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Chemical Characterization of Chitin from New Zealand Plants and their use as digestive aids

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A Dissertation
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Master of Science

at
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by
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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Master of Science.

Chemical Characterization of Chitin from New Zealand Plants and their use as digestive aids

by

Athira Jayasree Subhash

Natural polysaccharide, Chitin is a biopolymer isolated from shells and exoskeletons of fungal, algal, insect, molluscs and crustacean sources, and the extraction method varies among different sources. Among all these sources, crustaceans produce millions of tonnes of this polysaccharide every year, and are discharged in the form of snow in the sea floor, causing harm to the marine ecosystem. Known for their biocompatible, biodegradable and non-toxic nature, chitin and its monomers finds significant role in different sectors of economy. Even though, extraction of chitin from marine sources increases their economic value, one important factor, which is limiting this process, is the inconsistency in raw material supply annually and variability in physicochemical characteristics, owing to difference of process conditions. To overcome these problems and to develop clean labeled chitin polymers, alternative sources could be considered which offers consistent production. This work hence focuses on extraction of chitin from leaves of New Zealand plant- *Phormium tenax*, using *Agaricus bisporus* mushroom as positive control. Leaves were considered, as seeds of the same plant gave comparable results to standard chitin extraction, when carried out previously at Lincoln University. Leaves of *Phormium tenax* are known for their known fibre content and exhibits properties and applications similar to chitin. The leaf fibres are used by Maori community mainly for wound healing, and textile applications and are an important part of New Zealand bio-diversity.

. The fibre Approximately 37 g/100g and 38 g/100g of Chitin are obtained respectively from *Agaricus bisporus* (positive control) and pure chitin (standard chemical) after extraction, as quantified by HPLC-FLP method. Overall, the recovery is comparatively low, hence disproving the hypothesis that this extraction method works successfully in extracting chitin from all sources. Also, the chitin recovered from *Phormium tenax* leaf is non-quantifiable comparing to mushrooms and pure chitin, hence indicating the chance of some complex interaction between chitin and other bioactive components present in leaves. The last part of the study was the development of a fermented functional beverage, kefir and study of its physico-chemical properties. 5 different formulations of kefir (4 with milk as base and 1 with carrot juice as base); with roasted and un-roasted white and brown button mushrooms (*Agaricus bisporus*) were considered to study if mushrooms had a stabilization effect on kefir. The results of viscosity and pH clearly indicate that roasted white mushrooms offers a stabilization effect on kefir, compared to unroasted and roasted brown mushrooms. However no significant results were obtained in TSS and syneresis studies.

Keywords: Chitin, Chitosan, non-protein nitrogen, crustaceans, *Phormium tenax*, leaves, *Agaricus bisporus,* de-proteination, deacetylation, caharacteriation, biosynthesise, extraction, functional foods, Kefir, pH, TSS, viscosity, syneresis.

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Chapter 1
Introduction

Chitin is the principal source of carbon and nitrogen for chitinolytic organisms, which are largely marine organisms, soil fungi and soil bacteria. These organisms decompose chitin using their metabolic mechanisms by virtue of which they detect and modify small chitin oligosaccharides (Winkler, Dominguez-Nuñez et al. 2017). Chitosan, monomer of chitin, which is a more soluble biopolymer, is produced via deacetylation of one among the four GlcNAc units of the chitin polymer. Chitosan is a recognized biopolymer with antibacterial potential and acts as a source of nutrients for soil organisms (Zhao, Park et al. 2010). It is difficult to work with these complex biopolymers and hence researchers transformed these complex substances into low-molecular-weight oligosaccharides, chito-oligosaccharides (COSs) (Kim 2010), and exploited their potential by increasing their applications in diverse scientific fields. Glucosamine units (<100) acts as monomers of COSs, and contributes to their low viscosity and molecular sizes.

The chemistry of wound healing of chitin, when studied was found to be due to the fibrous nature of chitin particles. This chemical nature was found to be comparable to the chemical structure and anatomy of the leaves of native New Zealand plant, Phormium tenax. The crystalline microfibril nature of chitin, gives chitin containing compounds its rigid structural backbone. Therefore, there is a strong need to study the physico-chemical properties of the native *Phormium tenax* leaves which contributes to a greater portion of New Zealand biodiversity. If this research gap is justified, then a new source of chitin, plant chitin which was not identified before can be found out. This chitin from plant source if extracted successfully would act as a better clean labeled chitin, in terms of extraction and environmental pollution. Various studies have been carried out, not on the leaves, but on the seeds of *Phormium tenax* by various reserachers. Experimental trails have also been carried out as a part of FOOD 399, FOOD 699 during 2017 and 2018 at Department of Food, Wine and Molecular Sciences, Lincoln University, to find out the presence of chitin in the seeds of New Zealand Flax. The physico-chemical characterization of seeds showed the presence of some amounts of nitrogen, which were not essentially amino acids. Hydrolysis of the seeds, followed by their primary quantitative and qualitative studies indicated the presence of chitin. However, this result needs multiple trials for acceptance. For this, other parts of the same plant were considered. Leaves of *Phormium tenax*, which resembles chitin in terms of fibre

content, were considered in this study. An effort is made in this work to figure out if in any form or quantity, chitin is present in the leaves of *Phormium tenax*.

1.1 Mushrooms and functional foods

Functional food is the term given for everyday foods that are produced conventionally and could be consumed as a part of regular diet. These foods offer positive effects on our system by enhancing its functionality and thereby reducing the risk of diseases. There is a major misconception on this concept of functional foods that it prevents or stops the occurrence of any diseases. However, unlike neutraceuticals, functional foods do not prevent the outbreak of any diseases; rather reduce its intensity (Reis, Martins et al. 2017). Apart from their texture and flavour, nutritional value and medicinal properties are the other properties that drove researchers to find out more on if the fungal source could be made to a functional food. Various studies have reported multifunctionality of *Agaricus bisporus* mushrooms. They are known for their immunomodulating, antioxidant, anti-tumorous, anti-diabetic, cardio-vascular and even anti-microbial properties. Various bioactive compounds like catechin, gallic acid, caffeic acid, ferulic acid and myricetin were obtained by ethanolic extraction of *Agaricus bisporus* and hence contributed for all the above mentioned properties. The presence of phenolics, vitamins C and E and pigment carotene makes mushroom an excellent antioxidant. They are rich in polysaccharide fibres like chitin, hemicellulose, mannans, xylan and galactans, conferring their prebiotics potential (Jayachandran, Xiao et al. 2017). Even though mushrooms have been used in baked products like cake, bread etc, as carbohydrate replacers, none of them are marketised till date. An effort has been made in this work to develop a fermented beverage, with mushroom as chitin source, to find out its functionality in terms of stabilizing agent.

A new class of fermented beverage that came in recently is water kefir or sugary kefir. Being non-dairy kefir prepared out of other sucrose sources like molasses, fruit juices etc, it offers a better alternative to people with lactose intolerances. Kefir grains contains consortium of microbes (yeasts- Candida, Saccharomyces and lactic acid bacteria), in water soluble matrices named kefiran and aids to fermentation of the product (Randazzo, Corona et al. 2016). Vegetables and fruits, if consumed offer immense health benefits to people regardless of their age groups. However, post-harvest injuries and their complex chemistry affect their shelf life. However, if fermentation is carried out on fruit juices, they could be preserved for long.

Water, sugar and other bioactive compounds present in fruit juices makes them ideal candidate for development fermented beverages like kefir (Puerari, Magalhães et al. 2012).

1.2 Statement of Problems

(1) Studies done by researchers till date have failed to find out a successful method for identification, characterization and quantification of chitin from plant source, i.e. leaf here.

(2) Studies conducted by the Department of Wine, Food and Molecular Biosciences of Lincoln University on *Phormium tenax* seeds, found out some percentage of Nitrogen that was not essentially amino acids. Primary studies done after hydrolysis of the seeds indicated that compounds were chitin. Hence, there is a need to analyse other parts, like leaves, of the same plant to validate the previous studies.

(3) Functional foods developed with mushrooms as stabilizers and emulsifiers exist as solid food products. There is a need to figure out if they exhibit these properties in other food types including beverages. Fermented beverages form a new class of product preferred by people for their prebiotic functionality. Hence an approach is made for developing a fermented beverage, from plant source, and could aid in digestion.

1.3 Hypothesis

This study focuses on 3 main stages:

(a) As chitin have similar chemistry in all sources, methods used for chitin isolation from mushroom samples could also be applied to other plant-based samples like leaves.

(b) As chitin is fibrous in nature, resembling the structure of *Phormium tenax* leaves, chitin could be isolated from *Phormium tenax* leaves.

(c) Mushrooms are known to act as digestive aids in normal un- fermented foods and beverages, because of the chitin present in it. The potential of mushrooms as digestive aids and stabilizers, if used in fermented functional beverages will be studied.

1.4 Aims

This work is aimed at verifying the 3 hypotheses:

(a) To verify and apply mushroom based chitin quantification and extraction methods on *Phormium tenax* leaves, using chitin as control, mushroom as positive sample and *Phormium tenax* leaves as experimental sample.

(b) To quantify chitin, if present in New Zealand leaves using HPLC technique.

(c) Development of a fermented functional beverage, Kefir, using mushrooms and analysis of its physico-chemical properties.

1.5 Pitfalls

The observed drawbacks of the study were,

(a) The method gave quantitatively low concentration of standard chitin.

(b) Chitin content as obtained from HPLC of New Zealand Flax leaves were in non-quantifiable concentrations.

(c) Chitin, from mushrooms did not show a proper fermentation effect when used in the development of functional beverage, kefir.

Chapter 2
Review of Literature

Chitin, a linear homopolysaccharide of high molecular weight is known for its versatile and environment friendly nature (Khanafari, Marandi et al. 2008). The word Greek word chiton –coat of mail" marked the origin of chitin. Henri Braconnot, a French chemist in 1800's described the use of chitin for the first time (Elieh-Ali-Komi and Hamblin 2016), who identified chitin as an important component present in cell walls of fungal species, which exists as β 1, 4 branched N- acetyl glucosamine polymers. The monomers of chitin chitosan units, are obtained upon partial deacetylation of chitin (Nitschke, Altenbach et al. 2011). This compound's ability to serve as a supporting material especially in cell walls of crustaceans and mushrooms, contributing to the shape and rigidity was explained by Erdogern and coworkers. After cellulose, chitin is the second most abundant natural polysaccharide reported till date. Even though chitin resembles cellulose in terms of structure, the difference exists in the functional group present at their C-2 position. Acetamido occupies the C-2 position of chitin instead of hydroxyl in cellulose (Liao and Huang 2019). The >N-H group of one chain and >C=O group of the adjacent chain links together through intracellular hydrogen bonds links the chitin molecules together (Cabib 1981). Reserachers all over the world have identified number of sources for this biopolymer, but technological approaches for their extraction is still investigated, owing to their complex chemistry and biodiversity. Most commonly identified chitin sources till date are fungal and algal, insect exoskeleton, molluscs and crustaceans. Annual records have shown a production rate of 10^{11}-10^{12} tonnes of this polysaccharide by living organisms (Revathi, Saravanan et al. 2012) (Hamed, Özogul et al. 2016), most of which are discharged onto the sea floor by crustaceans. These were termed as marine snow by marine researchers. The decomposition of chitin is very significant in the natural soil ecosystem, and it removes tons of chitin that accumulate every year from dead insects and later used by soil biomass (Winkler, Dominguez-Nuñez et al. 2017). However chitin from all these sources is not commercialized as different sources exhibited different physicochemical properties for the compound. Commercial grade pure chitin is extracted from marine sources, namely crustaceans as a by- product and contributed to 10,000 tonnes production annually. Researches were carried out during initial period, to find out a best possible method to extract this polysaccharide, by de-proteination and de-mineralization of crustacean exoskeleton (Hamed, Özogul et al. 2016). This strategy helped reduce the large quantities of such by-products that were discarded, due to negligible economic value. Being

the second most available polysaccharide, chitin had been a source of interest of various researchers throughout these years. Chitin finds its use in every significant sectors of economy including textile, food, biomedical, waste water treatment and agriculture, due to its bio degradable, compatible and non-toxic existence. (Erdogan, Kaya et al.) (Khanafari, Marandi et al. 2008).

Cell wall of terrestrial crustaceans is composed of calcium carbonate, proteins and lipids, other than chitin. Insect cuticle, yet another chitin source however consists of melanin and protein, both of which are alkali soluble unlike chitin (Nemtsev, Zueva et al. 2004). Crustacean shells are composed of chitin, which exists in 4 layers of organic matrix. These matrices are composed of chitin–protein fibers along with calcium carbonate. The exo and endo cuticles are formed of parallel aligned complexes of calcium carbonate and chitin–protein fibers (Hild, Marti et al. 2008). One important factor, which is limiting the large scale manufacturing and use of chitin, is their water insoluble nature. However, considering their chemical structure, chitosan units formed as a result of deacetylation of chitin are relatively water soluble, and are the only known natural cationic polysaccharide. Another important challenge in the use of aquatic bio-wastes for the production of chitin is the inconsistency in raw material supply annually and variability in physicochemical characteristics owing to difference of process conditions. To overcome these problems and to develop a clean labeled chitin polymer, alternative sources and environment friendly approaches could be considered which offers consistent production rate.

Mushrooms were considered a novel source for chitin development, which was done using bio waste from irregularly shaped mushrooms and stalks. This source contributes to approximately 50 thousand metric tonnes of waste annually and results in consistent chitin production throughout the year. Mushrooms are composed of water, proteins, chitin, chitosan, glucans etc. Some mushrooms also forms chitin-glucan complexes, which when extracted could be used as food additives and enterosorbents in processed foods (Kim 2010). Chitin extracted from mushrooms is known to exhibit various benefits over those from crustaceans. Fungal chitin is devoid of tropomysin, myosin and arginine kinase, which are known allergens present in crustaceans. As mushrooms have relatively low impurities compared to crustaceans, chitin extraction from the former requires milder conditions over the latter. The use of mushrooms as a fungal source for chitin came into effect due to all the above mentioned factors. Moreover, mushrooms are known for their ease of growth without any restrictions to geographical conditions and harvesting period, compared to crustaceans and other sources (Liao and Huang 2019).

2.1 Biosynthesise of chitin

Highly complex, interconnected series of events which leads to the chitin biosynthesis occurs as intracellular reactions in crustacean's shells and fungal cell walls, which acts as chitin sources. The several steps involved in the sequential transformation are: i) conversion or assisted biotransformation of glucose sugars via phosphorylation, amination and formation of Uridine Di Phosphate -N-acetyl glucosamine. ii) Proper orientation of long chain chitin units. iii) Microfibril formation via crystallization and inter-cellular hydrogen bonding (Elieh-Ali-Komi and Hamblin 2016).

2.2 Characterization of chitin

Chitin and chitosan, even if derived from a single source most of the times, differ in characteristics. The complete characterization of chitin is often required for analyzing their physico-chemical properties and assigning them possible applications. The primary approach is the study of chemical structure of chitin from various sources to figure out the differences. Generally, Chitin exists in 3 forms - namely α, β and γ crystalline polymorphic forms (Aranaz, Mengíbar et al. 2009). These three forms differ in their microfiber orientation. Studies have shown that γ form does not exist in a polymorphic form, but as a distorted form of α and β chitin. α –chitin chains occur as stacks or sheets and the sheets along the central axis are anti-parallel. In β -chitin, adjacent sheets along the central axis are parallel, unlike other forms. The third form, γ- chitin, is different from other two forms such that, every third sheet takes the opposite direction of the preceding two sheets, or a one on 3 approaches. Chitin exists in pure crystalline form, whereas deacetylation of chitosan is required to make it crystalline. Characterization of chitin and chitosan were carried out using various methods. All the methods differ in principles, in which some methods characterized chitin and chitosan based on their degree of acetylation (DA) and polymerization (DP) respectively. In Asian countries, since last decade, Chitosan has been given the approval and standard as functional food. However in 2003, The Codex Alimentarius Commission considered the inclusion of chitin and chitosan as food additive. However further reports were not provided regarding the same, if chitin and chitosan is listed as Food Additives, or has been authorized by European Union as a food ingredient.

Characterization could be classified based on certain properties of chitin. These includes various attributes of chitin like deacetylation degree (DD), molecular weight distribution (M_W), crystallinity, moisture content, ash content and protein content. Till date several

methods have been employed to characterize chitin based on their degree of de-acetylation. One such approach was the use of Infrared Spectroscopy (Kasaai 2008). It works on the principle of determination of absorption ratio. It is mainly used for the characterization of chitin over chitosan in terms of DA, as properties of both polymers are closely related to the acetylation degree. Based on the method of (Kasaai 2008), DA was calculated as the ratio of AM and AR, where AM measures the N-acetyl content and AR measures the intensity of reference band. Quantitative evaluation of chitin is done by plotting a calibration curve with absorption ratio against DA of chitin sample. The DA of a known chitin standard was used as reference. H-NMR spectroscopy was chosen as precise and reliable method for DA determination for chitin by American Standard Test Method organization. The method was opted because of its sensitivity and precision, over other methods. The insoluble nature of chitin could be exploited for its characterization, based on which methods like Solid state NMR and Infrared Spectroscopy could be made use (Brugnerotto, Lizardi et al. 2001) (Brugnerotto, Desbrières et al. 2001). The IR showed perfect peaks which resembled chitin. This was due to the result of the 2 different of hydrogen bonds formed by amide groups in α-chitin. In case of β-chitin, their parallel chain alignment made them appear as a single peak.

Variable temperature Solid state NMR approach was made use for the investigation and characterization of α- chitins based on their structure and H- bond stability. Where diffraction methods fail to characterize mixed systems, Solid State NMR acts as an effective tool. The results indicated that for α-chitin, two types of hydrogen bond existed that was observed as 2 peaks at 173 and 175ppm. These hydrogen bonds were i) carbonyl carbon bonded to –NH group and ii) carbonyl carbon bonded to both NH and C–OH groups present in chitin (Kameda, Miyazawa et al. 2005).

Improved derivative UV method was used to characterize chitin based on degree of deacetylation in case of crustaceans like krill, crab, shrimp etc (Wang, Chang et al. 2013). Pre-treatment of samples were carried out by dissolving sample in H_3PO_4. This was further diluted to 100 ml using distilled water and then DD was measured using UV method. GlcNA and GlcN solutions for UV measurements were made using H_3PO_4, 0.85% at concentrations of ranging from 0-50µg/ml.

Studies by (Olofsson and Bylund 2016) showed a simple and rapid method for quantification of chitin and its monomers. The GlcN units of chitin were here quantified using LCMS and electrospray ionization, directly indicating their presence.

Degree of deacetylation as calculated by this method is expressed as:

$$DD\ (\%) = 1 - \frac{(m_1/203.21 \times 100)}{(m_1/203.21 + m_2/161.17)}$$

2- Dimensional FTIR spectroscopy was used for the characterization of chitin, due to its crystalline structure (Yamaguchi, Nge et al. 2005). The studies indicated 3 different bands in OH region of chitin and were characterized to be that of i) hydrogen bond between one C(6)OH group to the next C(6)OH group; ii) hydrogen bond between C(3)OH group and O(5); iii) hydrogen bond between C(6)OH group to next C(6)OH and C=O group. These results were according to the literature and hence helped in characterizing the crystalline nature of chitin. According to Yamaguchi and collaborators (2005), due to these intracellular hydrogen bonds in chitin, temperatures have varying effect on the chemistry of chitin. Unstable hydrogen-bonds get denatured at lower temperature range and with an increase in processing temperature the stable H-bonds get affected. This increase in temperature, also results in the breakdown of complex compounds like [C-OH…O==C] into C-OH, and double hydrogen-bonded compound into single bonded compounds. All the intertwined H-bonds are stable upto a temperature of 150-180 degree celcius.

2.3 Extraction of chitin

Commercial chitins were prepared using 2 step procedures, i.e. deproteinization followed by demineralization. Applying these conditions, the recovered chitin is in the form of collapsed chitin, which loses its native structure. However, if demineralization is followed by deproteinization, compacted chitin, in which the recovered chitin is intact and stable compared to the native chitin, is extracted. The table below indicates various comparison methods for chitosan extraction that were practiced traditionally.

Table 2.1 Chitin extraction methods used by researchers traditionally

Source	Process and Conditions
Lobster shell	Grounded shell was treated with dilute 1N NaOH solution at 100°C for deproteinization, followed by treatment with 10 % dilute HCl for demineralization. Ethanol was used for decolorization and production of a cream coloured chitin rather than a colored chitin (Hackman 1954).
Antarctic krill shell	Grounded shell was treated with dilute 3-3.5 percentage NaOH solution at 90-95°C for deproteinization (Anderson, De Pablo et al.), followed by treatment with 10% dilute HCl for demineralization. Chloroform was used for decolorization and production of a white coloured chitin.
Craw fish shell	Grounded shell was treated with dilute 3.5 percentage NaOH solution at 65°C for deproteinization (No, Meyers et al. 1989), followed by treatment with 10% dilute HCl for demineralization. Combined use of acetone and Sodium hypochlorite solution helped in decolorization and production of a white coloured chitin from shell of craw fish.
Shrimp shell	Grounded shell was treated with dilute i) 1N NaOH and ii) 3 % NaOH, at 100°C for deproteinization. 1% KOH at 90°C was used further for maximum protein separation (Bough, Salter et al. 1978). This was followed by treatment with 10% dilute HCl for demineralization. Hydrogen peroxide was used for decolorization of produced chitin.
Crab shell	Grounded shell was treated with dilute 1N NaOH, at 100°C for deproteinization. 2% KOH at 90°C was used further for maximum protein separation. This was followed by treatment with 22% dilute HCl for demineralization (Hackman 1954). Ethyl acetate was used for decolorization and hence final extraction of chitin (No and Meyers 1995).

The mainstream process for obtaining chitin or chitosan-derivatives involves various methods, most of which were finalized considering the nature of origin or source of chitin. Some of these methods include acid hydrolysis, deproteinization, deacetylation and purification by HPLC, wherein oligomers of high purity are produced (Winkler, Dominguez-Nuñez et al. 2017). Crustacean shell was of prime importance as a source for chitin traditionally. Chitin extraction from crustacean occurred in 3 main steps, acid treatment, alkaline extraction and de-pigmentation. Acid treatment was carried out, which aided in removal of calcium carbonate in shell. This was followed by alkaline extraction process to dissolve the proteins and depigmentation to remove astaxantine (form of carotenoid) and thereby producing colorless chitin (Aranaz, Mengíbar et al. 2009). Traditionally chitin extraction from shrimp waste was a proteination using 4% Sodium hydroxide NaOH, followed by demineralization using 4% HCl. However the expensive and non-environmental

friendly attribute made the process non-acceptable in the category of a good recovery option (Rao, Guyot et al. 2002).

Similarities exists in procedure for extraction of chitin and chitosan from insects cuticles and crustacean sources, where the most of the procedures includes demineralization followed by de-proteination and decolorization (Nemtsev, Zueva et al. 2004). Most of the demineralization to obtain chitin from insect cuticle was carried out using 2 N HCl, than the 1 N HCl used for demineralization process of aquatic crustacean materials. The demineralization of shrimp waste using 0.25 M HCl took around 15 minutes at room temperature (Roberts 2008). Zhang and coauthors (2000) found that when treated with 2N HCl, the acetyl groups present in insect cuticles were removed, displaying a prominent crystallinity. Therefore this study indicated that dilute HCl not only helped to remove the minerals present in insect cuticle, but also the acetyl groups. Pure chitin from silkworms were extracted by (Paulino, Simionato et al. 2006) using acetylation with 1N HCl. However, the yield was chitin was not in accordance to previous results obtained from crustacean chitin extractions and was comparatively low. This lower chitin yield may be considered as a negative effect of high temperature, strong acid treatment of insect materials. Therefore, modifications to acid hydrolysis must be made using weak or other organic acids that don't alter the chemistry of chitin by completely hydrolyzing it. However, de-proteination of the same samples done using consecutive treatments of bases, NaOH followed by mild Na_2CO_3 helped to completely remove the proteins (Zhang, Haga et al. 2000).

A Study by Varun and coauthors (2017) demonstrated another chemical extraction method for the production of chitin utilizing crab shell waste. The high temperature deacetylation of recovered chitin (12%) using NaOH solution yielded chitosan (10%). Fungal source of chitin and chitosan differ from crustacean source and even overweighs latter in terms of their year round availability. They also differ in molecular weight, acetylation degree (DA) and charged group distribution. From the current statistics of amount of mushroom waste accumulated annually and the chemical composition of mushrooms, it could be considered as a potential source of chitin. All the above mentioned and further proposed procedures could yield around 1000 metric tons of pure fungal chitin annually.

The extraction of chitin and chitosan from various mushrooms species are carried out till date. Some of the species includes *Agaricus bisporus*, *Auricularia auriculajudae*, *Lentinula edodes* and *Trametes versicolor*. Pochanavanich and Suntornsuk (2002) made use of farm grown mushrooms, carried out its deproteination using 1 M NaOH at 121°C for chitin extraction.

However, (Di Mario, Rapana et al. 2008) used the same chemical, 1 M NaOH but at a different temperature, 40°C for deproteination of chitin. (Yen and Mau 2007) followed a different order of process for extraction of chitin and chitosan- i) alkaline treatment, ii) decolorization iii) deacetylation using concentrated NaOH solution. All these extraction does not produce a significant yield of chitin as the chitin is in the form of a suspension in the mushroom bulk. Considering the organic matrix of mushroom, it could be understood that chitin is complexed or intertwined with polysaccharides including glucan and this could be a major challenge for extraction of chitin from mushroom sources, regardless of their species and origin (Kim 2010). However, the knowledge in this area is very limited, which may offer limitation in the successful chitin production from mushroom. In studies conducted by (Wu, Zivanovic et al. 2004), extraction and characterization of chitin and chitosan were carried out using freeze-dried mushroom (*Agaricus bisporus*) stalks as samples. The chitinous material was extracted after acid treatment, followed by alkali treatment and decolorization. Acid reflux of alkali insoluble material gave the final extract of chitin. The percentage of chitin yielded was 27, which consisted of 46% glucosamine and 21% of other polysaccharides. This result was consistent with the results of other researchers who found that crude chitin from *A. bisporus* is composed of a chitin- glucan complex in the form of polysaccharides and has the potential to be used as a bio pesticide, growth regulator, and feed additives.

Shell of Antarctic krill, was used for chitin extraction. The chitin extracted after a series of processes involving demineralization, deproteinization and decolorization was in white lamellar form (Wang, Chang et al. 2013). The chitin recovered was in the range of 27%. This yield was comparable to the yield of chitin from crab and shrimp shell 20% respectively for both. The SEM analysis of the krill shell after chitin extraction indicated a structure similar to the one before extraction. This indicated that the method preserved the native chitin structure, along with other components and could be used as a successful extraction method for chitin.

2.4 Novel methods for chitin extraction

All the above cited chemical methods used for chitin extraction generates a large amount of wastes which contributes to environmental-pollution as it results in partial deacetylation of chitin. Therefore, the use of biological extraction methods would be an interesting alternative. A recent study by (Marzieh, Zahra et al. 2019) was aimed to isolate chitin by using organic compounds, enzymes Trypsin and Ficin. Lactic acid solution was used for acid hydrolysis and shell demineralization and enzymes, Trypsin and Ficin were used for deproteinization. This was followed by treatment with 2% NaOH, a mild alkali. The steps indicated above are a combination of chemical and enzymatic treatments and displayed chitin extraction with an

efficiency of 92%. The deacetylation degree of extracted chitins ranged between 70-90% and can be considered good final products. The properties of the extracted chitin were comparable to commercial chitin and were interestingly similar in most cases. This enzyme-assisted extraction method offers a better and promising alternative for environment friendly chitin extraction using in future. The generated chitin is pure and of high grade, which even retained the exact smooth and high molecular packed structure of chitin (Marzieh, Zahra et al. 2019).

Studies by (Nitschke, Altenbach et al. 2011), demonstrated a new method for the quantification of chitin and chitosan. The reaction between polyiodide anions and chitosan, monomer of chitin was made use as the principle. Chitin could also be quantified using complex matrix systems, if de-acetylation is carried out. With this new spot assay, the chitin content of mycelia and fruiting bodies from several *basidiomycetes* and an *ascomycete* were analysed. The chitin content of the analysed species varied showed upto 9 g/100 g dry mass of chitin. The use of organic acid, lactic acid was considered a clean labeled approach for chitin extraction, as the recovered chitin retains all characteristics of pure chitin compared to traditional approach using strong acids, wherein the chitin quality was lost due to the aggressive chemical reactions. Adding to these, the use of inorganic acids for chitin extraction was also not preferred as they affected the intrinsic molecular weight and properties of recovered chitin. Various other studies have also been done for extraction of chitin. Partially fermented shrimp bio waste was also used as source for chitin extraction, wherein extracted chitin was characterized as crystalline, harmless and odorless (Khanafari, Marandi et al. 2008). This study also suggested considering lactic acid fermentation as a better alternative over chemical extraction of chitin due to the efficiency of the former method in terms of extraction and percentage recovery.

A first trial for chitosan extraction from shrimp waste using a fast, easy and efficient method was put forward by Knidri and collaborators (2016). The 3 steps in chitin extraction used by researchers previously were carried out in the assistance of microwave irradiation. Comparative studies indicated that chitosan was obtained with a deacetylation of 82% at a time period 1/16 times lesser than that required by conventional heating method. These results revealed that microwave irradiation, if done together with other chemical and organic solvents, could be considered energy saving, efficient and environment-friendly method for chitosan and chitin extraction.

Another approach was the development of a bio-remediation strategy using chitinolytic organisms (Kumar, Kumar et al. 2018). Commercial use of chitinase produced by bacteria as

well as fungi has restricted commercial uses, due to low enzyme yield and high production cost. They produce chitinase and even chitin-oligosaccharides using pure chitin as well as sea food waste as carbon source. Kumar and coauthors (2018) carried out their studies on *Paenibacillus*, *Paenibacillus sp. AD,* as indicated by 16s rRNA and biochemical analysis of bacterium. *Paenibacillus*, *Paenibacillus sp. AD*, isolated from seafood waste dumping sites is characterized for their ability to produce value added products like chitinase and chitin oligosaccharides by degrading marine waste, especially shrimp waste. Under optimum process conditions, the organism facilitated complete shrimp waste degradation yielding chitinase. SEM and FTIR results indicated that the extraction was possible due to breakage of bonds and thereby structural changes, both of which resembled the chemistry of bond breakage of chitin. The TLC also revealed the presence of chitin oligosaccharides of different degree of polymerization, indicating that this bioremediation approach could be used for degradation of chitin biopolymers too. Complete degradation of shrimp waste was exhibited by *Paenibacillus sp. AD*, making the microbe a potential scavenger of seafood waste.

2.5 Application of chitin

2.5.1 Bio-engineering

Nano crystals produced from chitin are known for their use for encapsulations and Pickering emulsions. Acid hydrolysis, centrifugation, precipitation, dialysis and ultra-sonication of chitin at variable time and temperature combinations were carried out to obtain chitin nano-crystals powder. They were characterized as nano biomaterials, as the SEM analysis of the obtained powder indicated that the fibrous crystals showed length upto several microns and width of few nanometers. The biocompatible nature of chitin had made researchers to work on it for the development of suitable biomaterials as bone regeneration scaffold (Tao, Cheng et al. 2019). Various methods were employed such as phase separation (thermally induced), electrospinning. The wide use of these scaffolds has been reported in drug delivery, wound dressings as well in other biomedical aspects.

2.5.2 Wound healing

The positive influence of chitin on wound-healing have been made use to develop dressing materials from chitin. These materials are made by deacetylation of chitin to chitosan by

treatment with a base. The medicated wound dressings made out of chitin are known to be non-toxic, biodegradable and compatible. Studies have also reported antimicrobial properties of such dressings made out of chitosan, which they believe is due to the presence of glucosamine which is evolved as a by-product of chitin to chitosan bioconversion (Qin 2015). Carboxymethylated chitosan fibres, known for its relatively higher liquid absorption rate is yet another form of chitin used for wound dressings. They are most likely used for the manufacturing of moisture resistant healings. (Murakami, Aoki et al. 2010) also developed wound dressings from a blend of alginate, chitin and fucoidan, in the form of a hydrated gel sheet and showed a greater wound healing potential compared to already known wound dressings. (Min, Lee et al. 2004)used electro spinning method for fabrication of a matrix of nano fibrous chitin as wound dressing material. SEM analysis showed the resultant matrix with a deacetylation degree of 85%.

2.5.3 Water treatment: a low cost adsorbent

Chitin has also found its application is waste water treatment as a low cost adsorbent. Nano particles made of chitin are known for their adsorption property (Kumar, Saxena et al. 2019). This is aided by innate properties of chitin such as effective surface area, non- toxic and environment friendly nature, functionality etc. chitin complexes have also been used successfully in waste water as well as color dye treatment processes.

Electrostatic interactions forms the basis of chitin or chitosan biosorptions used in waste water treatment (Sarode, Upadhyay et al. 2019). They act as metal chelating agents and adsorb heavy metals, dyes and protein molecules present in waste water, by protonating their derivatives. This reaction is essentially a chemisorption or rarely physisorption which occurs via metal ion chelation or ion-exchange mechanism. At pH 3, chitosan when used against several effluent dyes like Acid blue 9, food yellow 3 and FD&C, offered the best adsorption of all the dyes. Surface modified chitin displayed more efficient adsorption and recyclability of chitin, i.e., >7 times with same degree of adsorption (Ribeiro and dos Santos 2019). This was because; the ultrasound modification resulted in an increased surface area of chitin, making it more available for the dyes. Naturally occuring chitin and chitosan polymers are used in the adsorption of heavy metal ions like manganese, cobalt etc. This biosorption of heavy metals is aided by primary amino and hydroxyl groups present in chitin that causes electrostatic interactions between metals and effluent, thereby separating the metal ions from aqueous stream.

2.5.4 Food Packaging

Oxidized chitin nanocrystals, a food grade polymer known for their non-toxic, anti-bacterial, edible and biodegradable nature have been used for the development of packaging films recently (Wu, Li et al. 2020). An effective packaging film was developed combining anthocyanin from red cabbages and oxidized chitin nanocrystals, in which the later acted as reinforcement supports. The developed film displayed excellent water and UV resistance, antioxidant, antibacterial, and pH-sensitive properties, which in turn suggest that Oxidized chitin nanocrystal based films, could act as active component in food grade intelligent packaging systems.

A food packaging film using chitin fibre as a matrix was developed by Hai and collaborators, 2019, Yadav and collaborators, 2019. The developed film was characterized as green nano-composites. The film displayed excellent biodegradability, biocompatibility and mechanical properties because of the presence of chitin and bamboo-cellulose nano fibres. Chitin nano fibres were obtained using TEMPO mediated oxidation followed by acid hydrolysis and homogenization. The film bio-degradability was promising as it got completely degraded within a week of experimental trials and makes it a promising innovation in food packaging.

2.5.5 Food clarifying, thickening and stabilizing agent

Chitin finings have been reported as excellent clarifying agents in most of the citrus fruit juices. However studies showed that, even though these finings acted as clarifying agents in apple juice (Taştan and Baysal 2017), they did not show any direct influence on the microorganism (*Alicyclobacillus acidoterrestris*) present in the juice. The finest extracted form of chitin, chitosan, when used as finings in wine samples helped removing sulphides and polyphenols, both of which if oxidized effects the flavour and texture of wines. Chitin also displayed positive action against Ochratoxin a (OTA), major food contaminating mycotoxin produced by *Penicillium* species (Harkin, 2019).

Chitin also acts as texture modifying agents due to the presence of chitosan in it, in the form of hydrogels. This property of chitin derived chitosan adds to their emulsifying potential. When used as an emulsifier, chitosan assisted droplet formation and prevented aqueous phases from separating. Both degree of deacetylation and existence as a positively charged amphiphilic substance even influenced their emulsifying nature. Chitin and chitosan when added to a system, added to the system's viscosity by thickening them. This occurs via 3

stages. i) Fibres in chitin/chitosan act as ~~–b~~lockades" between emulsified droplets, ii) prevent molecular interaction by acting as a barrier and iii) preventing any coalescence/coagulation from occurring (Harkin, 2019).

Chitosan and chitin also acts as protective covering or coatings for natural colorants used in food industries. In either method, color gets encapsulated in the chitin/chitosan matrix. The no-toxic and biodegradable nature of both compounds are well known and hence the encapsulated color is considered clean labelled.

2.6 Phormium tenax

Phormium species belonging to genus Phormiaceae and order Asparagales used by the Maori community in New Zealand forms a major part of their biodiversity (Sims, 2006). Their resemblance to common flax seeds have given them their name as New Zealand Flax. The two species of Phormium, P. tenax and P. cookianum are known as harakeke and whaririki among Maori community. Phormium tenax is native to New Zealand and the Chatham Islands. As said by (Johnson 1989), the plant was said to be introduced by Maori primarily at Raoul Island belonging to the kermadec Islands groups. (Sparrow 1965)in his review stated that the plant was distributed in North Island in Wellington and South Auckland since ancient times. Marlborough, Canterbury, Westland, and Southland were identified as areas of high abundance of the plant in South island. Phormium tenax is a monocotyledonous herb, tall and grows in height. The leaves of the plant grows upto 3m in length ,and are made of very long fibers, which covers around 12–50% of the total area of the leaf. The length and fibre content varies with different varieties of Phormium. The leaves are characterized for their sword-like apperance and are joined together from the rhizome as clusters. In some cases, leaves are folded, keeled with long striations and grow upto a 120 mm, around times the width of normal leaf (Wehi and Clarkson 2007).These leaves are composed of sclerenchyma fibres, same as that of monocotyledons and are composed of ultimate fibres (Carr, Cruthers et al. 2005). Hemicelluloses and lignins form these sclerenchyma fibres, and hence extraction is complex process either involving the use of boiling water or alkali solutions. Extraction of the fibres is carried out by dissolving the bonds binding the fibres together in the leaf epidermis. An inconsistency exists in the fibre, lignin and cellulose contents in leaves of different Phormium cultivars. Cucurbitacin D, cucurbitacin I and farrerol were reported as phytochemicals found in the leaves of P. tenax (Cambie 1996). The leaf exudates are highly viscous due to an acidic xylan polysaccharide, pia harakeke (Tauwhare, Newman et al. 2006). P. tenax is reported to

have a notable Xylose contents than other Phormium species. The leaves are rich in cellulose and hemi-cellulose and are the most common weaving plant used by Maori community since ages (Sims and Newman 2006). Historically, the leaf was plaited and braded into many different items such as mats, receptacles, and bags, many of which continue to be made today. Although P. tenax is primarily known as a weaving plant, it is also associated with a number of medicinal remedies. The decoctions made out of the leaf base exudates are used as dressings for severe wounds and burns. Blanching of leaf bases were also done, which were sometimes pulped, roasted and used as remedies for tumours and abscesses. The root juices of *Phormium tenax* were used as purgatives (Wehi and Clarkson 2007). Researches done on *Phormium tenax* leaves all over the world have focussed on elastic and mechanical properties of the leaves, rather than their physico-chemical properties. No such data exists when it comes to chemical components or modifications if any present in leaves.

2.7 Non-protein nitrogen and tannin complexes: a new concept

Metabolites produced by plants vary in composition and functions. Where, primary metabolites functions as plant growth supporters, secondary metabolites are not essential for growth and development. However, the latter bears a more complex and chemically complex structure compared to the former. Most of the secondary compounds are produced as a defence mechanism against other pathogens and life forms. Apart from this function, they also acts as overall protectors of plant, especially leaves from different wild responses like UV, light, excess carbon and nitrogen cycling etc. Even though, wide varieties of secondary metabolites are studied and reported, tannins, the fourth abundant metabolite fascinates researchers with their chemical complexity. Tannin concentrations are found to be high in plant leaves which grow at areas where soil quality is low in terms of nutrition and pH. Tannins are reactive to increased temperature changes and their complex chemistry separates them into two groups, hydrolysable and condensed tannins (Adamczyk, Adamczyk et al. 2011, Adamczyk, Simon et al. 2017). Being a secondary plant metabolite, Tannins have a shown the unique ability to form protein complexes. This reaction proceeds via 2 step; i) binding of tannins to protein of interest, ii) formation of protein precipitate, via aggregation, by virtue of which covalently bonded and soluble complexes are formed. Tannins interaction is said to occur with proteins and enzymes, both belonging to the group of organic nitrogen compounds. However, recent studies have updated this list with non-protein organic nitrogen compounds. This includes reaction with nitrogenous bases, few amino acids, and chitin and chitosan units (Adamczyk, Simon et al. 2017). These groups form complexes with tannins via multiple hydrogen bonds, making their extraction difficult in the bound form.

Studies were conducted by (Wang, Li et al. 2016) by developing a film with chitin and tannic acid assembly, to figure out the possibility of hydrophobic interactions between chitin and tannic acid in the developed film. Strong and complex interactions were reported in this study, which they believe would be due to amphiphilic nature of chitin and also property of tannic acid to adsorb chitin (Adamczyk, Simon et al. 2017).

Chapter 3
Materials and Methods

3.1 Materials

3.1.1 Samples

Leaves of *Phormium tenax* (Harakeke) were obtained from Lincoln University campus and were freeze dried to prevent enzymatic modification. Button mushroom, *Aspergillus bisporus*, collected from Canterbury hills was taken as a positive control for all the analyses and flax seeds were taken as negative control. Mushrooms used for product development was obtained from local supermarket. All the samples were powdered using EM0405 coffee grinder (Sunbeam, Auckland, New Zealand) for 15 seconds and stored in plastic boxes. Samples were taken in triplicates throughout the research.

3.1.2 Chemicals

1 M NaOH, Concentrated KOH, 10% acetic acid, 1% Lugol's solution, standard chitin.

3.2 Methods

3.2.1 Extraction and qualitative estimation of chitin

Extraction of chitin

Chitin extraction was done following the method of Nitchke and others (2011). 2-3 grams of freeze dried New Zealand flax leaves samples were quantitatively measured and transferred to Erlenmeyer flask. This was then allowed to sit in a water bath equipped with magnetic stirrer for 2 hours, after the addition of 100ml of 1M sodium hydroxide. Samples were filtered either manually or using vaccum pump with RO water washes until the pH of the filtered material was slightly higher than 7. The residue was further treated with an alkali, 50ml of concentrated KOH for chitin extraction. This was then allowed to sit in a magnetic stirrer assisted water bath for three hours. The samples were then centrifuged, the residue were washed several times in order to maintain the pH slightly higher than pH7. The same procedure was

repeated for button mushrooms and standard chitin. All extracts were stored at freezer temperature for TLC and HPLC analysis.

Qualitative estimation of chitin by TLC method

Freeze dried samples were dissolved in 10% (v/v) acetic acid. Standard were formulated in a concentration range of 0.5-1 mg/ml of chitosan. TLC plates were obtained as pre-cast and were used for analysis. 2µl of standard solutions and triplicate samples were then pipetted into the plate using pipettes as spots. Spotted TLC plates were then sprayed with 1% Lugol's solution, ensuring the uniform distribution of solution on the plate, followed by hot air drying. The colour-complex formed on the plates was analyses to qualitatively determine the presence of the compound.

3.2.2 Quantitative estimation of chitin by HPLC

The extract stored under refrigerated condition was hydrolysed. Acid hydrolysis of chitin into its monomer glucosamine was achieved by mixing 200 mg of leaf powder, control chitin powder respectively with 10 ml of 5 M hydrochloric acid (Fisher) and heating at 100°C in a water bath. After cooling to room temperature, the hydrolysates were filtered by vacuum pump and collected for chromatographic analysis, following a modified version of the method by Yan and Evenocheck. A 1.0 ml aliquot of hydrolysate was dried by EZ2 Gene vacuum centrifuge (Genevac Ltd, USA) at 75°C for 1.5 hours. After cooling the tubes at room temperature, 1.0 ml of borate buffer (0.25 M, pH 10) was added to dissolve. Later, 200 µl ml of sample were combined with 200 µl of FMOC-OSu, (≥98%, Sigma-Aldrich) and let react for 4 hours at room temperature without agitation. After the derivitisation was completed, 1.2 ml of solvent mixture (equal volumes of HPLC solvents A and B) was added and the solution was filtered with 0.45 µl poly tetra fluoro ethylene syringe filters. Agilent 1100 HPLC (Agilent Technologies, Walbronn, Germany), equipped with a binary pump, auto-sampler with thermostat, kept temperature at 4°C, Software EzChrom Elite (version 3.3.2) was used, with a C-18 column (Agilent Zorbax SB C18, 5 µm x 150 mm x 4.6 mm). Column temperature was 30°C and a gradient elution was used: from 70% A (0.05% trifluoro acetic acid in water (pH 2.4) to 100% B (acetonitrile) in 11 minutes, then back to 70% A in 9 minutes, with a flow rate of 0.8 ml/min. A fluorescent detector was set at 260 nm. A calibration curve was built using glucosamine (≥99%, Sigma Aldrich) as standard.

3.2.3 Development of kefir and evaluation of its functional properties

Fresh brown and white button mushrooms were cleaned and cut in to small pieces, half of which were microwave assisted tray dried at 50˚C overnight and the rest half were left unroasted. Both roasted and unroasted brown and white button mushroom were ground finely. Procedure for developing normal kefir was followed. Kefir was made using 400ml of pasteurized standardized milk as base and 2 grams of kefir grains. 1 gram of sample respectively was added to it as a stabilizer. 6 different formulations for kefir were made. These included **C**-milk+kefir, **T_1**-milk+kefir+unroasted brown mushroom, **T_2**- milk+kefir+unroasted white mushroom, **T_3**- milk+kefir+roasted brown mushroom, **T_4**- milk+kefir+roasted white mushroom, **T_5**- carrot juice+kefir+roasted white mushrooms.

Physico chemical analysis of fermented kefir was carried out.

pH

The pH was measured with a pH-meter (Seven Easy pH, GLOBAL SCIENCE, China) (Wang, Chelikani et al. 2018).

Total Soluble Solids

The Total Soluble Solids (TSS), expressed as Brix value or ° BRIX was measured using refractometer.

Viscosity

The viscosity was measured using viscometer (RM100, Lamy Rheology, France), equipped with a 1–9 (cylinder-spindle) system. A shear rate of 6.4 s^{-1} for 30 s was used for the measurement (Wang, Chelikani et al. 2018).

Syneresis

30 g-sample of fermented beverage, kefir was centrifuged (Heraeus Multifuge XIR, Thermo Scientific, MA, USA) into 50-ml centrifuge tubes at 222 g for 10 min at 4 °C. Syneresis was expressed as the percentage of the supernatant weight by original weight (Wang, Chelikani et al. 2018).

3.2.4 Statistical Analysis

All analysis were performed in triplicates and data obtained were analyses using ANOVA at $p<0.05$.

Chapter 4

Results and Discussions

4.1 Extraction and qualitative estimation of chitin

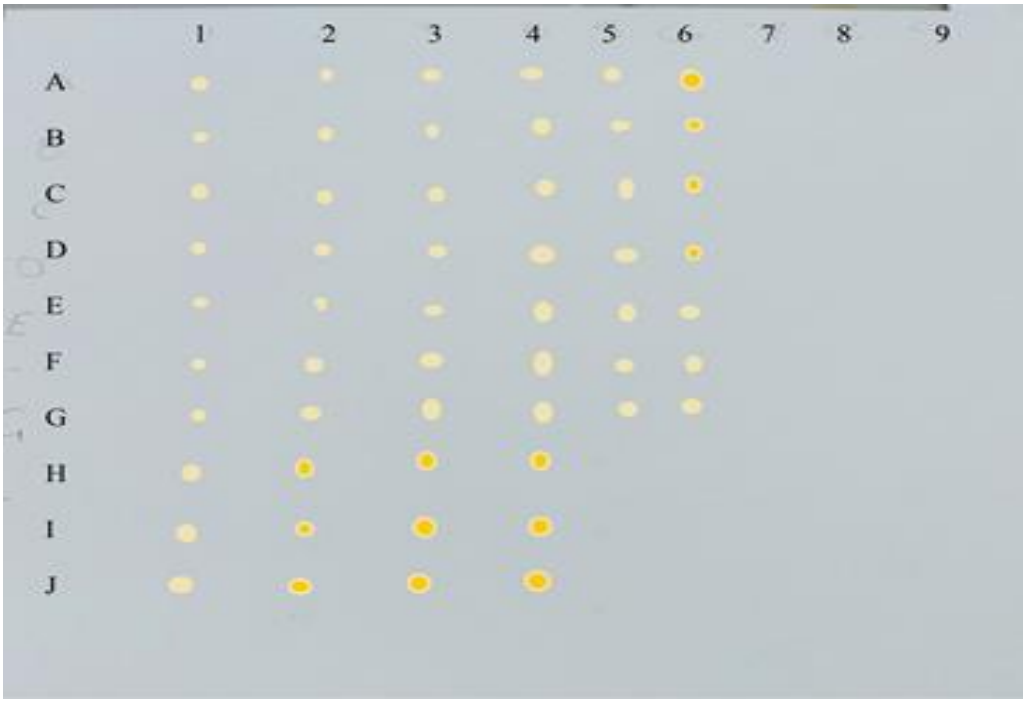

Figure 4.1 Picture of the spot assay: chitosan standards in a concentration range of 0.5-5 mg/mL of chitosan (from left to right)

The extracts of *Phormium tenax* leaves were prominently dark compared to standard chitin and mushroom extracts. This dark colour could be attributed to the presence of bio-active compounds like tannins, polyphenols, if any present in the leaves. The extract retained the same prominent and dark colour even after the lengthy extraction process, indicating that the compound responsible for this is not vulnerable to any chemical treatments. The figure indicates the TLC of extracts of *Phormium tenax* leaves, mushrooms and chitosan standards in the range of 0.5-1 mg/ml and is an depiction resembling the TLC results as obtained on plates. Significant difference in color was observed immediately after spraying with Lugol's solution. However, when it was allowed to stand for a few seconds, the color faded away. Darker spots were displayed by mushroom extract and chitosan standards indicating and validating that the method was successful in determining the chitosan content in the samples. However few spots containing leaf extract also displayed color intensity prominently. Being a preliminary qualitative analysis technique, TLC results indicates that there are chances that leaf extract may contain chitin or chitosan in pure or bound forms. These preliminary results

are in accordance with the findings of (Nitschke, Altenbach et al. 2011)), who described the complex of Lugol's solution and chitosan as insoluble and responsible for coloured spot formation. However, no studies have reported the scenario behind the instability of color as observed in this study. This rapid color reduction could be attributed to any cross reactions between Lugol's reagent and components present in leaves, standard chitosan and mushroom samples.

4.2 Quantitative estimation of chitin by HPLC

Table 4.1. Glucosamine, g/ 100 g of extract by HPLC-FLD studies

Sample name	Total Glucosamine (ppm)= mg/l	Glucosamine, g/ 100 g of extract
Phormium tenax leaves	13.34±0.05	0.05±0.00
Chitin	1503±56	38.1±1.4
Mushroom	2915±21	37.6±2.7 (wet basis)

The HPLC-FLD studies of samples with respect to standard chitin were done. *Phormium* leaves were analysed for negligible chitin content of 0.05 g/100 g. The recovery of standard chitin was 38.1 g/100 g. These results indicated very low recovery of pure chitin compared to other methods, where chitin being standard should have been recovered fully. Mushroom, Agaricus bisporus used in this study was observed to have 3.7% of chitin (dry weight) and was comparable to the results of Vetter and others (2007) who reported chitin content of Agaricus bisporus as 6.67%. Studies have also reported that the dry weight chitin contents of different fungal source, including different mushroom species belonging to different phylum vary in the range of between 2-42%. These results are in accordance to the fact that significant differences existed in mushroom species in terms of chitin contents. (Ifuku, Nomura et al. 2011) reported the presence of branched glucan compounds in the form of β-1,3 linkages in chitin in mushroom cell walls, which are absent in case of other chitin sources like crustaceans. These factors along with others like species, extraction method, age of thallus etc. could be attributed to low recovery of chitin from mushroom compared to those from other sources of chitin. However, studies by (Erdogan, Kaya et al.) on other mushroom species like *L. vellereus* and *P. ribis*, displayed a better yield of chitin, 11% and 7% (dry weight) compared to *Agaricus bisporus.*

Quantitative analysis of the sample under consideration, *Phormium tenax* leaves displayed negligible chitin content when compared to mushroom and chitin standard. This results if considered disproves the results of TLC of leaves. The TLC results for leaves could be considered false positive, and could be due to the presence of any other parts or

impurities present in the leaves used. Recent studies by (Adamczyk et al., 2019) on complex interactions between organic nitrogen components like chitin-chitosan with tannins could be a possible explanation of this. This is based on the principle that chemically tannins exists as hydrolysable and condensed tannins. Condensed tannins are polymers of procyanidins and prodelfinidins, both being three-ring flavonols joined through C-C bonds. Reviews by (Adamczyk, Adamczyk et al. 2011) first reported that condensed tannins can react with different organic N compounds like chitin, chitosan and amines and give dark color to the complex extracts formed. Tannins, in other form like tannic acids adsorbs to chitosan residues and form complexes. These findings stand in line with our observations in case of *Phormium tenax* leaves. Adding to this, Adamczyk and collaborators (2019) also indicated that chitins display this ability to form tannin complexes due to the presence of multiple intracellular hydrogen bonds, which are exhibited in the form of fibres by *Phormium tenax* leaves. Hence the method proposed by Nitschke and others (2011) cannot be considered as a standard method for extraction and quantification of chitin from all sources, irrespective of their claims regarding the same. Or in other words the hypothesis established, i.e. extraction method used for mushroom chitin extraction could be used as a standard method for chitin extraction is hence disproved. Further studies need to emphasise on proper methods to break the intercellular bonds forming condensed tannins, for a better and efficient extraction of chitin or chitosan from these complexes.

4.3 Development of kefir and evaluation of its functional properties

Kefir, the fermented functional beverage was developed by adding mushroom, in order to check the functionality of mushrooms as stabilizers. The texture and appearance of the beverage resembled to that of a stable colloidal system like yoghurt. All the samples were visually appealing; however degree of stability differed between samples. Considering stability as functionality, T_4 which consisted of roasted white mushroom as stabilizer, displayed a better and stable kefir. This could be due to a reduction in moisture content, which in turn increases the carbohydrate in form of dietary fibre in mushrooms as a result of roasting (Ayodele and Idoko 2011). Kefir, T_3 made using roasted brown mushrooms displayed orange like supernatant, which separated the solids from kefir. This indicated a prominent instability effect. Studies by (Asamoa, Essel et al. 2018), have suggested that upon roasting of mushroom (*T. Schimperi*), maillard reactions are caused by pigments present in them, which in turn results in inactivation of oxidizable enzymes

and antioxidant activity. The figure displays all the different kefir formulations developed and studied for physico-chemical properties.

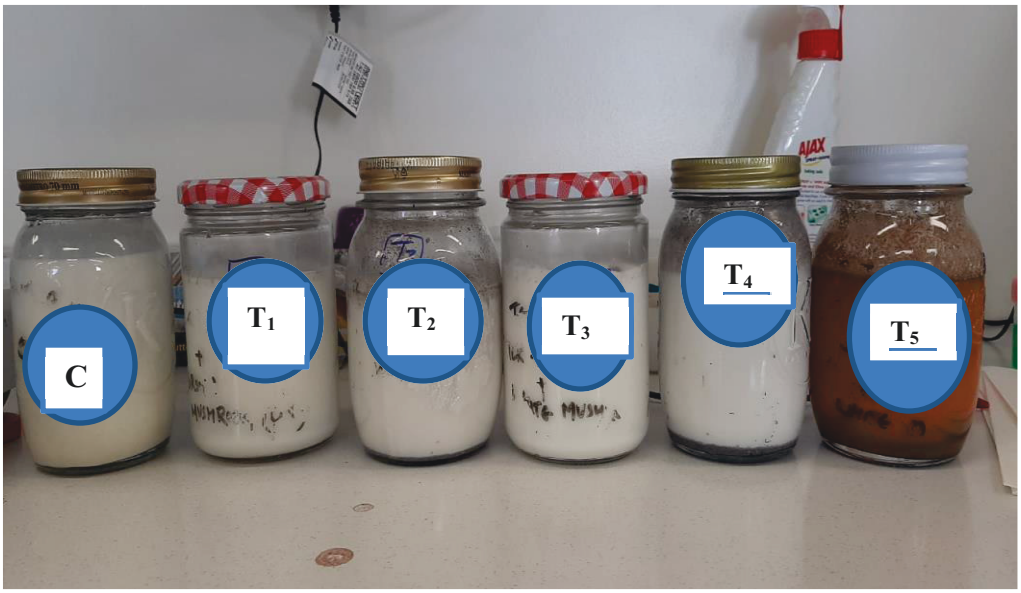

Figure 4.2 Developed kefir formulations

(These are denoted C-milk+kefir, T_1- milk+kefir+unroasted brown mushroom, T_2- milk+kefir+unroasted white mushroom, T_3- milk+kefir+roasted brown mushroom, T_4- milk+kefir+roasted white mushroom, T_5- carrot juice+kefir+roasted white mushrooms).

4.3.1 pH

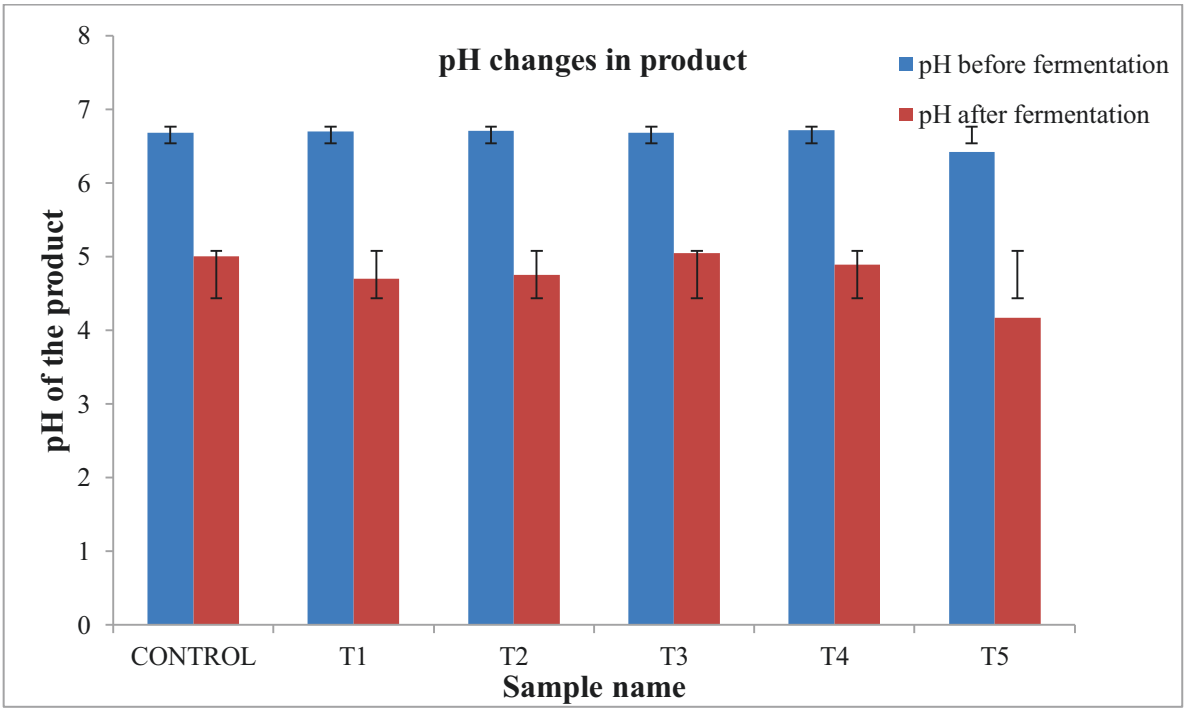

Figure 4.3 pH changes in product before and after fermentation

Table 4.2 pH of the product before and after fermentation

SAMPLES	pH before fermentation	pH after fermentation
C	6.68 ± 0.00^{A}	5.01 ± 0.00^{B}
T1	6.70 ± 0.00^{A}	4.7 ± 0.00^{B}
T2	6.71 ± 0.02^{A}	4.75 ± 0.01^{B}
T3	6.68 ± 0.00^{A}	5.05 ± 0.01^{B}
T4	6.72 ± 0.03^{A}	4.89 ± 0.02^{B}
T5	6.42 ± 0.01^{A}	4.17 ± 0.01^{B}

A significant difference clearly exists between the pH of kefir before and after fermentation (expressed as average± st.dev at $P<0.05$). This clearly indicates proper fermentability of kefir. Samples T_3 and T_4 displayed pH changes similar to that of control and is also in accordance to the pH of kefir reported as between pH 4.2 to 4.6 by (Otles and Cagindi 2003). T_5 also displayed a pH of 4.1, indicating a proper fermentation irrespective of the source used. Similar results were reported by reserachers who developed kefir from both dairy and non-dairy sources. This pH reduction is due to the combined action of microbes present in the kefir grains, which releases lactic acid during the process (Randazzo, Corona et al. 2016). The fermentability is clearly indicated with a decrease in lactose content in kefir, followed by rise in galactosidase level.

4.3.2 Total Soluble Solids

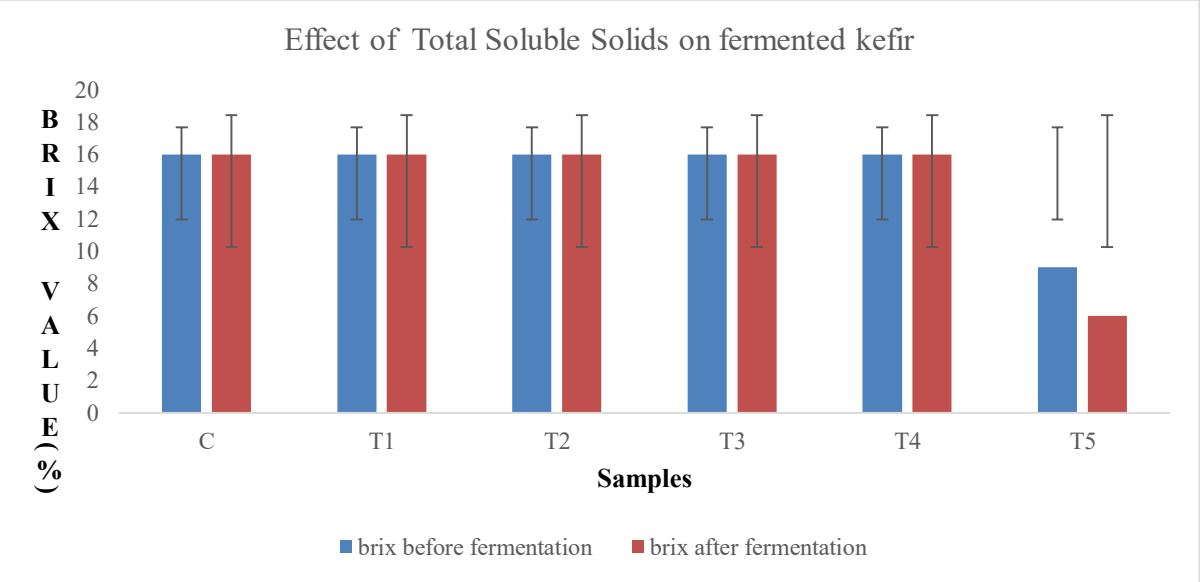

Figure 4.4 Effect of Total Soluble Solids on kefir fermentation

Table 4.3 Total Soluble Solids content of kefir before and after fermentation

Sample	TSS before fermentation(°Brix)	TSS after fermentation(°Brix)
C	16	16
T1	16	16
T2	16	16
T3	16	16
T4	16	16
T5	9	6

No significant difference existed between total soluble solids of control and products T_1-T_4 before and after fermentation. The fermented kefir also displayed the same TSS content as that of sample before fermentation. This could be due to the negligible or least conversion of solids in milk during kefir production. Increased galactosidase content along with reduced lactose (solid) in kefir during fermentation could be a possible explanation for this (Otles and Cagindi 2003). Also studies have indicated that TSS content remains stable during initial days of fermentation. Decrease in TSS content becomes prominent in fermented beverage, as their storage period increases. This is because more solids get converted as the fermentation proceeds and sugar gets converted to alcohol. Concentration and amount of kefir used would also be a possible reason for this, as studies have reported that when higher concentration of yeast was used for wine fermentation, more sugars were not utilized for conversion (Sevda and Rodrigues 2011). However, T_5 displayed a decrease in TSS content, indicating some amount of sugar was converted from carrot juice fermentation, owing to a reduction in TSS content. Similar results were reported by (Randazzo, Corona et al. 2016) on studies on kefir developed from fruit juices using kefiran grains.

4.3.3 Viscosity

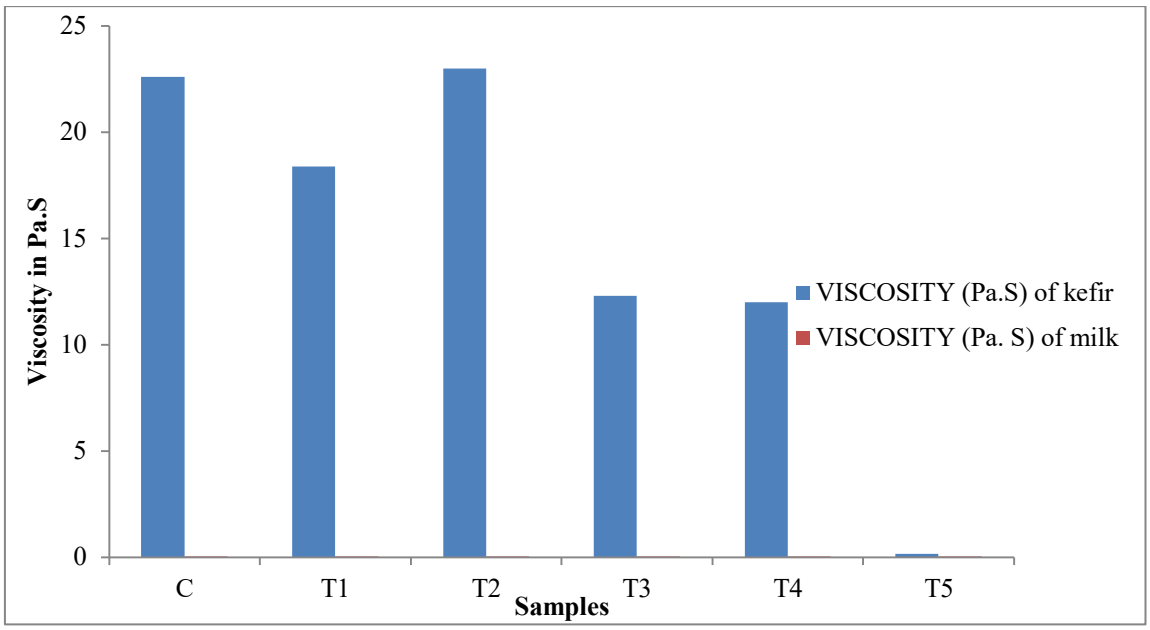

Figure 4.5 Viscosity studies of products before and after fermentation

Table 4.4 Viscosity of products before and after fermentation

Sample	Viscosity (Pa. S) of Kefir	Viscosity (Pa. S) of milk
C	22.2 ± 0.3^{A}	0.002 ± 0.0^{B}
T1	18.3 ± 0.2^{A}	0.002 ± 0.0^{B}
T2	23.1 ± 03^{A}	0.002 ± 0.0^{B}
T3	12.3 ± 0.2^{A}	0.002 ± 0.0^{B}
T4	12 ± 0.1^{A}	0.002 ± 0.0^{B}
T5	0.2 ± 0.0^{A}	0.0021 ± 0.1^{B}

Being non-Newtonian fluids, fermented milk beverages are characterized based on their viscosity. The viscosity of all such beverages is directly related to product quality when considered before and after fermentation and storage (Malbaša, 2009). Comparing viscosity of non-fermented and fermented product, a significant difference was observed. T_2, developed by addition of raw brown mushroom displayed viscosity similar to that of control. However, T3 and T4, which displayed prominence in other properties failed in terms of their viscosity. These results clearly indicate that, negligible amount of water soluble carbohydrates and protein present in kefir fermentation system used here results in the production of a kefir with

lower fermentability (Otles and Cagindi 2003). This variability in viscosity may be associated to weaker gel matrix that could differ with available proteins. Gel development aiding the rheological properties is also related to iso electric pH of proteins and type of interaction among particles (Wang, Chelikani et al. 2018). All our samples displayed a pH in the range of 4 to 5, whereas isoelectric point of most proteins is pH 4.6. This explains the reason behind weaker gel structure and thereby viscosity difference in different formulations of kefir. T_5, which was developed using carrot juice instead of milk as base for kefir, indicated significantly low viscosity values. This result clearly suggest that viscosity is directly influence by size of particles (Masson, 2011), in this case milk caseins and proteins exists as agglomerates of size prominent than fibres in carrot juice.

4.3.4 Syneresis

Table 4.5 Syneresis (in %) of fermented kefir

Sample	Syneresis (as %)
C	20±0.1
T_1	22±0.2
T_2	25±0.6
T_3	22±0.9
T_4	31±0.2
T_5	87±0.2

T_5, fermented kefir using carrot juice as base exhibited the maximum syneresis compared to rest of formulations. In contrast to all other findings T_4 exhibited highest value in terms of syneresis, compared to all formulations that used milk as base. Syneresis is associated to starch content of any component, if present in the formulation. They undergo rapid retro gradation and expel water from the gel matrix leaving the solids behind (Wang, Chelikani et al. 2018). Carrot and roasted mushrooms added products T_5, T_3 and T_4 respectively indicated the maximum syneresis percentage indicating the presence of starch or related components in it. The fine, powdered roasted mushroom, when used as an emulsifier, due to chitosan assisted droplet formation (Harkin, 2019), prevents aqueous phases of kefir from separating. It would also be a possible reason for forming weaker gel matrices compared to unroasted mushrooms.

Chapter 5
Conclusions

This study, in its primary view failed to prove the major hypothesis, i.e. presence of chitin in *Phormium tenax* leaves. One possible and strong reason, which must be taken into account, is the new concept of complex interaction between tannins and non-protein organic nitrogen compounds. The primary analysis and literature review of Phormium tenax leaves and their extracts clearly indicates the presence of a strong bio-active, yet stable compound that withstands the complex extraction process. Also, the wound healing potential of leaves, made used by the Maori community, makes its fibres comparable to chitin, for their functionality. Though this primary study was not able to prove the presence to tannins in any form in the leaves by normal extraction and quantification, the presence of a strong and complex interaction might be the reason behind it. If so, further studies need to rule out if this interaction is caused by tannin-chitin complexes and methods must be developed to extract chitin bound to these condensed tannins. This will open the possibility to find out the first plant based, organic source of chitin, which have the potential to replace chitin from other sources. *Phormium tenax*, important contributor to New Zealand biodiversity is underutilized even after knowing their potential effects. Advancements in nanotechnology and microencapsulation techniques, could also be made use to develop plant chitin based bio-films and edible films for food packaging and preservation. This approach not only would be a clean labelled one, but also would be economical.

The fermented beverage, kefir showed stabilizing and emulsifying effect, when added with roasted white button mushrooms (Agaricus bisporus). Further studies need to be carried out using different cultivars of white button mushrooms, in order to find out if this effect exists in all cases. Further sensory trials and nutritional profiling of the kefir must be carried out to figure out if the formulations are good to be marketised. Mushrooms, being the only source of fungal chitin resembling a plant source, are currently studied for their ability to be used as nootropic drinks. If this works positively, a new functionality could be added to mushroom other than clarifying, emulsifying and stabilizing agent. There are yet more functionalities and potential of mushrooms which are under exploited, even after their year around availability, which if done would make the fungal source rule our food sectors and markets.

Chapter 6

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Appendix A

Calculations

A.1 High Performance Liquid Chromatography

Table A.2. Calculations for quantification of chitin

Sample name	Total Glucosamine (ppm)= mg/l	weight after hydrolysis	mg of glucosamine per gram of extract	grams of glucosamine per gram of extract	grams of glucosamine per 100g of extract
L1	13.29	0.2961	0.539	0.001	0.054
L2	13.31	0.2961	0.539	0.001	0.054
L3	13.41	0.2961	0.544	0.001	0.054
C1	1446.46	0.0473	366.966	0.367	36.697
C2	1559.13	0.0473	395.552	0.396	39.555
M1	3126.88	0.0931	403.035	0.403	40.304
M2	2703.53	0.0931	348.468	0.348	34.847

A.2 Viscosity of Kefir

Table A.3. Viscosity calculations of product before and after fermentation

SAMPLE	CONTROL	T1	T2	T3	T4	T5
SHEAR RATE (S^{-1})	6.4	6.4	6.4	6.4	6.4	6.4
TORQUE (mNm)	11.6	9.9	11.8	6.33	6.17	0.077
VISCOSITY (Pa. S)-1	22.6	18.4	23	12.3	12	0.151
VISCOSITY (Pa. S)-2	22	18.6	22.8	12	12.1	0.148
VISCOSITY (Pa. S)-3	21.9	18	23.5	12.6	11.8	0.155
Average	22.2	18.3	23.1	12.3	12.0	0.2
St. Dev.	0.3	0.2	0.3	0.2	0.1	0.0