culture for horse gram sprout fermentation. Cleaned uncooked horse gram sprouts as well as freshly cooked sprouts were weighed and inoculated by addition of lactic acid bacterial cell culture suspension (2:3 w/v). Fermentation duration was carried out at 37 °C for 48 h. Fermentation time was finalized based on preliminary study that determined LAB

population showed decline after 48 h of sprout fermentation. All the fermentation samples were stored at -20°C until further analysis. For natural or spontaneous fermentation, a portion of cooked and uncooked horse gram sprouts were inoculated by sterile water, volume equal to the amount of culture suspension. Samples of cooked and uncooked, LAB mediated and naturally fermented sprouts were collected in triplicates.

3.3.4. Analytical Methods

3.3.4.1. Water Hydration Capacity and Germination Rate

Dry horse gram seeds were cleaned by discarding damaged or cracked seeds and one hundred and seventy seeds were counted (N) and weighed (W_1). Raw seeds were soaked in a mason jar filled with sterile water (1:5, w/v) for 8 h at ambient temperature. After 8 h, water was drained and superfluous water was removed using paper towel. The soaked were weighed (W_2) and hydration capacity was calculated using the following expressions

$$\text{Hydration Capacity (g/seed)} = \frac{W_2 - W_1}{N}$$

3.3.4.2. Microbiological analysis and Determination of pH

The fermented horse gram sprout samples (11g) were suspended in 99 ml autoclaved peptone water (0.1%) and mixed well. Serial dilutions of product suspension were prepared using sterile peptone water. One ml of appropriate dilutions was added to labelled sterile petri dishes followed by pouring molten MRS agar (Sigma, USA). After proper mixing of sample dilution and molten agar, all the petri dishes were overlaid by

approx. 5ml molten MRS agar and incubated at 37°C for 48 h ± 1 h. The selective colonies were counted and expressed as logarithmic colony forming units per gram (log cfu/g) of sample. pH of all fermented samples was determined using pH meter.

3.3.4.3. Extraction and Determination of changes in phenolic compounds

Phenolic compounds were extracted from horse gram raw seeds flour, crushed samples of soaked seeds, sprouts, cooked and uncooked fermented sprouts using 80% aqueous methanol containing 1% HCl (1:30, w/v) by reflux extraction in a boiling water bath for 90 min. The extracts were filtered through Whatman filter paper No. 1 and stored in -20°C until further analysis. The total phenolic content in the clear extracts were determined according to Folin-Ciocalteu method [72]. Gallic acid (GA) was used as the reference standard. The concentration of phenolic compounds in each sample was expressed as milligrams gallic acid equivalents (GAE)/g of dry weight of sample.

3.3.4.3.1. Quantification of Phenolic compounds by HPLC-DAD

Quantification of phenolic compounds was achieved by using analytical reversed-phase chromatography Waters HPLC system equipped with Waters 717 auto sampler, Waters binary pump coupled with Waters photodiode array detector, all controlled by Empower Pro software as data processor. The auto sampler was set at room temperature, while the chromatographic separation was done on Waters Symmetry® C18 (4.6µm x 250 mm) column with particle size of 5µm. The mobile phase included solvent A-30mM phosphoric acid and solvent B-acetonitrile: water (60:40, v/v). The flow rate was set to

0.5ml/min for total run time of 32 min and injection volume of 40µl. The elution of binary solvent was conducted in gradient pattern, starting 70% of solvent A for 3 min changing it to isocratic 100% solvent B for 26min and equilibrated for 6 min to start the next sample. Flavonoids and phenolic acids standards were co-eluted therefore UV absorbance of 340nm was finalized to quantify all phenolic compounds.

3.3.4.4. Determination of changes in free radical scavenging inhibition activity

The DPPH scavenging activity of the acidic methanolic extracts was determined using the modified method[73]. Sample extracts (10µl) was mixed with methanol (90ul) and sample blank by adding 270µl methanol. Ascorbic acid was used as standard (20 µg/mL) in varying concentration of 0.1, 0.2, 0.4, 0.8, 1.6, 2µg/ml. The absorbance decrease was measured at 515nm in a microtiter plate reader. Free radical scavenging activity was expressed as µg of ascorbic acid equivalent/g dry weight of sample.

3.3.4.5. Determination of changes in protein content and free amino acids

Protein extraction for raw horse gram seeds flour, sprouts, cooked and fermented sprout samples was performed by isoelectric precipitation method[74] with some modifications. The procedure involves adjustment of aqueous sample mixture (1:10) to pH 8.5 – 9.0 using sodium hydroxide (1N) solution followed by placing all alkaline sample mixture in shaking water bath for 60 min at room temperature for stabilization of protein. Centrifugation for 15min at 400g separates sample residues from the stabilized protein solution. Further adjustment of pH at 4.3–4.5 using hydrochloric acid (1N) led to

precipitation of protein in the mixture. Protein precipitate was separated by repeated centrifugation (400g for 15min) obtaining a pellet, which was later washed using acidic water (pH 4.5) and then neutralized in filtered deionized water (pH 6.6-7.0). The precipitate free clear solution was subjected to protein analysis. Lowry's Assay[75] was used to quantify total protein content in the raw seed flour, sprouts, cooked and fermented horse gram sprouts samples in order determine the effect of each process on assayable protein content. BSA (bovine serum albumin) at 1mg/ml was used as the reference standard. Absorbance at 650nm was recorded using microtiter plate reader (BioTek Instruments, Inc, USA). Free amino acids extraction was carried out using 80% methanol under ice cold conditions. The extraction procedure involved repeated high speed vortexing and sonication in ice cold water (60min) followed by high speed temperature controlled centrifugation at 4°C (1000rpm for 20min). A modified ninhydrin assay[76] was used to estimate the amount of free amino acids in the all the samples in order to determine changes occurring in peptide complexes due soaking, germination, cooking and fermentation processes. Glycine was used as the reference standard. The results for protein content and free amino acid is expressed in mg BSA/g of dry weight of sample and mg glycine equivalent/ g of dry weight sample respectively.

3.3.4.5.1. Quantification of Amino Acid Composition by HPLC

Sample extracts were further diluted by adding HPLC grade methanol (80%) and centrifuged for 15 min at 400g. Clear diluted extracts were split in two sample vials (100µl) for HPLC duplicate injections in order to minimize interaction time between the

derivatization and injection on to HPLC system thus limiting any loss in fluorescent signal intensity. Fluorescent derivatization of amino acids is completed using o-phthalaldehyde (OPA). Derivatizing solution was prepared dissolving 50mg of OPA in methanol, 40mM sodium borate buffer (pH 9.5), (3.1%) Brij-35 surfactant and 50µl 2-mercaptoethanol to make the final working solution, this solution should not be used prior to 8 h and post 72 h of preparation. The derivatization of each sample extracts was carried out in sample vials by addition of equal amount of OPA into each sample vials by an automated fashion using Perkin Elmer autosampler set at room temperature.

3.3.4.5.2. Chromatographic system

Perkin-Elmer HPLC system equipped with an auto sampler, binary pump coupled with a fluorescence detector and TotalChrom software (v. 6.3) as data processor was used for amino acid analysis. The HPLC method used for chromatographic quantification of 21 amino acids was adopted from method developed earlier with some modifications[77]. The auto sampler was set at room temperature, while the chromatographic separation of derivatized amino acids was done on a C8 column (Agilent Zorbax- Eclipse XDB-C8, 4.6 x 150mm) with particle size of 5µm and required two mobile phases which includes solvent A sodium acetate tetrahydrate buffer with pH adjusted to 5.7 with 2M NaOH and solvent B-acetonitrile. The injection volume was 5.0µl and the flow rate was 0.7ml/min for total run time of 28 min. The detection was performed flourometrically with an excitation of wavelength 240nm and an emission wavelength of 340nm.

3.3.4.6. Determination of α-amylase and α-glucosidase enzyme inhibitory activities

Methanol extracts used for free amino acids analysis were also subjected to enzyme inhibition activity analysis. The α-amylase inhibitory activity was determined by an assay modified from the Worthington Enzyme Manual[78] with some modifications. Twenty microliters of sample and substrate 45µl starch solution (1%) was added to only sample wells excluding sample blanks. Total volume was made up to 185µl using (0.9%) saline and incubated at room temperature for 5 min in 96-wells microtiter plate. After pre-incubation, 45µl of α-amylase enzyme solution (10mg/ml) was added to all sample wells including sample blanks. The reaction mixtures were then incubated at room temperature for 60min. The enzyme-substrate reaction was stopped with 50µl 3, 5-dinitrosalicylic acid color reagent and incubated at 75°C for 60min. The reaction mixture was then cooled down to room temperature prior to measuring absorbance at 540nm in a microtiter plate reader (BioTek Instruments, Inc. USA). The absorbance of sample blanks (buffer instead of enzyme solution) and control (buffer in place of sample extracts) was recorded and calculated to determine % inhibition activity of samples. Acarbose (1mg/ml) used as positive control. The α-glucosidase inhibitory activity was determined by an assay modified from the Worthington Enzyme Manual[78, 79] with some modifications. A volume of 50µl of sample extract diluted with 150µl 0.1M potassium phosphate buffer (pH 6.9) and varying concentration of acarbose (1mg ml^{-1}) containing 30µl α-glucosidase enzyme (0.1U ml^{-1}) was incubated in 96-wells microtiter plate at room temperature for 10min. After pre-incubation, 50µl of 4-Nitrophenyl-a-d-glucopyranosidase (NPGP) 2.5mM solution in 0.1M potassium phosphate buffer (pH 6.9) was added to all sample and

standard wells excluding sample blanks. The reaction mixtures were incubated at room temperature for 30min. Control wells containing 50µl of 0.1M potassium phosphate buffer (pH 6.9) were treated similar to standards and samples. The absorbance at 405nm was recorded by microplate reader (BioTek Instruments, Inc, USA). Acarbose (10 mg ml^{-1}) was used as positive control. The α-amylase and α-glucosidase inhibitory activity is expressed as percentage of inhibition and calculated according to the equation below.

$$\%\ Inhibition = \frac{Abs.(control)\ -\ Abs.(extract)}{Abs.(control)} \times 100$$

3.3.4.7. Statistical Analysis

All the experiments and analysis were performed in three independent replications with triplicate samples. The results were expressed as mean±standard error. The mean comparison was performed using JMP Statistical Discovery™ (SAS) Pro. v.12.0 software package and processed by one way analysis of variance (ANOVA) to evaluate significant differences (p<0.05) and Student;s t test to compares means of all samples.

3.4. Results and Discussion

Raw horse gram seeds are reported to be good source of dietary antioxidants as well as have compounds with potent antimicrobial activity against several pathogens. It has also been reported to possess functional metabolites against serious diseases like diabetes, hypercholesterolemia and obesity [12, 13]. Despite of numerous health benefits of dried edible seeds like horse gram, the major drawback in using the raw grains is that they are not readily rehydrated and are difficult to cook, as the seed coat is hard thus requires

longer cooking time. Dry horse gram seeds have been reported to contain high levels of anti-nutritional compounds like phytic acid, tannins, polyphenols that may challenge worldwide acceptance of horse gram for human consumption[80]. Moreover, there are several scientific convincing evidences validating conventional processing methods like soaking, germination and cooking which are capable of significantly reducing the content of undesirable compounds thus enhancing acceptability and nutritional value of the legume[13]. During such crucial though simple processes, dry seeds undergo critical chemical changes resulting in modification of texture, nutritional composition, digestibility and palatability [26, 30]. Therefore, these conventional processing methods were proposed for the present study in order to improve nutritional quality of this poor man's pulse. Soaking of grain legume is generally practiced before dry seeds are germinated or cooked, in order to render them edible and ensure sensory quality. Pre-soaking seeds facilitates upregulation of the water content in the seed that aids germination process to a greater extent, however response to soaking water or hydration varies with different species of pulses[81]. Such response can be measured in terms of hydration capacity (HC), explained as the amount of water that whole seeds absorb after soaking in excess water at room temperature. Knowledge of hydration capacity of an underexplored pulse like horse gram benefits prompt acceptance by food technologists and consumers, since it plays a major role in development of appetizing food products. In the present study, hydration capacity and germination rate were determined for horse gram seeds. It was observed that germination rate under laboratory conditions varied between 94% to 97% and hydration capacity was around 0.0334g/seed, similar results were observed in a previous study

assessing physiochemical and functional properties of four different varieties of horse gram[82]. Soaking of raw horse gram seeds also led to significantly increase free radical scavenging activity while soaked excess water also showed some antioxidant property that might be due to leaching of some polyphenols in the external medium during soaking (**Table 1**.). Soaked water may or may not be discarded in traditional practices, some scientific studies showed decrease in polyphenol content of horse gram and other lentils during soaking, hence it may be better to reuse the soaked water instead of draining[25-28, 83]. Furthermore, germination of horse gram soaked seeds in laboratory conditions, significantly increases DPPH scavenging activity (138.64 mg AA eq./g DW) of sprouts compared to raw seeds (95.97mg AA eq./g DW). Although results of total phenolic content did not appreciably showed differences amongst raw, soaked and germinated seeds (**Table 1**), this clearly indicates presence of certain metabolites other than phenolic compounds in sprouts with strong antioxidant activity pertaining to the escalation observed only in DPPH inhibition during analysis. Such results are in accordance of previous studies on horse gram[26, 84], soybean sprouts[37] and lentils[36]. Dry seeds store reserve nutrients like starch, proteins and lipids that are degraded in the cotyledons or endosperms during germination, products released are translocated to the developing axis that nourishes the sprout to grow into a seedling and subsequently into a new plant. During sprouting, certain compounds are also synthesized or released namely secondary metabolites that serve as defense mechanism of the small underdeveloped growing plant, protecting it from several stress reactive oxygen species. Therefore, the sprouted seeds or pulse sprouts have gained more importance over raw dry seeds not only due to it higher

Table 1 Changes in total phenolic content, DPPH free radical scavenging activity, protein content, free amino acids due to soaking, germination, cooking and lactic acid bacterial fermentation of horse gram seeds.

Samples*	Total Phenolic Content (mg GAE/g)	DPPH (mg AAE/g)	Protein Content (mg BSA eq./g)	Free Amino Acids (mg Gly.eq./g)	pH	Log_{10} CFU/ml
RS	4.67 ± 0.18^{de}	79.37 ± 3.42^{g}	30.54 ± 1.02^{a}	4.98 ± 0.22^{e}	6.32 ± 0.04^{bc}	-
SS	4.51 ± 0.22^{e}	132.47 ± 2.98^{f}	23.76 ± 0.64^{c}	6.18 ± 0.32^{de}	6.52 ± 0.04^{a}	-
SW	0.86 ± 0.06^{f}	23.38 ± 0.05^{h}	8.95 ± 0.51^{h}	0.30 ± 0.01^{f}	-	-
GS	5.57 ± 0.19^{de}	164.90 ± 6.30^{e}	27.25 ± 1.96^{b}	16.60 ± 1.02^{ab}	6.27 ± 0.02^{c}	-
CS	4.70 ± 0.19^{de}	228.18 ± 7.73^{d}	15.03 ± 1.13^{ef}	11.12 ± 0.78^{c}	6.43 ± 0.07^{ab}	-
Cooked Fermented sprouts						
CF4496	5.80 ± 0.20^{d}	261.51 ± 8.56^{c}	13.08 ± 0.85^{fg}	6.75 ± 0.23^{de}	4.25 ± 0.04^{d}	7.85 ± 0.11^{b}
CF1193	5.41 ± 0.16^{de}	264.37 ± 9.12^{c}	11.68 ± 0.61^{gh}	6.61 ± 0.24^{de}	3.76 ± 0.06^{e}	7.93 ± 0.06^{b}
CFN	7.78 ± 0.98^{c}	316.18 ± 12.55^{a}	14.52 ± 1.13^{ef}	7.06 ± 0.53^{d}	3.33 ± 0.04^{gh}	3.91 ± 0.23^{c}
Uncooked Fermented sprouts						
UCF4496	7.87 ± 0.24^{c}	265.43 ± 8.53^{c}	17.98 ± 0.77^{d}	16.50 ± 1.19^{ab}	3.48 ± 0.03^{f}	8.08 ± 0.08^{b}
UC1193	9.24 ± 0.15^{b}	291.26 ± 6.17^{b}	15.7 ± 0.60^{def}	16.16 ± 1.05^{b}	3.41 ± 0.03^{fg}	8.38 ± 0.07^{a}
UCFN	10.98 ± 0.87^{a}	330.27 ± 6.82^{a}	16.38 ± 0.99^{de}	18.50 ± 0.96^{a}	3.21 ± 0.07^{h}	4.19 ± 0.07^{c}

RS-Raw Seeds, SS-Soaked Seeds, SW-Soaked water, GS-Germinated Seeds, CF4496-Cooked sprouts fermented by *Lb. plantarum* NRRL-B-4496, CF1193-Cooked sprouts fermented by *Lb. plantarum* NCDO-1193, UCF4496- Uncooked sprouts fermented by *Lb. plantarum* NRRL-B-4496, UCF1193- Uncooked sprouts fermented by *Lb. plantarum* NCDO-1193, CS-Cooked sprouts, CFN-Cooked sprouts fermented naturally, UCFN- Uncooked sprouts fermented naturally.

Values are means of three independent fermentation batches ± standard error.

Lower case superscripts indicate significant differences ($p<0.05$*) among samples.

nutritional value but also due to health promoting benefits[32]. Such metabolic changes were extensively studied in past for common legumes like mung beans, soybeans, however there are several factors like genetic make-up of the seed, environment that affects the metabolic pathways a dormant seed undergoes during its development into a sprout. A study investigated changes in polyphenols and *in vitro* antioxidant activity during 24 h germination of horse gram (*Macrotyloma uniflorum*) and green gram (*Vigna radiata*)[31]. It was reported that total phenolic content in horse gram sprouts was higher compared to its raw seeds, as well as considerable increase in *in vitro* antioxidant activity was observed in both legumes during germination. Such an increase was accounted to the metabolic changes occurring in the dormant raw seeds during the process of sprouting that may have formed or released several bioactive compounds with good antioxidant activity. Therefore, the current study further adds to the rationale towards proposing that the germination process significantly increases antioxidant content in horse gram[17, 35]. In contradiction to this, one study reported lower total phenolic content in horse gram sprouts compared to its raw seeds, however such results can be due to protein-phenolic interactions that lead to formation of complexes exhibiting structural and functional changes in both the compounds. Such complexes formed influence determination of total phenolic content by reducing analytical recovery of analytes from food matrix like pulses with high amount of protein content. Moreover, taking into account that there are several phenolic compounds in horse gram seeds and sprouts investigating the individual phenolic compounds was of greater importance in order to clearly understand the effect of germination on horse gram seeds.

Cooking quality of seeds is one of the important parameters of legumes that varies to very high extent among all the legumes. Depending on the cooking quality of the legumes, cooking time differs widely among genotypes and is affected by the permeability of seed coat and the cotyledons to hot water [30]. Thermal processing of legumes modifies its structural and functional properties suggesting increase in the digestibility of foods but loss of certain heat-labile nutrients is inevitable[18]. Interestingly, present study showed conventional cooking increased DPPH scavenging activity of horse gram sprouts to 240.62 mg AA eq./g DW. Such can be attributed to either release of some bound non-phenolic compounds in sprouts during cooking [85] or heat mediated alteration of the some phenolic compound structure that plays a major role in reaction mechanism between the antioxidants and DPPH during analysis[18]. Therefore, we observe a significant increase in DPPH scavenging activity in cooked horse gram sprouts compared to uncooked sprouts. Another reason suggested could be due to interaction of other phytochemicals and thermally altered phenolic compounds[18]. In a previous study [25] it was observed that cooking significantly reduces the polyphenol content in horse gram sprouts, however the loss was much less in the horse gram sprouts compared to its raw seeds subjected to conventional cooking suggesting cooking sprouts could be a better option over cooking legume seeds. Such reduction might be due to either binding of polyphenols with other organic substances like protein or results from alterations caused in the chemical structure of polyphenols that render them incapable of giving the chemical color reaction measured by the analytical method. Two LAB strains *Lactobacillus plantarum* NRRL-B-4496 and *Lactobacillus plantarum* NCDO 1193 were used to ferment horse gram sprouts, cooked

and uncooked. The two strains were chosen as they were found to efficiently ferment horse gram sprouts in our preliminary study when compared with two other probiotic strains *Lactobacillus helveticus* ATCC 12046 and *Lactobacillus helveticus* ATCC 10797. Effect of fermentation on free radical scavenging activity of both cooked and uncooked sprouts was also determined. It was observed that DPPH inhibition was significantly higher in lactic acid bacterial fermented cooked sprouts compared to the non-fermented cooked sprouts, whereas total phenolic content did not cause much change in cooked sprouts subjected to lactic acid bacterial fermentation (**Table.1**). Hence such distinct relationship between total phenolic content and DPPH scavenging activity in fermented cooked sprouts might be due to antioxidative metabolites other than phenolic compounds synthesized during fermentation by *Lactobacillus plantarum* spp. Lactic acid bacteria are commonly used for fermentation of legumes and sprouts, Moreover, it has been reported that lactic acid fermentation have diverse effect on antioxidant activity of different legumes[48]. In addition the present study some other studies have reported significant increase in the antioxidant activity of lupin flour, autoclaved soybean seeds fermented by *Lactobacillus plantarum*.[48, 86, 87] while some studies showed no change or reduced antioxidant activity of *Lactobacillus plantarum* fermented lentil, kidney beans and soybean[15, 49]. Such assorted levels of antioxidant activity in fermented edible legumes suggest that modification of phytochemicals during fermentation largely depends on type of legume as well as type of starter culture.

Lactobacillus plantarum is one of those influential species of probiotics which is capable of producing antioxidants in the growth medium, two of such antioxidants were

isolated and identified in a recent study that demonstrated DPPH free radical scavenging activity from pure *Lactobacillus plantarum* cultures using MS, NMR and HPLC. 3-(4-hydroxyphenyl) lactic acid and indole-3-lactic were identified in this study, however it was also observed that their production by *Lactobacillus plantarum* is due to strain-specific mechanism[88]. Therefore, it was interesting to the identify antioxidants present in fermented horse gram sprouts in the current study.

Amongst both LAB fermented sprouts, uncooked sprouts fermented by *Lb. plantarum* NCDO 1193 showed highest total phenolic content (9.23 mg GAE eq./g DW) followed by uncooked sprouts fermented by *Lb. plantarum* NRRL B-4496 (7.87mg GAE eq./g DW), cooked sprouts fermented by *Lb. plantarum* NRRL B-4496 (5.8 mg GAE eq./g DW) and then cooked sprouts fermented by *Lb. plantarum* NCDO 1193 (5.4 mg GAE eq./g DW). Uncooked sprouts fermented using *Lactobacillus plantarum* showed higher levels of total phenolic content compared to cooked fermented sprouts, this might be due to nutrient depletion during thermal processing that may limits lactic acid bacterial growth during fermentation of cooked sprouts, leading to limited metabolic activities of LAB. Although no significant difference in total phenolic content was observed between two strains fermenting cooked sprouts, whereas both types of uncooked sprouts differed significantly (**Table.1**). Such results can be attributed to the changes occurring in phenolic compounds during cooking followed by different metabolic activities of two different strains of *Lb. plantarum* spp. as well as interfering microflora carrying out fermentation of fresh sprouts. Since this study is the first attempt of horse gram sprout fermentation, there are no reports available, however similar results were reported in eight common

edible fermented legumes including black gram, cowpea, kidney bean, lentil, rice bean, runner bean, black and yellow soy beans suggesting significant increase in the amount of phenolic compounds due fermentation by *Lb. plantarum* WCFS1[48].

Lactic acid bacteria like *Lactobacillus plantarum* spp. are able to decarboxylase the hydroxycinnamic acids, *p*-coumaric and caffeic acids[56]. Molecular characterization studies on *L. plantarum* revealed that this bacterium species possess inducible gene encoding *p*-coumarate decarboxylase (PadA) having *p*-coumaric acid decarboxylase (PAD) activity which was found to be different in structure, specificity of substrate, expression and N-terminal amino acid sequence compared to other characterized decarboxylases from *Saccharomyces cerevisiae, Bacillus pumilus* and *Psuedomonas fluorescens*. A study observed an increase in the bioactive compounds of soybean (*Glycine max* cv. Merit) and mung beans (*Vigna radiate* [L]) due to fermentation by *Lactobacillus plantarum* CECT 748 T that converted glycosylated isoflavones into bioactive aglycones [44]. Fermentation hydrolyzes complexes of polyphenols into simpler and biologically more active compounds due to the ability of fermenting bacteria of deglycosylation. Since conjugate glycosides are not absorbed intact across the intestine of healthy adults and they need to be hydrolyzed, releasing aglycones which are observed to be in more bioactive forms that can be easily absorbed by the intestinal lining[55], it is of greater importance to elucidate possible deglycosylation, decarboxylation during lactic acid bacterial fermentation in the present study.

Traditionally natural fermentation of legumes is widely practiced to produce many fermented popular streets foods like smelly bean curd, broad bean pastes in China. In this

study, a portion of freshly cooked and uncooked sprouts were inoculated with sterile water to allow natural fermentation by the microorganisms naturally inhabiting sprouts also showed significant differences in total phenolic content, although no significant differences were observed in free radical scavenging activity of cooked and uncooked naturally fermented sprouts. Despite of highest total phenolic content (10.97mg GAW/g DW) and highest antioxidant activity (356.36 mg AA eq. g DW) showed by uncooked naturally fermented sprouts, it contains strong stinky odor which indicates production certain volatile compounds during natural fermentation. Horse gram sprouts fermented using *Lactobacillus plantarum* had pleasant odor unlike sprouts fermented spontaneously. Such discrete aroma of two different types of fermented products in this study drives attention to evaluate metabolic activities involving production of volatile flavor during fermentation of horse gram sprouts.

Due to probable accelerated metabolic activity during germination and fermentation in horse gram seeds and sprouts, it was important to further investigate the changes occurring in the phenolic compounds in all the samples. Therefore, horse gram seeds, sprouts as well as fermented sprouts extracts were subjected to HPLC-DAD-ESI-MS analysis. In particular, changes in phenolic compounds of horse gram sprouts and fermented horse gram sprouts during germination and fermentation has not been described in literature so far, we have compared horse gram dry seeds with soaked seeds, horse gram sprouts, lactic acid bacterial fermented horse gram sprouts as well as naturally fermented sprouts. **Table 2** shows the list of chromatographic peaks, retention time of tentatively identified compounds in the horse gram samples with molecular ions in positive ionization

as well as concentration of each compound in different samples. It was observed that chromatographic peaks remained consistent in all samples throughout the all types of processing except peak 10 which was observed only on horse gram fermented and non-fermented sprouts. The chromatogram peak 2 (**Figure 1**.) showed molecular ion peak at m/z 309 $[M + H]^+$ and fragment ion at m/z 147 in its positive ionization mass spectrum. The difference between the molecular ion and fragment ion (162 mass units) revealed the occurrence of hexose sugar (glucose/galactose) attached to aglycone therefore identified as probable flavonoid glucoside. Peak 3, 4, 5, 6, 7, 8 and 9 showed same molecular ion at MS m/z 287 or MS/MS m/z 284, 255, 227 that represents to kaempferol fragments ions also descried in pollen typhae and fenugreek seeds[89]. Hence, all the above mentioned compounds peaks are flavonoid kaempferol glycosides except for peak 9 which was identified as kaempferol aglycone based on authentic standard retention time and mass spectrum. Peak 3 presents a molecular ion at m/z 741 $[M + H]^+$ followed by m/z 287 The MS data showed fragments ion signals in MS/MS at *m/z*: 284, 255, 227 $[M - H]^-$ so compound is tentatively identified as kaempferol-3-O-robinoside-7-O-rhamnoside. Peak 4 showed the molecular ion peak at m/z 595 with fragments at 449, 287 in its mass spectrum. The difference in the mass units between fragment ions with aglycone fragment ions of kaempferol indicates presence of kaempferol-7-rutinoside. Similar fragment ions were observed for peak 5 and 6 with molecular ion at m/z 595 followed by 449, 287 fragments that were tentatively identified as kaempferol-3-robinoside and kaempferol-3-glucoside-7-rhamnoside respectively.

Table 2 Changes in phenolic compounds (µg/g DW) due to soaking, sprouting, cooking and lactic fermentation of horse gram seeds.

								Cooked Fermented Sprouts			Uncooked Fermented Sprouts			
#	RT	Molecular ion	Tentatively Identified Compounds	RS	SS	SW	GS	CS	CF4496	CF1193	CFN	UCF4496	UCF1193	UCFN
1	2.1	**[118.086]+**	**Unknown compound**	381.60 ±18.71^{de}	203.72 ± 7.19^{ef}	8.39 ± 1.14^{f}	609.677 ±52.16^{bcd}	718.93 ±150.81^{abc}	813.834 ±170.64^{cd}	700.189 ±134.55^{abc}	592.978 ±68.86^{cd}	647.678 ±95.27^{bc}	834.170 ±77.04^{ab}	908.152 ±108.95^{a}
2	3.2	**[309.144]+**	**Unknown compound**	121.29 ± 14.7^{c}	93.35 ± 20.12^{c}	16.60 ± 1.96^{c}	766.62 ± 89.15^{ab}	586.05 ± 90.70^{b}	697.90 ± 139.65^{b}	546.34 ± 78.54^{b}	730.81 ± 92.65^{b}	701.40 ± 170.43^{b}	1021.22 ± 152.43^{a}	1036.68 ± 124.06^{a}
3	9.0	**[741.223]+**	**Kaempferol-3-O-robinoside-7-O-rhamnoside**	270.93 ± 26.10^{cd}	300.46 ± 29.52^{c}	ND	484.70 ± 17.01^{ab}	330.41 ±27.28^{c}	353.58 ± 23.50^{c}	261.81 ±27.38^{cd}	185.52 ± 47.17^{d}	547.44 ± 39.15^{a}	457.44 ± 37.45^{ab}	446.83 ± 56.36^{b}
4	12.1	**[595.163]+**	**Kaempferol-7-rutinoside**	16.0 ± 3.17^{d}	8.6 ± 4.13^{d}	ND	2616.2 ± 197.75^{ab}	2269.3 ± 165.05^{b}	2621.4 ± 129.67^{ab}	1717.1 ± 161.67^{c}	1470.1 ± 308.87^{c}	2775.3 ± 196.33^{ab}	2294.2 ± 186.34^{b}	2944.2 ± 304.60^{a}
5	12.9	**[595.167]+**	**Kaempferol-3-O-robinobioside**	143.53 ± 15.26^{c}	189.33 ± 18.44^{ab}	ND	159.80 ±10.09^{bc}	94.01 ± 10.77^{d}	99.50 ± 9.48^{d}	70.61 ± 13.17^{d}	15.24 ± 16.45^{e}	216.47 ± 15.30^{a}	165.86 ± 15.11^{bc}	169.11 ± 23.45^{bc}
6	13.5	**[595.166]+**	**Kaempferol-3-glucoside-7-rhamnoside**	46.25 ± 4.67^{e}	48.74 ± 7.65^{e}	ND	332.30 ± 15.38^{b}	296.63 ± 15.01^{bc}	245.99 ± 9.57^{c}	266.87 ± 16.31^{bc}	165.86 ± 31.78^{d}	459.44 ± 35.37^{a}	424.23 ± 44.00^{a}	436.05 ± 52.81^{a}
7	14.3	**[449.107]+**	**Kaempferol-3-O-glucoside**	25.3 ± 3.44^{d}	14.5 ± 3.93^{d}	ND	1219.3 ± 195.96^{a}	1226.0 ± 179.95^{a}	748.1 ± 146.29^{bc}	749.7 ± 109.35^{bc}	492.1 ± 102.42^{c}	850.6 ± 125.56^{bc}	931.3 ± 171.73^{ab}	934.8 ±159.21^{ab}
8	17.2	**[549.121]+**	**Kaempferol 7-(6''-p-succinylglucoside)**	16.262 ± 4.30^{cde}	2.071 ± 1.99^{e}	ND	79.81 ± 16.252^{e}	45.955 ± 11.51^{a}	32.240 ± 8.21^{bcd}	37.296 ± 9.82^{bc}	7.892 ± 11.03^{de}	44.480 ± 12.148^{b}	1.837 ± 14.03^{e}	ND
9	22.2	**[287.054]+**	**Kaempferol (aglycone)**	319.3 ± 28.62^{e}	374.3 ±41.51^{e}	ND	1180.5 ± 71.72^{d}	1932.9 ± 138.73^{c}	2036.1 ± 104.30^{c}	2224.5 ± 134.62^{bc}	2117.9 ± 323.13^{bc}	2323.6 ± 321.90^{bc}	2565.1 ± 287.99^{b}	3750.2 ± 187.93^{a}
10	27.2	**[389.082]+**	**Unknown compound**	ND	ND	ND	74.95 ± 12.5^{bc}	77.82 ± 17.12^{bc}	110.11 ± 29.77^{bc}	80.39 ± 24.14^{bc}	58.24 ± 24.07^{cd}	128.43 ± 33.91^{ab}	93.10 ± 18.22^{bc}	174.85 ± 36.54^{a}

RS-Raw Seeds, SS-Soaked Seeds, SW-Soaked water, GS-Germinated Seeds, CF4496-Cooked sprouts fermented by *Lb. plantarum* NRRL-B-4496, CF1193-Cooked sprouts fermented by *Lb. plantarum* NCDO-1193, UCF4496- Uncooked sprouts fermented by *Lb. plantarum* NRRL-B-4496, UCF1193- Uncooked sprouts fermented by *Lb. plantarum* NCDO-1193, CS-Cooked sprouts, CFN-Cooked sprouts fermented naturally, UCFN- Uncooked sprouts fermented naturally.
Values are means of three independent fermentation batches ± standard error.
Lower case superscripts indicate significant differences ($p<0.05$*) among samples.

Peak 7 showed molecular ion at m/z 449 with its fragment ions at m/z 287 which again represents kaempferol aglycone fragments in MS/MS m/z 284, 255, 227 $[M - H]^-$ tentatively identified as kaempferol-3-O-glucoside. Another probable kaempferol glucoside identified as kaempferol 7-(6”-p-succinylglucoside) as peak 8 with molecular ion at m/z 549 followed by aglycone fragment ion at m/z 287. Peak 9 was identified and confirmed based on authentic standard as kaempferol aglycone with molecular ion at m/z 287 $[M + H]^+$. Polyphenols of pulses mainly concentrated in the seed coat comprising of proanthocyanidins, flavonols, flavanones and hydroxycinnamic acids. The present study identified flavonols in horse gram seeds similar to those found in black beans, white beans and other beans[90]. Several studies have reported that qualitative as well quantitative phenolic compounds profile in pulses are influenced by processing conditions. Germination also have profound effect on polyphenols in legumes or pulses such as common beans (*Phaseolus vulgaris* L.)[91]. Maturity of the pod before subjecting the seeds to drying process for preservation purposes also significantly affects the amount of storage polyphenols[92]. With progressing stages of maturity concentration of both glucosides as well as aglycones increases significantly in broad bean pods. While germination or sprouting de novo synthesis of flavonols which in turn significantly increases the amount of glycosides as well as certain aglycone forms of flavonols in Mexican common beans[91]. In the present study, comparable effect of germination was also observed in horse gram seeds (**Table 2**.), where most of the kaempferol glucosides in horse gram sprouts extracts except for kaempferol-3-O-robinoside were higher compared to dry seed flour extracts.

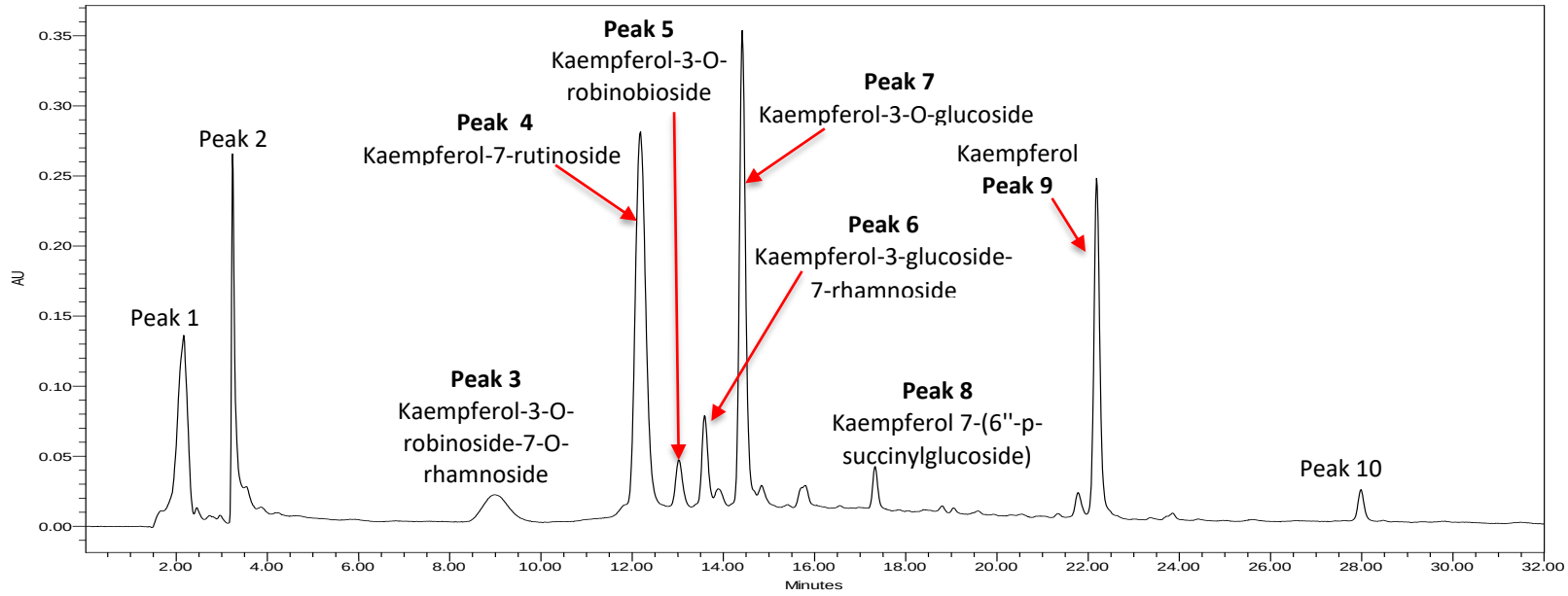


Figure 1 Chromatogram of tentatively identified phenolic compounds in horse gram sprouts using HPLC-DAD-ESI-MS Analysis

Since pulses are still considered under hard-to-cook group of foods, often subjected to vigorous cooking such as pressure cooking hence impact of such heat processing on polyphenol composition is matter of interest for several legume researchers. In the present study, cooked horse gram sprouts showed some of the glucosides showed significant increase while aglycone kaempferol reduced significantly during cooking. Similar increase in the kaempferol glucosides was observed in cooked common bean (*Phaseolus vulgaris* L.) whereas kaempferol aglycone observed a decrease by 5-51% [93, 94]. In contrast, fermentation of cooked sprouts although reduced the levels of some glucosides (**Table 2**.) while other increased significantly including kaempferol aglycone. Common dietary sources of kaempferol includes beans and soybeans apart from fruits and vegetables. Kaempferol exhibit anti-inflammatory, antitumor, antioxidant, antimicrobial, cardioprotective, neuroprotective as well as antidiabetic activities[95]. In particular, kaempferol-3-glucoside (astragalin) possesses potent neuroprotective properties against diseases such as Parkinson's and Alzheimer's[96, 97].

Horse gram protein content divides into albumin-globulin fraction about 75.27%-78.76%, while glutelin around 9.93-17.52% and residual 6.96-11.30% protein[98]. As discussed before, processes like soaking, germination reduces the amount of anti-nutritional constituents amongst which some are lectin and trypsin inhibitors. It is believed that proteases released during soaking and germination is responsible for such inactivation of proteinaceous anti-nutritional factors. There are three stages of proteolysis during germination. The first stage of hydrolysis describes liberation of amino acids and their subsequent use for the synthesis of enzymes responsible for conversion of reserves

substances into suitable form for transport, in the second stage bulk hydrolysis occur in which the reserve protein is hydrolyzed into amino acids for growing seeds. Finally, during the third stage, that occurs during the senescence of storage tissue, the cellular proteins and enzymes are broken down into amino acids which are subsequently used by the seedling before the onset of autotrophic growth[58]. In the present study, protein content in the precipitated fraction of soaked seeds extract showed lower protein content (23.76mg BSA eq./g DW) compared to raw seeds 30.54 mg BSA eq./g DW, in addition germination also lowered protein content (27.25mg BSA eq./g DW) compared raw seeds. Similar reduction in protein content was reported in mung, pea and lentil sprouts after 72 h and 120 h of germination[99].

Scientific evidences about the sprout pertaining optimal number of nutrients are substantial, recommending legume sprouts consumption for maximum health benefits to the consumers. Germination is known to have very little effect on crude protein content. A study reported 1.4% increase in protein content of green gram during germination, whereas a study[59] reported an increase of 2.5% in mung beans. Such an increase was attributed to biosynthesis of enzymes and protein during germination and compositional changes following the degradation of other constituents [35]. However, such an increase in crude protein content during seeds germination may not be due to an increase in true protein as most of analysis measure total nitrogen content of the samples that can be either due to presence of nucleic acid, free amino acids and polypeptides in legumes. Interestingly, present study showed remarkable increase in the amount of free amino acids along with a decrease in the protein content during soaking, germination and fermentation

(**Table.1**). Therefore, compositional analysis to evaluate the changes in amino acids profile during germination, cooking and fermentation is of greater interest for the present study. Mobilization of polymerized compounds such as protein into amino acids makes developing sprouts beneficial for human body as those simpler nutrients are readily used by the body for nutritional benefits. The essential amino acids could be amongst those free amino acids generated during germination, depending upon the biosynthesis pathway carried out by the seed. In the present study, amino acid chromatographic analysis was performed in order to determine effect of germination and lactic acid bacterial fermentation on essential amino acids. It was observed that horse gram raw seed flour extracts contain highest amount of arginine (2714.05µg/g DW) similar amino acid composition reported for horse gram[100].

Amongst essential amino acids tryptophan, phenylalanine, leucine, lysine, isoleucine, valine and histidine were detected while other two essential amino acids were either in trace amounts or not detected (**Table 3**.). Trace amounts of all amino acids was also found to be present in soaked water, which indicates onset of protein degradation during soaking prior to germination. Soaking of raw horse gram seeds significantly reduced amount of arginine accompanied by an increase in the amount of serine, histidine, β-alanine, methionine, phenylalanine. Arginine is degraded to ornithine and urea by the action of enzyme arginase (EC 3.5.3.1) in seedling cotyledons urea cycle. However, ornithine and citruline showed an increase in its concentration but non-significant compared to raw seeds, which clearly indicate lack of arginase activation during soaking. Arginase activity during germination have been reported in cotyledons of several species

like pea seeds[101], faba beans[102], soybeans[103]. In the present study, most of the amino acids significantly increased during germination (**Table 3**.). Arginine concentration significantly decreased to 328.27µg/g DW while dramatic increase in the concentration of asparagine was observed (254.93 µg/g DW to 2317.43 µg/g DW). Since arginine is one of the predominant amino acids in angiosperm seed protein of raw horse gram seeds, its breakdown is critical during germination for efficient mobilization of seed nitrogen towards growing new organs of sprouts[103]. Such an increase in the amount of other amino acids including essential amino acids as well as arginine reduction suggests arginine to be temporary nitrogen storage protein component in raw horse gram seeds predominantly utilized in biosynthesis of other amino acids during sprouting[103]. Another mechanism suggested in a previous study[104], that elucidates that dicots like legumes rely on storage proteins as sole nitrogen source for amino acid synthesis, nitrogen is transferred from arginine which is predominant in endosperm to asparagine in cotyledons which further aids the germinating sprouts (axis) (**Figure 2**). Major pathway of nitrogen flow in ASN-ARG-ASN cycle may be one of the possible reason for asparagine to be accumulated in 72 h germinated horse gram sprouts accompanied with significant reduction in arginine concentration during germination. Similar increase in the amount of asparagine was observed in soybean sprouts[105]. The proposed ASN-ARG-ASN cycle possibly will be mediated by enzymes like asparagine synthetase and glutamine synthetase encoded by gene gamily (*ASN1, ASN2, ASN3* and *GLN2*) that are present in legumes[104, 106].

Table 3 Changes in amino acids (µg/g DW) composition due to soaking, sprouting, cooking and lactic acid bacterial fermentation of horse gram seeds.

No.	Amino Acids	RS	SS	SW	GS	CF4496	CF1193	UCF4496	UCF1193	CS	CFN	UCFN
1	ASP	53.857^{def}	25.02^{ef}	3.842^{f}	180.638^{ab}	21.641^{ef}	21.346^{ef}	218.285^{a}	103.774^{bcd}	78.611^{cde}	26.1^{def}	150.527^{abc}
2	GLU	114.129^{def}	220.00^{abc}	5.116^{g}	194.803^{bcd}	54.44^{fg}	26.985^{fg}	273.911^{ab}	165.265^{cde}	77.734^{efg}	40.324^{fg}	295.444^{a}
3	ASN	254.93^{fg}	530.79^{efg}	22.71g	2317.43^{abc}	1733.54^{bcd}	1707.57^{bcd}	2907.16^{a}	2558.77^{ab}	3037.58^{a}	1392.66^{cde}	1014.51^{def}
4	GLN	84.812^{bcd}	67.421^{cd}	1.428^{d}	279.441^{a}	97.605^{bc}	126.453^{bc}	310.218^{a}	174.578^{b}	281.277^{a}	97.228^{bcd}	163.399^{b}
5	SER	32.702^{cd}	100.481^{b}	1.656^{d}	220.808^{a}	36.204^{cd}	35.589^{cd}	109.69^{b}	60.07^{bc}	97.219^{b}	34.473^{cd}	46.299^{c}
6	HIS	9.374^{e}	138.98^{cd}	1.823^{e}	422.131^{a}	70.077^{de}	133.299^{cd}	252.83^{b}	186.728^{bc}	263.575^{b}	140.815^{cd}	212.196^{bc}
7	CIT	27.181^{cd}	72.306^{cd}	2.053^{d}	82.247^{c}	45.448^{cd}	43.766^{cd}	215.615^{ab}	166.789^{b}	76.241^{c}	55.29^{cd}	250.209^{a}
8	GLY	35.631^{de}	35.869^{de}	0.944^{e}	157.503^{bc}	57.371^{d}	67.011^{d}	188.865^{ab}	126.429^{c}	125.093^{c}	58.101^{d}	205.6^{a}
9	THR	0.0489^{b}	0.0550^{b}	0.2099^{a}	0.0469^{b}	0.0310^{b}	0.0287^{b}	0.0291^{b}	0.0277^{b}	0.0372^{b}	0.0259^{b}	0.0289^{b}
10	ARG	2714.05^{a}	1878.9^{a}	9.32^{b}	328.27^{b}	158.03^{b}	146.32^{b}	401.12^{b}	236.53^{b}	293.08^{b}	143.5^{b}	246.45^{b}
11	ALA	57.911^{e}	85.878^{de}	4.253^{e}	386.622^{bc}	97.601^{de}	90.351^{de}	461.026^{ab}	341.835^{bc}	238.629^{cd}	105.575^{de}	580.531^{a}
12	BALA	28.1^{bc}	148.10^{a}	1.861^{c}	44.812^{bc}	20.25^{bc}	32.545^{bc}	66.533^{b}	43.908^{bc}	44.469^{bc}	20.747^{bc}	53.274^{b}
13	TYR	20.193^{ef}	27.737^{ef}	0.602^{f}	162.824^{bc}	50.701^{def}	47.446^{def}	280.828^{a}	203.679^{b}	104.637^{cd}	66.749^{de}	200.436^{b}
14	MET	1.1557^{d}	14.5512^{b}	0.0033^{d}	6.3642^{bcd}	0.0378^{d}	0.0349^{d}	3.7171^{cd}	13.9007^{bc}	3.1355^{d}	2.8913^{d}	34.4514^{a}
15	VAL	11.752^{d}	36.572^{d}	1.181^{d}	311.217^{a}	114.77^{c}	115.699^{c}	343.071^{a}	222.655^{b}	211.473^{b}	118.598^{c}	365.43^{a}
16	TRP	160.118^{ab}	124.505^{bc}	2.811^{d}	104.7^{bc}	44.19^{cd}	50.343^{cd}	137.867^{abc}	98.549^{bcd}	86.705^{bcd}	71.679^{bcd}	237.174^{a}
17	PHE	90.246^{ef}	315.203^{cd}	3.418^{f}	609.508^{ab}	204.676^{def}	221.185^{de}	722.283^{a}	481.603^{bc}	392.115^{cd}	189.927^{def}	646.022^{ab}
18	ILE	12.906^{ef}	26.212^{def}	1.168^{f}	199.289^{b}	70.315^{de}	70.639^{de}	278.679^{a}	202.441^{b}	138.942^{bc}	79.35^{cd}	271.845^{a}
19	LEU	20.602^{de}	44.101^{cde}	2.611^{e}	173.295^{b}	65.912^{cde}	70.683^{cde}	382.852^{a}	317.076^{a}	118.923^{bc}	91.428^{cd}	313.855^{a}
20	ORT	21.048^{bc}	48.423^{b}	2.714^{c}	54.691^{b}	33.208^{bc}	27.704^{bc}	65.01^{b}	49.356^{b}	58.405^{b}	46.207^{b}	139.861^{a}
21	LYS	18.863^{bc}	36.547^{bc}	1.291^{c}	80.95^{b}	38.299^{bc}	47.115^{bc}	249.246^{a}	173.455^{a}	71.799^{b}	43.209^{bc}	215.164^{a}

RS-Raw Seeds, SS-Soaked Seeds, SW-Soaked water, GS-Germinated Seeds, CF4496-Cooked sprouts fermented by *Lb. plantarum* NRRL-B-4496, CF1193-Cooked sprouts fermented by *Lb. plantarum* NCDO-1193, UCF4496- Uncooked sprouts fermented by *Lb. plantarum* NRRL-B-4496, UCF1193- Uncooked sprouts fermented by *Lb. plantarum* NCDO-1193, CS-Cooked sprouts, CFN-Cooked sprouts fermented naturally, UCFN- Uncooked sprouts fermented naturally. All values are mean of triplicate analysis. Different letters in same row indicate statistical significance ($p<0.05$).

Asparagine biosynthesis helps balancing carbon and nitrogen compounds in the growing sprout, which in turn is regulated by number of metabolite signals like sugar starvation and light. Unlike other amino acids, lack of simple sugars in the medium induces biosynthesis of asparagine, however presence of light during germination plays a key role in this pathway. Asparagine biosynthetic pathway is shutdown when germination is carried out under light as the enzyme asparagine synthetase is inhibited by light and also photosynthesis regulates sugars in the system[106]. Therefore, arginine and asparagine in the horse gram raw seeds as well as sprouts suggests not only nutritional but also agronomical benefits for being decent nitrogen source for the seedling before symbiosis by bacteria[107]. Release of proteolytic enzymes during germination leading increase in the amount of overall amino acids cannot be ruled out since it is one of the most important activities for translocation of simpler compounds to the growing sprout. Certain amino acids acts as antioxidants mainly aromatic amino acids like phenylalanine, tryptophan, tyrosine, histidine that renders about 75-80% of total antioxidative amino acids[35]. Increase in such amino acids can contribute to the total antioxidant activity, which might be the another reason of rise in free radical scavenging activity during germination in the present study. Cooking might have an adverse effect also on the levels of essential amino acids in horse gram sprouts, since cooked sprouts showed significantly lower amount of most of the amino acids compared to uncooked sprouts. During fermentation, several metabolic activities are carried out by the fermenting bacteria, one of them is amino acid metabolism.

All fermented samples in the present study also showed significant differences in the amount of free amino acids, certain essential amino acids showed significant increase

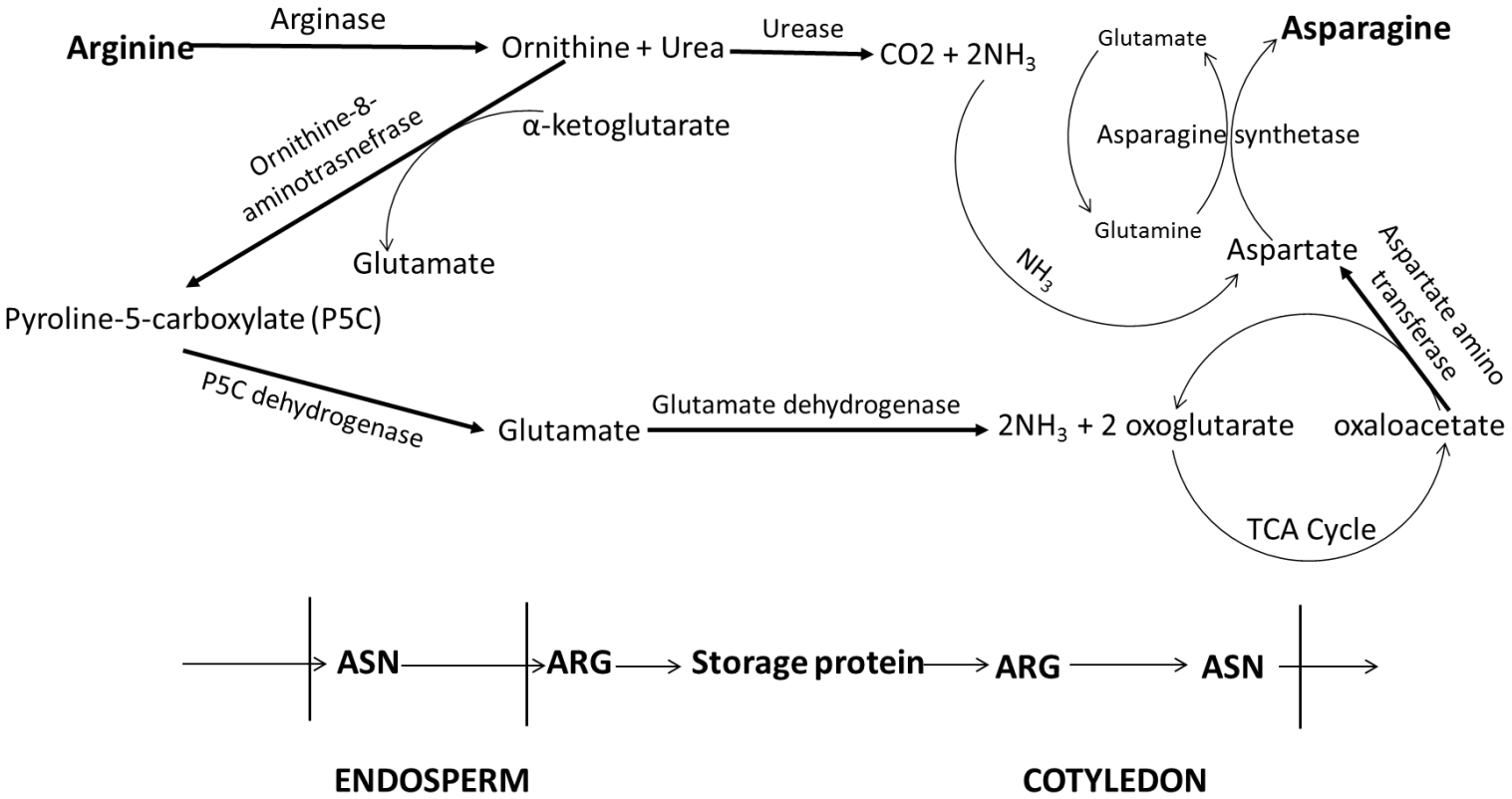


Figure 2 Proposed mechanism of ARG-ASN-ARG cycle for conversion of amine nitrogen of arginine to amide nitrogen of asparagine. [Reprinted from [104, 107, 108]].

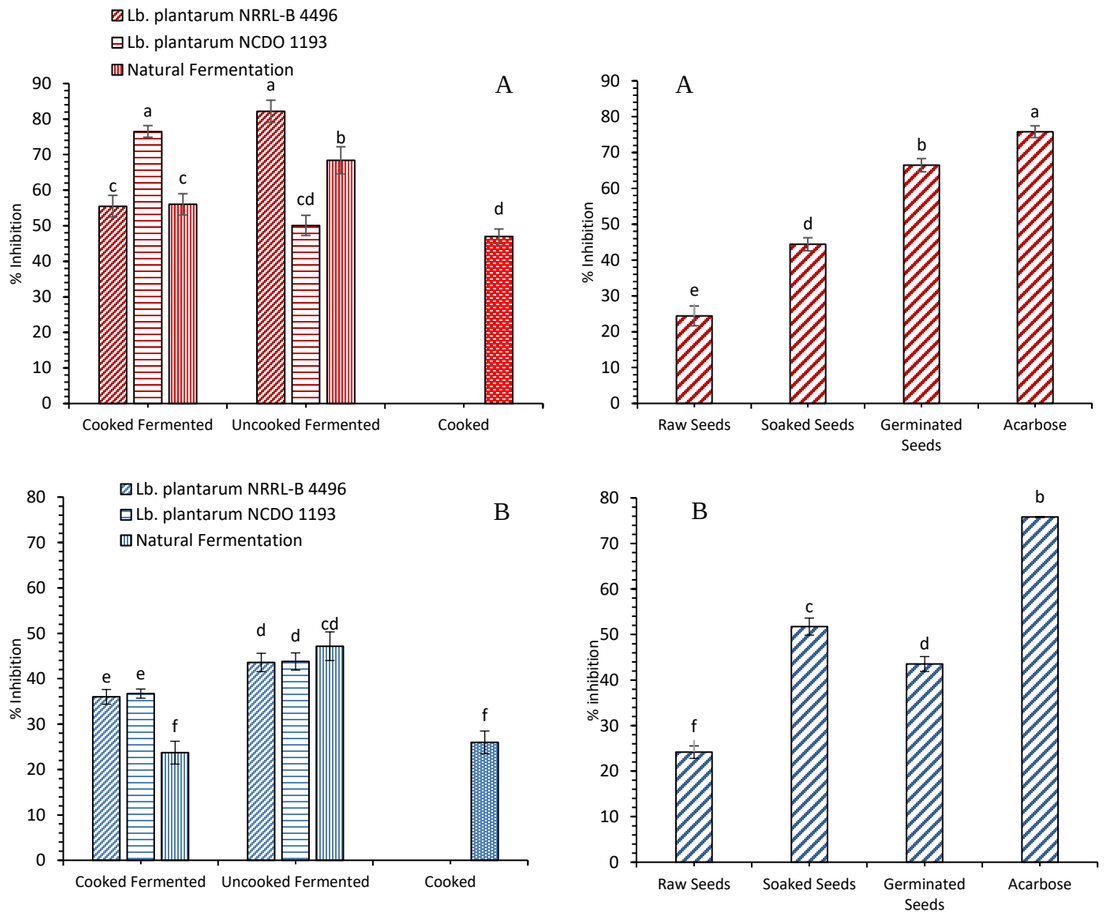
compared to the sprouts, while some showed significant decrease mainly sprouts fermented by lactic acid bacteria. Certain LAB are able to biosynthesize amino acids due to expression of certain enzymes involved in the anabolic pathways, while rise in the amount of majority of the amino acids is mainly due to proteolytic activity. A significant amount of decrease in essential amino acid content during fermentation in cooked sprouts can be attributed to the ability of lactic acid bacteria to utilize amino acids for their own survival and optimum growth during fermentation. Moreover, comparing two LAB strains *Lb. plantarum* NRRL B-4496 showed better amino acid metabolic activity in contrast to *Lb. plantarum* NCDO 1193. Lactic acid fermentation in general, has also been known to positively affect the amounts of amino acids in cereals and legumes, current study is an addition to such evidences. Several fermented foods comprising cereals and legumes are predominantly fermented using yeast, however bacterial fermentation usually involves proteolytic activity, while yeast mainly degrades carbohydrates. Hence the changes in amino acids composition during bacterial and yeast fermentation differ significantly; therefore, bacterial fermentation must be of higher interest in order to control nutritional improvement of legumes. Lactic acid bacteria particularly, are equipped with protein-degradation system, although some strains of *Lb. plantarum* lack genes encoding extracellular proteases required for primary breakdown of protein, but they possess 19 highly expressed genes encoding intracellular peptidases and uptake systems for peptides, which aids in further degradation of peptides into simpler amino acids. Despite of protein breakdown machinery, some genomic sequencing studies[52, 53] also reveals that *Lb.*

plantarum spp. possess genes that encodes for comprehensive pathways for biosynthesis of amino acids.

Amylase act as a catalyst in hydrolysis of starch comprising mainly glyosidic linkages, such activity is crucial for dietary starch metabolism and energy production. However, under high dietary consumption of starch such breakdown due to optimum pancreatic amylase activity may raise blood glucose levels in human metabolism which over time may increase hyperglycemia linked to overall management of type-2 diabetes. Hence compounds that can inhibit the activity of α-amylase are of greater interests in regards to hyperglycemic condition management through dietary solutions[18]. Non-dietary treatment methods includes therapeutic drugs such as acarbose that effectively reduced intestinal absorption of sugars[109]. However, there are some reported side effects due to oral consumption of acarbose mainly abdominal distention, flatulence, meteorism and possibly diarrhea[110]. For this reason, scientists, physicians and patients tend to look for some alternatives to therapeutic drugs that can effectively prevent rise in the blood sugar level without side effects. One of the most important groups of storage proteins found in legumes seeds are the enzyme inhibitors. These proteins are capable of forming stoichiometric complexes with various hydrolytic enzymes and cause the competitive inhibition of catalytic functions naturally occurring in the food system. In the present study, α-amylase inhibitory activity of horse gram raw seeds (24.41%) significantly (p<0.05) increases during soaking and germination, to (66.45%) in sprouts, however reduced ability (47%) of inhibition was observed in cooked sprouts, which indicates loss of some potent α-amylase inhibitors during conventional cooking. Further fermentation of

cooked sprouts regains α-amylase inhibitory activity with maximum inhibition (77%) exhibited by cooked sprouts fermented by *Lb. plantarum* NCDO 1193 (**Figure 3**). Amongst uncooked sprouts that were subjected to lactic acid bacterial fermentation, *Lb. plantarum* NRRL B-4496 fermented uncooked sprouts showed highest α-amylase inhibitory activity (82%) which is comparatively higher than positive control Acarbose (76%). These results do not correlate with total phenolic content which suggests that α-amylase inhibitory activity depends on compounds other than polyphenols. α-glucosidase enzyme is located in the brush border of the small intestine and is required for the breakdown of carbohydrates to release α-d-glucose that is readily absorbable[111]. Competitive and reversible inhibition of intestinal α-glucosidase enzyme limits the rate of absorption of glucose through intestine which in turn prevents hyperglycemic conditions in humans[111]. Acarbose also acts as an inhibitor of α-glucosidase enzyme, that is relevant for intestinal absorption of hydrolyzed glucose, but as discussed before due to adverse side effects natural alternatives are gaining higher importance. In the present study, we see α-glucosidase inhibitory activity of raw horse gram seeds (24%) is similar to α-amylase inhibitory activity. However, both enzymes inhibitory activity estimated for raw horse gram seeds in the present study is comparatively less than that of reported in previous study on horse gram raw seeds[23].

One of the major reason could be the analysis of methanolic extracts, since enzymes like α-amylase operates efficiently in aqueous phase, direct enzyme-inhibitor interaction is expected to be impacted in alcoholic phase during post processing analysis.



RS-Raw Seeds, SS-Soaked Seeds, SW-Soaked water, GS-Germinated Seeds, CF4496-Cooked sprouts fermented by *Lb. plantarum* NRRL-B-4496, CF1193-Cooked sprouts fermented by *Lb. plantarum* NCDO-1193, UCF4496- Uncooked sprouts fermented by *Lb. plantarum* NRRL-B-4496, UCF1193- Uncooked sprouts fermented by *Lb. plantarum* NCDO-1193, CS-Cooked sprouts, CFN-Cooked sprouts fermented naturally, UCFN- Uncooked sprouts fermented naturally. Data are expressed as means ± standard error of three independent experiments. Different letters in same column indicate statistical significance (p<0.05)

Figure 3 Changes in α-amylase inhibition activity (A), Changes in α-glucosidase inhibition activity (B) during soaking, germination, cooking and fermentation

Although we observe a rise in inhibition (43%) during germination, which suggests development or formation of certain compounds during germination that possess α-glucosidase inhibitory activity. Further significant (p<0.05) increase in such activity was observed due to lactic acid bacterial fermentation. While comparing both LAB strains, unlike α-amylase inhibitory activity both the strains showed non-significant differences in α-glucosidase inhibition (**Figure 3**). Surprisingly, it was found that fresh horse gram sprouts and fermented horse gram sprouts did not significantly differ in α-glucosidase inhibitory activity. This can be due to lactic acid bacterial fermentation leading to higher structural changes in protein peptides compared to phenolic compounds which are majorly acting as inhibitors to α-glucosidase activity[112]. It can be concluded that germination of horse gram sprouts significantly increases inhibitory potential of carbohydrates metabolizing enzymes compared to fermented sprouts, hence we suggest consumption of horse gram sprouts has relevance for dietary support for management of type 2 diabetes.

The pH of all the samples was recorded (**Table 1**.) and significant (p<0.05) decrease was observed during fermentation of horse gram sprouts with the lowest pH in uncooked naturally fermented sprouts which could be attributed to production of abundant organic acids during fermentation. The population of viable lactic acid bacteria after 48 h of fermentation of horse gram sprouts was also determined (**Table 1**.). In spite of horse gram sprouts being hostile ecosystem for LAB for optimal growth, population of both the strains *Lactobacillus plantarum* NRRL-B-4496 and *Lactobacillus plantarum* NCDO 1193 was higher than 7.99 $\log_{10}$cfu/ml without significant differences between the two strains. Naturally fermented sprouts showed significantly lower LAB count compared to lactic

acid bacterial intended fermented sprouts, presence might be either due to some LAB naturally present on sprouts or cross contamination. Such increase in the LAB count compared to raw seeds are consistent with decrease in the amount of protein content and increase in the amount of free amino acids (**Table 1**.). Overall population of lactic acid bacteria in all the LAB fermented samples suggest that horse gram sprouts can be considered as decent substrate to ensure high viability (>10^6 CFU/g) of lactic acid bacteria in concern and hence determines its potential to be utilized as a prebiotic in several functional probiotic food formulations.

3.5. Conclusions

Raw horse gram is a rich source of nutrients which are concentrated more in the seed coat and consumption of foods prepared with unprocessed raw horse gram seeds also have been reported to have health beneficial properties. However, present study demonstrates that the metabolic changes during germination and lactic acid bacterial fermentation positively affect major bioactive compounds with health promoting effects in addition to deliver higher nutritive value compared to raw horse gram seeds. Horse gram sprouts with abundant nitrogen rich amino acid like asparagine could also possibly help nitrogen poor soil cultivation of horse gram seeds in order for better agronomical acceptance of this poor man's pulse worldwide. Through present study, horse gram sprouts as well as fermented sprouts have shown to possess tremendous potential to be 'functional food ingredient' in several non-dairy based probiotic products. Transforming production systems with traditional processing methods like soaking, cooking, germination and

fermentation provides wide range of opportunities to exploit the actual potential of this underexploited legume Horse gram (*Macrotyloma uniflorum*). Focusing on elimination of hidden hunger has always helped to identify the crucial requirements of human body, which in turn proves to be challenging to meet the dietary goals of world's growing population. However, with the goal of making this little easier, further exploitation of this underutilized legume - Horse gram (*Macrotyloma uniflorum*) is gaining importance. Current research project must continue as it offers tremendous scope for future science and exploring diverse role of this legume in benefit of the mankind.

4. *Lactobacillus plantarum* AND NATURAL FERMENTATION-MEDIATED BIOTRANSFORMATION OF FLAVOR AND AROMATIC COMPOUNDS IN HORSE GRAM SPROUTS

4.1. Abstract

Bio-transformed volatile compounds in horse gram sprouts, *Lactobacillus plantarum* NRRL B-4496 and *Lactobacillus plantarum* NCDO 1193 mediated as well as naturally fermented horse gram sprouts were analyzed using gas chromatography-mass spectrometry coupled with headspace solid-phase micro extraction. A total of 40 compounds, including acids, alcohols, aldehydes, esters, ketones, sulfur containing compounds were identified in sprouts fermented for 1, 2, 3, 4 and 5 days. Fermentation-mediated volatile metabolites such as acetic acid, eugenol, benzyl alcohol, acetoin, 2,3-butanediol and ethyl palmitate were estimated to be the prime contributors to the sensory attributes of fermented samples. As the contents of alcohols, ketones, esters and volatile phenols increased during fermentation, some aldehydes significantly decreased. For the first time, eugenol was detected in a fermentation processing system as in fermenting horse gram, paving the way for new bioprocessing strategy for production. Therefore, fermentation of horse gram sprouts enhances bio-transformed volatile composition derived from primary metabolites which has relevance for improving value-added flavor and nutritional benefits while integrating potential probiotic benefits.

4.2. Introduction

Horse gram (*Macrotyloma uniflorum*) is an underutilized legume, however popular as an important pulse crop in poor communities of some parts of the world. It is empirically used in traditional therapeutic formulations for treatment of kidney stones, infections, obesity and diabetes [10, 11]. This Fabaceae family member is also an excellent source of proteins, antioxidants, carbohydrates, minerals as well as other health promoting compounds[20].

Legumes are generally processed prior to consumption, such processes mainly leads to certain physical as well as chemical alterations of the seeds, once such process is germination. Germination effectively changes levels of bioactive compounds in the sprouts and during such course, volatile flavor and aromatic compounds are also affected to a small extent[113]. However, remarkable changes in such volatile compounds is observed during the fermentation, a food preservation method commonly escorted by nutrition enhancement[113]. These changes are primarily due to the metabolic activity of fermenting microorganisms that bio-transform primary metabolites for nutritional benefits[15]. Such metabolic activity has major influence on acidity, antioxidant activity as well as protein digestibility of edible seeds while liberating flavor and aromatic compounds that relatively influences overall organoleptic quality of the fermented foods[114]. Some lactic acid bacteria are able to effectively bio-transform amino acids (sweetness, umami), peptides (bitterness) and organic acids (sourness) into flavor compounds such as aldehydes, alcohols, acids and sulfur compounds. Such bio-transformation occurs simultaneously with lipid degradation that also participate for aroma development in fermented foods [114, 115]. Besides

conventional starter cultures such as yeast, lactic acid bacteria and fungi, there is an increasing demand for products containing microorganisms providing additional health benefits *viz* probiotics[67]. Probiotics have been defined as nonpathogenic microorganisms that when ingested in adequate amounts exert a positive influence on human health, especially in the gastrointestinal tract[116]. Over few decades the most popular carrier choice of probiotics was believed to be only dairy products but lately due to changes in the lifestyle, preferences as well as dietary restrictions, non-dairy based probiotic product demand is rising[117]. Although cereal based fermented products are commonly considered as healthy and affordable choice in field of non-dairy based fermented probiotic foods, some legumes such as soybeans have also been demonstrated to be pertinent substrate for lactic acid bacterial fermentation and its bio-transformation potential to beneficial products. Since horse gram is a localized and important underutilized pulse crop in resource limited arid regions of the world, it has potential to provide food and value added nutritional security in the context of climate resilient food systems. Value added processing applications also require more biochemically relevant scientific evidences of relevant volatile composition of horse gram sprouts that can be targeted for multifunction benefits via lactic acid bacterial fermentation. Some studies have reported potential of legumes to serve as prebiotics for probiotic bacteria in fermented foods[43, 118]. Such evidences indicate the potential suitability of legumes such as horse gram for lactic acid bacterial fermentation, which can be used for development of bio-transformed functionally enhanced legume-based food products. Moreover, introducing novel functional food products in the wholefood market for fermented foods can be improved

by understanding the flavor and sensory profile of such fermented foods through controlled targeted fermentation as a bioprocessing strategy. Such value added factors would directly influence dietary preferences and the consistency of healthy food consumption[119]. Therefore, with deeper understanding of the biochemical processes conventional processing strategies such as soaking, germination and fermentation not only influences the levels of bioactive compounds in the seeds but most importantly it can positively influence palatability of the final product [26, 28, 120, 121].

Among the options for biotransformation of legumes natural or spontaneous fermentation of legumes is commonly practiced in some Asian countries to develop traditional fermented legumes with enhanced nutritional quality [122]. The volatile composition in terms of flavor and aroma remarkably differs in naturally fermented legumes compared to targeted lactic acid bacteria (LAB) fermented legumes, due to presence of certain undesirable flavor and off-odor compounds in naturally fermented products. However targeted species specific biotransformation has made it possible to commercially develop such traditional fermented products with value added benefits. For example products fermented using LAB and yeasts delivers flavor composition and organoleptic properties close to those of naturally fermented products[123]. Such targeted biotransformation can also improve food quality and safety leading to wider acceptance of such foods compared to natural sprouted and fermented products. Therefore, targeting of suitable lactic acid bacteria particularly potential probiotic starter cultures can advance development of value added sprouts from underutilized legumes such as horse gram with desirable nutritional, flavor and sensory quality. Based on this rationale specifically

targeted LAB was investigated in the present study to enhance volatile compounds for value addition through germination and fermentation of promising underutilized pulse horse gram.

4.3. Materials and Methods

4.3.1. Materials

Perillyl alcohol, n-alkane standards (C_{14}-C_{24}), acetic acid, ethanol, butyric acid, eugenol, ethyl palmitate, palmitic acid and 1, 3-butanediol was purchased from Sigma Aldrich (St. Louis, MO). Dry horse gram (*Macrotyloma uniflorum* var. *uniflorum* Paiyur 1) seeds were procured from supermarket (Patel Brothers, Dallas, TX).

4.3.2. Germination of Horse Gram Seeds

Cleaned horse gram seeds were washed with 70% ethanol and excess sterile water to remove the ethanol and soaked in sterile water (1:5) for 8 h at 25 °C. After soaking, seeds were placed over moist filter paper in petri dishes (150 mm) and incubated at 28 °C in the dark with sterile water sprayed at every 12 h for 5days period. After various known incubation times, germinated sprouts were crushed in mortar and transferred to 20 mL headspace GC-MS vails in triplicates under aseptic conditions.

4.3.3. Inoculum Preparation and Fermentation

Lactic acid bacterial (LAB) strains *Lb. plantarum* NRRL-B-4496 and *Lb. plantarum* NCDO-1193 were obtained from Prof. Shetty (Dept. of Plant Sciences, North

Dakota State University. ND). The frozen cell culture was sub cultured thrice using MRS broth (Sigma Aldrich, St. Louis, MO) by incubating at 37°C for 48 h under micro-aerobic conditions in order to be reactivated. The active cells were harvested and re-suspended in sterilized water in order to obtain 8 $\log_{10}$ cfu/mL. This cell suspension was used as starter culture for horse gram sprout fermentation. Cleaned horse gram sprouts were crushed using sterile mortar and pestle and transferred into sterile glass vials tightly capped with screw on cap and silicone septum. Lactic acid bacterial cell suspension (2:3, w/v) and sterile water for natural fermentation was added in respective labelled vials containing freshly crushed sprouts. Lactic acid bacterial-mediated fermentation was carried out at 37 °C 24 h (day 1), 48 h (day 2), 72 h (day 3), 96 h (day 4), 120 h (day 5) and immediately at the end of each day of the fermentation period, respective sample vials were subjected to GC-MS analysis.

4.3.4. Extraction of Volatile Compounds from Fermented Horse Gram Sprouts

Volatile compounds in horse gram seed flour, soaked seeds, sprouts, lactic acid bacterial fermented sprouts as well as naturally fermented sprout samples were subjected to head space solid-phase micro extraction using gray fiber. Each sample vial containing samples was spiked with 2 µL of perilyll alcohol as an internal standard (250 ppm in hexane). At the end of each fermentation period respective samples were taken out of incubator set at 37 °C, agitated using full speed vortex in order to reach an equilibrium state of headspace in each vial.

4.3.5. Gas Chromatography-Mass Spectrometry Analysis and Identification of Volatile Compounds

The volatile constituents of samples were analyzed by HS-SPME-GC-MS equipped with Triplus autosampler, Trace Ultra GC, and DSQ II mass spectrometer (Thermo Finnigan, Thermo Fisher Scientific, Inc., San Jose, CA, USA). Fermented samples containing internal standards were subjected to volatiles analysis using headspace – solid phase micro-extraction using 50/30 μm divinylbenzene /carboxen / polydimethylsiloxane (DVB/CAR/PDMS) fiber. The SPME fiber was initially conditioned in the GC injector at 225 °C for 1 h according to manufacturer's recommendations. The vial containing sample was preheated at 60 °C while agitating for 30 seconds. The SPME fiber was then exposed to the HS of the vial for 20 min at 60 °C for the adsorption of volatiles. The SPME fiber was introduced into the inlet of the GC for 2 min at 225°C to desorb the volatile compounds in splitless mode. Volatiles were separated on a fused silica Zebron ZB-WAXPlus capillary column (30 m × 0.25 mm, 0.25micron film (Phenomenex, CA, USA) coated with bonded 100% polyethylene glycol. Helium was used as a carrier gas at a flow rate of 1 mL/min and run time was 24 min. The oven temperature was programed from 60°C for 1 min, and then increased to 225 °C, at the rate of 15 °C/min with 5.0 min hold time at the end. The transfer line temperature and ion source temperature were maintained at 225 and 285°C respectively. The ionization voltage was 70 eV, the mass range was 45-450 amu and the scan rate was 12.82 scans/sec. The compounds peaks were identified by comparison of their Kovats indices (KI) relative to homologous alkane series (C_{10}-C_{24}), chemical ionization using methane gas, retention

times of authentic standards and matching the spectral fragmentation patterns in Wiley and NIST library database and published mass spectra [124-126]. All the volatile compounds were quantified by comparing their peak areas with that of the perilyll alcohol an internal standard. All the experiments were conducted three times with triplicate samples for quantification of volatiles and results were expressed as means and standard error (SE).

4.3.6. Statistical Analysis

Principal component analysis (PCA) using MetaboAnalyst 3.0 (Xia Lab @ McGill http://www.metaboanalyst.ca/) was conducted to evaluate changes in the volatile compounds of horse gram seeds and sprouts during germination and different fermentation periods. The results were expressed as mean ± standard error. The mean comparison was performed using JMP Statistical Discovery™ (SAS) Pro. v.12.0 software package and processed by one-way analysis of variance (ANOVA) to evaluate significant differences ($p<0.05$). All the experiments and analysis were performed in three independent replications with triplicate samples and significant difference between the samples was determined using Student's t test ($p<0.05$).

4.4. Results and Discussion

Bio-transformed volatile compounds present in dry (raw) horse gram seeds, fresh soaked seeds, germinated seeds as well as lactic acid bacteria *Lactobacillus plantarum* NRRL-B 4496 and *Lactobacillus plantarum* NCDO 1193 fermented horse gram sprouts and naturally fermented sprouts were analyzed by HS-SPME coupled with GC-MS. A

total of 40 volatile compounds were identified based on retention time of authentic standards, mass spectrum and Kovats retention index using *n*-alkanes C_{10}-C_{24} as external standards those were consistent with literature[124-126] as well as chemical ionization procedure using methane (**Table 4**). Amongst them 7 organic acids, 8 alcohols, 3 aldehydes, 8 esters, 6 ketones, 2 sulfur-containing compounds and certain aromatic compounds were detected during 24 h (day 1), 48 h (day 2), 72 h (day 3), 96 h (day 4), 120 h (day 5) of fermentation. Lactic acid bacterial fermentation had qualitative impact on the volatile composition of horse gram sprouts, particularly acids, alcohols, acid esters and some ketones. Similar to lactic acid bacterial fermentation, naturally fermented horse gram sprouts also showed significant changes in the levels of imperative volatile compounds which adds to the overall flavor and aromatic characteristics of fermented sprouts. Undesired aroma producing compounds such as *p*-ethylguaiacol and methionol was detected only in naturally fermented horse gram sprouts. Moreover, acetoin, acetic acid, 1-hexanol, benzaldehyde, linalool, methionol, phenylethyl acetate, hexanoic acid, 2-methanoxylphenol, phenethyl alcohol, octanoic acid as well as eugenol concentration considerably increased during natural fermentation of horse gram sprouts. Dry (raw) horse gram seeds extracts have been reported to deliver hepato-protective, diuretic, antiulcer and antioxidant activity in several animal study[22, 127]. Gas chromatography-mass spectroscopic analysis of such extracts of horse gram dry seeds revealed presence of n-hexadecanoic acid, linoleic acid, mome inositol, ethyl alpha-d-glucopyronoside, phenyl acetaldehyde, stigmasterol amongst which (3B)-stigmast-5-en-3-ol, linoleic acid indicated bioactive targets with antidiabetic, antioxidant and cholesterol lowering potential [128].

Table 4 Major identified volatile compounds detected in raw and soaked seeds, unfermented sprouts as well as fermented horse gram sprouts.

No.	RT(min)	Common Name	Chemical Formula	IUPAC Name	Chemical group	Identification
1	5.22	Acetoin	$C_4H_8O_2$	3-Hydroxybutan-2-one	Ketones	KI, CI
2	5.70	1-Hexanol	$C_6H_{14}O$	1-Hexanol	Alcohols	KI
3	6.52	Acetic Acid	$C_2H_4O_2$	Acetic acid	Acids	KI, CI, S
4	6.87	2-Ethyl-hexan-1-ol	$C_8H_{18}O$	2-Ethylhexan-1-ol	Alcohols	KI
5	7.28	Benzaldehyde	C_7H_6O	Benzaldehyde	Aldehydes	KI, CI
6	7.33	Linalool	$C_4H_{10}O_2$	3,7-Dimethylocta-1,6-dien-3-ol	Alcohols	KI, CI
7	7.58	2,3-Butanediol	$C_{10}H_{18}O$	Butane-2,3-diol	Alcohols	KI, CI
8	7.96	Butyric acid	$C_4H_8O_2$	Butyric acid	Acids	KI, S
9	8.20	Phenylacetaldehyde	C_8H_8O	2-Phenylacetaldehyde	Aldehydes	KI, CI
10	8.33	2-Methylbutanoic Acid	$C_5H_{10}O_2$	2-Methylbutanoic acid	Acids	KI, CI
11	8.42	Ethyl Benzoate	$C_9H_{10}O_2$	Ethyl benzoate	Esters	KI, CI
12	8.67	1-Hexadecyne	$C_{16}H_{30}$	Hexadec-1-yne	Others	KI, CI
13	8.75	Methionol	$C_4H_{10}OS$	3-Methylsulfanylpropan-1-ol	Sulfur containing compounds	KI, CI
14	8.85	2-Methyl-2-butenolide	$C_5H_6O_2$	4-Methyl-2H-furan-5-one	Ketones	KI, CI
15	9.29	Methyl Salicylate	$C_8H_8O_3$	Methyl 2-hydroxybenzoate	Esters	KI, CI
16	9.41	2-Tridecanone	$C_{13}H_{26}O$	Tridecan-2-one	Ketones	KI, CI
17	9.49	Dimethyl adipate	$C_8H_{14}O_5$	Dimethyl hexanedioate	Esters	KI, CI
18	9.52	Phenethyl acetate	$C_{10}H_{12}O_2$	2-Phenylethyl acetate	Esters	KI, CI
19	9.54	Ethyl Salicylate	$C_9H_{10}O_4$	Ethyl 2-hydroxybenzoate	Esters	KI
18	9.52	Phenethyl acetate	$C_{10}H_{12}O_2$	2-Phenylethyl acetate	Esters	KI, CI
19	9.54	Ethyl Salicylate	$C_9H_{10}O_4$	Ethyl 2-hydroxybenzoate	Esters	KI
20	9.60	(E)-β-Damascenone	$C_{13}H_{18}O$	(E)-1-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl)but-2-en-1-one	Ketones	KI, CI
21	9.63	Geraniol	$C_{10}H_{18}O$	(2E)-3,7-dimethylocta-2,6-dien-1-ol	Alcohols	KI
22	9.65	Hexanoic Acid	$C_6H_{12}O_2$	Hexanoic Acid	Acids	KI, CI
23	9.83	Guaiacol	$C_7H_8O_2$	2-Methoxyphenol	Volatile Phenols	KI, CI

Table 4 Continued

No.	RT (min)	Common Name	Chemical Formulae	IUPAC Name	Chemical Group	Identification
24	9.94	Benzenemethanol	C_7H_8O	Benzenemethanol	Alcohols	KI, CI
25	10.20	2-Phenylethanol	$C_{10}H_8O$	2-Phenylethanol	Alcohols	KI, CI
26	10.43	*β*-Ionone	$C_{13}H_{20}O$	(E)-4-(2,6,6-trimethylcyclohexen-1-yl)but-3-en-2-one	Ketones	KI
27	10.63	Benzothiazole	C_7H_5NS	1,3-Benzothiazole	Sulfur containing compounds	KI, CI
28	10.80	Phenol	C_6H_6O	Phenol	Acids	KI
29	10.93	Pentadecanal	$C_{15}H_{30}O$	Pentadecanal	Aldehydes	KI, CI
30	11.00	*p*-Ethylguaiacol	$C_9H_{12}O_2$	4-Ethyl-2-methoxyphenol	Volatile Phenols	KI
31	11.04	Ethyl tetradecanoate	$C_{16}H_{32}O_2$	Ethyl tetradecanoate	Esters	KI, CI
32	11.06	γ-Nonalactone	$C_9H_{16}O_2$	5-Pentyloxolan-2-one	Ketones	KI, CI
33	11.10	Octanoic Acid	$C_8H_{16}O_2$	Octanoic Acid	Acids	KI, CI
34	11.17	2-Pyrrolidinone	C_4H_7NO	Pyrrolidin-2-one	Others	KI, CI
35	11.80	1-Tridecanol	$C_{13}H_{28}O$	Tridecan-1-ol	Alcohols	KI
36	11.89	Eugenol	$C_{10}H_{12}O_2$	2-Methoxy-4-prop-2-enylphenol	Volatile Phenols	KI, CI, S
37	12.06	4-Vinylguaiacol	$C_9H_{10}O_2$	4-Ethenyl-2-methoxyphenol	Volatile Phenols	KI
38	12.11	Methyl Palmitate	$C_{17}H_{34}O_2$	Methyl hexadecanoate	Esters	KI
39	12.35	Ethyl Palmitate	$C_{18}H_{36}O_2$	Ethyl hexadecanoate	Esters	KI, CI, S
40	12.46	Decanoic acid	$C_{10}H_{20}O_2$	Decanoic acid	Acids	KI, CI

ID-Identification of volatile compounds was according to as follows: KI- Kovats Retention Index were determined using *n*-alkanes C_{10}-C_{24} as external standards, CI-Chemical Ionization, S-Authentic Standards Injection. Mass spectrum and kovats retention index matches with the authentic compounds under similar condition (standard-S); B-mass spectrum and kovats retention index consistent with those of the literature[124-126] and NIST Mass Spectral Database.

These compounds were not detected in the present study except phenyl acetaldehyde. Such variation can be attributed to differences in the extraction methods involved as in the present study freshly ground raw seed flour subjected to solid phase micro-extraction was analyzed whereas previous study involved defatting, ethanol extraction of raw horse gram seeds by soxhlation prior to GC-MS analysis.

4.4.1. Effect of soaking, germination, and fermentation

The changes in the concentration of major volatile compounds in horse gram seeds and fermented sprouts during germination and fermentation for 1, 3 and 5 days are presented in **Table 5**. and effect of 2 and 4 days fermentation is presented in **Table 6**. Among 40 volatile compounds identified, only 13 compounds were found in raw horse gram seeds, out of which 2-ethyl-hexan-1-ol, benzaldehyde, phenylacetaldehyde, 2-methyl-2-butenolide, geraniol, benzoyl alcohol, 2-phenylethanol, β-ionone and benzothiazole showed significant increase during germination, while methyl salicylate and dimethyl adipate showed decrease in their levels during sprouting. In addition to these, some compounds that were not detected in raw seeds were found in soaked seeds and sprouts such as acetoin, benzaldehyde, 1-hexanol, acetic acid, octanoic acid and ethyl palmitate, while pentadecanal present only in sprouts. Soaking and germination showed significant changes in the levels of some volatile compounds found in raw horse gram seeds including 2-methyl-2-butenolide (known to be a germination stimulant), phenylacetaldehyde and benzoyl alcohol, 2-phenylethyl alcohol (aromatic compounds) and geraniol (monoterpenoid). In addition to these compounds, germination also seems to

Table 5 Impact of hydration, germination and fermentation on the concentration (µg/g FW) of major volatile flavors and aromatic compounds in horse gram seeds and sprouts fermented for 24h (day 1), 72h (day 3), 120h (day 5) by *Lb. plantarum* spp. and natural fermentation process.

						Fermentation by *Lb. plantarum* NRRL B-4496			Fermentation by *Lb. plantarum* NCDO 1193			Natural Fermentation		
No.	Volatile Compounds	KI	RS	SS	GS	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
1	Acetoin	1226	ND^{d}	0.57 ± 0.21^{a}	0.23 ± 0.06^{bcd}	0.14 ± 0.05^{bcd}	0.33 ± 0.13^{abc}	0.34 ± 0.18^{abc}	0.30 ± 0.16^{abcd}	0.22 ± 0.06^{bcd}	0.18 ± 0.11^{bcd}	0.23 ± 0.06^{bcd}	0.29 ± 0.13^{abcd}	0.19 ± 0.06^{bcd}
2	1-Hexanol	1308	ND^{f}	0.21 ± 0.06^{ef}	1.12 ± 0.28^{abc}	0.65 ± 0.15^{de}	0.67 ± 0.18^{cde}	0.67 ± 0.14^{cd}	0.67 ± 0.09^{cde}	0.65 ± 0.08^{de}	0.80 ± 0.11^{bcd}	1.02 ± 0.19^{abcd}	1.38 ± 0.26^{a}	0.80 ± 0.23^{bcd}
3	Acetic Acid*	1417	ND^{e}	1.15 ± 0.29^{de}	0.87 ± 0.14^{de}	3.0 ± 0.70^{bcd}	3.94 ± 0.86^{bc}	4.83 ± 0.87^{b}	3.90 ± 0.78^{bc}	4.87 ± 0.42^{b}	5.04 ± 0.70^{b}	2.34 ± 0.49^{cde}	7.65 ± 1.53^{a}	9.55 ± 1.98^{a}
4	2-Ethyl-hexan-1-ol	1440	0.01 ± 0.002^{gh}	0.05 ± 0.01^{efg}	0.09 ± 0.02^{bcde}	0.11 ± 0.02^{abc}	0.09 ± 0.01^{bcd}	0.09 ± 0.01^{bcd}	0.04 ± 0.01^{fgh}	0.04 ± 0.01^{fg}	0.06 ± 0.01^{def}	0.13 ± 0.03^{a}	0.12 ± 0.01^{ab}	ND^{h}
5	Benzaldehyde	1465	ND^{f}	1.56 ± 0.29^{bcd}	1.05 ± 0.35^{def}	0.44 ± 0.09^{ef}	0.31 ± 0.09^{ef}	0.83 ± 0.18^{def}	0.43 ± 0.12^{ef}	0.49 ± 0.13^{def}	0.43 ± 0.11^{ef}	1.12 ± 0.28^{de}	2.51 ± 0.88^{ab}	2.26 ± 0.85^{abc}
6	Linalool	1468	0.07 ± 0.01^{e}	0.64 ± 0.38^{cde}	0.06 ± 0.03^{e}	0.46 ± 0.14^{de}	0.40 ± 0.10^{e}	ND^{e}	0.23 ± 0.09^{e}	0.13 ± 0.04^{e}	0.17 ± 0.05^{e}	0.74 ± 0.25^{bcde}	1.59 ± 0.96^{abc}	1.38 ± 0.54^{abcd}
7	2,3-Butanediol	1483	ND^{e}	ND^{e}	ND^{e}	1.21 ± 0.61^{de}	2.10 ± 0.90^{bcde}	1.51 ± 0.75^{cde}	0.45 ± 0.29^{e}	0.39 ± 0.12^{e}	0.20 ± 0.10^{e}	2.97 ± 1.11^{abcd}	4.21 ± 1.63^{ab}	3.60 ± 1.13^{abc}
8	Butyric acid*	1606	ND^{h}	ND^{h}	ND^{h}	0.07 ± 0.02^{a}	0.03 ± 0.01^{def}	0.04 ± 0.01^{bcdef}	0.01 ± 0.01^{fgh}	0.01 ± 0.06 ^{gh}	0.02 ± 0.004^{defg}	0.04 ± 0.01^{bcde}	0.05 ± 0.012^{abcd}	ND^{h}
9	Phenylacetaldehyde	1623	0.01 ± 0.06^{f}	0.33 ± 0.06^{ab}	0.18 ± 0.03^{de}	0.12 ± 0.02^{e}	0.16 ± 0.02^{e}	0.21 ± 0.02^{cde}	0.20 ± 0.04^{cde}	0.18 ± 0.02^{de}	0.14 ± 0.01^{e}	0.22 ± 0.01^{bcde}	0.21 ± 0.03^{bcde}	0.15 ± 0.01^{e}
10	2-Methylbutanoic Acid	1633	ND^{f}	ND^{f}	ND^{f}	ND^{f}	0.08 ± 0.02^{cde}	0.08 ± 0.02^{cde}	0.05 ± 0.02^{ef}	0.14 ± 0.03^{ab}	0.11 ± 0.03^{bcd}	ND^{f}	ND^{f}	ND^{f}
11	Ethyl Benzoate	1639	ND^{f}	ND^{f}	ND^{f}	0.06 ± 0.02^{e}	ND^{f}	0.14 ± 0.03^{a}	0.09 ± 0.03^{bcde}	0.13 ± 0.01^{ab}	0.08 ± 0.01^{cde}	0.13 ± 0.03^{ab}	0.09 ± 0.02^{bcde}	0.12 ± 0.02^{abc}
12	1-Hexadecyne	1656	ND^{c}	ND^{c}	ND^{c}	0.48 ± 0.07^{b}	ND^{c}	0.74 ± 0.11^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
13	Methionol	1662	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	0.07 ± 0.0^{b}	0.13 ± 0.03^{a}	0.06 ± 0.03^{b}
14	2-Methyl-2-butenolide	1669	0.05 ± 0.01^{d}	0.18 ± 0.05^{bcd}	0.20 ± 0.05^{bc}	0.21 ± 0.04^{abc}	0.21 ± 0.04^{abc}	0.23 ± 0.03^{abc}	0.19 ± 0.05^{bcd}	0.14 ± 0.01^{cd}	0.14 ± 0.01^{cd}	0.27 ± 0.03^{abc}	0.28 ± 0.09^{abc}	0.35 ± 0.08^{a}
15	Methyl Salicylate	1698	0.04 ± 0.01^{ef}	ND^{f}	ND^{f}	0.22 ± 0.05^{bcd}	0.26 ± 0.05^{bc}	0.24 ± 0.05^{bcd}	0.34 ± 0.07^{ab}	0.31 ± 0.06^{ab}	0.24 ± 0.04^{bcd}	0.14 ± 0.02^{cde}	0.12 ± 0.02^{def}	0.12 ± 0.04^{def}
16	2-Tridecanone	1807	ND^{d}	ND^{d}	ND^{d}	0.12 ± 0.01^{bc}	0.09 ± 0.02^{c}	0.16 ± 0.02^{ab}	0.12 ± 0.03^{bc}	0.09 ± 0.02^{c}	0.11 ± 0.01^{c}	0.17 ± 0.03^{ab}	0.13 ± 0.03^{c}	0.13 ± 0.02^{bc}
17	Dimethyl adipate	1813	0.01 ± 0.002^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
18	Phenethyl acetate	1815	ND^{f}	ND^{f}	ND^{f}	ND^{f}	0.10 ± 0.05^{cde}	0.16 ± 0.02^{abc}	0.02 ± 0.01^{ef}	0.05 ± 0.02^{def}	0.11 ± 0.03^{cde}	0.06 ± 0.01^{def}	0.23 ± 0.05^{a}	0.23 ± 0.04^{a}
19	Ethyl Salicylate	1817	ND^{e}	ND^{e}	ND^{e}	ND^{e}	0.09 ± 0.05^{bcde}	0.19 ± 0.03^{abcd}	0.03 ± 0.01^{e}	0.23 ± 0.11^{ab}	0.24 ± 0.09^{a}	0.07 ± 0.01^{cde}	0.21 ± 0.10^{abcd}	0.01 ± 0.01^{e}
20	(E)-*β*-Damascenone	1821	ND^{g}	ND^{g}	ND^{g}	0.09 ± 0.04^{ef}	0.13 ± 0.05^{cde}	0.30 ± 0.04^{a}	0.06 ± 0.01^{efg}	0.20 ± 0.02^{bcd}	0.24 ± 0.04^{ab}	0.11 ± 0.03^{def}	0.06 ± 0.03^{efg}	0.06 ± 0.03^{efg}
21	Hexanoic Acid	1823	0.01 ± 0.003^{e}	0.24 ± 0.06^{d}	0.05 ± 0.03^{e}	0.29 ± 0.04^{bcd}	0.24 ± 0.06^{d}	0.30 ± 0.05^{bcd}	0.33 ± 0.04^{bcd}	0.31 ± 0.03^{bcd}	0.29 ± 0.06^{bcd}	0.23 ± 0.05^{d}	0.41 ± 0.10^{abc}	0.42 ± 0.07^{ab}
22	Geraniol	1823	0.01 ± 0.06^{c}	0.29 ± 0.05^{a}	0.26 ± 0.04^{a}	ND^{c}	0.01 ± 0.01^{c}	ND^{c}	0.09 ± 0.02^{b}	0.26 ± 0.03^{a}	0.26 ± 0.05^{a}	ND^{c}	0.27 ± 0.05^{a}	0.03 ± 0.02^{bc}
23	Guaiacol	1838	0.09 ± 0.02^{c}	0.14 ± 0.03^{c}	0.08 ± 0.02^{c}	0.14 ± 0.03^{c}	0.20 ± 0.10^{c}	0.16 ± 0.05^{c}	0.11 ± 0.03^{c}	0.14 ± 0.03^{c}	0.10 ± 0.02^{c}	0.36 ± 0.07^{c}	0.96 ± 0.19^{ab}	0.93 ± 0.19^{ab}
24	Benzenemethanol	1846	0.05 ± 0.02^{g}	0.11 ± 0.03^{fg}	1.43 ± 0.25^{a}	0.28 ± 0.05^{defg}	0.24 ± 0.03^{defg}	0.31 ± 0.06^{defg}	0.34 ± 0.11^{defg}	0.25 ± 0.03^{defg}	0.18 ± 0.02^{efg}	0.39 ± 0.07^{cdef}	0.64 ± 0.18^{bc}	0.42 ± 0.09^{bcde}

Table 5 Continued

						Fermentation by *Lb. plantarum* NRRL B-4496			Fermentation by *Lb. plantarum* NCDO 1193			Natural Fermentation		
No.	Volatile Compounds	KI	RS	SS	GS	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
25	Benzeneethanol	1865	0.04 ± 0.01^{c}	0.80 ± 0.13^{c}	1.14 ± 0.27^{c}	0.94 ± 0.25^{c}	0.97 ± 0.28^{c}	1.29 ± 0.76^{c}	1.30 ± 0.57^{c}	0.69 ± 0.21^{c}	0.78 ± 0.24^{c}	6.92 ± 1.42^{b}	15.66 ± 2.75^{a}	14.88 ± 3.40^{a}
26	*β*-Ionone	1882	0.02 ± 0.01^{g}	0.21 ± 0.04^{a}	0.11 ± 0.03^{bcd}	0.11 ± 0.03^{bcd}	0.08 ± 0.02^{cdefg}	0.03 ± 0.03^{fg}	0.07 ± 0.02^{defg}	0.15 ± 0.01^{ab}	0.12 ± 0.02^{bcd}	0.04 ± 0.004^{efg}	0.09 ± 0.02^{cdefg}	0.06 ± 0.03^{defg}
27	Benzothiazole	1895	0.03 ± 0.01^{e}	0.20 ± 0.04^{abcd}	0.11 ± 0.02^{cde}	0.18 ± 0.03^{bcd}	0.09 ± 0.03^{de}	0.22 ± 0.03^{abc}	0.30 ± 0.08^{a}	0.22 ± 0.06^{abc}	0.12 ± 0.01^{cde}	0.15 ± 0.03^{cd}	0.13 ± 0.04^{cde}	0.17 ± 0.04^{bcd}
28	Phenol	2009	ND^{g}	0.04 ± 0.02^{fg}	ND^{g}	0.33 ± 0.12^{def}	0.23 ± 0.08^{defg}	0.40 ± 0.14^{bcde}	0.24 ± 0.13^{defg}	0.15 ± 0.12^{efg}	0.06 ± 0.04^{fg}	0.72 ± 0.13^{ab}	0.66 ± 0.13^{abc}	0.51 ± 0.11^{bcd}
29	Pentadecanal	2019	ND^{e}	ND^{e}	2.94 ± 0.75^{a}	0.43 ± 0.08^{cde}	0.17 ± 0.06^{de}	0.28 ± 0.09^{cde}	0.26 ± 0.04^{cde}	0.24 ± 0.10^{cde}	0.20 ± 0.05^{cde}	1.01 ± 0.35^{b}	0.56 ± 0.15^{bcd}	0.53 ± 0.12^{bcde}
30	*p*-Ethylguaiacol	2025	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	0.07 ± 0.04^{bc}	0.43 ± 0.13^{a}
31	Ethyl tetradecanoate	2028	ND^{h}	0.18 ± 0.06^{bcde}	ND^{h}	0.19 ± 0.06^{bcde}	0.11 ± 0.04^{efgh}	0.29 ± 0.05^{abc}	0.04 ± 0.01^{gh}	0.07 ± 0.03^{efgh}	0.13 ± 0.04^{efg}	0.17 ± 0.06^{cdef}	0.30 ± 0.06^{ab}	0.31 ± 0.06^{a}
32	*γ*-Nonalactone	2030	ND^{e}	ND^{e}	0.02 ± 0.01^{e}	0.20 ± 0.07^{abc}	0.08 ± 0.02^{de}	0.13 ± 0.04^{cd}	0.11 ± 0.01^{cde}	0.16 ± 0.03^{bcd}	0.18 ± 0.03^{bcd}	0.16 ± 0.03^{bcd}	0.20 ± 0.11^{abcd}	0.29 ± 0.06^{a}
33	Octanoic Acid	2033	ND^{i}	0.08 ± 0.02^{hi}	0.22 ± 0.06^{lh}	0.36 ± 0.11^{jk}	0.20 ± 0.05^{lhi}	0.31 ± 0.06^{jkg}	0.27 ± 0.07^{dkgh}	0.29 ± 0.04^{jkg}	0.49 ± 0.14^{abcd}	0.13 ± 0.03^{ghi}	0.64 ± 0.13^{a}	0.50 ± 0.09^{ab}
34	2-Pyrrolidinone	2039	ND^{f}	ND^{f}	ND^{f}	ND^{f}	ND^{f}	0.03 ± 0.01^{ef}	0.05 ± 0.01^{def}	0.08 ± 0.01^{de}	0.25 ± 0.03^{a}	0.04 ± 0.01^{def}	0.24 ± 0.06^{a}	0.22 ± 0.02^{ab}
35	1-Tridecanol	2088	ND^{d}	ND^{d}	0.13 ± 0.04^{ab}	0.09 ± 0.03^{abc}	0.06 ± 0.02^{bcd}	0.11 ± 0.03^{abc}	0.12 ± 0.04^{abc}	0.13 ± 0.05^{ab}	0.12 ± 0.03^{abc}	0.08 ± 0.02^{abcd}	0.09 ± 0.02^{abc}	0.08 ± 0.02^{abcd}
36	Eugenol*	2095	ND^{f}	ND^{f}	ND^{f}	3.40 ± 0.83^{ef}	4.18 ± 1.17^{e}	5.19 ± 1.12^{de}	3.42 ± 0.91^{ef}	2.90 ± 0.58^{ef}	5.30 ± 1.36^{de}	9.36 ± 1.07^{c}	7.83 ± 2.23^{cd}	13.81 ± 2.37^{ab}
37	4-Vinylguaiacol	2208	ND^{b}	ND^{b}	ND^{b}	0.11 ± 0.05^{b}	0.13 ± 0.05^{b}	0.21 ± 0.11^{b}	0.14 ± 0.08^{b}	0.15 ± 0.05^{b}	0.10 ± 0.03^{b}	1.07 ± 0.42^{a}	1.49 ± 0.60^{a}	0.96 ± 0.34^{a}
38	Methyl Palmitate	2211	ND^{g}	0.12 ± 0.05^{def}	0.10 ± 0.02^{defg}	0.12 ± 0.05^{def}	0.12 ± 0.04^{defg}	0.13 ± 0.02^{def}	0.11 ± 0.04^{defg}	0.11 ± 0.03^{defg}	0.17 ± 0.08^{cde}	0.10 ± 0.03^{defg}	0.31 ± 0.06^{ab}	0.40 ± 0.08^{a}
39	Ethyl Palmitate*	2228	ND^{f}	1.05 ± 0.33^{cde}	0.23 ± 0.10^{ef}	0.36 ± 0.10^{ef}	0.41 ± 0.12^{ef}	0.66 ± 0.20^{def}	0.31 ± 0.09^{ef}	0.49 ± 0.17^{def}	0.46 ± 0.12^{ef}	1.51 ± 0.54^{bcd}	2.93 ± 0.94^{a}	2.58 ± 0.64^{a}
40	Decanoic acid	2236	ND^{d}	ND^{d}	ND^{d}	0.70 ± 0.26^{abc}	6.0 ± 0.12^{bcd}	0.56 ± 0.18^{abc}	0.70 ± 0.22^{abc}	0.56 ± 0.23^{abc}	0.49 ± 0.09^{abc}	0.38 ± 0.16^{abcd}	0.27 ± 0.04^{bcd}	1.36 ± 0.07^{bcd}

Results are expressed as mean ±SE
RS: Raw seeds-RS; SS: Soaked seeds; GS: Germinated seeds; ND: not detected
Significant differences ($p<0.05$) among samples analyzed using Student's t test between the samples represented with different letters
(*) compounds are identified by comparing of retention time and mass spectrum of authenticated standards
All compounds were identified by mass spectral data, comparing KI from literature ([124-126]) by EI and CI mode.

Table 6 Concentration (µg/g FW) of identified volatile compounds in horse gram raw, soaked and germinated seeds in addition to 48h (Day 2) and 96h (Day 4) fermented sprouts influenced by germination as well as *Lb. plantarum* and natural fermentation process.

						Lb. plantarum NRRL B-4496		*Lb. plantarum* NCDO 1193		Natural Fermentation	
No.	Volatile Compounds	KI	RS	SS	GS	Day 2	Day 4	Day 2	Day 4	Day 2	Day 4
1	Acetoin	1226	ND^{d}	0.57 ± 0.21^{a}	0.23 ± 0.06^{bcd}	0.14 ± 0.08^{bcd}	0.44 ± 0.19^{ab}	0.15 ± 0.06^{bcd}	0.10 ± 0.04^{cd}	0.35 ± 0.15^{abc}	0.39 ± 0.12^{abc}
2	1-Hexanol	1308	ND^{f}	0.21 ± 0.06^{ef}	1.12 ± 0.28^{abc}	1.31 ± 0.21^{a}	1.17 ± 0.13^{ab}	0.67 ± 0.14^{cd}	0.73 ± 0.09^{bcd}	0.93 ± 0.19^{abcd}	1.02 ± 0.21^{abcd}
3	Acetic Acid*	1417	ND^{e}	1.15 ± 0.29^{de}	0.87 ± 0.14^{de}	4.00 ± 0.63^{bc}	4.65 ± 0.65^{bc}	4.23 ± 0.41^{bc}	4.64 ± 0.21^{bc}	4.42 ± 1.18^{bc}	9.22 ± 1.74^{a}
4	2-Ethyl-hexan-1-ol	1440	0.01 ± 0.002^{gh}	0.05 ± 0.01^{efg}	0.09 ± 0.02^{bcde}	0.10 ± 0.02^{abcd}	0.11 ± 0.02^{abc}	0.04 ± 0.01^{fg}	0.07 ± 0.01^{cdef}	0.08 ± 0.01^{bcde}	0.11 ± 0.01^{abc}
5	Benzaldehyde	1465	ND^{f}	1.56 ± 0.29^{bcd}	1.05 ± 0.35^{def}	0.41 ± 0.07^{ef}	0.61 ± 0.20^{def}	0.37 ± 0.07^{ef}	0.39 ± 0.07^{ef}	1.21 ± 0.36^{cde}	2.83 ± 0.91^{a}
6	Linalool	1468	0.07 ± 0.01^{e}	0.64 ± 0.38^{cde}	0.06 ± 0.03^{e}	0.41 ± 0.10^{de}	0.28 ± 0.13^{e}	0.14 ± 0.04^{e}	0.15 ± 0.03^{e}	2.12 ± 0.72^{a}	1.71 ± 0.93^{ab}
7	2,3-Butanediol	1483	ND^{e}	ND^{e}	ND^{e}	1.01 ± 0.39^{de}	1.46 ± 0.76^{cde}	0.18 ± 0.04^{e}	0.29 ± 0.15^{e}	3.14 ± 1.29^{abcd}	4.83 ± 1.59^{a}
8	Butyric acid*	1606	ND^{h}	ND^{h}	ND^{h}	0.06 ± 0.01^{ab}	0.03 ± 0.01^{cdef}	0.02 ± 0.01^{efgh}	0.02 ± 0.004^{efgh}	0.04 ± 0.01^{bcde}	0.06 ± 0.01^{abc}
9	Phenylacetaldehyde	1623	0.01 ± 0.06^{f}	0.33 ± 0.06^{ab}	0.18 ± 0.03^{de}	0.28 ± 0.06^{abcd}	0.30 ± 0.06^{abc}	0.15 ± 0.02^{e}	0.12 ± 0.01^{e}	0.12 ± 0.02^{ef}	0.35 ± 0.10^{a}
10	2-Methylbutanoic Acid	1633	ND^{f}	ND^{f}	ND^{f}	0.06 ± 0.01^{de}	0.13 ± 0.04^{abc}	0.17 ± 0.04^{a}	0.08 ± 0.02^{cde}	ND^{f}	ND^{f}
11	Ethyl Benzoate	1639	ND^{f}	ND^{f}	ND^{f}	ND^{f}	0.14 ± 0.02^{a}	0.12 ± 0.02^{abc}	0.09 ± 0.01^{bcde}	0.08 ± 0.01^{de}	0.10 ± 0.01^{abcd}
12	1-Hexadecyne	1656	ND^{c}	ND^{c}	ND^{c}	ND^{c}	0.73 ± 0.17^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
13	Methionol	1662	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	0.08 ± 0.03^{b}	0.17 ± 0.04^{a}
14	2-Methyl-2-butenolide	1669	0.05 ± 0.01^{d}	0.18 ± 0.05^{bcd}	0.20 ± 0.05^{bc}	0.23 ± 0.05^{abc}	0.23 ± 0.04^{abc}	0.16 ± 0.03^{bcd}	0.19 ± 0.03^{bcd}	0.29 ± 0.08^{ab}	0.18 ± 0.09^{bcd}
15	Methyl Salicylate	1698	0.04 ± 0.01^{ef}	ND^{f}	ND^{f}	0.31 ± 0.07^{ab}	0.43 ± 0.13^{a}	0.28 ± 0.05^{b}	0.24 ± 0.05^{bcd}	0.07 ± 0.01^{ef}	0.13 ± 0.02^{cdef}
16	2-Tridecanone	1807	ND^{d}	ND^{d}	ND^{d}	0.13 ± 0.03^{bc}	0.17 ± 0.02^{ab}	0.13 ± 0.01^{bc}	0.10 ± 0.01^{c}	0.10 ± 0.02^{c}	0.20 ± 0.04^{a}
17	Dimethyl adipate	1813	0.01 ± 0.002^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
18	Phenethyl acetate	1815	ND^{f}	ND^{f}	ND^{f}	ND^{f}	0.22 ± 0.05^{ab}	0.17 ± 0^{d}.06^{abc}	0.13 ± 0.02^{bcd}	0.12 ± 0.03^{cd}	0.24 ± 0.06^{a}
19	Ethyl Salicylate	1817	ND^{e}	ND^{e}	ND^{e}	ND^{e}	0.21 ± 0.04^{abc}	0.06 ± 0.01^{e}	0.25 ± 0.10^{a}	0.08 ± 0.03^{cde}	0.30 ± 0.07^{a}
20	(E)-β-Damascenone	1821	ND^{g}	ND^{g}	ND^{g}	0.13 ± 0.04^{cde}	0.24 ± 0.05^{ab}	0.19 ± 0.04^{bcd}	0.21 ± 0.04^{bc}	0.03 ± 0.01^{fg}	0.19 ± 0.04^{bcd}
21	Hexanoic Acid	1823	0.01 ± 0.003^{e}	0.24 ± 0.06^{d}	0.05 ± 0.03^{e}	0.33 ± 0.06^{bcd}	0.37 ± 0.06^{bcd}	0.30 ± 0.04^{bcd}	0.25 ± 0.04^{cd}	0.32 ± 0.08^{bcd}	0.54 ± 0.10^{a}
22	Geraniol	1823	0.01 ± 0.06^{c}	0.29 ± 0.05^{a}	0.26 ± 0.04^{a}	ND^{c}	ND^{c}	ND^{c}	0.29 ± 0.04^{a}	ND^{c}	0.29 ± 0.05^{a}

Table 6 Continued

No.	Volatile Compounds	KI	RS	SS	GS	*Lb. plantarum* NRRL B-4496		*Lb. plantarum* NCDO 1193		Natural Fermentation	
						Day 2	Day 4	Day 2	Day 4	Day 2	Day 4
23	Guaiacol	1838	0.09 ± 0.02^{c}	0.14 ± 0.03^{c}	0.08 ± 0.02^{c}	0.11 ± 0.03^{c}	0.20 ± 0.05^{c}	0.11 ± 0.03^{c}	0.10 ± 0.01^{c}	0.70 ± 0.22^{b}	1.19 ± 0.33^{a}
24	Benzenemethanol	1846	0.05 ± 0.02^{g}	0.11 ± 0.03^{fg}	1.43 ± 0.25^{a}	0.36 ± 0.07 ^{cdef}	0.36 ± 0.05^{cdef}	0.13 ± 0.04^{fg}	0.18 ± 0.02^{efg}	0.70 ± 0.23^{b}	0.50 ± 0.10^{bcd}
25	2-Phenylethanol	1865	0.04 ± 0.01^{c}	0.80 ± 0.13^{c}	1.14 ± 0.27^{c}	1.44 ± 0.47^{c}	1.82 ± 0.89^{c}	0.74 ± 0.34^{c}	0.61 ± 0.18^{c}	10.36 ± 1.65^{b}	14.23 ± 3.01^{a}
26	*β*-Ionone	1882	0.02 ± 0.01^{g}	0.21 ± 0.04^{a}	0.11 ± 0.03^{bcd}	0.10 ± 0.01^{bcd}	0.10 ± 0.03^{bcde}	0.11 ± 0.02^{bcd}	0.09 ± 0.01^{bcdef}	0.04 ± 0.01^{efg}	0.13 ± 0.03^{bc}
27	Benzothiazole	1895	0.03 ± 0.01^{e}	0.20 ± 0.04^{abcd}	0.11 ± 0.02^{cde}	0.28 ± 0.10^{ab}	0.14 ± 0.03^{cde}	0.19 ± 0.06^{abcd}	0.14 ± 0.03^{cde}	0.11 ± 0.03^{cde}	0.18 ± 0.04^{bcd}
28	Phenol	2009	ND^{g}	0.04 ± 0.02^{fg}	ND^{g}	0.16 ± 0.02^{efg}	0.34 ± 0.14^{cdef}	0.25 ± 0.14^{defg}	0.34 ± 0.15^{cdef}	0.53 ± 0.12^{bcd}	0.99 ± 0.25^{a}
29	Pentadecanal	2019	ND^{e}	ND^{e}	2.94 ± 0.75^{a}	0.37 ± 0.12^{cde}	0.36 ± 0.16^{cde}	0.07 ± 0.02^{de}	ND^{e}	0.72 ± 0.13^{bc}	0.42 ± 0.11^{cde}
30	*p*-Ethylguaiacol	2025	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	0.14 ± 0.09^{b}
31	Ethyl tetradecanoate	2028	ND^{h}	0.18 ± 0.06^{bcde}	ND^{h}	0.16 ± 0.05^{defg}	0.13 ± 0.02^{efg}	0.05 ± 0.02^{fgh}	0.10 ± 0.05^{efgh}	0.18 ± 0.04^{bcde}	0.26 ± 0.06^{abcd}
32	*γ*-Nonalactone	2030	ND^{e}	ND^{e}	0.02 ± 0.01^{e}	0.11 ± 0.03^{cde}	0.21 ± 0.05^{abc}	0.13 ± 0.02^{cd}	0.14 ± 0.03^{cd}	0.17 ± 0.04^{bcd}	0.25 ± 0.06^{ab}
33	Octanoic Acid	2033	ND^{i}	0.08 ± 0.02^{hi}	0.22 ± 0.06^{lh}	0.23 ± 0.05^{fgh}	0.34 ± 0.06^{bcdefg}	0.29 ± 0.06^{cdefgh}	0.24 ± 0.03^{efgh}	0.45 ± 0.11^{abcde}	0.50 ± 0.11^{abc}
34	2-Pyrrolidinone	2039	ND^{f}	ND^{f}	ND^{f}	0.06 ± 0.03^{def}	0.08 ± 0.02^{de}	0.04 ± 0.01^{def}	0.16 ± 0.04^{bc}	0.03 ± 0.01^{ef}	0.10 ± 0.04^{cd}
35	1-Tridecanol	2088	ND^{d}	ND^{d}	0.13 ± 0.04^{ab}	0.03 ± 0.003^{cd}	0.11 ± 0.04^{abc}	0.13 ± 0.05^{ab}	0.16 ± 0.04^{a}	0.09 ± 0.02^{abc}	0.10 ± 0.02^{abc}
36	Eugenol*	2095	ND^{f}	ND^{f}	ND^{f}	3.06 ± 0.49^{ef}	4.57 ± 0.57^{de}	3.06 ± 1.03^{ef}	2.97 ± 0.53^{ef}	11.25 ± 2.30^{bc}	15.63 ± 2.80^{a}
37	4-Vinylguaiacol	2208	ND^{b}	ND^{b}	ND^{b}	0.07 ± 0.02^{b}	0.18 ± 0.04^{b}	0.08 ± 0.04	0.11 ± 0.03^{b}	0.95 ± 0.39^{a}	1.20 ± 0.35^{a}
38	Methyl Palmitate	2211	ND^{g}	0.12 ± 0.05^{def}	0.10 ± 0.02^{defg}	0.09 ± 0.02^{defg}	0.21 ± 0.04^{bcd}	0.04 ± 0.01^{fg}	0.08 ± 0.01^{efg}	0.19 ± 0.03^{cde}	0.27 ± 0.05^{bc}
39	Ethyl Palmitate*	2228	ND^{f}	1.05 ± 0.33^{cde}	0.23 ± 0.10^{ef}	0.53 ± 0.14^{def}	0.61 ± 0.14^{def}	0.33 ± 0.11^{ef}	0.50 ± 0.16^{def}	1.95 ± 0.54^{abc}	2.39 ± 0.65^{ab}
40	Decanoic acid	2236	ND^{d}	ND^{d}	ND^{d}	0.86 ± 0.35^{a}	0.60 ± 0.22^{abc}	0.71 ± 0.23^{ab}	0.70 ± 0.25^{abc}	0.22 ± 0.07^{cd}	0.09^{d}

Results are expressed as mean ±SE
RS: Raw seeds-RS; SS: Soaked seeds; GS: Germinated seeds; ND: not detected
Significant differences ($p<0.05$) among samples analyzed using Student's t test between the samples represented with different letters
(*) compounds are identified by comparing of retention time and mass spectrum of authenticated standards
All compounds were identified by mass spectral data, comparing KI from literature ([124-126]) by EI and CI mode

affects certain organic acids, acid esters and fatty acids. Acetoin and 2,3-butanediol are considered as plant growth-promoting compounds while benzaldehyde is a GRAS food additive used as flavoring agent and in aromatic applications[129]. Furthermore, fermentation process of horse gram sprouts also showed major impact on the volatile composition of horse gram sprouts. With increasing relevance of probiotic fermented foods for providing nutritional quality and therapeutic health benefits[130] targeted lactic acid bacterial-mediated fermentation in the current study as a bioprocessing strategy can ensure optimum consistent quality and potential food safety to develop acceptable fermented food product. In the present study, *Lactobacillus plantarum* NRRL-B 4496 and *Lactobacillus plantarum* NCDO 1193 were selected based on the preliminary experiments that determined the optimum growth parameters suitable for this legume system. Application of potential probiotic fermentation to dry edible seeds could be limited due to lack of bioconversion of key nutrients such as amino acids that are readily available in dairy products[131]. Therefore, to improve nutrients for bacterial growth soaking and germination prior to fermentation supports nutrients needs essential for bacterial growth and biotransformation. During germination, several metabolic activities such as proteolysis facilitates breakdown of protein into peptides and free amino acids which itself impart some flavor to the sprouts and also provide nutrients for lactic acid bacteria [132]. Therefore, in the present study, germination prior to fermentation of horse gram sprouts facilitated optimum growth of the two LAB strains, which further led to production of array of flavor and aromatic compounds during fermentation.

4.4.2. Changes in flavor and aromatic compounds during fermentation

GC-MS analysis of horse gram sprouts fermented by *Lactobacillus plantarum* NRRL-B 4496 and *Lactobacillus plantarum* NCDO 1193 showed presence of acetic acid and butyric acid (**Table 5 and 6**). Generally these aliphatic organic acids are produced under anaerobic condition by human gut microbiota due to degradation of dietary fibers [133]. Since human metabolic pathways lack critical enzymes to metabolize the dietary fibers, colonic microbial population aids such degradation and produces short chain fatty acids (SCFAs) mainly acetate, propionate and butyrate in the average molar ratio (60:25:10 mmol/l). These major SCFAs might play an important role in the prevention and potential management of disease conditions such as metabolic syndrome, bowel disorders and also certain types of cancer [134]. It seems that *Lb. plantarum* spp. possess the ability to consume lactic acid and releases an equivalent amount of acetic acid under micro-aerobic conditions[135]. Accordingly, the concentration of acetic acid found in the fermented horse gram sprouts in the present study indicates that *Lb. plantarum* can effectively metabolize organic acids into SCFAs such as acetic acid and butyric acid. Other acids such as 3-methylbutanoic acid, hexanoic acid, octanoic acid and decanoic acids are fatty acids derived from either amino acids or carbohydrate metabolism. Some of these acids were not detected during the initial stage of fermentation, instead appeared as the fermentation proceeded. Complex metabolic activities occurring during fermentation led to significant changes in the concentration of each of those compounds, mainly acids, alcohols and esters. Among 7 acids, all the samples showed high amount of acetic acid and octanoic acid during lactic acid bacterial fermentation and hexanoic acid

increased during natural fermentation while other acids remained unchanged. Naturally fermented horse gram sprouts for 4 and 5 days showed highest amount of organic acids content, followed by *Lactobacillus plantarum* NCDO 1193 fermented sprouts and *Lactobacillus plantarum* NRRL-B 4496 fermented sprouts.

On the other hand, contents of aldehydes except benzaldehyde were observed to be highest only during the initial stage of all types of sprouts fermentation. Phenyl acetaldehyde and benzaldehyde might be products of aromatic amino acid phenylalanine metabolism, while some sulfur-containing compounds such as methionol derived from methionine as nitrogen source[136, 137]. Microorganisms are able to reduce certain type of aldehydes to alcohols or oxidize to acids[138]. In the present study, aldehyde content particularly pentadecanal decreased during fermentation, possibly converted into alcohols and acids during the fermentation process. Most of the aldehydes decreased during *Lb. plantarum* fermentation of cherry, pineapple, carrot and tomato juices with corresponding increase in the level of alcohols[139]. Alcohols forms the largest group of volatile compounds identified in fermenting horse gram sprouts which suggests its importance in the organoleptic properties of fermented horse gram sprouts. As mentioned previously, 2,3-butanediol plant growth promoting compound is a product of glucose fermentation by *Lb. plantarum* via acetoin reduction, stimulated by lower pH of the fermenting medium [140]. Hence content of 2, 3-butanediol increased during *Lb. plantarum* fermentation of horse gram sprouts with corresponding effect on acetoin concentration. Other alcohols (2-phenylethanol) are also a product of aromatic amino acid metabolism while benzyl alcohol impart pleasant odor to the product. In addition, 1-hexanol, 1-tridecanol and 2-

ethylhexanol are condensation products of alcohols, whereas linalool and geraniol are monoterpene alcohols present as secondary metabolites in legumes[141]. Several ketones are produced during fermentation but very few have sensory significance such as acetoin. Some nor isoprenoid ketones such as β-damascenone that were not detected in raw, soaked seeds or sprouts were observed after fermentation while β-ionone, a common flavor constituent in wines was also detected in trace amount. These compounds are known to be products of carotenoid degradation[142]. It was also observed that the 2-methyl-2-butenolide which increased during germination remained unchanged throughout fermentation period. Other ketones, such as γ-nonalactone and 2-tridecanone were not detected in raw, soak seeds and sprouts but were remarkably identified in fermented horse gram sprouts samples. Acetoin (3-hydroxy-2-butanone) was not detected in raw seeds but gradually increased during germination and the highest amount was found in naturally fermented horse gram sprouts followed by sprouts fermented by *Lactobacillus plantarum* NRRL-B 4496. Lactic acid bacteria are able to anaerobically metabolize substrates such as citrate and pyruvate as product of fermentation to produce acetoin and diacetyl in fermented cereals [143].

Esters are widely known as most important flavor constituents of alcoholic beverages which impart fruity flavors to the fermented products. These are produced either by condensation reaction between alcohols and acids or due to enzymatic activity of certain microorganisms, commonly yeasts [144]. In the present study, 2-phenyl ethyl alcohol being the most abundant alcohol formed during fermentation resulted in increasing phenyl ethyl acetate levels. Other acid esters such as ethyl benzoate, ethyl salicylate, methyl

salicylate, ethyl palmitate, methyl palmitate are volatiles imparting pleasant flavor and aroma to fermented products and are derived from respective acids and alcoholic reactions. Dimethyl adipate which is an ester of adipic acid was only detected in sprouts fermented by *Lactobacillus plantarum* NRRL-B 4496 at 5 days, whereas it was absent in all other samples, which explains the rare occurrence of adipic acid in foods.

4.4.3. Volatile Phenols – Biosynthesis Proposed Pathways

Volatile phenols are aromatic compounds commonly reported as major concerns in white and red wine sensory quality. Each of these volatile phenols such as *p*-ethylguaiacol, 4-vinylguaiacol, guaiacol, 4-ethyl phenol, eugenol and vanillin imparts distinct aroma varying from bacon to smokey and spicy like clove to the fermented products. In the present study, eugenol was the second major volatile compound detected in fermented horse gram sprouts but only trace amounts was found in raw, soaked and sprouts (**Table 5 and 6**). This indicates biosynthesis of eugenol during fermentation that might be dependent either on the fermenting lactic acid bacteria or certain precursors that are absent in raw seeds but formed during germination. It is well known that eugenol is a major constituent in essential oils such as clove, nutmeg and cinnamon. Previously, L-methyl-eugenol and eugenol was detected in extracts of soybeans and mung beans, while their strong antioxidant activity was also assessed[145]. Presence of such phenyl propenes in the vegetative parts of the plant represents to be a crucial part of defense mechanism against herbivorous, parasitic bacteria and fungi. However, when the seeds enter dormant stage during dehydrating stage the defense mechanism is apparently suppressed.

Therefore, the most likely mechanism for biosynthesis of eugenol exclusively during fermentation in the present study is the conversion of hydroxycinnamic acids coenzyme-A thioesters to eugenol via products of phenyl propropanoid pathway obtained from horse gram sprouts (**Figure 4**). Based on some reported eugenol production mechanisms [146, 147], a brief description for the proposed biosynthesis pathway for the eugenol detected in the present study is as follows: when germinating horse gram seeds are metabolically active in the form of sprouts several accelerated metabolic pathways such as glycolysis, pentose phosphate pathway and shikimate pathways are expressed. These pathways in the sprouts produces phenylalanine and tyrosine, which are further acted upon by phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), respectively as first committed enzymes in the pathway towards other phenypropanoids which may be then be used in subsequent eugenol synthesis by lactic acid bacteria. These two potential horse gram sprout enzymes channel the aromatic amino acids from the primary metabolic pool towards the biosynthesis of hydroxycinnamic acids. Such activity of PAL and TAL mainly occur in all the plants, certain type of fungi and some bacteria but not detected in animals [148]. It is likely that in the present study, PAL and TAL catalyzing reactions occurred during seed germination followed by fermentation by lactic acid bacteria as well as sprouts native microflora with further conversion of hydroxycinnamic acids and related precursors to respective coenzyme A thioester that are substrates for further condensation, reduction and transformation type reactions producing coniferyl alcohol, which can then be further reduced to coniferyl acetate or some related esters for the final stage of eugenol synthesis (**Figure 4)** [149].

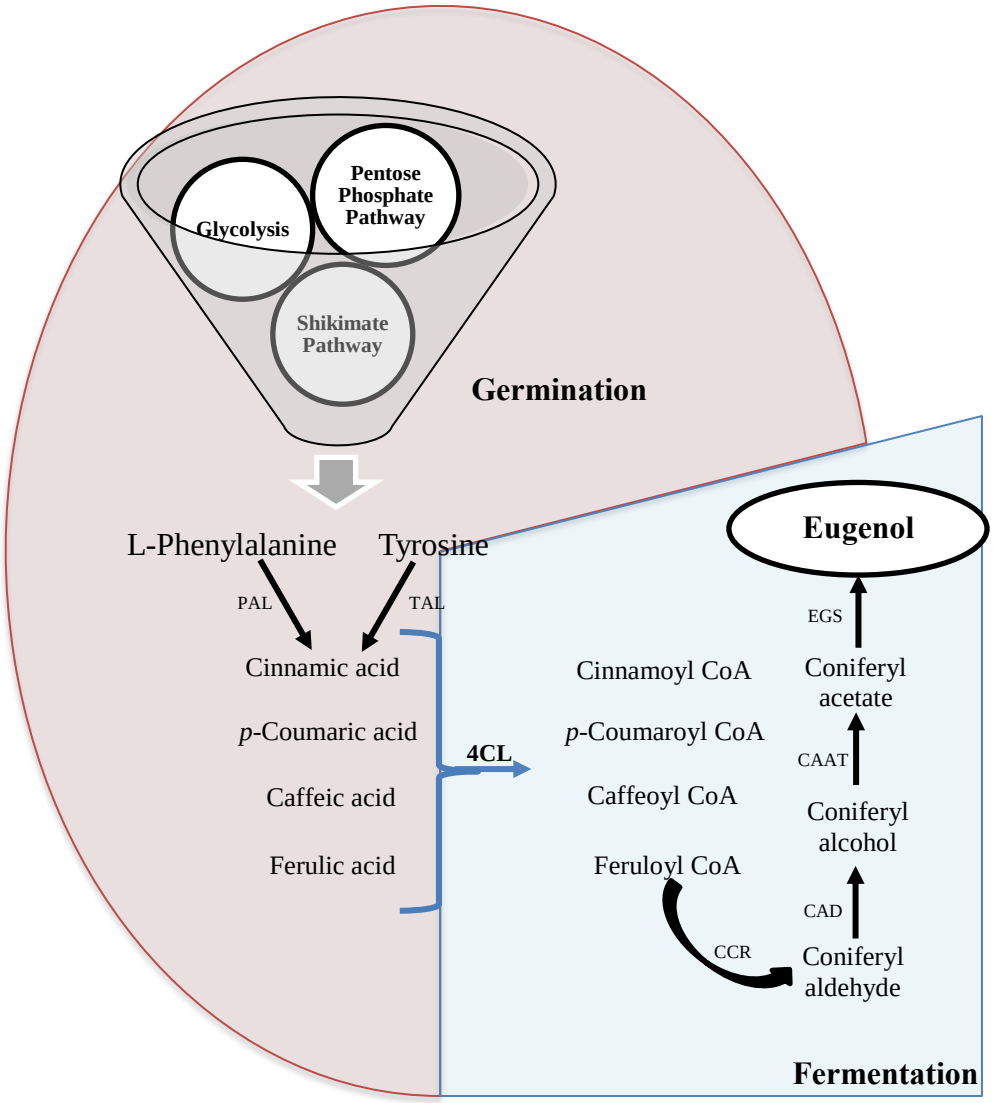


Figure 4 Proposed biosynthetic pathway of eugenol in fermented horse gram sprouts. (PAL Phenylalanine ammonia-lyase; TAL Tyrosine ammonia-lyase; 4CL 4-coumarate CoA ligase; CCR cinnamoyl-CoA reductase; CAD cinnamyl alcohol dehydrogenase; CAAT coniferyl alcohol acetyl transferase; EGS eugenol synthase) [Reprinted from *147, 150*].

Phenylpropanoids such as eugenol are integral part of plant defense mechanism and may also serve as the building blocks of phenolic based polymer lignin [151] but requires special sequestered glandular vesicles to be formed in plants. An important enzyme catalyzing the formation of coenzyme thioesters of hydroxycinnamic acids and subsequently phenylpropanoids production is 4-coumarate: CoA ligase (4CL; EC 6.2.1.12). However, since biosynthesis of phenylpropanoids in plants such as legumes is strictly regulated by glandular vesicular developmental processes and by environmental elicitors such as microorganisms [152] also lactic acid bacteria that are involved in the horse gram fermentation were most likely the source of eugenol after key precursors were generated during the germination process Since there is no clear information about genetic markup of horse gram seeds, scientific evidences for closely related legume such as soybean seedlings provides some insights on eugenol precursor production during horse gram sprouts and later conversion by lactic acid bacteria during fermentation. Unlike other plants, isoenzymes of 4CL in soybean contain distinct substrate affinities that are catalytic specific that subsequently leads to branched phenylpropanoid pathway to produce precursors for lignin [150]. Distribution of 4CL expression pattern in soybean seedling to some extent supports the results observed in the present study. In a previous study, it was reported that amongst the four 4CL mRNAs isolated from soybean seedlings (*4CL1, 4CL2, 4CL3* and *4CL4*), two *4CL1* and *4CL2* mRNA amounts were maximum in hypocotyls, stem and young roots, while others *4CL3/4* mRNA were observed to be in lower amount in roots and hypocotyls of soybean seedling under study [152]. Such scientific evidences related to the present study led to the suggestion that elicitor specific 4CL

catalytic activity in legume such as horse gram may play a vital role for development of substrates for subsequent condensation, reduction and transformation type reactions involved in biosynthetic pathways of phenylpropanoids such as eugenol [152].

There is little information available related to these pathways and genomic information for both the strains of *Lactobacillus plantarum* used in the present study, though closely related LAB strains have been reported to possess certain enzymes facilitating conversion of phenylpropanoid pathway metabolites and therefore LAB could use the plant-based precursors to bio transform eugenol during the active fermentation stage [70, 139, 153]. Therefore, high concentration of eugenol bio-transformed during fermentation could be due to the horse gram precursors from initial biosynthesis of aromatic amino acids such as phenylalanine via shikimate pathway during germination which in turn further led to production of eugenol by natural and lactic acid bacterial fermentation aligning to the above proposed mechanism. Phenylpropenes such as eugenol are potent antioxidants, also possess strong antimicrobial activity, particularly anti-fungal due to which it has potential application relevance as natural preservatives or flavor ingredients in foods. The known biochemical pathways for synthesis of eugenol is limited and present study potentially offers a new bioprocessing strategy for producing eugenol that links germination and natural fermentation. This is one of the most interesting findings of this study, since the highest concentration of eugenol about 80-90% which is approximately 125929 µg/g FW is found in clove glandular vesicular systems [154] and compared to this, the concentration of eugenol produced during horse gram germination/natural fermentation process was about 3.06 – 15.63 µg/g of fresh weight of

fermented horse gram sprouts. Such level of eugenol is several folds lower to the amount related to severe effects on hepatotoxicity due to consumption of clove oil which is rich in eugenol[155]. This creates an interesting new opportunity for further detailed transcriptomic and molecular studies of horse gram seeds and its products from a combination of germination and natural fermentation as an innovative bioprocessing strategy.

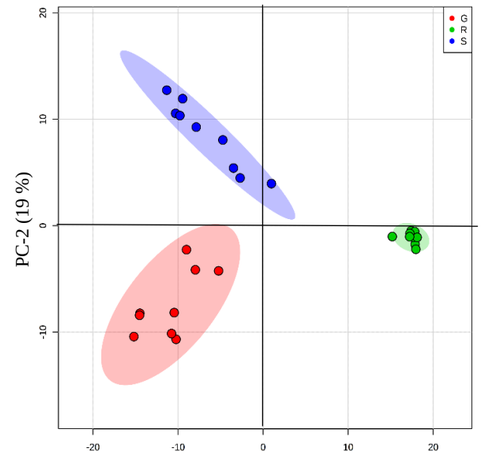
4.4.4. Organoleptic Importance

In the present study, acetic acid, hexanoic acid, 2, 3-butanediol, octanoic acid, eugenol, 4-vinylguaiacol, methyl palmitate, ethyl palmitate, decanoic acid were predominant in *Lb. plantarum* spp. fermented horse gram sprouts. These compounds have been previously identified as crucial aromatic compounds in foods fermented by *Lactobacillus plantarum* strains [156]. It was observed that *Lactobacilli* strains produce high amount of certain volatile compounds including acetaldehyde, diacetyl, ethyl acetate, acetone and ethanol which can strongly affect the organoleptic qualities of the fermented products. While there were some important volatile compounds detected only in naturally fermented horse gram sprouts, such as methionol (3-(methylthio)-1-propanol) and *p*-ethylguaiacol but these were not detected in LAB fermented sprouts. A volatile sulfur compound such as methionol is commonly found in alcoholic beverages and widely distributed as an aromatic compound imparting off-odor "cauliflower aroma" to fermented foods especially in dry wines during ageing[157]. The other volatile compound *p*-ethylguaiacol is generally found in fungi and produced during pathogenic fungus

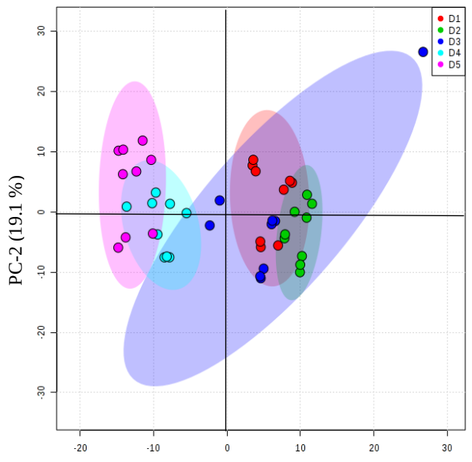
Phytophthora cactorum-mediated infection of cucurbit crops in southeastern United States. Its presence indicates leather rot disease in strawberry plants and gives unpleasant odor to strawberries affecting nearly 50% of strawberries produced in the U.S. [158]. In the current study, presence of such undesirable volatile compounds in naturally fermented horse gram sprouts is reflection of unpleasant aroma. The fact that these undesirable volatiles are absent in targeted LAB fermented horse gram sprouts indicates potential to improve sensory characteristics through targeted LAB-mediated fermentation systems.

4.4.5. Principal Component Analysis (PCA)

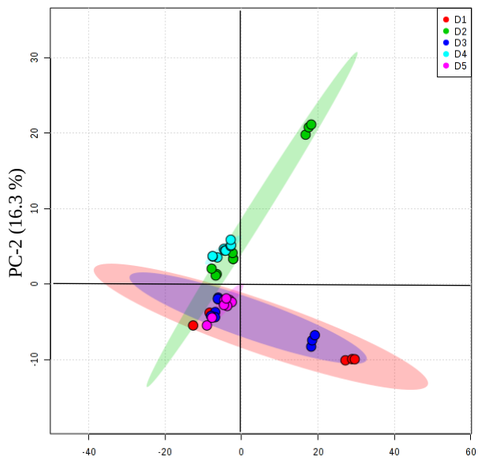
PCA is an effective tool for compressing large data sets into a summarized form of variables, which can be presented as simplified and visual data [159]. The volatile compound data from all the treatments were analyzed to understand the variation of principle components during germination and fermentation process (**Figure 5**). Germination distinctly differentiates volatile compounds profile of sprouts from raw and soaked seeds. With PC2, some volatile compounds like hexanol and linalool were located along the axis and these compounds were of importance during hydration of dry seeds. The levels of aldehydes such as pentadecanal was observed to be highest in sprouts (**Figure 6**), thus was higher at the beginning of fermentation and positioned corresponding to the germinated seeds. However, positioning of pentadecanal only towards initial fermentation stages indicate that they originated mainly from the raw materials of seeds or sprouts and were utilized as precursors for the subsequent fermentation by microorganisms (**Figure 6**). This corresponds to reduction in the levels of pentadecanal



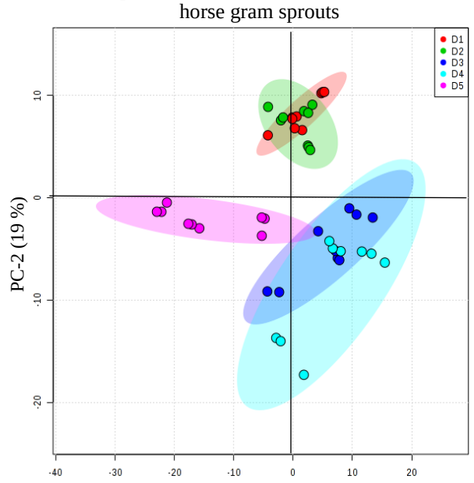
Raw, soaked and germinated horse gram seeds



Lb. plantarum NRRL B-4496 fermented horse gram sprouts

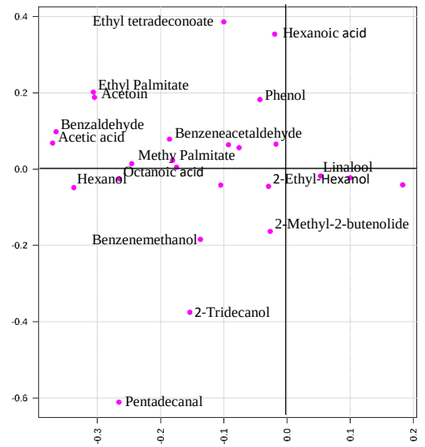


Lb. plantarum NCDO-1193 fermented horse gram sprouts

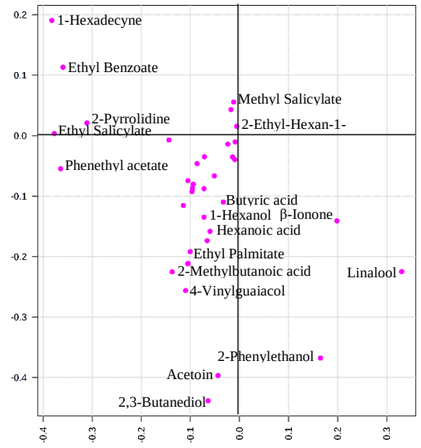


Naturally fermented horse gram sprouts

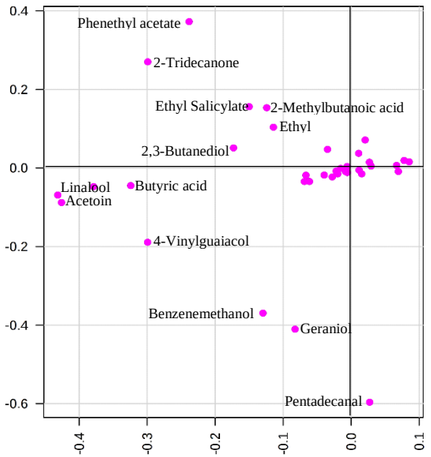
Figure 5 The score plots of PCA: horse gram dry raw seeds (R), soaked seeds (S) and germinated seeds (G), horse gram sprouts fermented by *Lb. plantarum* NRRL B 4496 from one to five days (D1-D5), horse gram sprouts fermented by *Lb. plantarum* NCDO 1193 from one to five days (D1-D5), horse gram sprouts naturally fermented from one to five days (D1-D5).



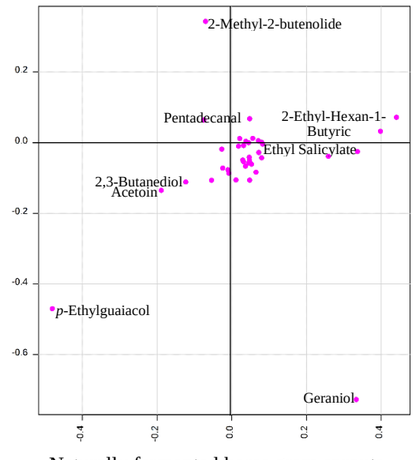
Raw, soaked and germinated horse gram seeds



Lb. plantarum NRRL B-4496 fermented horse gram sprouts



Lb. plantarum NCDO-1193 fermented horse gram sprouts



Naturally fermented horse gram sprouts

Figure 6 The loading plot for all the identified volatile compounds in horse gram dry raw seeds, soaked seeds and germinated seeds, horse gram sprouts fermented by *Lb. plantarum* NRRL B 4496 from one to five days (D1-D5), horse gram sprouts fermented by *Lb. plantarum* NCDO 1193 from one to five days (D1-D5), D-horse gram sprouts naturally fermented from one to five days (D1-D5).

with the increasing fermentation period (**Table 5**). Classifying the fermented sample plots according to fermentation time revealed that the profiles of the volatiles moved from right to left along the PC1 dimension with increasing fermentation time in both LAB fermented sprouts samples, accounting for the observed steady changes in profiles as fermentation proceeded. The *Lb. plantarum* NRRL B-4496 fermented horse gram samples could be differentiated primarily according to their PC1 score, with the initial fermentation period (day 1, 2 and 3) being positioned on the positive side of the axis, opposite to later fermentation period (day 4 & 5). In particular, mid-stream fermentation of about day 2 and 3 lined along with PC2 axis in the middle similar to later fermentation stages (day 4 and 5) being positioned close to each other on the positive side of axis. As shown in (**Figure 5**), positive axis on PC1 was highly influenced by volatile compounds such as acids, acid esters, volatile phenols and alcohols which exhibited marked concentration changes during the initial fermentation stage especially the levels of acetic acid and eugenol. Compounds with the highest concentration were eugenol and acetic acid apparently increasing during final days of fermentation (day 4 and 5) seemingly clustered along with other volatile compounds on the side corresponding to later fermentation stages. Moreover, we observed dense cluster of volatile compounds at the center of both axis, which signifies that most of the flavor and aromatic compounds were produced in all the fermented horse gram samples, irrespective of fermentation period. This also signifies LAB fermentation resembles natural fermentation of horse gram sprouts except for the production of some off-odor producing compounds such as *p*-ethylguaiacol in natural fermentation. The duration of fermentation by *Lb. plantarum* NRRL B 4496 and *Lb.*

plantarum NCDO 1193 mainly affects the concentration of volatile compounds rather than the composition.

Certain volatile compounds such as acetoin, 2,3-butanediol, phenylethyl acetate and acetic acid were formed from pyruvate metabolism and contributed to the negative PC1 axis for naturally fermented horse gram sprouts and their concentrations increased as fermentation proceeded (**Figure 6**). These compounds were previously identified as key aroma compounds in *Lactobacilli* fermentation of barley and malt substrates [156]. These results indicated that the horse gram sprouts fermented using *Lb. plantarum* spp. at different fermentation times could be clearly differentiated at the initial and middle fermentation stages (day 1, 2, 3, 4 and 5) positioned on the negative PC1 axis due to their relatively high contents of aldehyde compounds, and the later fermented samples positioned on the positive PC1 axis due to the presence of acids, alcohols, esters and ketones. As discussed before, HS-SPME GC-MS benefits in detection and identification of volatile compounds in fermented horse gra sprouts (**Figure 7**) with authentic standards injections for comparison of retention time (**Figure 8**).

4.5. Conclusions

The principal component analysis distinguished the raw horse gram seeds from soaked as well as germinated seeds. It also differentiated stages of fermentation into five groups divided by the concentration of volatile compounds produced enduring fermentation. Significant levels of pyruvate fermentation products such as acids, alcohols were identified in the fermented samples which were not detected in raw seed flour or

sprouts. Detection of ketones, esters and acids also indicates possible lipid oxidation during fermentation. Since horse gram seeds are rich source of proteins, germination and fermentation induced proteolysis potentially releasing major aromatic amino acids, along with those from shikimate pathway in sprout primary metabolism, and these are likely precursors for biosynthesis of eugenol through biotransformation by natural and lactic acid bacterial fermentation. In addition, to eugenol presence of off-odor producing compounds in naturally fermented sprouts make it unlikely choice for commercial method of fermentation. Therefore, strains of *Lb. plantarum* spp. with probiotic potential as targeted fermenting bacteria would advance innovative bioprocessing strategies to produce desirable flavor and aromatic volatile compounds including a potent antioxidant, eugenol that also adds to therapeutic benefits along with strong anti-fungal activity of fermented horse gram sprouts.

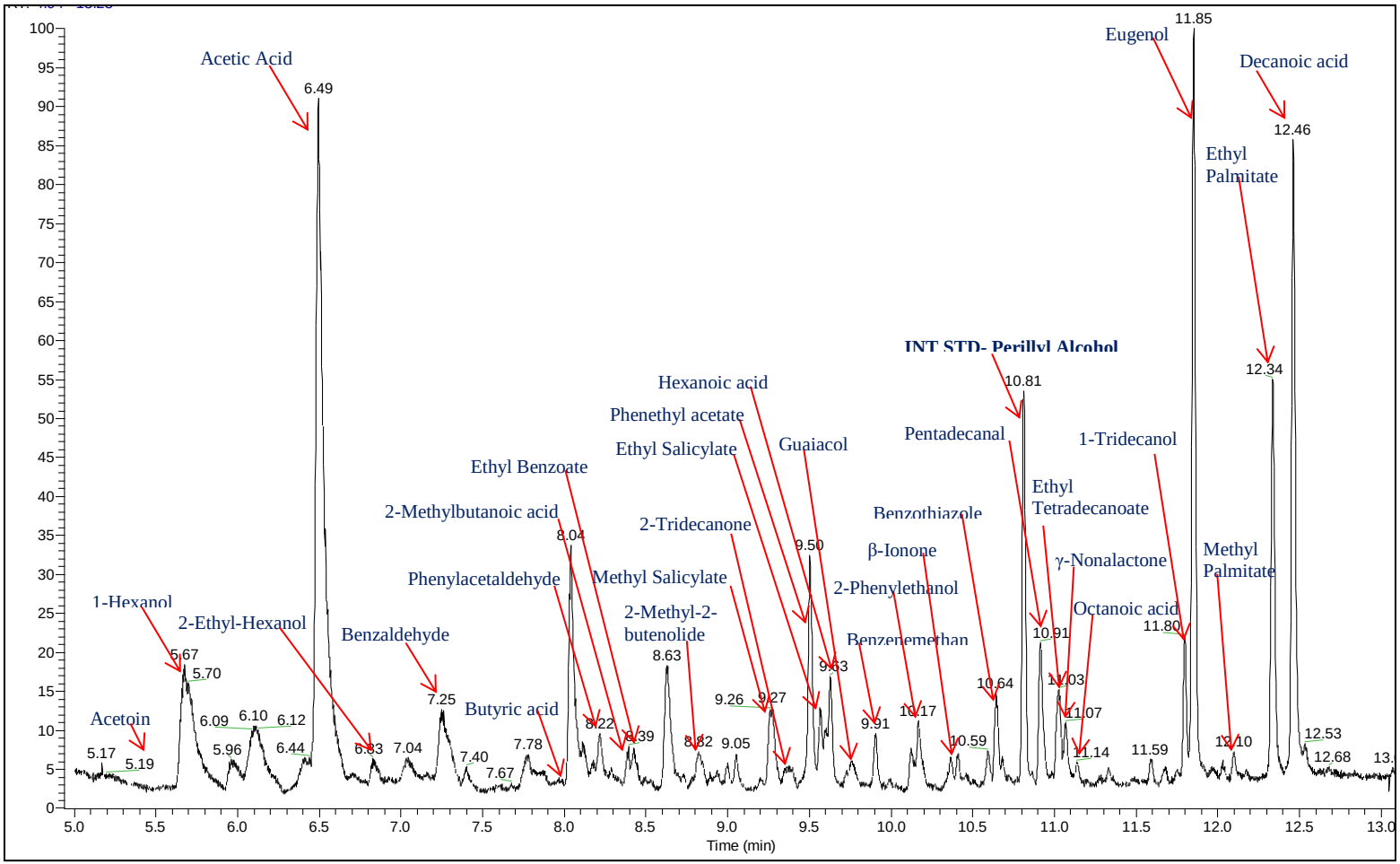


Figure 7 Peaks in a GC-MS chromatogram flagged with respective volatile compounds identified in fermented horse gram sprouts.

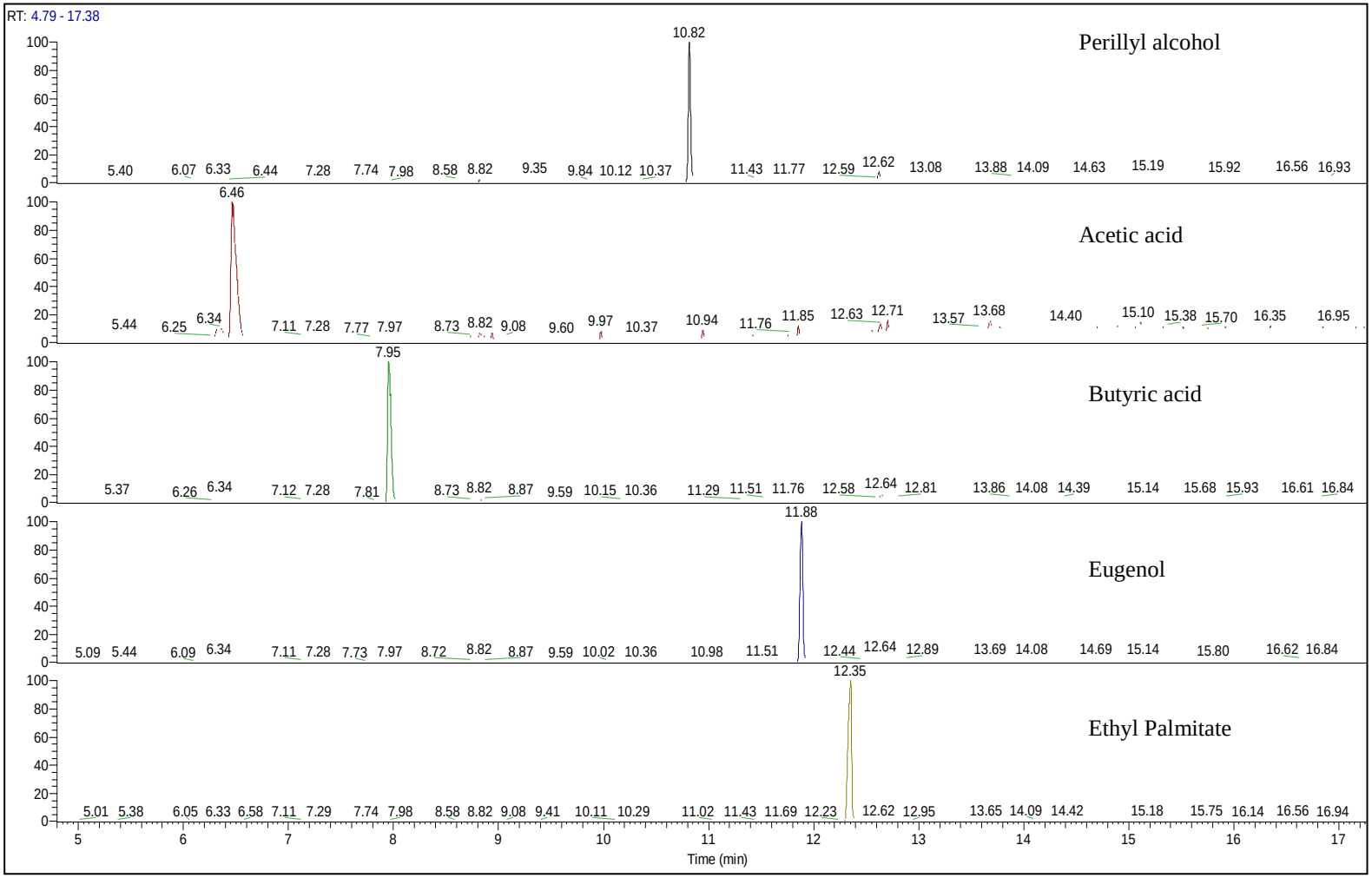


Figure 8 GC-MS chromatograms of pure standards with their retention time (mins).

5. CONCLUSIONS

Horse gram traditionally grown in arid regions of the developing countries is wholesome food that should be added to diet on regular basis. Raw horse gram seeds have been studied by several researchers for various health benefits. The present study showed that conventional processes such as germination, cooking and fermentation significantly ($p<0.05$) influences the levels of essential amino acids, protein content, free radical scavenging activity, α-amylase and α-glucosidase inhibition activity as well as imperative volatile compounds. Germination and lactic acid fermentation significantly increases levels of essential amino acids, free radical scavenging activity, carbohydrates hydrolyzing enzymes compared to raw seeds. Heat treatments such as cooking in significantly reduces the levels of essential amino acids and protein content while free radical scavenging activity was observed to be increasing during cooking. Lactic acid fermentation overall increases the amount of total phenolic content corresponding to the increase in free radical scavenging activity of fermented horse gram sprouts. Volatile compounds, importantly flavor and aromatic compounds increased with increasing fermentation time. Amongst all the volatile compounds identified, acetic acid and eugenol are the major compounds quantified during lactic acid fermentation as well as natural fermentation. Fermented legumes are significant part of the diet of large population belonging to some developing countries, since it the most economical and convenient method of processing and preserving foods. However, lactic acid bacteria fermentation led to desirable changes in the bioactive compounds as well as flavor aromatic compounds.

Furthermore, the current study is an addition to the existing science of horse gram and it possibilities to be further explored beyond food and medicinal aspects for its chemo profile, pharmacology, biological evaluation, toxicological consequences and agronomical benefits.

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