Aalto University
School of Chemical
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BIOPROCESSING WITH ENZYMES AND LACTIC ACID BACTERIA FOR PRODUCTION OF NEW FUNCTIONAL FABA BEAN INGREDIENTS

Master's thesis for the degree of Master of Science in Technology submitted for inspection, Espoo, 19 October, 2015.

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Abstract of master's thesis

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Abstract

Faba bean (*Vicia Faba* L.) is a nutritious high protein crop widely cultivated for uses of both food and feed. Its use has limited due to presence of anti-nutritional factors, including phytic acid, bitter taste and poor technological functionality. Phytic acid is the major storage of phosphorus in cereals and legumes lowering the bioavailability of proteins and micronutrients. The aim of this master's thesis was to study the impacts of bioprocessing with enzymes and lactic acid bacteria on both nutritional and textural value of faba bean flour. Food-grade phytase was applied for degradation of phytic acid in faba bean matrix. Fermentation technology with lactic acid bacteria was studied in aim to improve both technological and nutritional quality of faba bean. The specific objective studied with the aim of improving the technological properties of faba bean matrix with *in situ* exopolysaccharide, dextran production. The final step was to study the functionality of bioprocessed faba bean ingredients in extrusion. Extrusion process is a high-temperature short-time, modern and versatile operation widely used commercially to produce high-value expanded breakfast and snack foods based on cereals.

Enzyme treatments were performed with food-grade phytase with three different enzyme activity doses (2, 10 and 20 U) and three treatment times (1, 2 and 4 h). Lactic acid bacteria fermentation was performed with *Weissella confusa* VTT E-143403, *Leuconostoc lactis* VTT E-032298 and *Lactobacillus plantarum* VTT E-78076. Fermentations were carried in small (0.5 kg) and in large (10-15 kg) scale. Extrusion was performed with twin-screw extruder using the bioprocessed faba bean as a raw material. Raw a material was mixed with rice flour (100 %, 50 % and 25 % addition levels of faba bean).

Bioprocessing modified the nutritional value and texture of faba bean flour. Phytase treatment reduced phytic acid over 80 % improving also mineral and amino acid profile of faba bean. Faba bean flour was an excellent material for *in situ* dextran production. With *W. confusa*, 6.5 % dextran yield was achieved. Fermentation reduced the flatulence causing oligosaccharides and improved the amino acid and mineral composition. Bioprocessed faba bean flour showed moderate improvement on mechanical properties of extrudates. Maillard reactions caused challenge in extrusions with protein-rich material, especially in fermented samples with *in situ* produced dextran. However, fermentation with *L. plantarum* gave 55 % increase in crispiness index and reduced hardness 45 % at addition level of 25 %.

Keywords Anti-nutritive factors, Dextran, Exopolysaccharide, Extrusion cooking, Faba bean (*Vicia faba* L.), Lactic acid bacteria fermentation, Phytase, Phytic acid

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Tiivistelmä

Härkäpapu (*Vicia Faba* L.) on ravinteikas proteiinipitoinen viljelykasvi, jota käytetään laajasti sekä eläin- että ihmisravintona. Härkäpavun käyttöä joudutaan kuitenkin rajoittamaan sen sisältämien antinutritiivisten tekijöiden vuoksi. Näihin kuuluu muun muassa fytiinihappo, joka on pääasiallinen fosforivarasto vilja- ja palkokasveissa. Lisäksi käyttöä rajoittavat heikot teknologiset ominaisuudet. Tämän diplomityön tavoite oli tutkia entsyymien ja maitohappobakteerien vaikutuksia härkäpavun ravitsemuksellisiin ominaisuuksiin ja rakenteeseen. Fytiinihapon hydrolysoinnisa hyödynnettiin elintarvikkeiden valmistukseen sopivaa fytaasikäsittelyä. Tavoitteena oli parantaa härkäpapumatriisia *in situ* – tuotetulla eksopolysakkaridilla, dekstraanilla. Lopuksi tutkittiin bioprosessoidun härkäpavun toimivuutta ekstruusiossa. Ekstruusioteknologia on moderni ja monipuolinen prosessointimenetelmä, joka perustuu korkeisiin lämpötiloihin ja lyhyeen prosessointiaikaan. Ekstruusion avulla voidaan tuottaa viljapohjaisia puffattuja aamu- ja välipalatuotteita.

Entsyymikäsittelyt toteutettiin kolmella entsyymiaktiivisuusannoksella (2, 10 ja 20 U) sekä kolmella käsittelyajalla (1, 2 ja 4 h). Maitohappobakteerikäyminen toteutettiin *Weissella confusa* VTT E-143403-, *Leuconostoc lactis* VTT E-032298- sekä *Lactobacillus plantarum* VTT E-78076-kannoilla. Fermentoinnit tehtiin sekä pienessä (0,5 kg) että isossa (10–15 kg) mittakaavassa. Ekstruusio toteutettiin kaksiruuviekstruuderilla käyttäen bioprosessoitua härkäpapua raaka-aineena. Raaka-aine sekoitettiin riisijauhon kanssa 100 %, 50 % ja 25 % härkäpapumateriaalipitoisuuksilla.

Bioprosessointi vaikutti sekä ravitsemuksellisiin ominaisuuksiin että härkäpapumateriaalin rakenteeseen. Fytaasikäsittelyllä vähennettiin fytiinihapon määrää jopa 80 %. Käsittely paransi samalla härkäpavun mineraali- ja aminohappoprofiilia. Härkäpapujauho oli erinomainen alusta dekstraanin *in situ*-tuottamiseen. *W. confusa*-kannalla saavutettiin 6,5 % dekstraanisaanto. Lisäksi fermentoinnilla vähennettiin ilmavaivoja aiheuttavia oligosakkarideja sekä parannettiin aminohappo- ja mineraalikoostumusta. Bioprosessoidulla härkäpavulla saavutettiin kohtalaisia parannuksia ekstrudaattien mekaanisiin ominaisuuksiin. Lisäksi tutkittiin valikoitujen maitohappobakteerien vaikutuksia härkäpapujauhon ravitsemuksellisen arvon ja teknologisen toimivuuden parantamisessa. Fermentointi *L. plantarum*-kannalla lisäsi rapeusindeksiä 55 % sekä vähensi tuotteen kovuutta 45 % (25 % lisäystasolla).

Avainsanat Antinutritiiviset tekijät, Dekstraani, Eksopolysakkaridit, Ekstruusio, Fytaasi, Fytiinihappo, Härkäpapu, Maitohappobakteerikäyminen

Preface

This Master's Thesis was carried out at VTT Technical Research Centre of Finland Ltd., Espoo. The study was a part of the EU Eranet project (Novel multifunctional plant protein ingredients with bioprocessing (Bioprot). National funding was obtained from the Ministry of Agriculture and Forestry. The experiments were performed during the period of December 2014 and June 2015.

I address my sincerest gratitude to my instructors at VTT, Dr. Arja Laitila and Dr. Nesli Sozer for their excellent supervision. I would like to specially acknowledge Arja Laitila for the constant guidance and encouragement and all the interest she has shown in my master's thesis study. I would like to warmly thank Nesli Sozer for all the guidance and advices through the extrusion study. I am also grateful to professor Heikki Ojamo for supervising my master's thesis. Due to retirement, a change in official supervisor to professor Katrina Nordström was made towards the writing phase of this thesis and I warmly thank her for the supervision.

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Anni Karsma

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Abbreviations

ANF	Anti-nutritive factor
C_i	Crispiness index
CFU	Colony forming unit
dm	Dry matter
EPS	Exopolysaccharide
ER	Expansion ratio
FAA	Free amino acids
F_{Cr}	Crushing force
GABA	γ-aminobutyric acid
GRAS	Generally Recognized As Safe
HOPS	Homopolysaccharide
$InsP_6$	*myo*-inositol hexakis phosphate
LAB	Lactic acid bacteria
l_s	Specific length
PD	Piece density
P_i	Inorganic phosphate
PA	Phytic acid
RFO	Raffinose family oligosaccharide
SD	Standard deviation
U	Unit

Introduction

Food legumes are potential novel and low cost plant sources for proteins, dietary fibre, vitamins and minerals. Faba beans are a minor arable pulse crop that are historically important as a food crop for humans and animals throughout Europe, Africa and Asia (Smith and Hardacre, 2011). Globally, there is a lack of protein and it is important merchandise in world trade. Currently, the most important plant protein source is soybean but its use has been questioned by genetically modified organism (GMO) and protein independency issues in Europe. Therefore, production of home-grown plant protein, such as peas and faba beans could be considered. Other advantages of faba beans are their mild "beany" flavour compared to other pulses and their low cost which is the half of the price of soy bean and peas. Faba bean is also suitable for in gluten-free products.

The protein content of faba beans is 28-30 %. Due to presence of some anti-nutritional factors (ANF), faba bean cannot be utilized efficiently at the moment; only 9-14 % of faba bean grown are currently used for human consumption. Furthermore, plants are the major food source especially in developing countries, but unrefined cereal and legume foods have a low bioavailability of micronutrients (Gupta *et al.*, 2015). Phytic acid (PA) is the major storage of phosphorus in cereals and legumes, also in faba beans. It is known as a food inhibitor which chelates minerals and makes protein complexes. Therefore, phytic acid is considered as an antinutrient and controlling it in food is vital (Kenndler 1995).

Faba bean can be considered as important source of protein. Degradation of PA could further improve protein bioavailability. Different processing methods for reducing ANFs of faba bean have been applied including soaking, dehulling, heat treatment, air classification, fermentation and extrusion cooking (Jezierny *et al.*, 2010). Fermentation technology with lactic acid bacteria (LAB) is a potential option in improving both technological and nutritional quality of faba bean. Food associated LAB are granted the status of Generally Recognized As Safe (GRAS).

During the LAB fermentation, the bean constituents could be modified by enzymes with the aim of modifying their structure, bioactivity and bioavailability. Some LAB are able to produce polysaccharide polymers (Gänzle and Schwab, 2009). These exocellular polysaccharides (EPS), secreted by LAB are of interest as natural and economically important additives improving textural properties in food products (Welman and Maddox, 2003). EPS-producing LAB could be used as multifunctional starter cultures in order to modify the physicochemical and sensorial characteristics of faba bean. Consumer demands for low levels of additives, as well as cost factors, make EPS an important alternative for synthetic hydrocolloids, for instance.

Applications of faba bean ingredients in extruded products could in addition improve the bioprocessed faba bean. Extrusion technology is a high-temperature short-time, modern and versatile operation widely used commercially to produce high-value expanded breakfast and snack foods based on cereals (Berrios *et al.*, 2013). Extrusion processing could offer great potential for the development of high protein faba bean snack products improving their nutritional value as well as chemical properties and physical functionality.

The objective of this master's thesis study was to improve functionality of faba bean by bioprocessing with LAB and phytase. The functionality of bioprocessed faba bean was studied in extruded products. The growing need in novel plant protein sources and especially the increasing interest in grain legumes (e.g. The UN declared 2016 to be an *International Year of Pulses*) as well as consumer interest for increased protein content, and more natural and unmodified food were the key motivators of this study.

LITERATURE REVIEW

1 Faba bean

Faba bean (*Vicia faba* L.), also known as broad bean, field bean, horse bean, or tick bean is a high protein crop that belongs to the botanical family of grain legumes *Fabacea* (Jezierny *et al.,* 2010). Cultivation of faba bean has a long history of valuable uses in feed and food based on rich protein and energy of their seeds (Figure 1) and ability to grow in various climatic zones (Crépon *et al*. 2010). The scanning electron micrograph (Figure 2) of faba bean shows the composition of the bean seed cotyledon and embryonic root. Nutritious faba beans are used as pulses or as vegetables and the beans can be dried and canned or eaten as fried and salted crunchy snack (Lim, 2012). Nutrient composition of faba bean compared to that of soybeans is shown in Table 1.

Figure 1. Faba bean seeds (Romano, 2011).

Table 1. Nutrient composition of raw faba bean in pod and raw mature faba bean seeds and soybean seeds for comparison (USDA 2011).

Nutrient composition/ 100 g edible portion	**Raw faba bean in pod**	**Raw mature faba bean seeds**	**Raw mature soybean seeds**
Proximates			
Water (g)	72.6	**11.0**	8.5
Energy (kcal)	88.0	**341.0**	446.0
Protein (g)	7.9	**26.1**	36.5
Lipid, total (g)	0.7	**1.5**	19.9
Ash (g)	1.1	**3.1**	4.9
Carbohydrate (g)	17.6	**58.3**	30.2
Fibre, total dietary (g)	7.5	**25.0**	9.3
Sugars, total (g)	9.2	**5.7**	7.3
Minerals			
Ca (mg)	37.0	**103.0**	277.0
Fe (mg)	1.6	**6.7**	15.7
Mg (mg)	33.0	**192.0**	280.0
P (mg)	129.0	**421.0**	704.0
K (mg)	332.0	**1062.0**	1797.0
Na (mg)	25.0	**13.0**	2.0
Zn (mg)	1.0	**3.1**	4.9
Vitamins			
Vitamin C (mg)	3.7	**1.4**	6.0
Thiamin (mg)	0.1	**0.6**	0.9
Riboflavin (mg)	0.3	**0.3**	0.9
Niacin (mg)	2.2	**2.8**	1.6
Vitamin B-6 (mg)	0.1	**0.4**	0.4
Folate, total (µg)	148.0	**432.0**	375.0
Vitamin A (IU)	333.0	**53.0**	22.0
Vitamin K (µg)	40.9	**9.0**	47.0
Lipids			
Fatty acids, total saturated (g)	0.1	**0.3**	2.9
Fatty acids, total monounsaturated (g)	0.1	**0.3**	4.4
Fatty acids, total polyunsaturated (g)	0.3	**0.6**	11.3
Essential amino acids			
Phenylalanine (g)		**1.1**	2.1
Valine (g)		**1.2**	2.0
Threonine (g)		**0.9**	1.8
Tryptophan (g)		**0.2**	0.6
Methionine (g)		**0.2**	0.5
Leucine (g)		**2.0**	3.3
Isoleucine (g)		**1.1**	2.0
Lysine (g)		**1.7**	2.7
Histidine (g)		**0.7**	1.1

Faba bean is highly nutritious and rich in proteins, amino acids, dietary fibre, carbohydrates, folate, niacin, panthoenic acid and plenty of minerals such as Ca, Fe, K, Mg, P, Zn and Se (Lim, 2012). Positive health effects of faba bean also include the decrease in plasma total lower-density lipoproteins (LDL)-cholesterol levels and their antioxidant activity (Lim, 2012). Based on Lim (2012), faba bean was found to be rich in levo-dihydroxy phenylalanine (L-DOPA), which is a dopamine precursor that plays an important role in the treatment of Parkinson's disease.

Faba beans contain several secondary metabolites that can be considered as anti-nutritional factors, including glycosides like vicine and convicine, alkaloids, as well as tannins, trypsin and protease inhibitors and phytic acid (Lim, 2012; Crépon et al., 2010; Luo & Xie 2013). Due to those factors, its use in human nutrition is thus far limited.

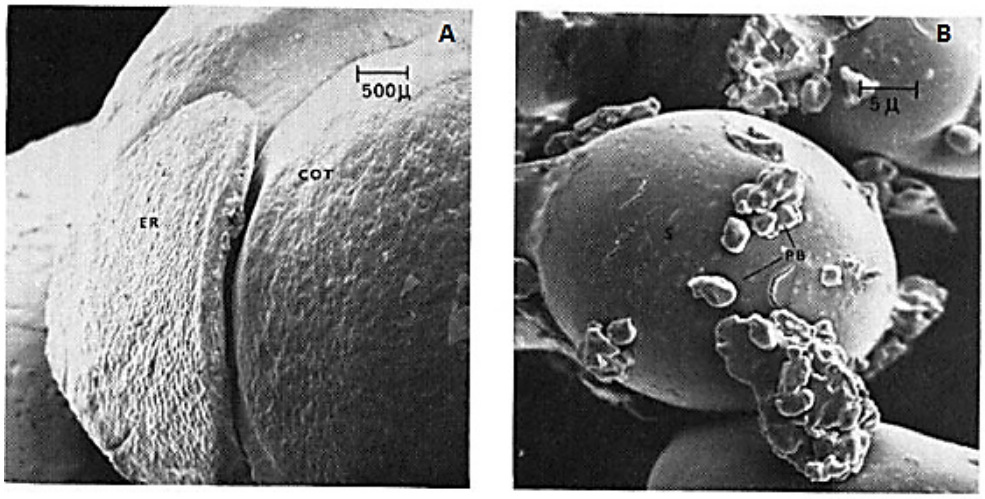

Figure 2. Scanning electron micrograph of a faba bean seed (A) with the seed coat removed showing the cotyledons (COT) and the embryonic root (ER) and of faba bean flour (B) showing the starch granules (S) and protein body aggregates (PB). (Mc Ewen *et al.*, 1974)

Three types of varieties are cultivated; 1) *V. faba major*, or broad beans, are mainly grown in southern Europe, 2) *V. faba minor*, or field beans or horse beans which grow in larger range of regions, and 3) *V. faba equina* (Jezierny *et al.*, 2010). *V. faba major*

are used for human nutrition, either as fresh or dried seeds. *V. faba equina* and *V. faba minor* are used both for food and feed. The size of faba beans can vary considerably. (Crépon *et al.*, 2010)

Faba beans have been an important and common staple food in the Mediterranean area and in continental areas like Iraq, Syria, Iran, India and Southern China and North Africa (Crépon *et al.*, 2010). In these regions dry faba beans are usually soaked to soften and then fried or ground to a paste (Smith and Hardacre, 2011). Faba bean flour is also used as a substituent in spaghetti-type pasta making. Giménez *et al.*, (2012) observed an increase in the nutritional quality of wheat pasta with 30 % addition of faba bean flour without affecting the product texture, flavour or physicochemical properties. In addition, faba bean is also cultivated as a fodder and stock feed for poultry, pigeons, pigs, horses (Lim, 2012) and dairy cows (Volpelli *et al.*, 2010). It has also used as a green manure crop in industrialised countries. The faba bean straws are also used as a fuel and brick making in Sudan and Ethiopia (Lim, 2012).

1.1 Anti-nutritive factors of faba bean

The use of faba bean is limited due to the presence of ANFs in the seeds, which might have negative impacts in human nutrition. The ANF are divided into two categories based on their structure: 1) proteins such as lectins and protease inhibitors, and 2) others such as phytic acid (PA) and tannins (Luo and Xie, 2013a). Trypsin-inhibitor is a crystalline globular protein that decreases the growth rate of mammals, chickens, and fish as well as cause pancreatic hypertrophy (Adamidou *et al.*, 2011). Tannins are secondary composites of chemical structures divided into hydrolysable and condensed tannins. For instance, some specific enzyme inhibitors like trypsin- and α-amylase inhibitor can have an impact in the digestion of starch and protein, whereas PA and tannins can also effect on mineral utilisation (Luo *et al.*, 2010).

Faba beans are rich in two glucosidic amino pyrimidine derivatives, vicine and convicine which are known to be responsible for favism, a type of potentially lethal

haemolytic anemia (Duranti and Gius, 1997). Vicine and convicine with their hydrolytic derivatives have found to render the red blood cells of glucose-6-phosphate dehydrogenase deficient patients vulnerable to oxidation and destruction (Lim, 2012). Favism is an acute hemolysis caused by the ingestion of faba beans and occurring only in humans that are glucose-6-phosphate dehydrogenase deficient. It may cause rapid destruction of up to 80 % of circulating red blood cells (Crépon *et al.*, 2010).

Furthermore, legumes are well known inducers of intestinal gasses (flatulence), due to presence of the raffinose family oligosaccharides (RFOs) which are α-galactosyl derivatives of sucrose. Most common are the trisaccharide raffinose, tetrasaccharide stachyose, and pentasaccharide verbascose (Adeyemo, and Onilude, 2014). Monogastric species, like humans, are not capable in digesting these oligosaccharides due to the absence of α-galactosidase in the intestinal mucose. Therefore, α-galactosides pass into the colon and are fermented by the intestinal bacteria, such as *Clostridium* spp. and *Bacteroides* spp. (Djaafar *et al.*, 2013) with production of gas (Duranti and Gius, 1997). According to the study of Lattanzio *et al.* (1986), the total content of α-galactosides in dry mature whole seeds of *Vicia faba L.* is 2.16 ± 0.09 %.

1.2 Phytic acid as an anti-nutritive factor in faba bean

Phytic acid (PA, *myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate, IP_6) is a natural plant compound formed during maturation of the plant seed (Kumar *et al*. 2010; Patel *et al*. 2007). PA can be found in many forms since it has 12 replaceable protons that allows it to complex with multivalent cations and positively charged proteins. It can exist as free acid, phytate (salt of phytic acid) or phytin, depending on metal ions present and physiological pH (Oatway *et al.*, 2007). Monogastric species have a limited capability to hydrolyse PA salts and release phosphate for absorption (Urbano *et al.*, 2000). Majority of the phosphorus in plant legume seeds appears as PA and phytates. Molecular formula of phytic acid is $C_6H_{18}O_{24}P_p$ and molecular mass 660.04 g mol^{-1} (Kumar *et al.*, 2010). The basic chemical structure of PA is presented in Figure 3.

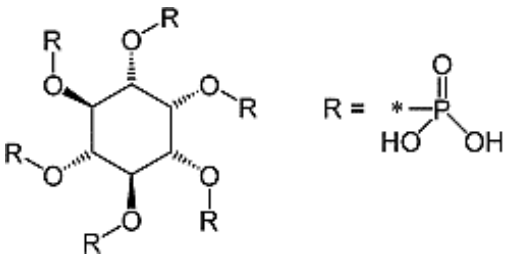

Figure 3. Chemical structure of phytic acid (Kumar et al. 2010).

PA can be found in most legumes, cereal grains, nuts, oilseeds, pollen, tubers, spores, and organic soils (Oatway *et al.*, 2007). In dormant seeds it represents 60-90 % of the total phosphate, so it is a common constituent of plant derived foods (Kumar *et al*. 2010). It constitutes 1-3 % of cereal grains, legume seeds, and nuts. In legume seeds, phytate is founded in the protein bodies in the endosperm (Sandberg, 2002). PA is utilised during seed germination, when it supports seedling growth and cell wall formation. According to Luo *et al*. (2012), the typical phytic acid content in faba beans is approximately 8.6 mg/ g. PA content in several legumes is presented in Table 2. The interaction of PA with proteins and some vital minerals is considered to be one of the principal factors that limits the nutritive value of cereal grains and legume seeds (Lopez *et al.*, 2000).

Table 2. Content of phytic acid in legumes.

Name	Type	Phytic acid (mg g^{-1} dry matter)	References
Faba bean, green	raw seeds	8.4	Luo and Xie, 2013
Faba bean, white	raw seeds	8.6	Luo and Xie, 2013
	flour	8.4	Luo et al., 2010
Green pea	cooked	1.8-11.5	Kumar *et al*., 2010
Soybean		9.2-16.7	Kumar *et al*., 2010
Chickpea	cooked	2.9-11.7	Kumar *et al*., 2010
Black bean	cooked	8.5-17.3	Kumar *et al*., 2010
White bean	cooked	9.6-13.9	Kumar *et al*., 2010
Kidney bean	cooked	8.3-13.4	Kumar *et al*., 2010
Lentil	cooked	2.1-10.1	Kumar *et al*., 2010

1.2.1 Effect of phytic acid on protein solubility and digestibility

Phytate tends to form strong complexes (Figure 4) with some proteins and inhibit their proteolysis. Several studies indicate that there is a negative correlation between phytic acid content and *in vitro* protein digestibility (Pallauf and Rimbach, 1997). Phytate-protein interaction is pH dependent; at a pH value lower than the isoelectric point of protein, the phosphoric acid groups of phytates bind with the cationic group of basic amino acids. These insoluble complexes could affect the protein structures and hinder protein solubility, enzymatic activity and protein digestibility. Complexes dissolve only below pH 3.5 (Kumar *et al.* 2010.) So far, the significance of phytate-protein complexes in human nutrition is not clear. Based on Kumar *et al.* (2010), several studies have shown that proteolytic and digestive enzymes, such as pepsin, trypsin, chymotrypsin, amylase and lipase are inhibited by phytate. Furthermore, phytic acid also effects on carbohydrate and lipid utilisation (Kumar *et al.*, 2010).

1.2.2 Effect of phytic acid on mineral availability

The antinutritive effect of PA is due to its molecule structure (Pallauf and Rimbach, 1997). PA causes weak absorption of essential electrolytes and minerals due to its tendency to be highly negatively charged, and make complexes or bind to positively charged molecules such as metallic cations and proteins at pH values normally encountered in food (Deak and Johnson, 2007). Phytate decreases the bioavailability of vital minerals and nutrients, such as zinc, iron, calcium, magnesium manganese, and copper by chelating them (Konietzny and Greiner 2002; Luo *et al*. 2012).

Considering the minerals, zinc seems to be the most affected by phytic acid since it forms the most stable and insoluble complexes (Oatway *et al.*, 2007). The stability and solubility of phytase complex depends on the pH value, the individual cation, the phytate to cation molar ratio, and presence of other components in the solution. Phytate seems to be more soluble at lower than higher pH value. Calcium, zinc and copper salts are soluble at below pH 4-5, while magnesium salt is soluble above pH 7.5. At the same time, ferric phytate is insoluble at pH values 1-3.5 and solubility increases above pH 4. In addition, the synergistic effect of cations needs to be noted. (Kumar *et al.*, 2010)

Furthermore, there is a lack of phytate degrading enzyme in human small intestine and it is also limited in upper part of the digestive track. Thus, the complex remains partially hydrolysed in the human gut. (Kumar *et al.*, 2010)

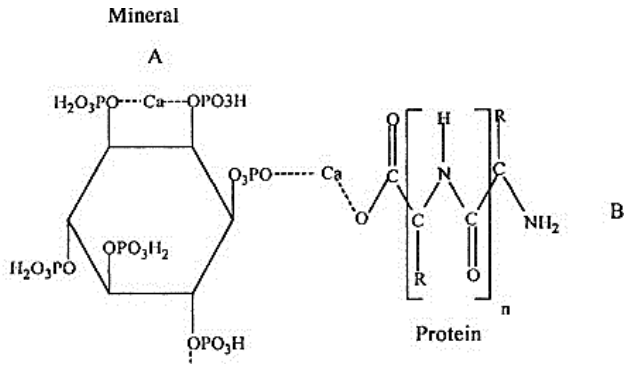

Figure 4. Interactions between PA and mineral (A) and proteins (B) (Modified from Oatway *et al.* 2007).

1.3 Reduction of anti-nutritive factors and improvement of technological functionality of faba bean by different processing methods

Raw legumes contain much higher levels of ANF than their processed forms, thus, processing before using them in food or feed diets is necessary (Luo and Xie, 2013b). The nutritional value of legumes could be improved by implementing of large range of processing technology including soaking, germination, boiling (Márquez and Alonso, 1999), dehulling, cooking, roasting, autoclaving, microwaving (Adamidou *et al.*, 2011), supplementation with enzymes (Adamidou *et al.*, 2011), fermentation, and extrusion cooking (Luo and Xie, 2013; Abd El-Hady and Habiba, 2003). Heat treatment in feed used faba bean is also considered (Volpelli *et al.*, 2010).

In a study of Khalil and Mansour (1995), heat processing and germination showed reduction in phytic acid, tannins, vicine, stachyose, and trypsin inhibitor activity in faba bean. Besides, Luo *et al.* (2010) reported 95 % reduction of phytate content after 3 h incubation with exogenous phytase. Luo and Xie (2012) also reported that treatment of faba bean flour with phytase led to a significant improvement of iron absorption in growing rats. In addition, cooking, soaking and germination have been reported to reduce the PA contents of faba bean (Luo *et al.*, 2012).

Bioprocess is a specific process that uses complete living cells or their components to obtain desired products. These include bacteria, yeast, mould, enzymes, and chloroplasts for instance. This study is focused on bioprocessing with enzymes and lactic acid bacteria. In the study of Coda *et al.* (2015), fermentation with *L. plantarum* reduced vicine and convicine contents and trypsin inhibitory activity, and condensed tannins in faba bean flour. RFO concentration in legumes has been reduced by treating with α-galactosidase or microorganisms capable of degrading RFOs (Teixeira *et al.*, 2012). Several lactic acid bacteria produce α-galactosidase, and have been used for elimination of RFOs in food prepared from pea and bean flours. Adeyemo and Onilude (2014) observed 30, 28 and 37 % reductions in stachyose, raffinose and verbascose levels when fermenting soybeans with *L. plantarum*. In a study of Djaafar *et al.* (2013), *L. plantarum-pentosus* species were able to use raffinose for their growth in *kerang* milk.

In addition to decrease ANF, LAB fermentation can result in enhanced content of free amino acids (FAA) and γ-aminobutyric acid (GABA), as well as improve *in vitro* protein digestibility of some legumes. Coda *et al.* (2015) obtained increase in the amount of essential amino acids and GABA.

Recently, some studies about the effect of *in situ* produced EPS on quality and functionality of cereal food products. Di Monaco *et al.* (2014) obtained positive effect on sensory perception, sensory quality of bread during shelf life of sourdough breads using EPS-positive *Lc. lactis* and *Lactobacillus curvatus* strains as starters.

This study focused mainly on phytic acid, as well as, oligosaccharides as an ANF in faba bean processing.

2 Enzymatic degradation of phytic acid

Phytases (IP_6-phosphohydrolases) are enzymes that catalyse the hydrolytic phosphate splitting of phytic acid (IP_6) to lower inositol phosphate esters (IP_5-IP_1) and inorganic phosphate (P_i) (Pallauf and Rimbach, 1997). Phytate-degrading enzymes were first proposed as animal feed additives enhancing the plant material value in feed by liberating orthophosphate (Mitchell *et al.*, 1997). Phytases are classified as 3-phytases (EC 3.1.3.8), and 6-phytases (EC 3.1.3.26) based on the location of first phosphate that is hydrolysed (Gupta *et al.*, 2015). Phytates have now been studied because of an interest in their capability of reducing phytate content in human food as well (Konietzny and Greiner, 2002). The description of the hydrolytic action of a microbial phytase on PA is shown in Figure 5.

2.1 Degradation of phytates in food processing

Several methods have been developed to reduce PA content in food with the aim of improving the nutritional value of cereal, including genetic improvement, (Gupta *et al.*, 2015) as well as various biological processing techniques that increase the activity of native enzymes in legumes and cereals. The studies of Luo *et al*. (2012) have shown that traditional processing methods such as soaking and germination of faba bean, can decrease its phytate content. Fermentation has also been studied to reduce the PA content of cereal foods (Gupta *et al.*, 2015). The effect of these processing techniques is a result of activation of phytate-degrading endogenous phytase naturally present in plants and microorganisms (Kumar *et al.*, 2010).

Furthermore, phytate can be degraded by adding exogenous phytase (Kumar *et al.*, 2010). In several experiments (Luo *et al.*, 2012), the *in vitro* iron bioavailability of faba beans was higher after phytase and tyrosinase incubation. Moreover, the phytate content in faba beans were significantly decreased due to the addition of phytase. In their study, phytate was degraded by 58-65% when faba bean was incubated with 2.0-4.0 U of phytase. The effect of phytase was even better with cooked, soaked or

germinated samples. Phytase acts by releasing the phosphorus from plant-based diets and makes minerals, protein and lipid available.

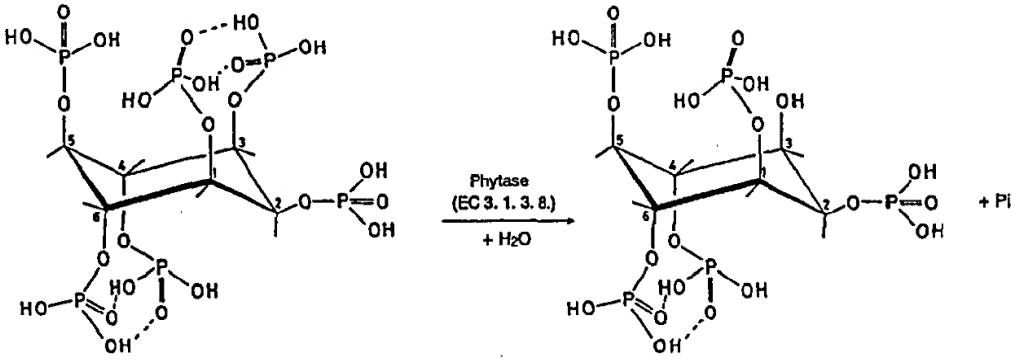

Figure 5. Hydrolysis of phytic acid by microbial phytase generating dihydrogen phosphate and inorganic phosphate (Pallauf and Rimbach, 1997).

2.2 Sources of phytases

Two main types of phytate-degrading enzymes have been identified so far, phytases with an optimum pH around 5 and alkaline phytases with pH optimum around 8.0. Most of the phytate-degrading enzymes have their pH optimum around 4.5 to 6.0. Several bacterial phytases, like those from *Bacillus*, have pH optimum at 6.0-8.0. The optimum temperature of phytate-degrading enzymes ranges from 35 to 77 °C and in general, phytate-degrading enzymes from plants display maximum activity at lower temperatures compared with microbial phytases (Konietzny and Greiner, 2002). The four general sources of phytase are plant, microbes (fungi and bacteria), gut-associated microbiota and the small intestinal mucosa (Kumar *et al*. 2010; Sandberg and Andlid 2002).

Plant phytases

Plants may consist high levels of phytases in their seeds as energy storage for growth of the germinating plant (Sandberg and Andlid, 2002). Phytases have been isolated

from plant sources, such as rice, rape seed, soybean, maize, wheat, rye (Kumar *et al.*, 2010), barley, buckwheat and amaranth (Konietzny and Greiner, 2002). In cereal grains, phytases are mainly located in the aleurone layers and are inactive in dry cereals (Oatway *et al.*, 2007). Most of the plant phytases are 6-phytases (Kumar *et al.*, 2010). Activities of both acidic and alkaline phytases have been reported in legumes (Konietzny and Greiner, 2002).

Microbial phytases

Various fungi, yeast and bacteria contain phytase (Pallauf and Rimbach, 1997). Since phytate is present in most living cells, intracellular phytate hydrolysing enzymes must be present in most microorganisms. Yet for fermentation or food application production with phytase expressing microorganisms, the extracellular phytases, enzymes that are secreted to the surroundings, are the main producers (Sandberg and Andlid, 2002).

One of an important microbial source of phytases is fungi and the most explored genus is *Aspergillus* from which the commercially produced, also food grade, phytases mostly originate. Furthermore, according to the study of Sandberg and Andlid (2002), the ability of phytase synthesising and secretion seems to exist with majority of the yeast species.

Few bacteria, such as *Escherichia coli, Bacillus subtilis, Klebsiella terringa,* have been shown to be able to degrade phytate during growth and then produce extracellular phytases (Sandberg and Andlid, 2002). The largest group of phytases found in bacteria and fungi are the 3-phytases (Gupta *et al.*, 2015).

3 Lactic acid bacteria fermentation as a technology in food processing

Lactic acid bacteria (LAB) fermentation is a traditional and natural bioprocessing technology that is used for processing and preserving food and beverage products. Its benefits are low cost, low energy required, and high yields (Martin-Cabrejas *et al.*, 2004). LAB fermentation contributes beneficially to processing technology and to quality of the end-products such as flavour, shelf-life and product safety.

LAB are a group of bacteria having an ability to synthesize lactic acid. They are typically gram-positive, catalase-negative, non-sporing, anaerobic, but aerotolerant cocci or rods. They are acid-tolerant and strictly fermentative producing lactic acid as the major end product of carbohydrate-fermentation. LAB can be divided into homo- and heterofermentative species (Table 3) based on the products of their sugar metabolism. Homofermentative LAB convert sucrose entirely to lactic acid, whereas heterofermentative species produce also ethanol, carbon dioxide, and acetic acid. Heterofermentative LAB are *Leuconostoc, Oenococcus, Weissella*, and a subgroup of *Lactobacillus* genus. Other LAB genera are homofermentative. (Axelsson, 2004)

Table 3. Principal LAB genera (Axelsson, 2004).

Heterofermentative LAB	**Homofermentative LAB**
subgroup of *Lactobacillus*	*Aerococcus*
Leuconostoc	*Carnobacterium*
Oenococcus	*Enterococcus*
Weissella	*Lactobacillus*
	Lactococcus
	Pediococcus
	Streptococcus
	Tetragenococcus
	Vagococcus

Some *Leuconostoc* strains are reported to have the ability to degrade PA during fermentation of cereals (Björkroth and Holzapfel, 2006). During lactic acid fermentation, the pH decreases due to bacterial production of lactic and acetic acids,

which is favourable for phytase activities and can reduce phytic acid contents (Kumar *et al.*, 2010). For example, Lopez *et al.* (2000) obtained degradation of phytate and improvement of calcium and magnesium solubility during 9 hours fermentation of whole wheat flour with *Leuconostoc mesenteroides* strain 38. Moreover, Najafi *et al.* (2012) reported reduction in PA levels and increase in zinc bioavailability when using *Saccharomyces cerevisiae, L. plantarum* and *L. mesenteroides* in 30 % sourdough replacement in baking of traditional Iranian wheat bread.

3.1 Exopolysaccharide producing lactic acid bacteria

Sugar catabolism is not only vital for the generation of energy and biomass but also for the production of polysaccharides with many bacterial species (Boels *et al.*, 2001). LAB convert sugars, proteins, organic acids or fats into the aroma and flavour active components as well as antimicrobial substances. In addition, they are able to produce few types of polysaccharides classified based on their location in the cell (Welman and Maddox, 2003). Some LAB strains are also capable of the synthesis of exopolysaccharides (EPS), which are primarily involved in cell adhesion and protection in different environments (Monsan *et al.*, 2001). Furthermore, bacterial EPS have technological and functional importance. They can improve the viscosity and texture of fermented products (Ruas-Madiedo *et al.* 2002; Welman and Maddox 2003).

EPS are long-chain polysaccharides which are produced extracellularly from sucrose by glycansucrases, or intracellularly by glycosyltransferases from sugar nucleotide precursors (Patel *et al.* 2012; Gänzle and Schwab 2009). LAB synthesize polysaccharides which are utilised as cell wall components, or secreted from the cell (Gänzle and Schwab, 2009). EPS can be classified according to whether they are composed of different kinds (heteropolysaccharides) or only one kind (homopolysaccharides) of sugar monomers (Waldherr and Vogel, 2009). EPS contribute to the products texture, mouthfeel, taste and stability (Duboc and Mollet, 2001) and have a huge role in the production of fermented dairy products and cheese (Katina *et al.*, 2009). Recently, fermentation with HOPS has shown both technological

and nutritional benefits in sourdough baking (Katina *et al.*, 2009), cereal based drinks (Waldherr and Vogel, 2009) and carrot applications (Juvonen *et al.*, 2015).

3.1.1 Homopolysaccharides

Homopolysaccharides (HOPS) are glucan or fructan polymers that are composed of glucose or fructose units (Patel *et al.*, 2012) and are synthesised from sucrose by extracellular glucansucrases or fructansucrases (van Hijum *et al.*, 2006). The HOPS can be divided into four groups: α-D-glucans, β-D-glucans, fructans and others (Ruas-Madiedo *et al.*, 2002). The difference depends on the degree of branching and different linking sides, which are strain-specific characteristics (Ruas-Madiedo *et al.*, 2002). The assembly of monosaccharide units takes place outside the bacterial cell, and the production of HOPS requires the source of substrate, such as sucrose (Ruas-Madiedo *et al.*, 2002). Extracellular HOPS synthesis does not consume metabolic energy of the producer organism and the activity of glycansucrases is therefore separate from growth (Gänzle and Schwab, 2009).

This study focused on the HOPS, especially on dextran. When introduced into sourdough products, HOPS influenced structural quality and baking ability, and reduced staling of bread (Waldherr and Vogel, 2009).

Hydrocolloids are food additives that are currently used as a substitute to gluten and to improve sensory properties in gluten-free bread. EPS produced by LAB from sucrose have been studied as a promising natural alternative for non-bacterial, E-coded hydrocolloids used as additives in industrial baking (Galle *et al.*, 2010) and vegetable containing products (Juvonen *et al.*, 2015). Advantages in *in situ* produced (by the starter cultures) EPS includes that there will be no additional costs with conventional process. In addition, the EPS produced by the LAB starter cultures does not need to be mentioned in ingredients.

3.1.2 Dextran

Dextrans are made of a main chain containing α-(1→6)-linked glucolsyl units and α-(1→2), α-(1→3), or α-(1→4)-linked single unit or lengthened side chains (Juvonen *et*

al., 2015). Dextrans feature usually more than 50 % α-1,6-linkages of the total linkages in their major chains. The basic chemical structure of dextran is shown in Figure 6. The precise structure of each type of dextran depends on its specific producing LAB strain and on the type of dextransucrase concerned (Naessens *et al.*, 2005).

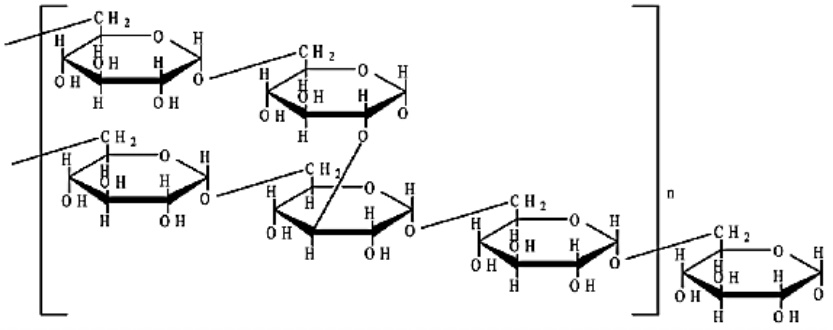

Figure 6. Chemical structure of dextrans (Khan *et al.*, 2007). Dextran is composed of glucose units that are linked with α-(1→6)-bonds in their main chain.

Most of the dextrans are synthesised from sucrose by extracellular enzymes, dextransucrases, which are mainly secreted by species of genera *Leuconostoc* (Galle *et al.*, 2010*;* Naessens *et al.*, 2005), *Lactobacillus*, *Streptococcus* (Malunga *et al.*, 2012) and *Weissella* (Galle *et al.*, 2010; Katina *et al.*, 2009). It has been reported that dextransucrase synthesised dextran is formed from sucrose via glucosyl intermediates that are then transferred to the glucanosyl chain, which is covalently linked to the active site of the enzyme (Naessens *et al.*, 2005). This mechanism involves two nucleophiles at the active site, which attack sucrose and displace fructose with the aim of giving two β-glucosyl intermediates. In addition, the reaction requires the transfer of a hydrogen ion to the displaced fructosyl moiety of sucrose. Naessens *et al.* (2005) reported that two imidazolium groups of amino acid histidine are essential for dextran synthesis. As shown in Figure 7, these groups donate their hydrogen ions to the leaving fructose units following a step in which the resulting

imidazole group gets reprotonated by abstracting a proton from the OH group of the enzyme intermediate. This step facilitates the formation of the-(1→6)-linkage (Naessens *et al.*, 2005).

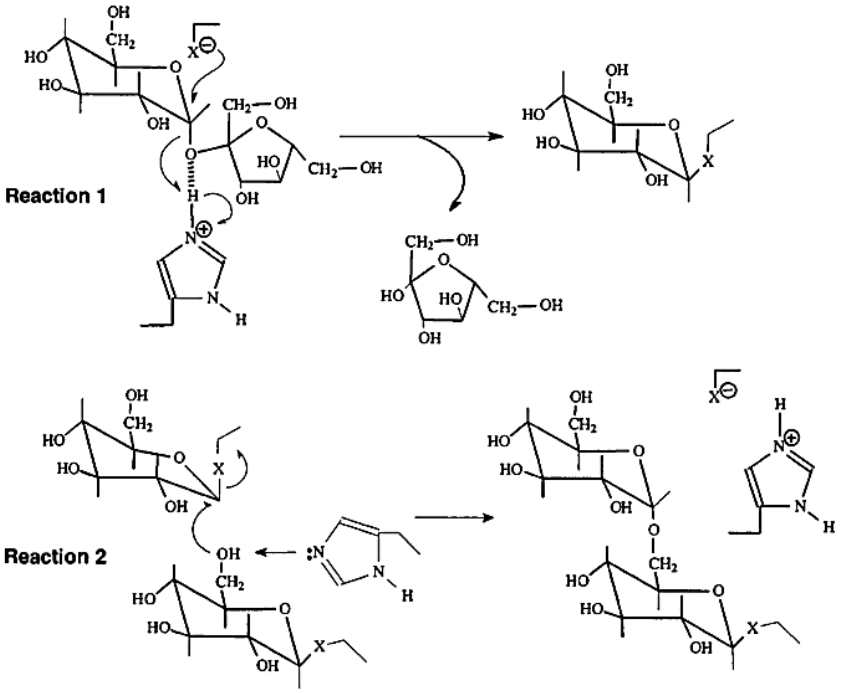

Figure 7. Mechanism of the cleavage of sucrose and formation of α-(1→6)-glycosidic bond by dextransucrase. **1**: Nucleophilic displacement and protonation of the fructose moiety to form a glucosyl-enzyme intermediate. **2**: Formation of α-(1→6)-bond by attack of a C-6 hydroxyl group on to the C-1 of glucosyl-enzyme complex. The reaction is facilitated by abstraction of a proton from the hydroxyl group by the imidazole group. (Naessens *et al.*, 2005)

3.1.3 Genera Leuconostoc and Weissella

Leuconostoc species have coccoid to ovoid-like morphology, whereas *Weissella* strains vary from ovoid cells to irregular rods. *W. confusa* (used in the present study) are reported to be short rods and produce DL-lactate, while *Leuconostoc* species produce the D(-)-lactate isomer from glucose. In contrast to the genus *Leuconostoc*,

most *Weissella* species have tendency to produce ammonia from arginine (Arg) via the arginine-deaminase pathway. (Björkroth and Holzapfel, 2006)

Weissella and *Leuconostoc* strains are reported to be suitable starter cultures for dextran-like EPS production. Katina *et al.* (2009) reported *Weissella confusa* to be a promising strain for effective *in situ* production of dextrans in sourdoughs. The study of Galle *et al.* (2010) also proved that *Weissella* and *Leuconostoc* strains synthesised EPS in the presence of sucrose and maltose as a sourdough starter. Furthermore, Park *et al.* (2013) studied the dextran-like EPS producing *Leuconostoc mesenteroides* and *W. confusa* strains from Korean traditional fermented vegetable food Kimchi. In a recent study of Juvonen *et al.* (2015), use of EPS-producing LAB starter strains was studied in fermentation of pureed carrots. They obtained 1.6 % and 1.9 % (w/w) production of water-soluble low-branched dextran with *Lc. lactis* E-032298 and *W. confusa* E-90392. Summary of beneficial EPS-applications is shown in Table 4.

3.1.4 Viscosity as an indicator of EPS production

Viscosity values are used in evaluating the rheology and EPS production of fermented products, yet clear correlation between EPS concentrations and viscosities is not easy to form because the polysaccharides of different LAB vary in composition, charge, spatial arrangement and protein interactions (Jolly *et al.*, 2002). Nevertheless, some relationship between high viscosity and high molar mass and stiffness of the polysaccharide chain have been obtained (Jolly *et al.*, 2002). Dextran results in carrot functions correlated with the viscosity values (Juvonen *et al.*, 2015). In addition, Katina *et al.* (2009), showed that addition of sucrose in *W. confusa* sourdough baking significantly increased viscosity.

Table 4. Applications of EPS-producing *Weissella* and *Leuconostoc*.

	Species	**Applications**	**Benefits**	**Reference**
Weissella	*confusa* E-90392	*In situ* production of dextran in wheat sourdough	Dextran increased the viscosity of the sourdoughs, improved volume and crumb softness of wheat bread	Katina *et al.* (2009)
	cibaria	Fruit and vegetable smoothies	Higher value of viscosity was probably due to EPS-synthesis	Di Cagno *et al.* (2011)
	confusa E-90392	Pureed carrots	Produced dextran correlated with thick texture, pleasant odour and flavour	Juvonen *et al.* (2015)
Leuconostoc	*lactis* E-032298	Pureed carrots	Produced dextran correlated with thick texture, pleasant odour and flavour	Juvonen *et al.* (2015)
	lactis 95A	Sourdough	Sourdough obtained with EPS-producing LAB preserved hardness, chewiness, and springiness of bread during storage.	Di Monaco *et al.* (2014)

4 Extrusion cooking as a multipurpose food processing method

Extrusion cooking process is a high-temperature short-time modern and multipurpose food operation that converts agricultural commodities, usually in a granular or powdered form, into cooked food products. It has found important uses in both food and feed products, such as cereals, snacks, baby food, dairy applications, as well as pet nutrition and ruminant feed (Berrios *et al.*, 2013). With food products, extrusion process offers a cost competitive alternative allowing flexibility in choice of materials and the resultant product structure. Extrusion cooking offers a combined batch process of mixing, heating, forming and drying with single equipment (Lillford, 2008). Extrusion process can be operated continuously with high throughput. Its advantages are also low processing costs and high productivity (Riaz, 2001). In addition, extrusion is studied to reduce microbial population, inactive enzymes, and minimize the nutrient and flavour losses (Berrios *et al.*, 2013).

The processing unit of an extruder is divided into three main sections, which are feeding/ mixing zone, cooking or transition zone, and high pressure/ forming zone. The basic structure of an extruder is presented in Figure 8. The main components of an extruder are screws, which convey the premixed ingredients through the barrel (Riaz, 2001). There are two different types of extruders: 1) single-screw extruders and 2) twin-screw extruders. Single-screw extruders consume more energy, are simpler in design and cost less than twin-screw extruders. In addition, when dealing with complex formulation, the functionality of a single-screw extruder is limited. Consequently, use of twin-screw extruders has increased over the years and its major advantages include better process flexibility and productivity as well as higher versatility of handling raw materials.(Berrios *et al.*, 2013) Twin-screw extruders are further categorized into counter-rotating and co-rotating extruders based on the direction of screw rotation and degree to which the screws knit (Riaz, 2001) .

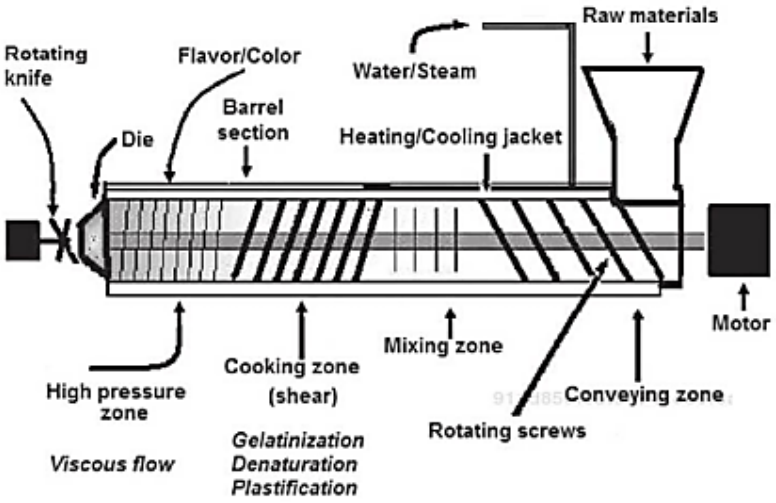

Figure 8. Basic components of extruder (Berrios *et al.*, 2013).

Normally, granulated dry and starchy material is fed into the extruder barrel forced by rotating spiral screws. The expansion happens when the product leaves the die at high temperature and residual water in the starchy melt forms a low density and crispy foam (Smith and Hardacre, 2011). A wild range of reactions and structural changes occur during the process, including the Maillard reaction between amino acids and reducing sugars (Zilić *et al.*, 2014), protein denaturation, texturisation (Giménez *et al.*, 2013) and starch, vitamin and pigment degradation (Smith and Hardacre, 2011). These changes are due to the high shear and temperatures, and are important not only for the development of the structure but also for technological and nutritional functionality of the end product.

4.1 Functional properties of extrudates

Process variables, which affect product quality during extrusion processing as well as sensory properties, are screw speed, water injection rate, feed rate, barrel temperature profile and speed of rotating knife (Chessari and Sellahewa, 2010). The screw-speed affects the amount of shear introduced, the residence time, the viscosity of melt and amount of frictional energy generated, as well as the barrel fill. The

water-injection rate determines the barrel moisture content, which in turn controls the frictional energy and the melt viscosity. The feed rate affects the generated energy, the residence time and the barrel fill. The temperature at the barrel profile has an effect on product temperature, which again affects the melt viscosity. (Riaz, 2001) The main controlling variables of an extruder are specific mechanical energy (SME), melt temperature and pressure in the die and flow rate through the die (Chessari and Sellahewa, 2010).

Value of extruded products is usually evaluated based on expansion rate, bulk density, colour, and microstructure (Berrios *et al.*, 2013). Texture is one of the most important sensory features of extrudates and it depends on microstructural features such as on cell size distribution, cell wall thickness, (Chessari and Sellahewa, 2010), as well as phase-state transitions, ingredients and process parameters. Mechanical properties (including hardness, crushing force and crispiness) can be used to predict sensory attributes of the end products (Alam *et al.*, 2013).

4.2 Effect of extrusion on nutritional value

Effects of extrudates on both functionality and nutritional value of legumes are presented in Table 5. Extrusion technology has been used to improve both the textural, nutritional and sensory characteristics, as well as to reduce the ANF of the raw material of several legumes. Extrusion cooking reduces the nutritional loss of the products compared to ordinary cooking methods and reduces the enzymes responsible for off-flavour and ANF of pulse material via denaturation (Zilić *et al.*, 2014). However, typical extrusion conditions (high barrel temperatures and low moisture) also promote Maillard reactions, which are increased by acidic pH, reducing sugars and free amino acids, especially lysine, in the product composition (Camire, 2010).

Extrusion have also been reported to reduce the flatulence-causing RFOs in pinto beans (Borejszo and Khan, 1992) compared to non-extruded products, which can improve the consumer acceptance even more. Furthermore, extrusion cooking can

improve protein digestibility of the product by destruction of trypsin inhibitors, haemaglutins, tannins and PA (Singh *et al.*, 2007).

4.3 Effect of extrusion on technological functionality

Different results have been obtained when studying the effect of extrusion on legume processing. Shirani and Ganesharanee (2009) for instance, examined incorporation of chickpeas with rice flour in extrusion and obtained decrease in product expansion and increase in bulk density of the products. Berrios *et al.* (2013) reported that high protein content in cereals might decrease expansion of the final product. On the other hand, Meng *et al.* (2010) obtained expanded chickpea flour-based snack products with low bulk density and low hardness. Frohlich *et al.* (2014) examined the suitability of different dehulled pulse flours in extruded snacks and reported high protein and fibre contents in extrudates containing 100 % of pea, lentil or chickpea flours. In their study, extruded snacks made from yellow pea flour had the closest expansion ratio (2.8) and bulk density (0.1 g cm^{-3}) to the corn meal reference sample (3.3; 0.1 g cm^{-3}). In a study of Berrios *et al.* (2008), screw speed lift from 500 to 600 rpm lowered the hardness of extrudates made from black bean flour. Furthermore, Smith and Hardacre (2011) made extruded snack products from *V. faba minor*, and observed a reduction in product hardness when increasing the screw speed from 200 to 300 rpm. The hardest products were also the least crispy. In their study, texture analysis showed that the extrudates made from dehulled bean flour were less hard than those made from wholegrain flour, yet the results from the sensory panel were opposite. Onyango *et al.* (2004) studied the effect of extrusion variables on lactic acid fermented maize-finger millet blend used in the sub-Saharan Africa food *Uji*.

4.4 Protein-rich raw material

Functionality of proteins in food products result from interactions with other proteins, polysaccharides, lipids, phenols, and PA (Lampart-Szczapa *et al.*, 2006). Extrusion cooking provides conditioning, shear heat, and pressure that promote cross-linking and polymerization among proteins and starches to form the expansion.

During extrusion, proteins are unfolded, hydrolysed and denatured and align themselves along the streamlines in the laminar flow occurring in the extruder screw and die. Macromolecules tend to crosslink and reform at high temperatures. The cross-linking sites increase when increasing the protein concentration, which can affect the textural quality of the end products. Increasing shear strain and temperature-time in the extruder have been reported to improve cross-linking between protein molecules, while increasing shear through the die is presented to disrupt the linkages. (Day and Swanson, 2013)

Among the factors above, amino acid composition affect the proteins secondary structure, hydrophobicity, net charge, molecule flexibility and isoelectric point. The functionality of proteins also depends on pH, ionic strength, denaturants, other macromolecules and enzyme activities (Lampart-Szczapa *et al.*, 2006). Della Valle *et al.* (1994) reported that SME influenced starch and protein solubility more than the temperature. Lampart-Szczapa *et al.*(2006) compared the protein functionality with extrudates from lupine and reported that protein solubility was higher with products that were fermented (blend starter of *Lc. mesenteroides*, *L. plantarum*. *Lactobacillus brevis*) fermented, than ones without LAB fermentation.

Table 5. Effects of extrusion processing on functionality and nutritional value of legumes.

Material	Results/ Impact	References
Two faba bean varieties (*Vicia faba minor)*	Extruded faba bean snacks had a high protein and fibre levels. Screw speed ↑hardness ↓ crispiness ↓	Smith and Hardacre, 2011
Faba bean	Soaking and extrusion: antinutrients (PA, tannins, phenols α-amylase trypsin inhibitor)	Abd El-Hady and Habiba, 2003
Soybean residue	Extrusion reduced the concentrations of total cholesterol	Chen *et al.*, 2014
Pea and kidney bean	Extrusion increased the Fe, Ca and P absorption	Alonso *et al.*, 2001
Chickpea flour (60 %)	Desirable products were obtained at low feed moisture, high screw speed and medium to high barrel temperature.	Meng *et al.*, 2010
Kodo millet-chickpea flour	Desirable extrudates were obtained at higher screw speed (293 rpm), lower feeder speed (19 rpm) and medium to high temperature (123 °C)	Geetha *et al.*, 2012
Maize-finger millet blend in the production of *uji*	Feed moisture ↑ expansion ↓ specific volume ↓ bulk density ↑ darkness of the product ↑	Onyango *et al.*, 2004
Lupin	Protein solubility was higher with extrudates which were fermented (*Lc. mesenteroides, L. plantarum. L. brevis*) fermented, than ones without LAB fermentation	Lampart-Szczapa *et al.*, 2006

↑ increase; ↓, decrease

EXPERIMENTAL PART

This study was part of the EU Eranet project: Novel multifunctional plant protein ingredients with bioprocessing (BIOPROT). The overall objectives of the project were improving protein and nutritional functionality of bran and faba bean by bioprocessing with microbes and enzymes and establishing technological functionality of modified plant protein sources in several food categories.

The key aim of this master's thesis was to improve functionality of faba bean by bioprocessing with exocellular polysaccharide producing LAB and phytase. The functionality of bioprocessed faba bean was studied in extruded products. The experimental part of this study was divided into four main sections.

1. Application of phytase in faba bean bioprocessing

The aim of this task was to study the potential of commercial food-grade phytase to degrade phytic acid.

2. Selection of exopolysaccharide (EPS) producing LAB

This task selected the best EPS producing LAB for faba bean fermentation. The test panel included *Leuconostoc* and *Weissella* and *Pediococcus* strains. The selection criteria included growth kinetics, sensorial observations and capability of producing dextrans during fermentation.

3. Optimization of EPS-production

Selected EPS producers were cultivated in a larger scale (10-15 kg) in order to produce raw materials for extrusion trials.

4. Extruded products from bioprocessed faba bean

The aim of this task was to study the functionality of fermented faba beans as a raw material in extrusion. Hardness and crispiness were used as important selection criteria. The experimental plan of this study is presented in Figure 9.

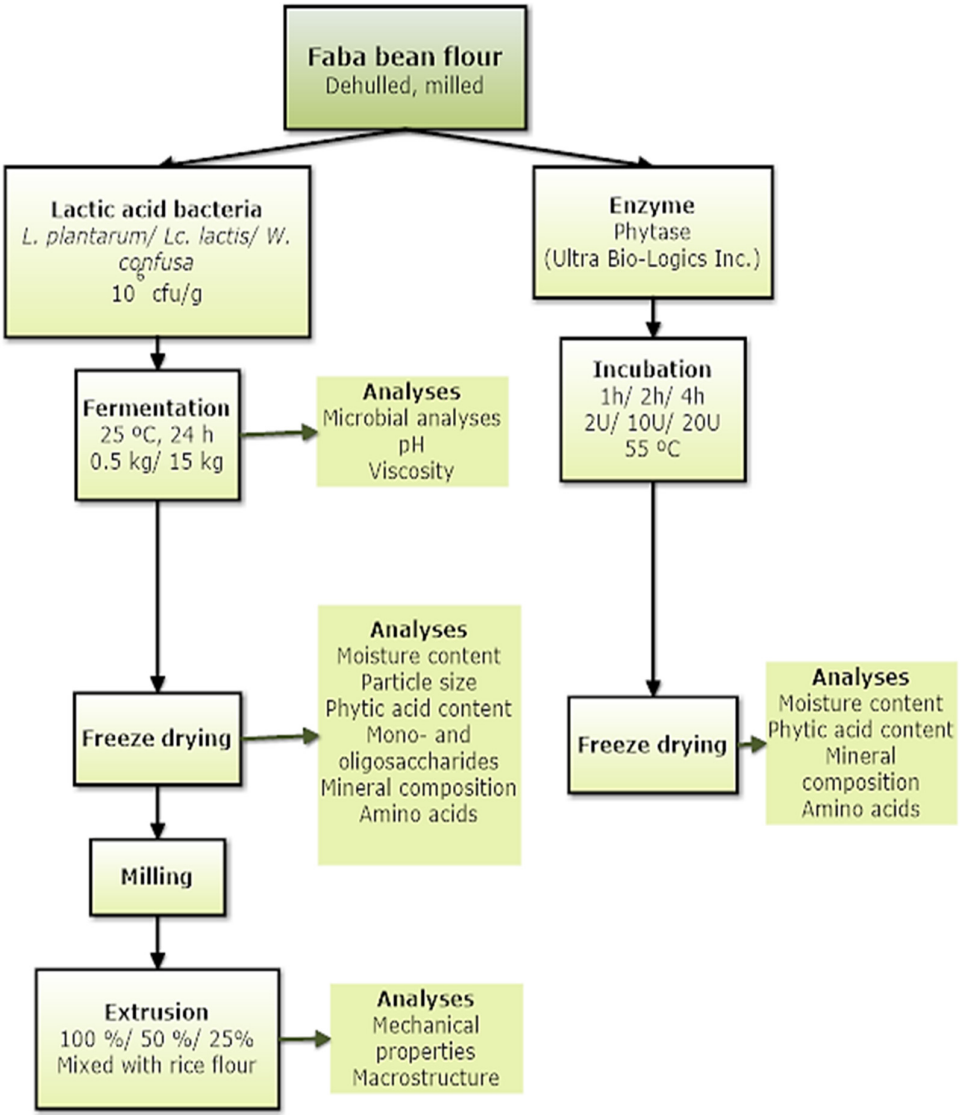

Figure 9. The experimental plan of the study.

5 Materials and methods

5.1 Raw material

Italian variety of faba bean (*V. faba major*) was used as a raw material in this study. The beans were first de-hulled and successively milled at a particle size of ca. 600 µm by Cereal Veneta (Italy). The analysed composition of the raw material is presented in Table 6. A batch of the raw material was sent for irradiation in order to inactivate the indigenous microorganism of the flour. Irradiation was performed at Scandinavian Clinics Estonia OÜ, Tallinn, Estonia, with gamma radiation dose of 11.8 kGy.

Table 6. Composition of faba bean flour used as a raw material.

	Content (g/ 100g dm)	Analysed at
Starch	44.8 ± 0.2	a
Protein	29.9 ± 0.1	b
Dietary fibre	9.9 ± 0.4	a
Fermentable sugars		a
Fructose	ND[1)]	b
Glucose	0.2 ± 0.1	b
Sucrose	2.9 ± 0.0	a
	Content (mg/kg dm)	
Ca	463.0 ± 4.0	c
Mg	999.5 ± 11.5	c
Zn	41.6 ± 0.5	c
Fe	46.0 ± 1.1	c

[1)]ND: not detected

[a] Central Research institute of Food and Feed Control, Turkey

[b] VTT

[c] SGS Inspection Services, Germany

5.2 Reagents

The reagents used (Table 3, Appendix 1) were analytical quality, except the phytase, that was food approved.

5.3 Enzyme treatment

Commercial food-grade phytase was obtained from Ultra Bio-Logics Inc. (Canada). It was produced by *Aspergillus niger*. Phytase (2, 10 or 20 units (U)) was added to 1:3 faba bean flour-water suspension. The mixture was incubated in covered beakers in a water bath at 55 °C (recommended conditions of the supplier) in a magnetic stirrer (540 rpm) for 1, 2 and 4 hours. After incubation, the pH of the samples was measured and the samples were placed into a freezer (-20 ° C) and were freeze-dried (Christ Alpha, B. Braun, Biotech International, Germany) prior to later analyses.

In addition to 40 g studies, a larger scale phytase treatment was performed with a 9 kg batch at 25 °C for 6 with 20 U enzyme dose.

5.4 Lactic acid bacteria (LAB) fermentations

5.4.1 LAB strains

Table 7 shows the LAB strains included in this study. All the strains were obtained from the VTT Culture Collection (Espoo, Finland). After preliminary screening of six LAB strains, three strains were chosen for further studies. *Weissella confusa* VTT E-143403 (E3403), isolated from faba bean flour was selected based on the EPS producing capability. *Leuconostoc lactis* VTT E-032298 (E2298) was also a potential EPS producer. *Lactobacillus plantarum* VTT E-78076 was used as an EPS-negative reference strain. Furthermore, *L. plantarum* had shown improved stability and functionality in previous faba studies (Coda *et al.*, 2015).

Table 7. The LAB strains used as fermentation starter cultures.

Species	Strain	VTT code	Source
Lactobacillus plantarum	E-78076	E76	beer, UK
Leuconostoc mesenteroides subsp. mesenteroides	E-91461T	E461	fermenting olives
Leuconostoc lactis	E-032298	E2298	a syrup sample, Finland
Pediococcus claussenii	E-032355T	E2355	spoiled beer, Canada
Weissella confusa	E-90392	E392	soured carrot mash
Weissella confusa	E-143403	E3403	faba bean flour

5.4.2 Isolation of lactic acid bacteria strains grown in the raw material

Various types of LAB were isolated and characterised from the spontaneous fermentations as well as from the LAB fermentation started with *Lc. lactis*. Different species for isolation were chosen based on the different cell and colony morphology. The colonies were isolated and pure cultures were made on MRS medium. Isolates were amplified with end-point PCR using BSF7/20 (5'-AGATTGATYMTGGCTCAG-3') and BSR1541/20 (5.-AAGAGGTGATCCAGCCGC) primers (Wilmotte *et al.*, 1993). PCR products were sent to Macrogen Inc. for DNA sequencing using EZ-seq Purification service (Macrogen Inc., The Netherlands). The received DNA sequences were assembled and manually edited using the Kodon software version 1.0 (Applied Maths Inc., Austin, TX, US). The edited sequences were compared with the sequences of the GenBank DNA database by using the BLASTN algorithm.

5.4.3 Small scale fermentations

LAB strains were first propagated at 30 °C in de Man, Rogosa and Sharpe (MRS) broth (Table 2, Appendix 1). Then, cell suspension for faba fermentations was prepared in general edible medium (GEM) broth (Table 4, Appendix 1) either containing sucrose (2 %, w/w) or no sucrose, and incubated at 30 °C for 24 h. Cells were harvested by centrifugation (4000 rpm, 10 000 g, 20 min), and resuspended in sterile tap water. Inoculation was then done by adding cell suspension (target cell density 10^6 CFU/ ml) to 1:3 (1 portion of flour; two portions of sterile tap water) faba bean flour-water suspension containing 5.1 % (w/w) sucrose. The suspension contained 150 g faba

bean flour, 23 g sucrose and 277 g water. Then, the faba bean flour-water suspension was incubated at 25 °C for 14 or 24 h, static or mixing. Spontaneous fermentation without added bacteria was performed in order to study the growth and effect of indigenous microbes. The experiment was carried out in 2 L beakers with aluminium foil top layers (Figure 10 A).

5.4.4 Large scale fermentations

The fermentation set-up with selected three LAB strains was scaled-up in order to study the function of fermented faba bean flour in extrusion cooking. The volume in larger scale fermentations was 10…15 kg and fermentation was carried out in 30 l plastic buckets with lids, with light mixing (magnetic bar). The overview of fermentation series is presented in Table 8.

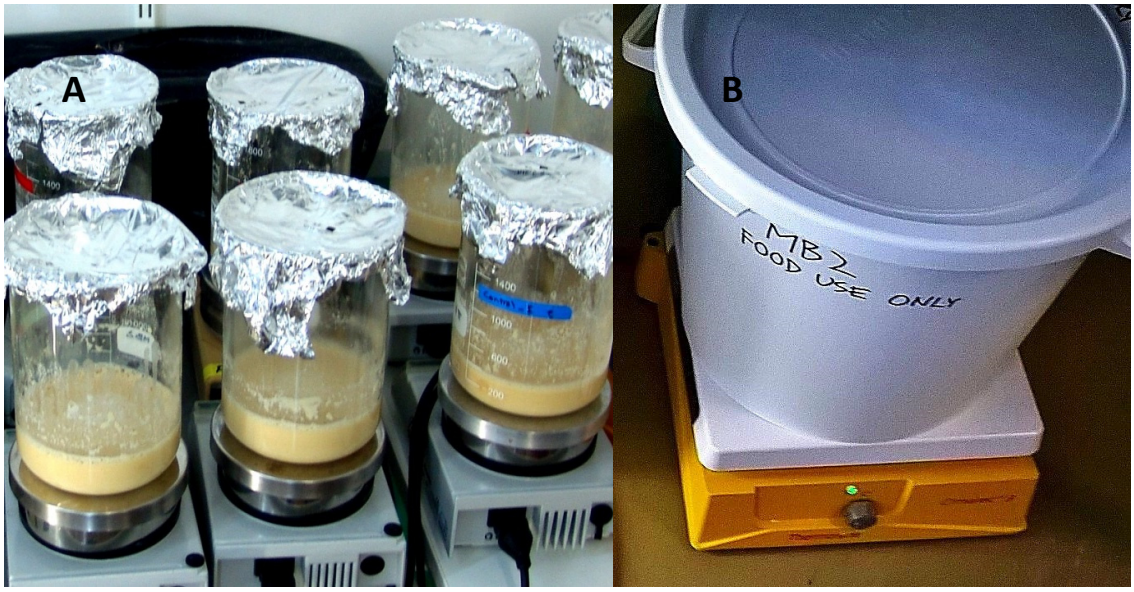

Figure 10. The set-up of the experiments: small scale fermentation (A) and large scale-up fermentation (B).

Table 8. List of fermentation series.

Series	Descriptions		
Series A First screening			
L. plantarum + suc[1)] *L. mesenteroides* subs. mesenteroides	static 24 h	native flour	small scale
L. lactis + sucrose			
P. claussenii + sucrose			
W. confusa E392 + suc			
W. confusa E3403 + suc			
Series B			
L. plantarum	mixing 24 h	native flour irradiated flour	small scale
Series C			
Lc. lactis + suc	mixing 24 h	native flour irradiated flour	small scale
Lc. lactis		native flour irradiated flour	
Series D			
W. confusa + suc	mixing 24 h	native flour irradiated flour	
W. confusa		native flour irradiated flour	
Series E			
W. confusa + suc	mixing 14 h	native flour irradiated flour	small scale
W. confusa		native flour irradiated flour	
Series G			
L. plantrum[a)]	native flour	mixing 24 h	large scale
Lc. lactis + suc[b)]	native flour	mixing 24 h	
Lc. lactis[b)]	native flour	mixing 24 h	
W. confusa + suc[b)]	native flour	mixing 24 h	
W. confusa[c)]	native flour	mixing 24 h	

[1)] suc = 5.1 % (w/w) addition of sucrose

5.4.5 Grinding and particle size determination

Fermented samples were freeze-dried (Christ epsilon 2-25DS, Christ) 2-3 d and ground at room temperature at 2500 rpm with SM 300 cutting mill (Retsch, Düsseldorf, Germany) using a 6-disc rotor and 4x4 mm bottom sieve. The particle

diameter of the milled samples was measured using a Coulter LS 230 Particle Size Analyser (Beckman Coulter Inc., Indianapolis, USA).

5.5 Extrusion

5.5.1 Production of extrudates

Extrusion was done using the bioprocessed, freeze-dried and grounded faba bean raw material from large-scale fermentation series. Faba bean raw material were mixed with rice flour (Beneo, Germany) in ratios of 100:0, 50:50 and 25:75 (faba bean: rice). A control sample with added fructose (2.5 % dm) and dextran (2.5 % dm) were also made as an *ex situ* dextran control sample. Extrusion was performed using co-rotating twin-screw extruder (Figure 11) (APV MPF, Baker Perkins Group Ltd, Peterborough, UK) with a die of diameter of 2mm and screw speed of 450 rpm. Temperature profile used was 125-110-90-80 ^{o}C. The extrudates were collected in trays and dried at 100 ^{o}C for 10 min in an oven drier. The samples were stored at 14 ^{o}C in sealed pouches. All the extrusions were done in duplicate. Extrusion parameters are shown in Appendix 7.

Figure 11. Twin-screw extruder.

5.6 Analyses

5.6.1 Measuring phytic acid (PA) content

The phytic acid content was measured by a colorimetric method, according to Latta and Eskin (1980), modified by Vaintraub and Lapteva (1988). The freeze dried samples were homogenised in a mortar and suspended in 2.4 % hydrochloride acid. The soluble compounds were extracted by agitating in a Vari mix test tube roller (Thermo Scientific) at room temperature for 2 h. The extracts were centrifuged at 12880 rpm for 30 min. Supernatant was collected and dilutions with distilled water were made in Eppendorf tubes. Wade reagent was added and the tubes were vortexed. The assay was followed with centrifugation for 10 min at 10000 rpm (Eppendorf minispin, Eppendorf). After that, absorbance of the samples was red at 500 nm with spectrophotometer (Shimadzu UV-1800). A standard curve was made with 0.0-41.5 µg/ml phytic acid.

5.6.2 Microbial growth

For determination of viable cell counts, 2.5 g of sample was added to 22.5 g Ringer's solution, vortexed, and serially diluted and cultivated on several different selective medium plates (Table 2, Appendix 1). The colonies were counted after incubation and the results were expressed as colony forming units (CFU) per g of wet weight. All the analyses were carried out as triplicates, except in small scale series 1.

5.6.3 pH

pH was measured in order to monitor the bioprocess of the raw material and other possible changes in the microbiota. pH was determined with pH detector (Basic Meter, Denver Instrument Company, USA) from 3 g of fresh sample. The determinations were carried out in triplicate.

5.6.4 Viscosity

The main method in analysing the texture of the fermented samples and an indicator of dextran production in this study was viscosity determination (Figure 12). Changes in viscosity were followed with AR-G2 reometer (TA Instruments Ltd., UK) with

vanned rotor. During the measuring, the shear rate developed between 2…200 1/s, and the viscosity values represented in this study were taken at shear rate 50 1/s. Viscosity was analysed at 22 °C in triplicate from fresh 300 ml sample.

Figure 12. Viscosity determination of fermented faba bean sample with reometer.

5.6.5 Determination of dextran

Dextran yield of fermented samples was determined at University of Helsinki. The specific enzyme-assisted (dextranase and α-glucosidase) method developed for analysing dextran was based on the method of Katina *et al.* (2009).

5.6.6 Mono- and oligosaccharide analysis

Analysis of mono- and oligosaccharides of the fermented freeze-dried samples was performed with HPLC anion-exchange method using Dionex ICL-3000 equipment and PA 20 column. The pre-treatment of samples was done with 50 % water-ethanol extraction based on the method of Xiaoli *et al.* (2008). 1 g of sample was mixed with 20 ml water-ethanol (50 %:50 %). Then, the tubes were incubated in a water bath at 50 °C for 30 min and centrifuged three times (Sorvall, 10000 rpm, 10 min). Supernatants were combined and the sample was concentred into 5 ml with

Rotavapor. Then, the sample was filtered through 0.45 µm. HPLC analysis was performed from freezed samples.

5.6.7 Free amino acids analysis

Free amino acids (FAA) were analysed from freeze-dried samples by Dr Carlo Rizzello at University of Bari, Italy, Department of Plant Protection and Applied Microbiology. Water/salt-soluble extracts of samples were prepared according to Weiss *et al.* (1993) and used to analyse FAA. FAA were analysed by a Biochrom 30 series Amino Acid Analyser (Biochrom Ltd., Cambridge Science Park, England) with a Na-cation-exchange column (20 by 0.46 cm internal diameter), as described by Rizzello *et al.* (2010).

5.6.8 Mineral composition

Freeze-dried samples were shipped to SGS Inspection Services (Hamburg, Germany) for mineral analyses.

5.6.9 Microscopy of extrudates

Extrudates were cut to thin pieces and examined and micrographs were obtained with stereomicroscope (Zeiss Discovery V8, Carl Zeiss) and Cell^P imaging software (Olympus).

5.6.10 Macrostructure of extrudates

From each extrusion, 15 samples were collected and length and diameter in three different points of each sample were measured using a vernier calliper. Also the weight of each sample was determined.

Expansion ratio (ER) of extrudates was calculated with equation 1.

$$Expansion\ ratio\ (\%) = \frac{D_e}{D_d} \times 100\ \% \qquad (1)$$

Where D_e = Average diameter at three different points of the sample (mm)

D_d = Diameter of the die (2 mm).

Specific length (l_{sp}) was calculated with equation 2.

$$Specific\ lenght\ \left(\frac{m}{kg}\right) = \frac{L_e}{m_e} \quad (2)$$

Where L_e = Length of the sample (m)

M_e = Mass of the sample (kg).

Piece density (PD) was calculated with equation 3.

$$Piece\ density\ \left(\frac{kg}{m^3}\right) = \frac{4 \times m_e}{\pi \times (D_e)^2 \times L_e} \quad (3)$$

Where m_e = Mass of the sample (kg)

D_e = Average diameter at three different points of the sample (mm)

L_e = Length of the sample (m).

5.6.11 Mechanical properties of extrudates

Ratio of linear distance, crushing force, crispiness work, and crispiness index were calculated to describe the hardness and crispiness of samples. Samples for texture analysis were cut in 10 mm pieces with an electric saw (Power ST-WBS800, Taiwan Sheng Tsau Industrial Co. Ltd., Taiwan). Measurements were carried out 20 times for each sample. Mechanical properties of the extrudates were analysed by uniaxial compression test using a texture analyser (TA-XT plus, Stable Micro Systems) equipped with a 5 kg load cell and a cylindrical aluminium probe. Each measurement was performed with 20 replicates. The values of actual and smoothened curve length, area under the F/D-curve and number of peaks and hardness were obtained with Texture Exponent software v.5.1.2.0 (Stable Micro Systems, UK). Number of peaks is the number of cell wall ruptures during the compression and hardness (F_{max}) is the maximum force that is needed to cause the cell wall rupture.

Crushing force (F_{Cr}) was calculated with equation 4.

$$Crushing\ force\ (N) = \frac{A}{l} \quad (4)$$

Where A = Area under the F/D curve (Nmm)

l = Distance of compression (mm).

Crispiness index (C_i) was calculated with equation 5.

$$C_i = \frac{L_N}{A\ X\ F_{mean}} \quad (5)$$

Where L_N = Normalized curve length (Actual curve length (Nmm)/F_{max} (N))

A = Area under the F/D – curve

F_{mean} = the sum of the actual force values in the data file divided by the number of data points.

5.6.12 Statistical analyses

Data was processed using Microsoft excel (Microsoft, USA) and IBM SPSS Statistics 22 (IBM Corporation, Somers, NY, USA). There were two independent experiments of phytase incubation, and all the samples were analysed as triplicate. The results were presented as means and standard deviations and statistical analysis was performed using analysis of variance (ANOVA).

All extrusions (except for the samples E2298_EPS, E2298_Control and E3403_Control) were performed in duplicate. The parameters of macrostructure and mechanical properties of each extrudate were calculated as means of 15-20 results as described in sections 5.6.10 and 5.6.11. The means and standard deviations of means were calculated. Analysis of variance was determined by factorial two-way multivariate analysis of variance (MANOVA). Mean values of both PA and extrudates were compared by Tukey's HSD test. Significance was accepted at a probability of $P < 0.05$.

6 Results

6.1 Suitability of food-grade phytase in faba bean bioprocessing

The phytic acid (PA) and mineral contents of phytase-treated faba samples was measured in order to study the effect of phytase in degrading PA and as well as the effect in mineral availability. Table 9 presents the average phytic acid content ± SD (as mg per 100 g dm) for faba bean flour samples after 1, 2, and 4 hour incubation with different phytase activity levels (2, 10, and 20 U). Untreated faba flour (0 U) and native faba flour was used as references.

The initial PA content of the raw material was 9.98 ± 0.57 mg/g. As shown in Table 9, with only 1 h and 20 U of phytase activity, it was possible to reduce the phytic acid content 88 % compared to native faba bean flour. Both the enzyme activity and the incubation time had a significant ($P \leq 0.05$) effect on PA content, although the mean difference between 1 and 2 h treatments was only slightly significant (Appendix 8, Tables 1-2).

The phytase treatment was also performed in large scale (9 kg). In this study, faba bean was incubated with 20 U phytase dose at 25 ° C for 6 h. The PA content effectively reduced from 9.98 ± 0.57 mg/g to 1.37±0.12 mg/g. Furthermore, the raw material costs of phytase application were rather small; 20 U phytase treatment of 10 kg faba bean requires 40 g phytase, which has raw material costs of approximately 7 €.

Table 9. Effect of Phytase in phytic acid content (means ± SD; mg per 100 g dm) of faba bean flour.

Incubation time	Enzyme concentration		Average PA content (mg/g dm)	Reduction (compared to native faba flour) %
	(U)	nkat		
Small scale series, 55 °C				
1h	0.0	0.0	8.01 ± 0.13	19.7
	2.0	33.3	5.11 ± 0.17	48.8
	10.0	166.7	1.97 ± 0.07	80.3
	20.0	333.4	1.22 ± 0.22	87.8
2h	0.0	0.0	8.15 ± 0.14	18.3
	2.0	33.3	4.76 ± 0.17	52.3
	10.0	166.7	1.73 ± 0.07	82.6
	20.0	333.4	1.09 ± 0.06	89.0
4h	0.0	0.0	7.78 ± 0.02	22.0
	2.0	33.3	3.52 ± 0.04	64.7
	10.0	166.7	1.47 ± 0.06	85.2
	20.0	333.4	1.11 ± 0.29	88.7
Large scale series, 25 °C				
6h	20.0	333.4	1.37 ± 0.12	86.3

Mean values and standard deviations of two individual experiments analysed in triplicate (n=3). The enzyme activity is presented as units (U) and nano katals (nkat). One Phytase unit is the amount of enzyme that liberates inorganic phosphate at 1 µmole per minute. 1 U = 16.67 nkat.

Furthermore, the purpose was to determine the effect of enzyme treatment on protein composition and amino acid profile. Total free amino acid (FAA) content of faba bean flour was 5.7 g/kg. Phytase treatment improved the FAA profile of the raw material (Figure 13). Total FAA increased 43 % during the incubation with phytase. There was an increase in all essential amino acids, except threonine. The release of amino acids was a result of degradation of phytate-protein complexes. No protease activity was observed with the phytase used in this study (data not shown).

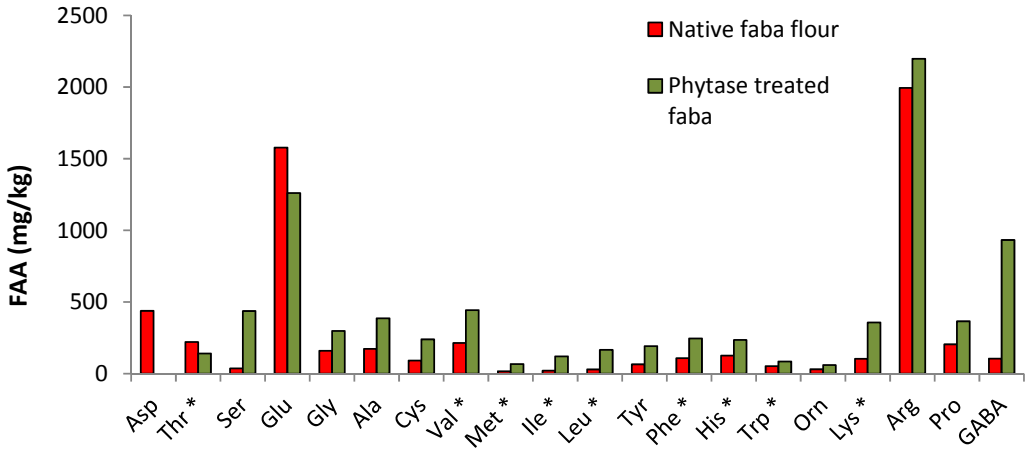

Figure 13. Free amino acids (FAA) of phytase treated (20 U, 6 h, 25 °C) faba bean flour compared to native flour. * Essential amino acids are indicated on the x-axis.

6.1.1 Effect of phytase on mineral composition

Phytase treatment improved the calcium, magnesium, iron, and zinc availability of faba bean (Table 10). The most remarkable increase was observed in calcium (91 %) level.

Table 10. Mineral content (mg/kg) of native and phytase treated faba bean.

mg/kg	Native faba bean flour	Phytase treated faba bean flour	Change (%)
Calcium	463 ± 4	885 ± 3	91
Magnesium	1000 ± 12	1109 ± 46	11
Iron	46 ± 1	61 ± 6	32
Zinc	42 ± 1	47 ± 2	13

The results are average ± SD of duplicate analysis.

6.2 Characterisation of the faba bean raw material

The indigenous microbial community consisted of LAB, gram-negative bacteria, sporeforming *Bacillus* spp and yeast (Figure 14). Notable increase of coliforming bacteria and 3 log unit increase of *pseudomonas* spp. was observed during 24 h incubation. The growth of indigenous LAB community was remarkable. The number of LAB after 24 h was 10^9 cfu/g. Intensive LAB growth led to the acidification of faba mixtures. pH was lowered from 6.4 ± 0.3 to 5.0 ± 0.1.

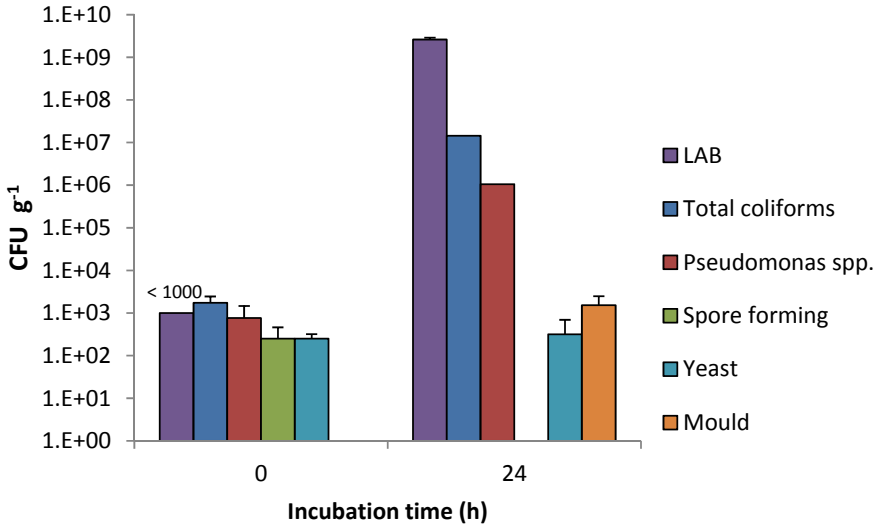

Figure 14. Growth of microorganisms in faba bean raw material. The results are expressed as means + SD (n=3).

In order to study the impacts of added starter cultures without background microbiota, part of the flour samples were shipped to be irradiated. Samples were cultivated after 11.8 kGy treatment which confirmed that all the microbial activity had been eliminated by the irradiation. Different LAB were isolated from MRS culture plates. Selection was based on different colony morphology (diameter, shape, surface). Isolates were identified by partial 16S ribosomal RNA gene sequencing (Table 11) and the strains were deposited in the VTT Culture Collection.

Table 11. Sequencing results of isolated from faba bean fermentations.

Isolate	Source	Nearest phylogenetic neighbour (BLAST)	Lenght of sequence (bp)	Similarity (%)	VTT number
Bp1	Fermentation with *L. Lactis* E2298 slimy colony on MRS	*Weissella confusa*	451	100 %	E-153482
		Weissella cibaria		100 %	
Bp3	Spontaneous faba bean fermentation after 24h	*Pediococcus pentosaceus*	1079	99 %	E-153483
		Pediococcus claussenii		99 %	
		Pediococcus acidilactici		99 %	
Bp4	Spontaneous faba bean fermentation after 24 h	*Leuconostoc kimchii*	1170	99 %	
		Leuconostoc gelidium		100 %	E-153484
		Leuconostoc gasicomitatum		100 %	
		Leuconostoc lactis		99 %	
Bp5	Slimy colony co-occurring with *L. plantarum* E76	*Weissella cibaria*	513	100 %	E-153485

This study indicated that faba beans are a natural source of EPS-producing LAB. Obligatory heterofermentative LAB, mainly *Weissella* spp. and *Leuconostoc* spp. dominated fermentations. In addition, *Pediococcus* sp. was identified from spontaneous fermentation.

6.3 Fermentation with different LAB

Fermentation with different LAB including *Lactobacillus plantarum* E76 and exopolysaccharide producing strains such as *Leuconostoc lactis* E2298 and *Weissella confusa* E3403 were carried out at the scale of 0.5 kg and 15 kg.

6.3.1 Small scale fermentations

The slimy colonies observed in MRS-sucrose agar medium were different between the two EPS-producing starter strains *Lc. lactis* and *W. confusa*, as shown in Figure 15. Growth of LAB and formation of slimy colonies (EPS) is presented in Table 12.

Initial LAB counts were in the range 6 x 10^4 (*L. plantarum*) – 7 x 10^6 (*W. confusa*). The initial pH value was 6.4. 2-3 log unit increase in LAB counts was measured after 24 h fermentations. Fermentations led to the acidification of the faba bean material decreasing the pH value from 6.4 to 4.5. Fermentation with *W. confusa* resulted in the least acidic medium. The growth of LAB starters was higher in samples with irradiated faba bean flour, in which the natural microbiota had been inactivated. pH after 24 h of fermentation varied between 5.1-4.5 (small scale, native flour). The indigenous microbiota effected on the growth and activity of added LAB starters. Elimination of the background microbiota created the best growth conditions, which was observed in cell counts and acidity. The growth of different microorganisms during LAB fermentations is presented in Appendix 2.

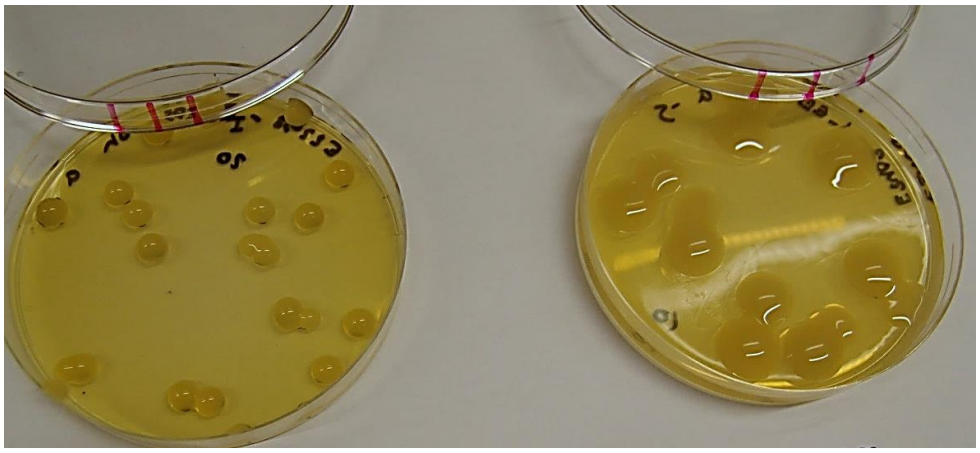

Figure 15. Slimy colonies of *Lc. lactis* E2298 (left) and *W. confusa* E3403 (right) from faba bean fermentations on MRS-sucrose medium plates.

Table 12. Growth of LAB, and pH after fermentation of 24 h (series A, B and C) and 14 h (series D).

			LAB[1)]	EPS[2)]	
	Type of fermentation		CFU g^{-1}		pH
Series A First screening					
L. plantarum + suc	static 24 h	native flour	$4 \cdot 10^8$	$3 \cdot 10^6$	4.61
L. mesenteroides subs. mesenteroides			$4 \cdot 10^8$	$5 \cdot 10^9$	4.69
L. lactis + sucrose			$4 \cdot 10^9$	$5 \cdot 10^9$	4.76
P. claussenii + sucrose			$1 \cdot 10^9$	$1 \cdot 10^8$	5.38
W. confusa E392 + suc			$5 \cdot 10^8$	$4 \cdot 10^8$	5.56
W. confusa E3403 + suc			$8 \cdot 10^8$	$3 \cdot 10^8$	5.45
Series B					
L. plantarum	mixing 24 h	native flour	$3 \cdot 10^9$	$4 \cdot 10^8$	4.7 ± 0.0
		irradiated flour	ND[3)]	ND	4.5 ± 0.0
Series C					
Lc. lactis + suc	mixing 24 h	native flour	$3 \cdot 10^9$	nd[4)]	5.1 ± 0.5
		irradiated flour	$4 \cdot 10^9$	$2 \cdot 10^9$	4.8 ± 0.5
Lc. lactis		native flour	$3 \cdot 10^9$	$2 \cdot 10^9$	4.5 ± 0.0
		irradiated flour	$2 \cdot 10^9$	$2 \cdot 10^9$	4.2 ± 0.0
Series D					
W. confusa + suc	mixing 24 h	native flour	$2 \cdot 10^9$	$2 \cdot 10^9$	4.7 ± 0.1
		irradiated flour	$3 \cdot 10^9$	$1 \cdot 10^8$	4.5 ± 0.0
W. confusa		native flour	$2 \cdot 10^9$	$2 \cdot 10^9$	5.0 ± 0.0
		irradiated flour	$3 \cdot 10^9$	$1 \cdot 10^9$	4.9 ± 0.1
Series E					
W. confusa + suc	mixing 14 h	native flour	$2 \cdot 10^9$	$1 \cdot 10^9$	5.1 ± 0.1
		irradiated flour	$2 \cdot 10^9$	$2 \cdot 10^9$	5.2 ± 0.2
W. confusa		native flour	$3 \cdot 10^9$	$2 \cdot 10^9$	5.4 ± 0.1
		irradiated flour	$4 \cdot 10^9$	$2 \cdot 10^9$	5.3 ± 0.1

The results are mean values(± SD) of independent experiments A: n=1; B- E: n=3

[1)] LAB: colonies enumerated from MRS agar

[2)] EPS: slimy colonies on MRS + sucrose agar

[3)] ND: not detected

[4)] nd: not determined

[5)] suc: 5.1 % (w.w) sucrose in the medium

Viscosity

Viscosity values of small scale fermentation series are presented in Table 13. Addition of EPS-producing LAB led to notably increased viscosity. After 24 h fermentation with *Lc. lactis* the viscosity had increased from 160 mPas to 3200 mPas (series A). The highest viscosity was measured with *W. confusa* E3403 being almost 7500 mPas (series A). The lowest viscosity was measured with EPS-negative strain *L. plantarum*. In series A, viscosity values were higher compared to series B-E. Series A included only one sample per each strain, whereas series B-D (Appendix 3) included three individual fermentations and consequently there were notable deviations in the viscosity values.

Table 13. Viscosity values after LAB fermentation of faba bean flour. Values reported have been taken at time point of shear rate 50 s^{-1}. Series A-E.

	Viscosity (mPa s) at shear rate 50 s^{-1}			
	Native flour		Irradiated flour	
	sucrose	no sucrose	sucrose	no sucrose
Series A, first screening				
L. plantarum	364	nd		
Lc. mesenteroides	1424	nd		
Lc. lactis	3152	nd		
P. claussenii	1632	nd		
W. confusa E392	5306	nd		
W. confusa E3403	7486	nd		
Series B-D				
L. plantarum	nd	439 ± 129	nd	428 ± 62
Lc. lactis	437 ± 243	310 ± 23	589 ± 281	587 ± 86
W. confusa E3403	812 ± 299	453 ± 68	382 ± 209	80 ± 24
Series E				
W. confusa E3403	2204 ± 273	649 ± 231	4545 ± 192	717 ± 83

[a] nd – not determined.

6.3.2 Impacts of fermentation conditions on EPS production

After the small scale series A-E, the effect of mixing and fermentation time was studied in more detail with *W. confusa* E3403 (series F) with both native and irradiated faba bean flour, with and without added sucrose in the medium. Samples were taken from three time points; 0 h, 14 h and 24 h. Mixing improved growth of LAB as observed in growth curve (Figure 16) and acidification rate (Table 14). However, mixing seemed to have negative impact on viscosity (Figure 17) although some contradictory results were observed. Highest viscosity values were obtained with samples of irradiated faba bean flour with sucrose addition.

Table 14. pH development of *W. confusa* E-143403 fermentation (series F).

	0h	14h	24h	Δ pH
EPS_radiated_static	6.42	5.83 ± 0.0	5.06 ± 0.0	1.36
EPS_radiated_mixing		5.03 ± 0.0	4.62 ± 0.0	1.80
EPS_native_static	6.47	5.86 ± 0.0	4.95 ± 0.0	1.52
EPS_native_mixing		4.70 ± 0.0	4.60 ± 0.0	1.87
Control_radiated_static	6.33	5.95 ± 0.0	5.62 ± 0.0	0.71
Control_radiated_mixing		5.14 ± 0.0	5.22 ± 0.0	1.11
Control_native_static	6.40	5.98 ± 0.0	5.58 ± 0.1	0.82
Control_native_mixing		5.34 ± 0.0	4.94 ± 0.0	1.46

Results are average ± SD of triplicate measurements. EPS: sucrose addition in the fermentation medium; Control: no sucrose addition.

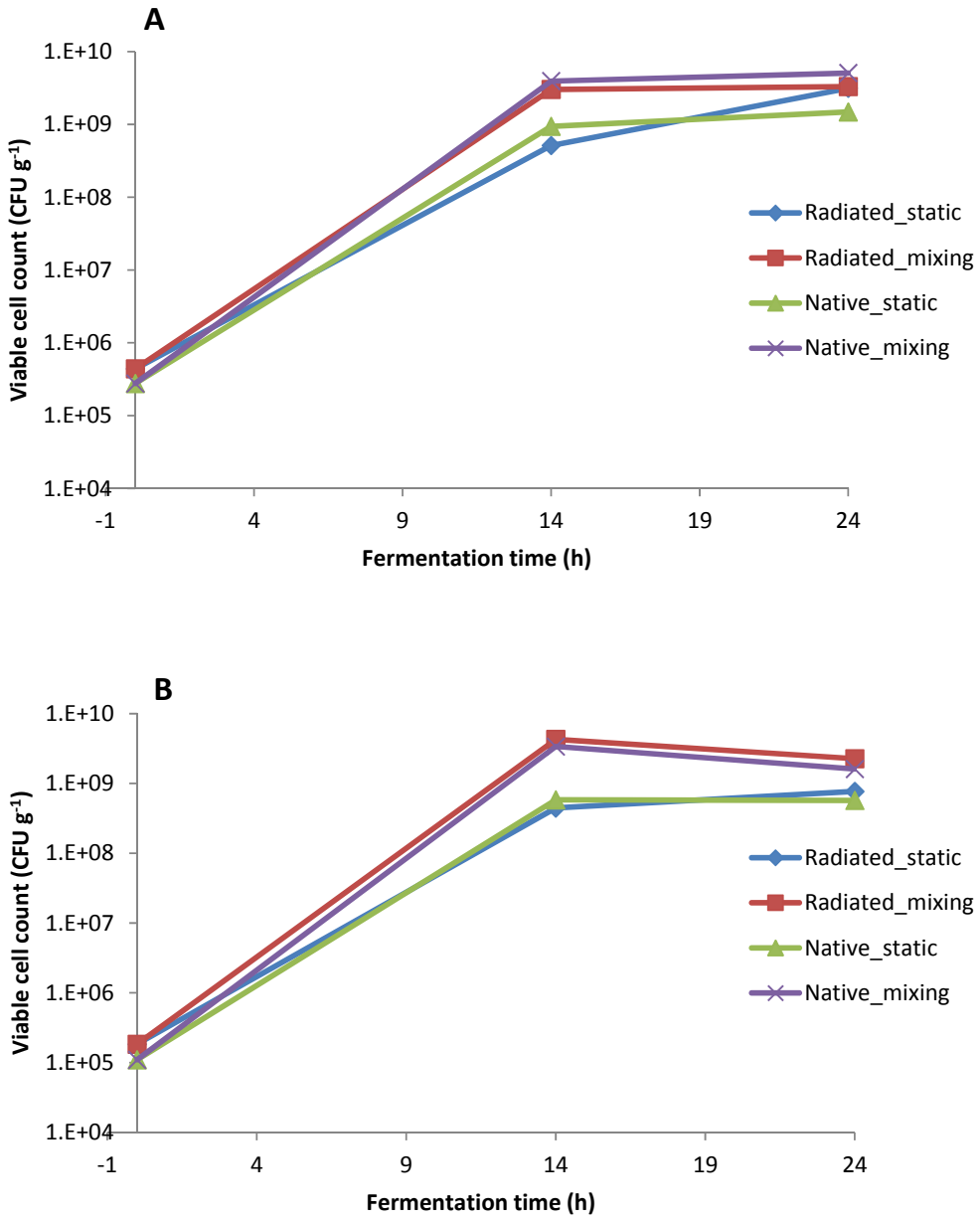

Figure 16. Impacts of mixing and elimination of indigenous microbiota on growth of *W. confusa* E3403 in the presence of sucrose (A) and in control fermentations (B).

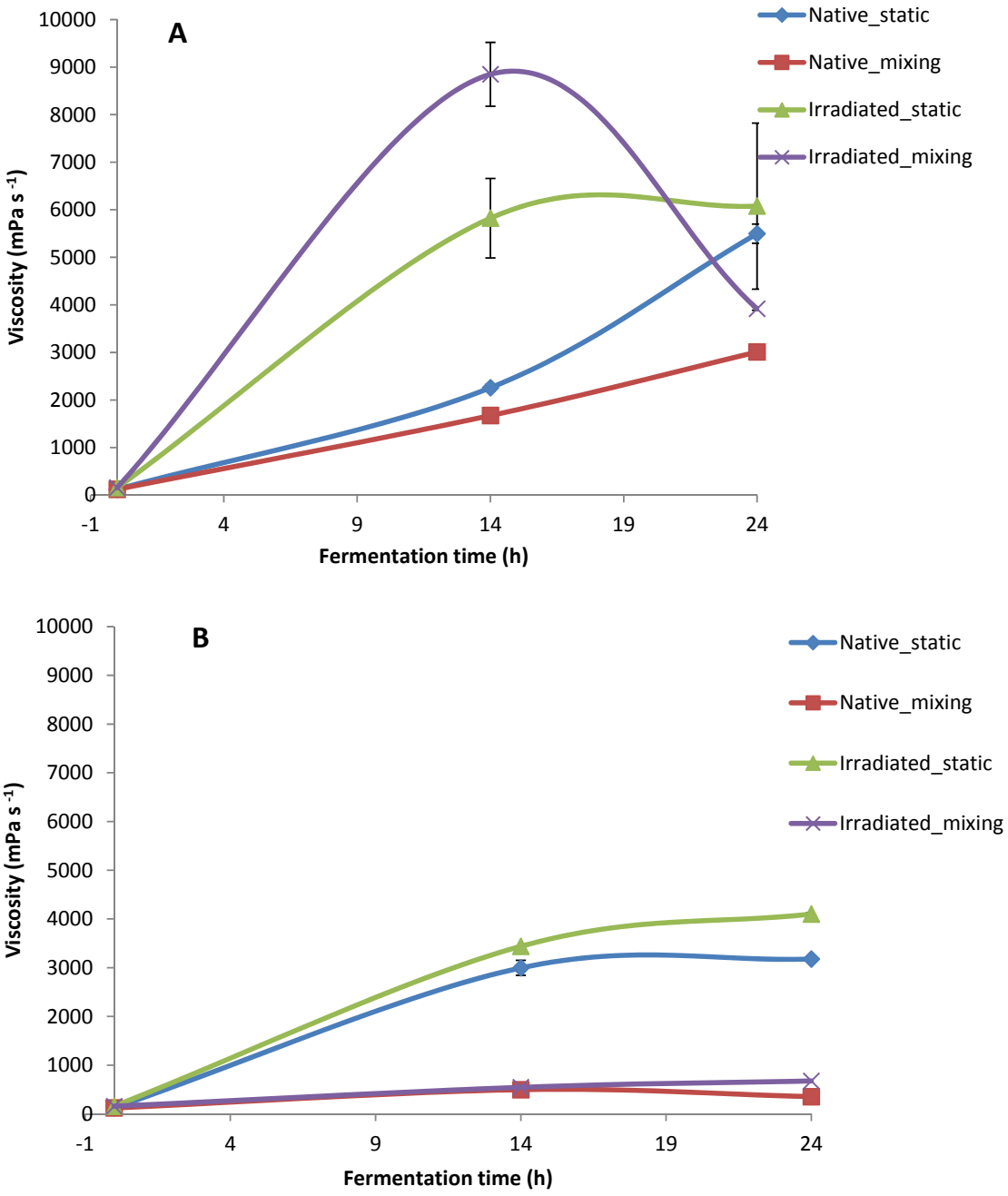

Figure 17. Impacts of mixing and elimination of indigenous microbiota on viscosity development of fermentations with *W. confusa* E3403 in the presence of sucrose (A) and in control fermentations (B).

6.3.3 Large scale fermentations

Large scale fermentations were done with the aim of producing raw material in extrusion study as well as to study the effect of scale-up on the properties of the bioprocessed material. The LAB growth and acidification rate is presented in Table 15. *L. plantarum* grew from 10^6 to 10^8, *Lc. lactis* from 10^5 to 10^9 and *W. confusa* from 10^6 to 10^8 cfu/g. The addition of sucrose had a positive effect on the growth of *Lc. lactis* and *W. confusa*.

Table 15. Growth of LAB, and pH after large scale fermentations of 24 h (series G).

	Type of fermentation		LAB[1)] CFU/g 0 h	LAB[1)] CFU/g 24 h	EPS[2)] CFU/g 24 h	pH
Series G						
L. plantrum[a)]	native flour	mixing 24 h	$2{\cdot}10^6$	$5{\cdot}10^8$	$2{\cdot}10^7$	4.68 ± 0.0
Lc. lactis + suc[b)]	native flour	mixing 24 h	$6{\cdot}10^5$	$3{\cdot}10^9$	$3{\cdot}10^9$	4.87 ± 0.1
Lc. lactis[b)]	native flour	mixing 24 h	$4{\cdot}10^6$	$1{\cdot}10^9$	$1{\cdot}10^9$	5.11 ± 0.1
W. confusa + suc[b)]	native flour	mixing 24 h	$3{\cdot}10^6$	$3{\cdot}10^9$	$2{\cdot}10^9$	4.94 ± 0.1
W. confusa[c)]	native flour	mixing 24 h	$4{\cdot}10^6$	$6{\cdot}10^7$	$6{\cdot}10^7$	5.60 ± 0.0

The results are mean values ± SD of triplicate analysis.

Amount of individual replicates: [a)] n=3; [b)] n=2; [c)] n=1

[1)] LAB: colonies enumerated from MRS agar

suc: 5.1 % (w/w) sucrose in the medium

6.4 Dextran production

Viscosity was generally used as indicator of EPS production. Furthermore, enzyme assisted dextran analyses were performed on samples of large scale fermentations. Faba bean fermentations with *W. confusa* and *Lc. lactis* promoted dextran production. Even in LAB started control fermentations (no added sucrose) dextran was produced. When sucrose was present in fermentations, 6.5 ± 0.03 g/100g dextran was produced into the faba bean flour. Best conversion (Figure 18) compared to theoretical yield (all glucose from sucrose converted to dextran) was obtained with *W.* confusa with no external sucrose addition before the fermentation. The results were in line with viscosity trend of large scale samples (Figure 18 B) as well as with the sensory observations of the texture. Within the large scale fermentation series, the viscosity was even 7314 ± 829 mPas after 24 h making almost 50-fold increase to

the initial viscosity (148 ± 32 mPas). Viscosity values in more detail as well as the viscosity curves are presented in Appendix 3.

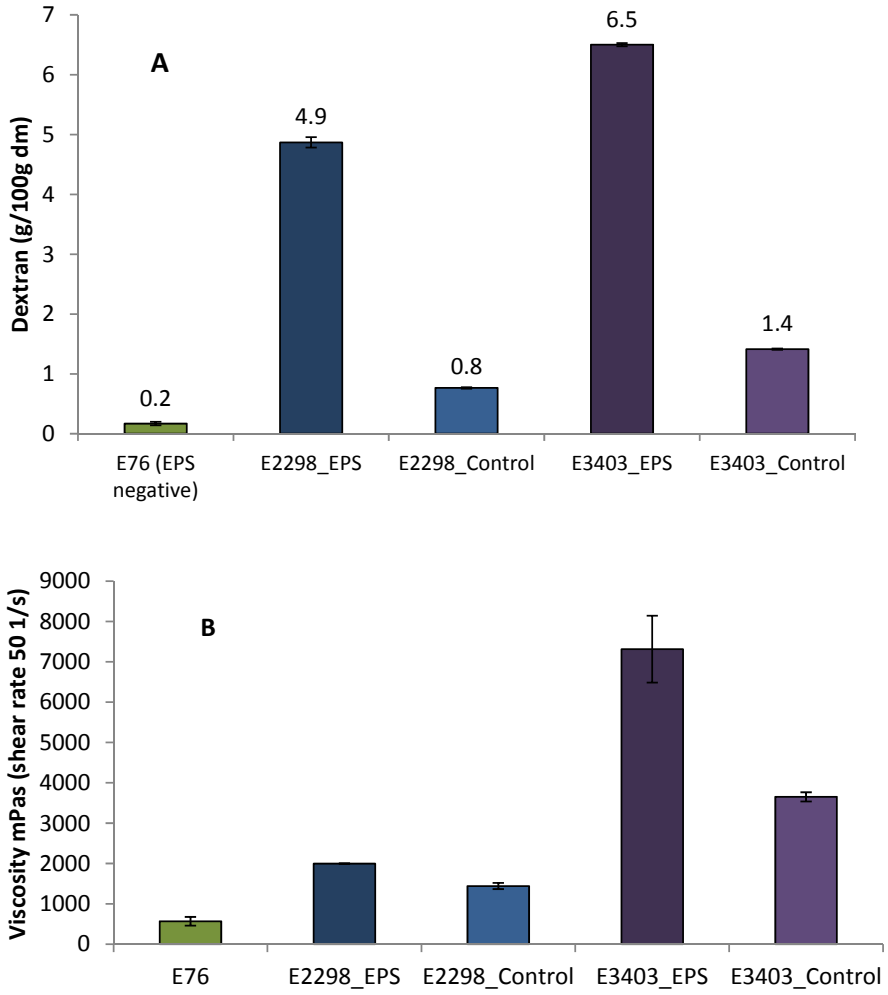

Figure 18. Dextran yield (A) and viscosity (B) and after large scale series of 24 h LAB fermentation. Viscosity values reported have been taken at time point of shear rate 50 s^{-1}. E76: *L. plantarum* (EPS-negative); E2298: *Lc. lactis*; E3403: *W. confusa*. EPS: sucrose added in the medium; Control: no sucrose added.

The texture of samples from *W. confusa* fermentations in the presence of sucrose was thickened due to dextran production (Figure 19). The texture of fermentations

with *Lc. lactis* had a gel-like appearance whereas those of EPS-negative *L. plantarum* samples were runny. Brown-coloured top layer was observed in fermentations with *W. confusa*. A thin brown layer (Figure 20) was observed in spontaneous fermentations as well. The brown layer was most marked in samples with no mixing during the fermentation, potentially due to the ammonia formation during the fermentation with *W. confusa*.

Figure 19. Faba bean matrix with *W. confusa* E3403 with sucrose (left) and control medium (right) after 24 h fermentations.

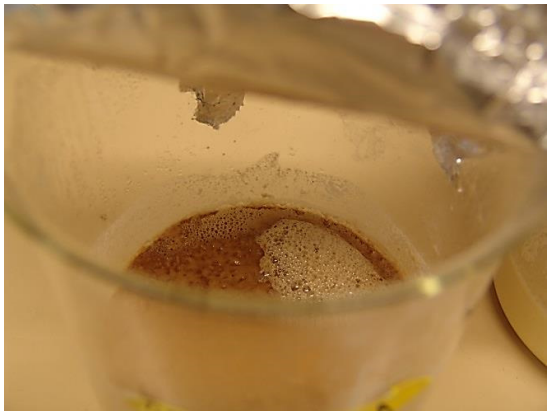

Figure 20. Brown-coloured layer after 14 h fermentation with *W. confusa* E3403.

The sucrose addition level in EPS-samples was 5.1 % of the total weight, thus 15 % of dry weight. Moreover, the initial sucrose content of faba bean flour was 2.9 %.

Increase of fructose in samples of fermentations started with both EPS-producing strains, *Lc. lactis* and *W. confusa* shows that glucose from sucrose was used for dextran production and fructose was left free in the medium. Fructose was not detected in the raw material nor in fermentations with EPS-negative *L. plantarum*. Detailed composition of monosaccharides is presented in Appendix 4.

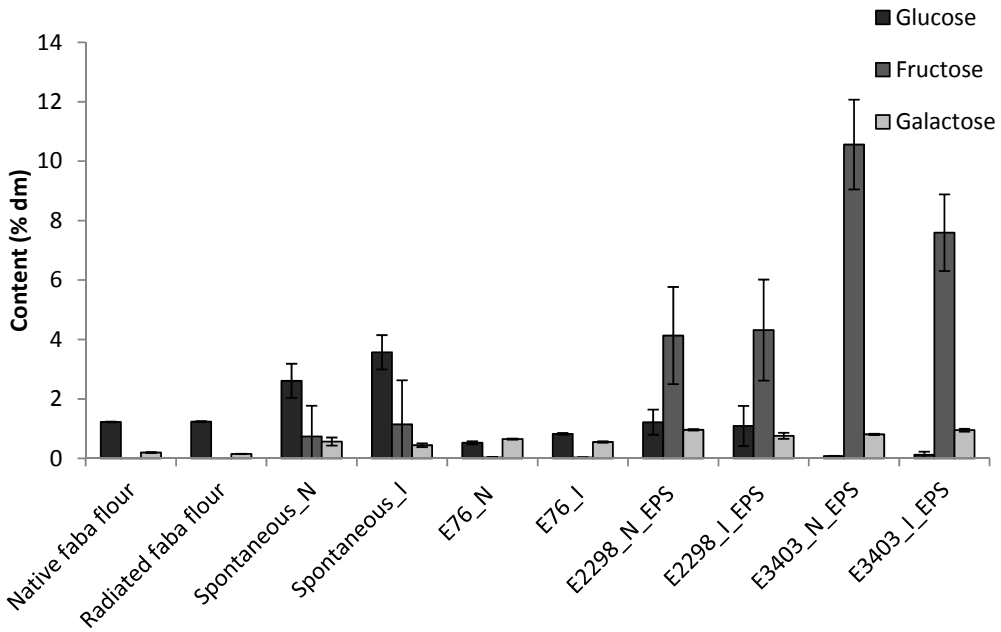

Figure 21. Glucose, fructose and galactose contents (% dm) of 24 h small scale fermentations (series B-D). EPS-negative *L. plantarum* (E76), *Lc. lactis* (E2298), and *W. confusa* (E3403). N: native flour; I: irradiated flour.

6.4.1 Effect of LAB fermentation on nutritional value of faba bean matrix

Phytic acid content

With LAB fermentation, it was possible to reduce the PA content of the raw material (Figure 22). The effect was more pronounced with samples of native flour, obviously due to endogenous phytase activity of the raw material. In addition, indigenous microbial activity was involved in the PA degradation. 57 % degradation was observed in spontaneous fermentations with native flour and 16 % degradation with irradiated flour after 24 h fermentation. The highest reduction (67 %) was measured in small

scale fermentations with *Lc. lactis* without added sucrose. Moderate PA reduction effect was observed in large scale fermentations with of *Lc. lactis* and *W. confusa* with sucrose added in the fermentation medium (Appendix 5).

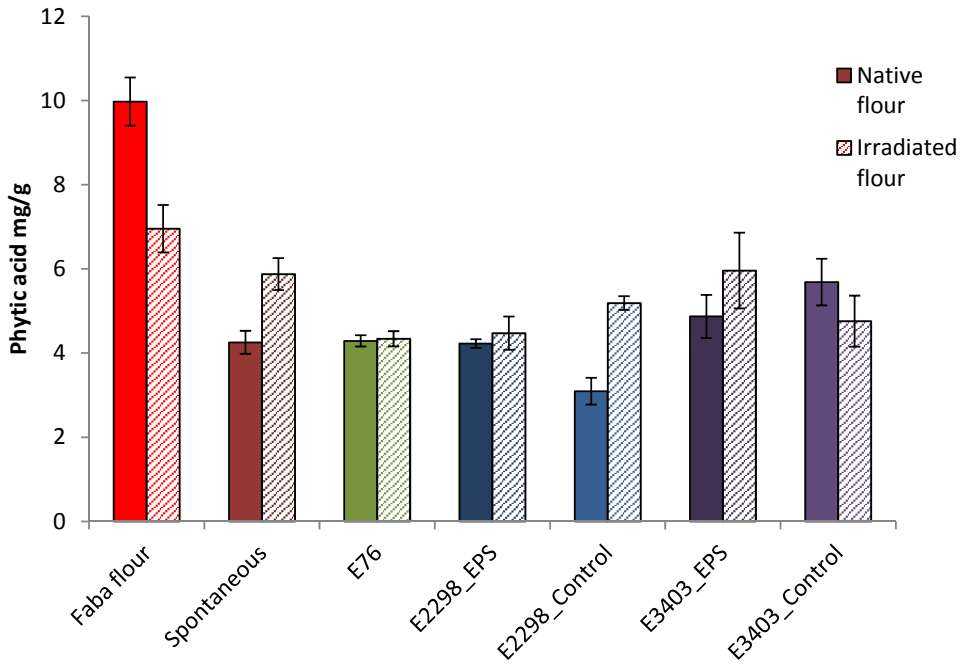

Figure 22. Phytic acid content of 24 h fermented faba bean flour. Spontaneous: natural fermentation without starter culture; E76: *L. plantarum*; E2298: *Lc. lactis*; E3403: *W. confusa*. EPS: sucrose added in the medium; Control: no sucrose added.

Impact of LAB fermentation on raffino-oligosaccharides (RFOs)

As seen in Table 16, α-galactosidase activity was mainly microbial origin. Total α-galactosides was reduced from 4.5 to 3.4 % in 24 h incubation with irradiated flour, whereas in spontaneous fermentation with native flour, 59 % reduction was observed. LAB fermentation notably reduced RFOs with all three bacterial strains. With *W. confus*a E3403, it was possible to achieve even 74 % reduction in total α-galactoside content (raffinose, stachyose, verbascose).

Table 16. Oligosaccharide content (% on dm) in LAB fermented faba bean flour. Native flour and radiated flour have used as reference samples.

	Oligosaccharide content (% dry matter)			
	Raffinose	Stachyose	Verbascose	Total α-galactosides
Native faba flour[b]	0.32 ± 0.01	1.52 ± 0.01	2.33 ± 0.06	4.16 ± 0.82
Radiated faba flour[b]	0.34 ± 0.00	1.58 ± 0.03	2.55 ± 0.10	4.47 ± 0.90
Spontaneous_I [a]	0.32 ± 0.12	1.32 ± 0.05	1.73 ± 0.10	3.37 ± 0.59
Spontaneous_N[a]	0.16 ± 0.01	0.83 ± 0.11	0.73 ± 0.14	1.72 ± 0.30
E76_I [a]	0.15 ± 0.02	1.19 ± 0.05	1.28 ± 0.04	2.63 ± 0.51
E76_N [a]	0.14 ± 0.01	1.09 ± 0.04	1.02 ± 0.06	2.25 ± 0.43
E2298_I_EPS [a]	0.10 ± 0.05	0.86 ± 0.09	1.12 ± 0.06	2.08 ± 0.43
E2298_N_EPS [a]	0.08 ± 0.01	0.88 ± 0.18	0.89 ± 0.06	1.85 ± 0.38
E3403_I_EPS [a]	0.04 ± 0.01	0.73 ± 0.08	1.00 ± 0.16	1.78 ± 0.40
E3403_N_EPS [a]	0.05 ± 0.03	0.29 ± 0.24	1.74 ± 0.05	1.09 ± 0.29

[a] Results are means ± SD of three individual fermentations (n = 3).

[b] Results are means ± SD of analytical triplicates.

Mineral composition

Fermentation with LAB starters showed moderate improvement in mineral (Ca, Mg, Fe, Zn) availability (Table 17). Highest relative increase was observed in calcium levels. In general, fermentation without sucrose addition (EPS-production) was more effective in mineral improvement.

Table 17. Mineral composition (mg/kg) of faba bean samples from large scale fermentations (25 °C, 24h).

	mg/kg			
Sample	Calsium	Magnesium	Iron	Zinc
Native faba bean flour [a]	463 ± 4	1000 ± 12	46 ± 1	42 ± 1
L. plantarum E76 [a]	548 ± 21	1096 ± 18	52 ± 1	46 ± 0
Lc. lactis E2298 (EPS) [b]	460 ± 23	936 ± 36	49 ± 7	39 ± 1
Lc. lactis E2298 (Control) [b]	535 ± 23	1112 ± 72	52 ± 3	46 ± 3
W. confusa E3403 (EPS) [a]	490 ± 30	982 ± 11	49 ± 2	41 ± 1
W. confusa E3403 (Control) [a]	538 ± 18	1093 ± 19	51 ± 1	46 ± 1

[a] The results are average ± SD of duplicate analysis.

[b] The results are average ± SD of duplicate analysis from two individual replicates

Free amino acids (FAA) and γ-aminobutyric acid (GABA)

FAA and GABA composition was analysed from large scale (series F) faba bean samples after 24 h fermentation with three different LAB starter strains with and without sucrose addition in the fermentation medium. Results were compared to unfermented native faba bean flour, in which the total FAA composition was 5.6 g/kg. Fermentation with LAB increased the FAA content of faba bean matrices (Figure 23). Total FAA content of fermented samples varied from 7.6 g/kg (*L. plantarum* E76) to 10.4 g/kg (*Lc. lactis* E2298 Control). From essential amino acids, amounts of isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), and valine (Val) were increased in fermented samples. Histidine (His) was increased in all samples, except with *W. confusa* E3403 (EPS). A notable increase was obtained in GABA content (Table 18), which increased approximately 10 times compared to unfermented native faba bean flour.

Furthermore, notable increase of both ornithine (Orn) and ammonia (Amm) levels was observed in both *Weissella* fermentations, which indicates that *W. confusa* showed activity in producing ammonia from arginine. Approximately 30-fold increase of ammonia was measured in *W. confusa* fermentations (Appendix 6).

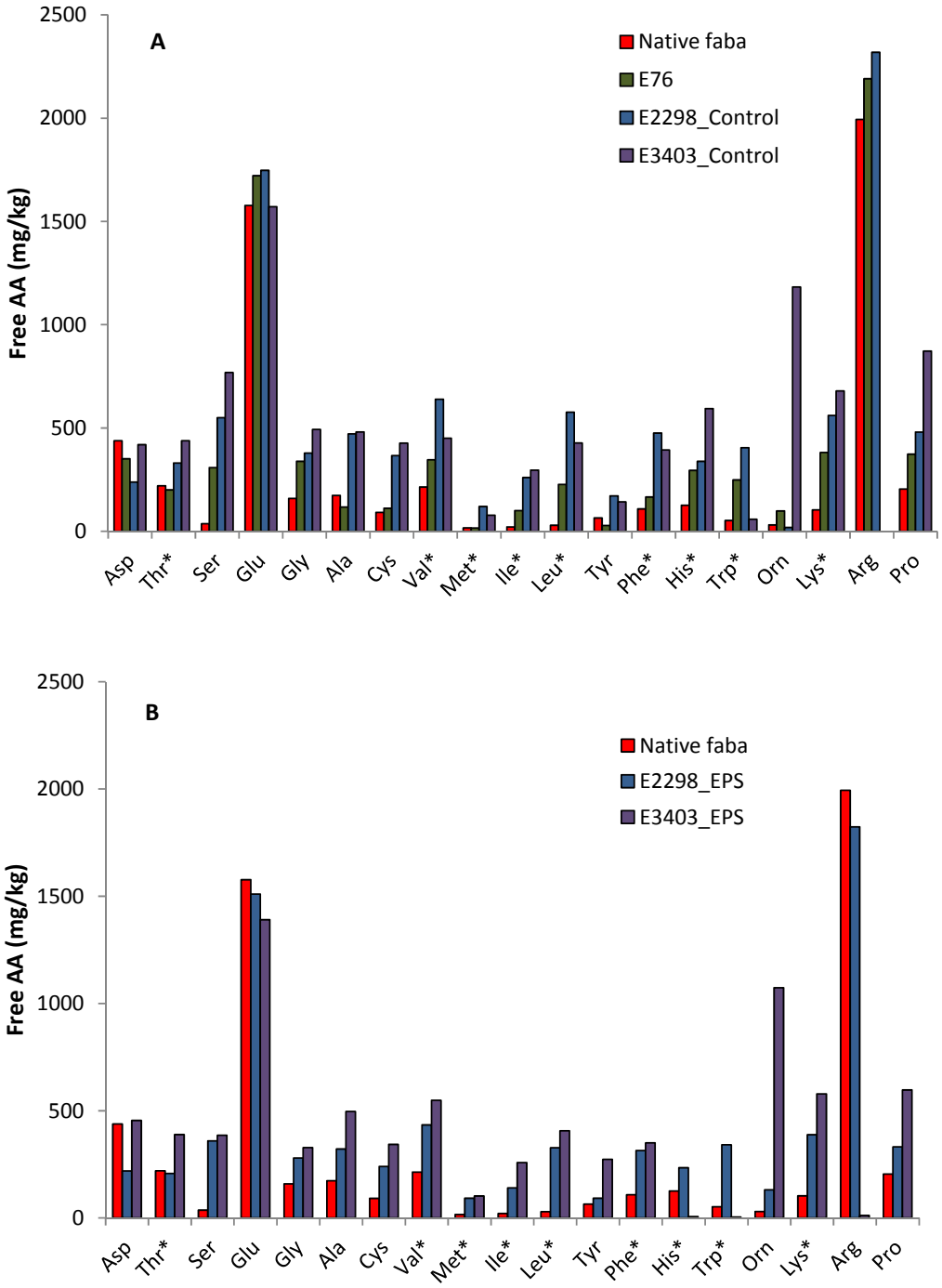

Figure 23. Free amino acids (FAA) of fermented faba bean flour without sucrose (A) and with sucrose (B) in the fermentation medium (mg/kg). E76: *L. plantarum* (EPS-negative); E2298: *Lc. lactis*; E3403: *W. confusa*. Results are from one independent analysis. * Essential amino acids indicated on the x-axis.

Table 18. γ-aminobutyric acid (GABA) content of native and fermented faba bean.

	GABA (mg/kg)
Native faba	105
E76	1164
E2298_EPS	1004
E2293_Control	1162
E3403_EPS	1082
E3403_Control	1156

E76: *L. plantarum* (EPS-negative); E2298: *Lc. lactis*; E3403: *W. confusa*. EPS: sucrose added in the medium; Control: no sucrose added. Results are from one independent analysis.

6.5 Extrusion processing

Cross-sectional stereomicroscopy images of extruded snacks with 0:100, 50:50 and 25:75 faba bean-rice flour ratios are presented in Figure 24. Extrudates cooked from 100 % addition levels of bioprocessed faba bean, especially samples with EPS-production were relatively less expanded and brown coloured compared native faba flour extrudates. The brown colour was probably due to Maillard reactions between protein and monosaccharides under high temperatures. The effect was milder with extruded samples of 50 % and 25 % addition level.

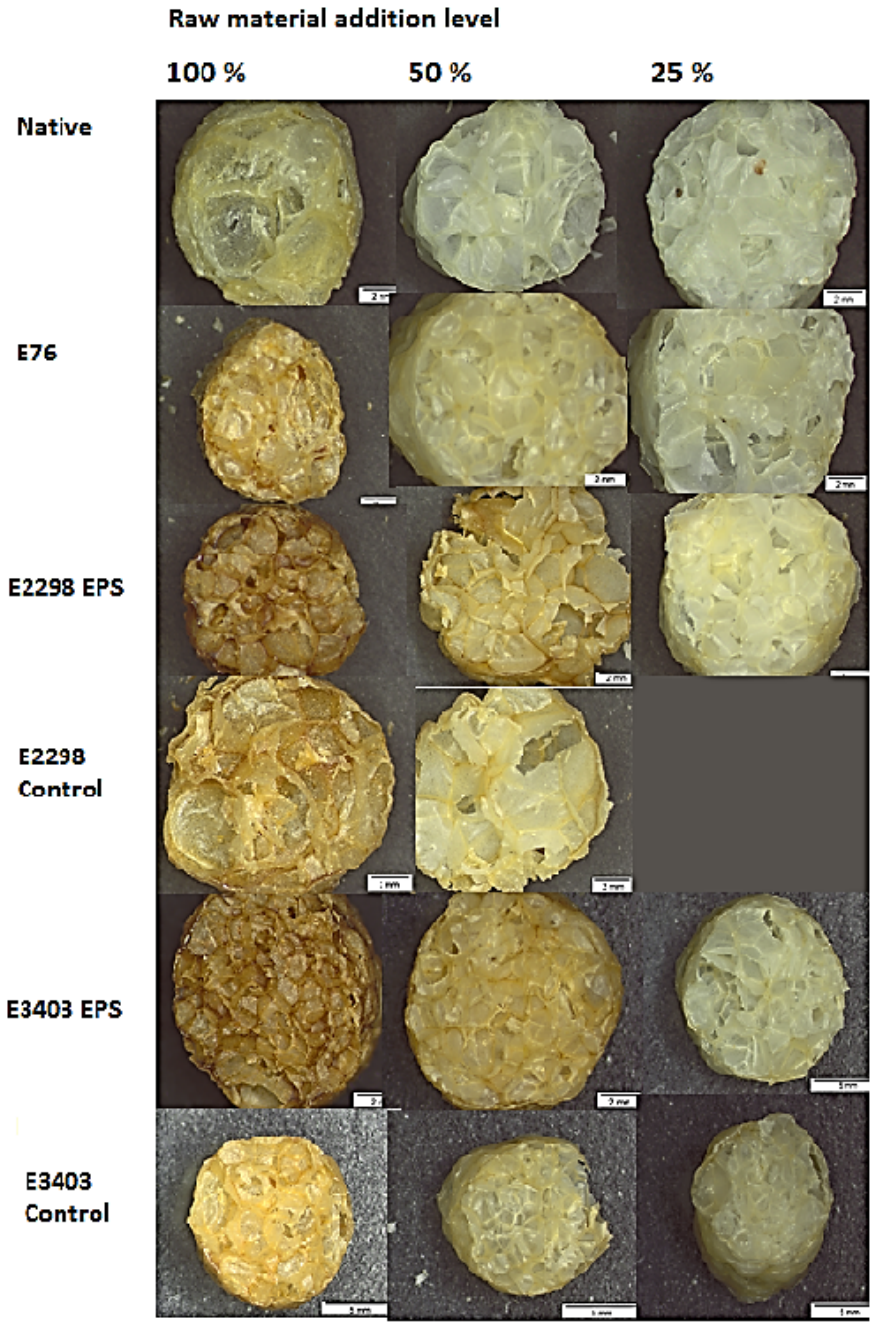

Figure 24. Cross-section pictures of extrudates obtained with stereomicroscope. Native: native faba bean; E76: *L. plantarum*; E2298*: Lc. lactis*; E3403*: W. confusa*. EPS: sucrose added in the medium; Control: no sucrose added.

6.5.1 Macrostructure of faba bean extrudates

Macrostructural parameters (expansion rate, specific length and piece density) effect textural and sensory properties. Desirable products have high expansion rate and low piece density. The native faba bean flour was suitable for extrusion processing due to its better processability and easier controllability of the extrusion parameters compared to the fermented samples. Macrostructural properties of faba bean extrudates made from bioprocessed faba bean flour mixed with different ratios of rice flour are shown in Table 19. The addition level had significant effect ($P \leq 0.05$) on all macrostructural outcomes, except on specific length between 50 % and 100 % levels (Appendix 8, Tables 3-4). The highest expansion rates (644 %, 608 % and 624 %) were obtained with 50 % addition levels of native faba flour and *L. plantarum* E76 fermented faba bean flour, as well as with *Lc. lactis* E2298 fermented faba bean flour in 100 % addition level. Specific length of extrudates ranged between 104 and 624 m/kg and highest values were obtained with major of the bioprocessed samples at 50 % addition level. Piece density of extrudates varied markedly, however the lowest value (53 kg/ m^3) was obtained with *L. plantarum* fermented faba flour in the 50 % addition level.

Table 19. Macrostructural properties of faba bean extrudates.

Sample	Addition level	Expansion rate (%)	Specific length (m/kg)	Piece density (kg/ m^3)
Native faba flour [2]	100 %	515 ± 15	130 ± 5	94 ± 1
	50 %	644 ± 12	124 ± 3	62 ± 1
	25 %	510 ± 6	104 ± 9	119 ± 13
E76 [1]	100 %	444 ± 1	135 ± 24	125 ± 21
	50 %	608 ± 6	164 ± 3	53 ± 0
	25 %	514 ± 2	133 ± 4	92 ± 2
E2298_EPS [2]	100 %	476	150	94
	50 %	514 ± 21	141 ± 9	86 ± 1
	25 %	513	123	98
E2298_Control	100 %	624	133	61
	50 %	573	119	83
	25 %	nd	nd	nd
E3403_EPS [1]	100 %	439 ± 26	172 ± 0	97 ± 11
	50 %	471 ± 29	159 ± 15	92 ± 3
	25 %	497 ± 18	134 ± 17	98 ± 7
E3403_Control	100 %	498	177	73
	50 % [2]	543 ± 16	156 ± 1	70 ± 4
	25 %	477	137	103

E76: *L. plantarum*; E2298: *Lc. lactis*; E3403: *W. confusa*. EPS: sucrose added in the fermentation medium; Control: no sucrose added

nd: not determined

[1] Results are average ± SD of two replicate fermented samples.

[2] Results are average ± SD of two replicate extrusions.

6.5.2 Mechanical properties of faba bean extrudates

Mechanical properties (hardness and crispiness) of extrudates can be used to predict sensory attributes of products. Hardness indicates the maximum force needed for cell wall rupture during compression. Extrudates of *Lc. lactis* E2298 and *L. plantarum* E76 fermented extrudates represented lowest hardness (Figure 25 A) and highest

crispiness (Figure 25 B) values compared to the extruded non-bioprocessed faba flour. Fermentation with *L. plantarum* gave 55 % increase in crispiness index and reduced hardness 45 % at addition level of 25 %. In general, hardness values were the lowest in addition level of 50 %, whereas hardness of extrudates was the highest at 25 % addition level. The promising effects of *Lc. lactis* fermented flour (even 84 % increase in crispiness index and 25 % reduction of hardness) are results of one extrusion trial, thus a replicate experiment will be needed. *In situ* produced dextran did not present improvements in extrusion cooking of faba beans. Control samples with *ex situ* added dextran and fructose were hardest with lowest crispiness.

In general, samples presented a correlation in hardness-crispiness ratio: samples with high crispiness were also less hard. The addition level showed significant ($P \leq 0.05$) effect between 100 % and 25 % as well as between 50 % and 25 % addition levels (Appendix 8, Tables 5-6).

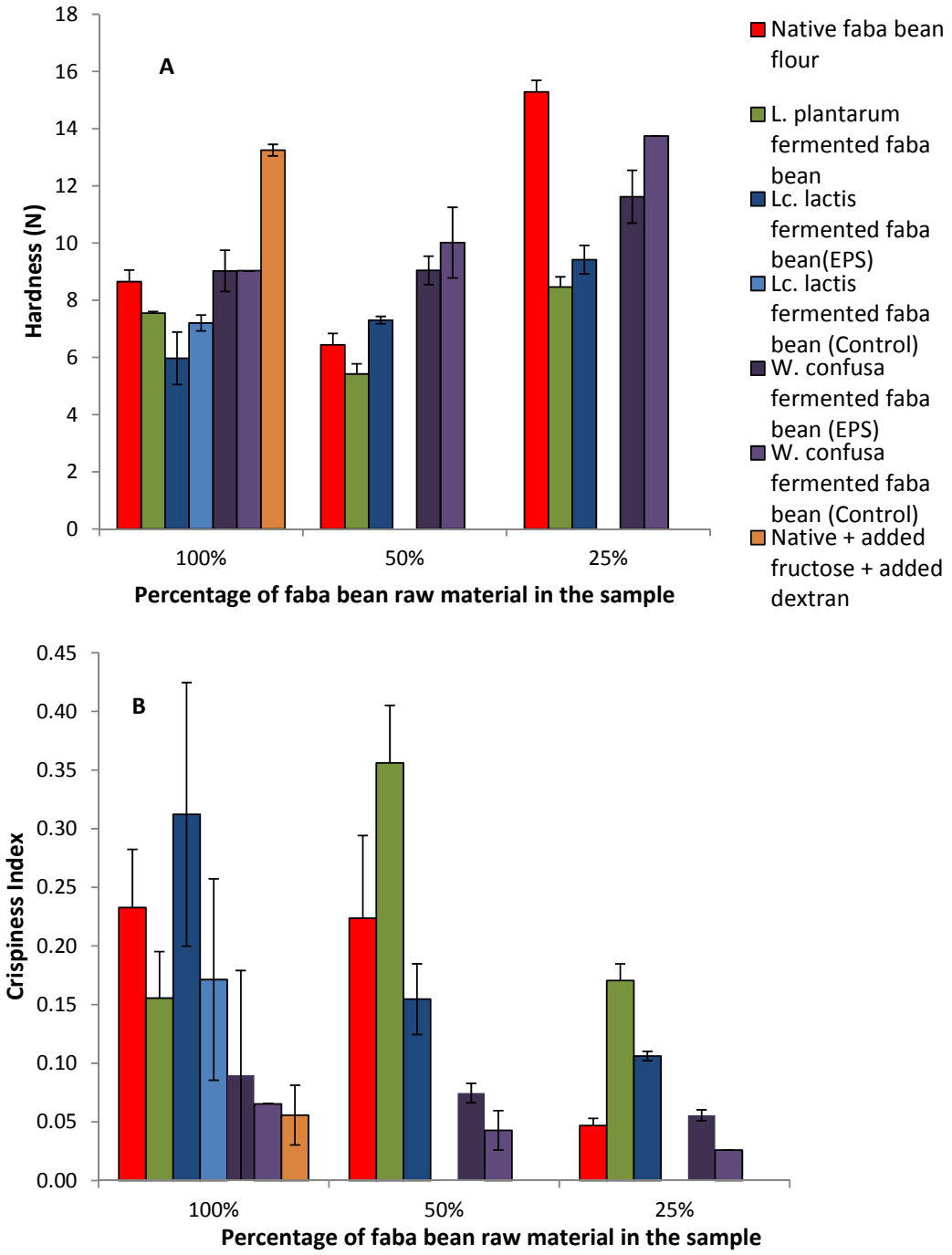

Figure 25. Hardness (A) and crispiness index (B) of faba bean extrudates.

[a] Results are average ± SD of two biological replicates (two replicate fermented samples).

[b] Results are average ± SD of two replicate extrusions.

7 Discussion

The aim of this master's thesis study was to study the effects of bioprocessing with phytase treatment and LAB fermentation on faba bean. The study showed that bioprocessing offers many beneficial effects: with enzymes and LAB it is possible to influence the nutritional value of the faba bean ingredients, as well as to produce functional ingredients for possible later food applications.

7.1 Bioprocessing of faba beans with food-grade phytase

This study revealed that food grade phytase (Ultra Bio-Logics Inc., Canada) from *A. niger* effectively degraded phytic acid in faba bean matrix. Over 80 % reduction in PA levels was obtained with 20 U phytase activity dose at 55 °C with only 1 h incubation. Phytase activity levels had more significant impact than the incubation time. The results were in agreement with Luo *et al.* (2009) who measured almost 80 % decrease in PA content after 30 min soaking with 500 U/kg dose of fungal phytase at 50 °C. Phytase treatment was effective not only in its optimum conditions (at 55 °C) but also at room temperature. Incubation at 25 °C for 6 h with 20 U phytase resulted in 87 % reduction in phytic acid level. PA level after the incubation was then 1.2 ± 0.2 mg/g. The initial PA content in the raw material was 9.98 ± 0.57 mg/g, which is in line with studies of Luo and Xie (2012), who obtained a value of 8.36 ± 0.06 mg/g.

Phytase treatment improved nutritional value of faba bean. This study showed that phytase treatment led to increased mineral availability. In large-scale, 6h phytase incubation led to increases of approximately 90 % in calcium, 30 % in iron, and 10 % in magnesium and zinc levels. In contrast, Luo and Xie (2012) obtained 10 % reduction in iron content after 60 min treatment with 800 U/kg wheat-derived phytase at 37 °C. Also in a study of Luo *et al.* (2009), phytase treatment decreased the losses of iron, zinc and calcium and they suggest the mineral losses to be due to dissolving of minerals during soaking of faba bean material.

Faba bean matrix showed to have endogenous phytase activity from both microbes and the plant itself. Incubation of faba bean flour at 55 °C without external addition of enzyme reduced the PA level by 25 %. However, based on study of Luo *et al.* (2009), endogenous phytases of faba bean did not have a significant effect on PA under the conditions used in the experiment. Furthermore, plant-origin phytases have optimum conditions at lower temperatures than microbial phytases (Konietzny and Greiner, 2002).

PA content could be also modified by LAB fermentation. In small scale fermentations, approximately 65 % degradation was observed when *Lc. lactis* was applied. However, the same effect was not detected in the large scale fermentations. This can partly be due to the weaker mixing during the large scale fermentations compared to the mixing in small scale. The highest depletion, 65 %, was obtained with 24h fermentation with *Lc. lactis* E2298 at 25 °C. No significant effect was observed in PA content after fermentation with *L. plantarum* by Coda *et al.* (2014) either. The degradation during fermentation is mostly a consequence of the low pH due to the production of organic acids during the LAB fermentation which favours the endogenous phytases.

PA may form complexes with proteins influencing in protein release and digestibility (Luo and Xie, 2013a). Total FAA content was increased by 43 % with phytase treatment. The influence of phytase treatment on *in vitro* protein digestibility was studied at VTT in the M.Sc. thesis of Margherita Re (2015) done simultaneously in the project. Her study showed that *in vitro* protein release was notably higher in faba bean samples treated with phytase. PA tends also to inhibit the phytate-degrading and other digestible enzymes, such as amylase, pepsin and trypsin (Urbano *et al.*, 2000), which can also influence the protein availability of the raw material.

In the future, a tailored bioprocessing using both LAB and phytase in order to maximise the positive effects would be interesting. However, the conditions should be carefully optimized as Marklinder *et al.* (1995) combined *A. niger* phytase with *L. plantarum* fermentation of oatmeal for 15 h resulting a significant degradation of PA,

but also leading to negative side effects as in lowering of viable cell counts and a bitter aroma.

7.2 EPS production of LAB in faba bean matrix

To our knowledge, this was the first study in which dextran was produced in faba bean matrix. Viscosity was used as a main indicator of EPS-formation. Dextran yields were analysed later from samples of large scale fermentations. Based on the viscosity results, the preliminary screening of six strains showed that the *Leuconostoc* and *Weissella* strains have the ability to produce EPS in faba bean material. Two selected EPS-producing LAB strains, *W. confusa* E3403 and *Lc. lactis* E2298*,* and a non-EPS-producing *L. plantarum* E76 were used to carry out the faba bean fermentations. Fermentation of *W. confusa* led to the thick texture and high viscosity. The increase of fermentation time from 14 h to 24 h did increased the viscosity with most of the *W. confusa* started fermentations, whereas the LAB growth was not significantly higher after 24 h fermentation compared to 14 h fermentation. However, the acidification continued notably during the last 10 h lowering the pH values, which is vital considering the microbial safety. Mixing during the fermentation lead to greater decrease of viscosity in fermentations with native faba bean flour compared to static fermentations. Mixing on the other hand presented almost 100-fold increase in LAB count compared to samples fermented in static conditions, which might be due to better availability of nutrients in the fermentation medium. Mixing during the fermentations led to more intense acidification than in static fermentations. Juvonen *et al.* (2015) showed that moderate pH decrease is favourable in dextran synthesis. The results in this study are in line with studies of Katina *et al.* (2009) and Juvonen *et al.* (2015) in which the fermentation with both *W. confusa* sp. and *Lc. lactis* sp. starters resulted in high viscosities. In addition, Di Cagno *et al.* (2011) obtained also high viscosity values in fermented vegetable smoothies started with *Weissella cibaria*. Consequently viscosity measurements can be used as a marker in *in situ* EPS-production in food materials.

Dextran is synthesised extracellularly from the glucose residue of sucrose (Ruas-Madiedo *et al.*, 2002). Therefore, sucrose was added in the fermentation medium in this study. High amounts of dextran was produced into the faba bean fermentations. Yield was 6.5 % of dm with *W. confusa* and 4.9 % with *Lc. lactis*. Dextran was also produced in LAB started fermentations without additional sucrose. This study revealed that the faba bean flour itself contains ca. 2.9 % sucrose and this was effectively converted to dextran. Approximately 1.4 % of dextran was obtained after fermentation with *W. confusa*. In wheat sourdough the dextran yield obtained with *W. confusa* E392 was 1.1-1.6 % (Katina *et al.*, 2009) , but the sucrose addition levels were lower (10 %) than in this faba bean study (17 %).

Glucose and fructose consumptions were analysed from small scale fermentations. Fermentation with EPS-producing strains led to high fructose concentrations after 24 h fermentation of faba bean flour. A sample fermented with *W. confusa* with sucrose addition resulted in even 10 % (dm) fructose content, which was approximately half the level of the total sucrose content in the beginning of the fermentation. The fermentations in question reduced the α-galactosides, which are hydrolysed to D-galactose and sucrose. Hence, the actual theoretic glucose-to-dextran conversion cannot be estimated in more detail.

The dextran results correlated with viscosity trend, monosaccharide contents, as well as with the sensory observations of the texture. Juvonen *et al.* (2015) have also reported logical correlations between the low-branched dextran of *W. confusa* E392 and *Lc. lactis* E2298 in carrot fermentations. The sucrose addition in EPS-fermentations was almost 20 % in dry matter, thus the next step should be the optimisation of sucrose concentration. In contrast to rye bran fermentations (Sozer *et al.* 2014) , additional sucrose is probably not needed at all in the fermentation medium. In conclusion, this study indicated that with dextran-producing LAB starter strains could provide a natural approach in improved textural and nutritional characteristics of faba bean raw material.

7.3 Nutritional value of fermented faba bean

This study showed that with LAB fermentation, it was possible to improve the nutritional value of faba bean. LAB fermentation notably reduced flatulence causing raffino-oligosacchaides (RFOs) with all three bacteria strains. With *W. confusa,* it was possible to achieve 73 % reduction in total RFO content. The initial α-galactoside content of faba bean raw material was two-fold the concentration Lattanzio *et al.* (1986) had obtained, although the proportions of raffinose, stachyose and verbascose remained the same. Previous studies have shown that *L. plantarum* has α-galactosidase activity, that hydrolyses the RFOs (Adeyemo, and Onilude, 2014). Similar effects of α-galactosidase activity have been reported when using *L. plantarum* as a starter in legume fermentations in previous studies (Djaafar *et al.,* 2013).

This study indicated that LAB fermentation was effective also in improving the amino acid profile of faba bean. *Lc. lactis* fermentation without sucrose addition gave 85 % increase in total FAA content. LAB fermentation increased the amount of almost all essential amino acids. Approximately 2-4 fold increase was measured. In a study of Coda *et al.* (2014), faba bean fermentation with *L. plantarum* VTT E-133328 increased the total FAA from 2 up to 3.5 times. In this study a 1.3-1.8 fold increase was obtained with *L. plantarum* E76. A notable increase was obtained also in γ-aminobutyric acid (GABA) content, which increased from 100 to 1100 g/kg with all three LAB compared to unfermented faba bean flour. This result differed from the results of Coda *et al.* (2014), who obtained increase from 540 to 630 mg/kg. However, it should be noticed that different *L. plantarum* strain as well as different faba bean raw material was used in this earlier study. In addition to microbial enzymatic activity, the increase of GABA might be due to the endogenous glutamic acid decarboxylase-activity of the faba bean flour (Coda *et al.*, 2015).

The liberation of amino acids effected on the colour formation during the fermentation. In this study brownish upper layer formation was observed in *Weissella* initiated fermentations. Fermentation with *W. confusa* produced high amounts of

ammonia, which is a part of arginine degradation and ornithine formation via the arginine-deaminase pathway (Liu *et al.*, 1995). The ammonia concentrations correlated with high amounts of ornithine and almost a total drop in arginine. Several species of *Weissella*, including W*. confusa,* are reported to have the activity of producing ammonia from arginine, whereas *Leuconostoc* species are arginine-negative (Björkroth and Holzapfel, 2006). The results could explain the brownish upper layer formed during the faba bean fermentations with *W. confusa,* probably due to ammonia that has reacted with oxygen. Thin brown layer was observed during the spontaneous, unstarted fermentations as well, which together with the sequencing results from isolated strains reinforces that the faba bean flour is rich in endogenous *Weissella* species. The arginine-deaminase pathway contributes to pH-homeostasis and acidic tolerance of LAB (Gänzle *et al.*, 2007).

A possible future study could be fermentation with co-starter culture of both *L. plantarum* and *W. confusa* using the antimicrobial advantages of *L. plantarum* and functional EPS-production of autochthonous LAB such as *W. confusa* for instance.

7.4 Functionality of bioprocessed faba bean ingredients in extrusion

The aim of the present study was also to elucidate the impact of bioprocessed faba bean ingredients in extruded snacks. Sozer *et al.* (2014) showed positive impacts of *W. confusa* fermented rye bran ingredients in extrusion. One of the aims of this master's thesis was also to produce faba bean extrudates that are less hard, crispier, and have increased expansion rate. The improved mechanical properties were obtained with fermented samples without added sucrose (less or no dextran at all). Extrudates of *L. plantarum* E76 and *Lc. lactis* E2298 represented lowest hardness and highest crispiness values compared to the extruded non-bioprocessed faba flour. Fermentation with *L. plantarum* gave 55 % increase in crispiness index and reduced hardness 45 % at addition level of 25 % with rice flour. The extrudates made from *Lc. lactis* fermented faba bean flour at 50 % addition level led to even 84 % increase in crispiness index and 25 % reduction of hardness (data not shown). However the

results of *Lc. Lactis* are from one extrusion trial, thus a replicate experiment will be needed.

This study showed that *in situ* produced dextran did not show any additional impacts in extrusion cooking of faba beans, unlike in the rye bran studies of Sozer *et al.* (2014). However, LAB fermentation without sucrose addition resulted in rather functional extrudates, which did not get as burned as extrudates made from faba bean ingredients containing *in situ* dextran and free fructose. The properties did not vary that much from the extrudates made from native faba bean flour, yet the mechanical properties were better than with the extrudates made of EPS-samples.

Due to Maillard reactions faba bean extrudates, especially samples with EPS-production were relatively less expanded and brown coloured. The raw material was easily burned during the process. The conditions of extrusion process are known to favour Maillard reaction, which is a chemical reaction between amino and carbonyl groups of reducing sugars resulting in browning colour and flavour production (Singh *et al.*, 2007). Fermentation positively increased the amount of FAA, which on the other hand jointly with free sucrose and fructose probably led to strong Maillard reaction in high temperatures, low moisture, and acidic pH. Especially lysine is reported to be the most reactive amino acid having two available amino groups (Singh *et al.*, 2007). In this study, bioprocessing with LAB generally increased the availability of lysine in faba bean raw material. Increase in lysine was also obtained in the study of Coda *et* al. (2014). Properties of extrudates improved when the material was mixed with starch-fortified rice flour. Fermented faba bean flour without EPS-production and no added sucrose, were more suitable in extrusion.

In spite of the overall improvement in FAA, LAB fermentation of faba bean on the contrary decreased the content of asparagine, amino acid that contributes to acrylamide (group 2A carcinogen) formation in the presence of reducing sugars at high temperatures (Stadler *et al.*, 2002). In future studies, bioprocessed faba bean ingredients in snack products could be improved by optimising the extrusion

conditions, the mixture made with rice flour as other ingredients, and the sucrose levels in fermentations.

Despite the challenges of the EPS-material in faba bean extrusions, other food applications could be considered as a next step. *In situ* EPS have already shown promising effects in bread baking (Katina *et al.*, 2009; Di Monaco *et al.*, 2014). EPS can offer improvements in sensory quality and shelf life properties without additives for example in gluten-free products. Faba bean raw material with *in* situ produced EPS could be a natural alternative for synthetic hydrocolloids used as a substitute improving the sensory properties of gluten-free products. Furthermore, EPS-producing LAB fermentations with selected starter strains could be studied in other plant based food applications, such as smoothies, providing additive-free high protein plant-based products from domestic cultivated raw material.

8 Conclusions

The aim of this master's thesis was to study impacts of bioprocessing with food-grade phytase and lactic acid bacteria (LAB) on nutritional and textural value of the protein-rich faba bean raw material. Furthermore, the objective was to study the functionality of bioprocessed material in extrusion process.

Phytase treatment was highly effective in degrading phytic acid in faba bean flour in a notable short time (1-2 h). With the commercial food-grade phytase, phytic acid was reduced almost entirely, which was related to better protein and mineral availability results.

Faba bean flour was shown to be a source for EPS-producing LAB strains. Fermentation with exopolysaccharide producing *W. confusa* and *Lc. lactis* showed novel promising results in *in situ* dextran production in faba bean matrix. The conversion from sucrose to dextran was highest with *W. confusa*. Even the native faba bean flour without added sucrose, offered a good medium for dextran production. Dextran production was observed as increased viscosity. Static cultivations provided better EPS production compared to mixed fermentations. Even 14 h fermentation time would be enough to obtain EPS production. Selected LAB starters *W. confusa*, *Lc. lactis* and *L. plantarum* enriched the nutritional value of faba bean by reducing the flatulence causing α-galactosidase content and by increasing the composition of essential amino acids and GABA.

Extrusion cooking of EPS-produced faba bean flour did provide additional value in faba bean extrusions, even though it was evident that faba bean can be used as a raw material in expanded extrudates. Native faba bean flour functioned in extrusion as well as *L. plantarum* fermented faba bean without sucrose addition and showed to be suitable material for extrusion cooking. Especially fermentation with *L. plantarum* could be considered for further studies of raw material for extrusion due to both the positive impacts on the mechanical properties of extrudates, and the antimicrobial feature of the bacterium.

Recently published protein self-sufficiency survey (Kaukovirta-Norja *et al.*, 2015) predicted that replacing soy with faba bean and pea in Finland would be profitable. Future studies of bioprocessed faba bean in food applications will be considered. A possible next step could be controlled fermentation with selected combination of LAB starters with phytase incubation. Subsequent studies could include experiments of EPS-produced faba bean in baking, pastes and protein-rich smoothies. This fusion processing could provide raw material for nutritive, high-protein, stable and additive-free plant-based food products from domestic raw materials meeting the growing consumer demands as well as the global malnutrition issues.

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Appendices

Reagents and medium compositions

Table 1. Growth media and conditions used in the study.

Microbial group	Medium composition	Incubation conditions
Total aerobic heterotrophic bacteria	Plate count agar (PCA)	Aerobic, 30 °C, c. 48 h
LAB	MRS agar	Anaerobic, 30 °C, c. 48 h
LAB + EPS (slimy colonies enumerated)	MRS + sucrose agar	Anaerobic, 30 °C, c. 48 h
Aerobic spore forming bacteria	Tryptone soy agar (TSA). Heat treatment in a 80 °C water bath for 10 min	Aerobic, 30 °C, c. 3 d
Pseudomonas spp.	C-F-C agar	Aerobic, 30 °C, c. 48 h
Gram-negative coliforms	Chromocult agar	Aerobic, 37 °C, c. 48 h
Yeast and mould	YM agar	Aerobic, 25 °C, c. 3 d

Table 2. Ingredients of selective growth media.

Medium	Manufacturer
de Man, Rogosa and Sharp medium (MRS)	
MRS agar	Oxoid Ltd.
0.01% actidione	Sigma Aldrich
MRS sucrose agar	
2 % (w/v) sucrose	Dansukker
Trypticase soy agar (TSA)	Oxoid Ltd.
C-F-C pseudomonas agar	
500 ml:	
24.2 g pseudomonas agar base	Oxoid Ltd.
5 ml glycerol	
5 ml mixture of 50 % ethanol + sterile filtered water	Lab M
Chromocult	
1000 ml: 26.5 g Chromocult agar	Merck
Yeast Malt (YM) extract agar	Difico laboratories
0.01 % chloramphenicol	Sigma Chemical
0.01 chloramtetracycline	Sigma Chemical
0.02 Triton-X 100	Koch-Light Ltd, UK

Table 3. Reagents and raw materials.

Reagent	Manufacturer	City/ country
Phytase enzyme		
Food grade Phytase	Ultra Bio-Logics Inc.	Canada
Phytic acid determination		
Hydrochloric acid 1.0 mol/L (1.0N)	Oy FF-Chemicals Ab	
5-sulfosalicylic acid (dihydrate) M = 254.21 g/mol	Sigma Aldrich	India
Phytic acid (inositol hexaphosphoric acid) from corn	Sigma Chemical Co	USA
Iron(III) chloride, hexahydrate	Sigma Aldrich	Canada
Faba bean fermentations		
MRS[1)] broth	Oxoid	England
GEM[2)] + 2 % sucrose broth		
GEM broth		
Ringer's solution	Merck	Germany
Sucrose (Taloussokeri)	Dansukker	Finland
Exstrusion		
Dextran T2000	Pharmacosmos A/S	Denmark
Fructose	Danisco Sweeteners Oy	Finland

[1)] MRS = de Man, Rogosa and Sharp medium

[2)] GEM = General edible medium

Table 4. General edible medium (GEM), based on Saarela *et al.* (2004).

Composite	Manufacturer	L^{-1} of medium
Glucose (dextrose)	LAB M	20 g
Sucrose	Dansukker	20 g
Soypeptone	LAB M	30 g
Yeast extract	Difco	7 g
Magnesiumsulphate $7{\cdot}H_20$	Uniferm	1 g
0.01 M Potassium phosphate buffer pH 6.3		Up to 1000 ml

APPENDIX 2

Cell counts

Table 1. Growth of different microorganisms (CFU/g) in the faba bean matrix during the small scale fermentation series oh 24 h and 14 h at 25 °C. Results obtained as viable cell count.

	Spore forming		*Pseudomonas* spp.		Total coliforms		Yeast		Mould	
	0h	24h/ 14h	0h	24h/ 14h	0h	24h/ 14h	0h	24h/ 14h	0h	24h/ 14h
Spontaneous_N	250	<1000	767	1050000	1733	14400000	250	317	< 100	1525
Spontaneous_I	< 100	<1000	<100	<100	<100	< 1000	<100	< 1000	< 100	< 1000
*L. plantarum*_N	nd	<1000	nd	nd	nd	nd	nd	150	nd	575
*L. plantarum*_I	nd	<1000	nd	nd	nd	nd	nd	< 100	nd	< 100
*Lc. lactis*_N_EPS	nd	<1000	nd	<100	nd	nd	nd	267	nd	1000
*Lc. lactis*_I_EPS	nd	<1000	nd	<100	nd	nd	nd	< 100	nd	< 100
*Lc. lactis*_N_control	nd	< 1000	nd	<100	nd	nd	nd	533	nd	2000
*Lc. lactis*_I_control	nd	< 1000	nd	<100	nd	nd	nd	< 100	nd	< 100
*W. confusa*_14_I_EPS	nd	<1000	nd	<100	nd	< 10000	nd	200	ND	< 100
*W.confusa*_14_I_EPS	nd	<1000	nd	<100	nd	< 100	nd	< 100	ND	< 100
*W. confusa*_14_N_control	nd	1000	nd	<100	nd	> 300000000	nd	267	ND	< 100
*W. confusa*_14_I_control	nd	<1000	nd	<100	nd	< 10000	nd	< 100	ND	< 100
*W. confusa*_N_EPS	nd	<100	nd	15667	ND	18233	nd	250	ND	< 100
*W. confusa*_I_EPS	nd	<100	nd	<100	ND	< 100	nd	< 100	ND	< 100
*W. confusa*_N_control	nd	<100	nd	311667	ND	66950	633	533	150	100
*W. confusa*_I_control	nd	<100	nd	<100	ND	< 100	< 100	< 100	<1 00	< 100

Results are average of three independent fermentations (n=3). nd: not detected; ND: not determined. N: native faba bean flour; I: irradiated faba bean flour.

Viscosity diagrams and curves

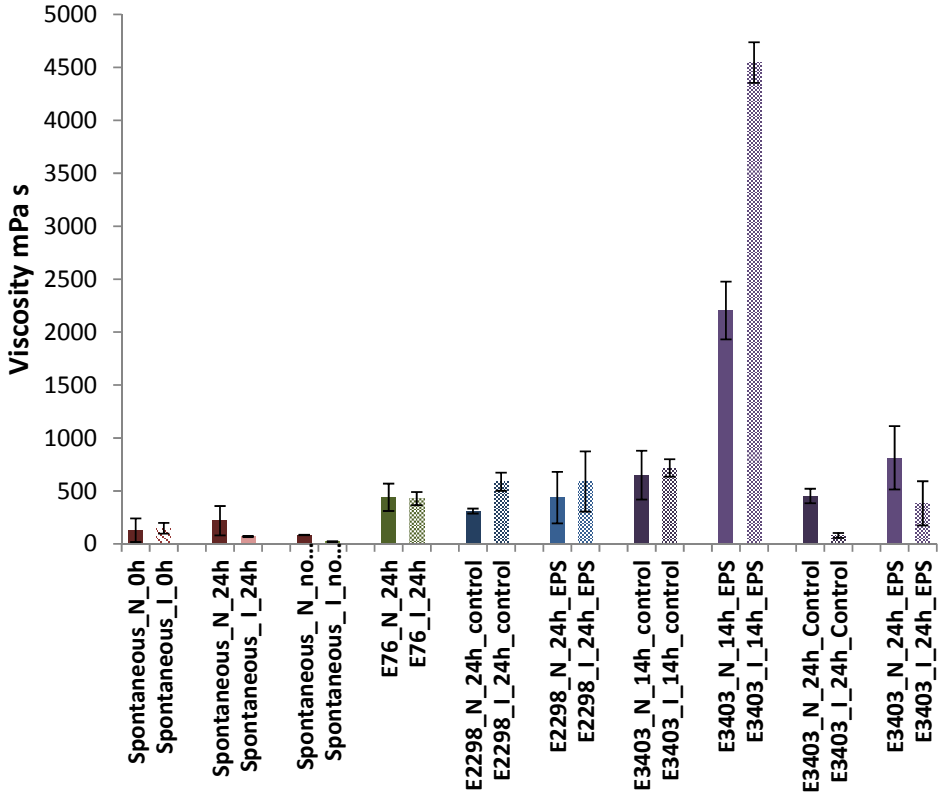

Figure 1. Viscosity of fermented faba bean samples from small scale series A-D. Results are mean values ± SD of individual triplicate analyses (n=3). N = native faba flour; I = Irradiated faba flour. EPS = 5 % (w.w) sucrose addition in the fermentation medium; Control = no added sucrose.

Table 1. Viscosity of fermented faba bean samples from large scale series.

Sample		**mPa s**	**Change (%)**
Medium 0h E2298		116.1	
Medium 0h E3403		180.5	
	average	**148.3**	
	SD	32.2	
E76 #3	a	665.2	
E76	b	588.4	
	c	451.9	
	average	**568.5**	283
	SD	108.0	
E2298_EPS #1	a	1999.9	
E2298_EPS	b	1994.8	
	average	**1997.4**	1247
	SD	2.6	
E2298_Control #1	a	1529.3	
E2298_Control	b	1390.8	
	c	1407.5	
	average	**1442.5**	873
	SD	75.6	
E3403_EPS #2	a	7498.0	
E3403_EPS	b	8224.8	
	c	6219.6	
	average	**7314.1**	4831
	SD	828.9	
E3403_Control #2	a	3573.7	
E3403_Control	b	3811.5	
	c	3571.9	
	average	**3652.4**	2363
	SD	112.5	

APPENDIX 3 (3/3)

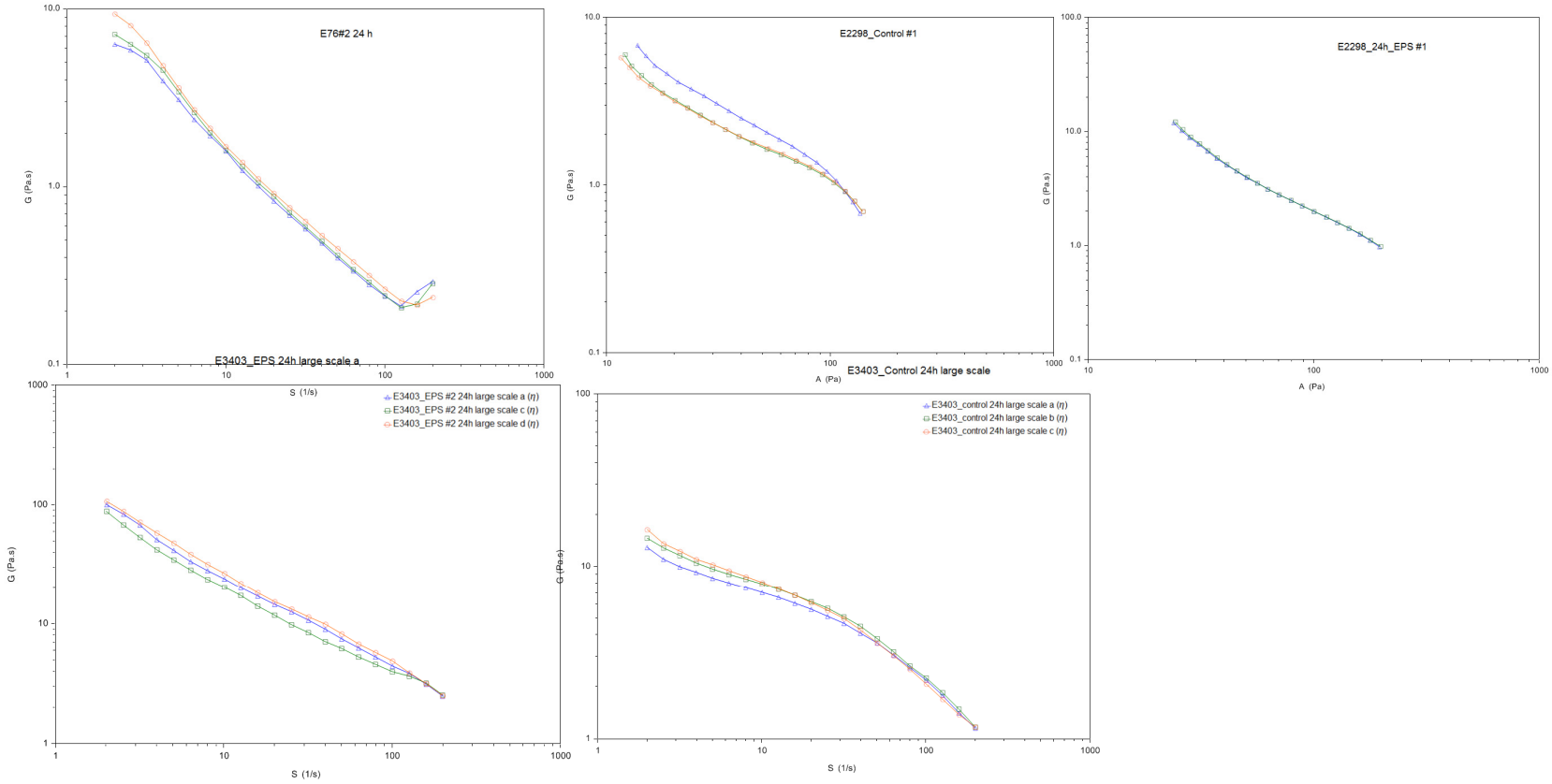

Figure 2. Viscosity curves of faba bean samples from large scale fermentations.

Monosaccharide content of fermented samples

Table 1. Monosaccharide content (% dm) of fermented samples from series B-D.

	Rhamnose + Arabinose	Galactose	Glucose	Mannose	Fructose
Native faba flour	nd	0.20 ± 0.01	1.22 ± 0.01	nd	0.01 ± 0.00
Radiated faba flour	nd	0.15 ± 0.01	1.23 ± 0.02	nd	0.01 ± 0.00
Spontaneous_N	0.02 ± 0.03	0.57 ± 0.14	2.61 ± 0.58	nd	0.74 ± 1.03
Spontaneous_I	0.03 ± 0.03	0.44 ± 0.06	3.57 ± 0.58	nd	1.15 ± 1.48
E76_N	0.01 ± 0.04	0.65 ± 0.02	0.53 ± 0.05	nd	0.03 ± 0.00
E76_I	0.09 ± 0.01	0.55 ± 0.03	0.83 ± 0.03	nd	0.03 ± 0.00
E2298_N_EPS	0.02 ±0.00	0.20 ± 0.03	1.22 ± 0.42	0.01 ± 0.00	4.13 ± 1.64
E2298_I_EPS	0.05 ± 0.04	0.76 ± 0.10	1.09 ± 0.67	0.01 ± 0.00	4.32 ± 1.70
E3403_N_EPS	0.02 ± 0.02	0.81 ± 0.02	0.08 ± 0.00	0.02 ± 0.01	10.56 ± 1.51
E3403_I_EPS	0.1 ± 0.00	0.95 ± 0.05	0.12 ± 0.11	0.02 ± 0.00	7.60 ± 1.29

Results are means ± SD of three individual fermentations.

Xylose was not detected.

Phytic acid content of fermented samples

Table 1. Phytic acid content of fermented samples from faba bean fermentation series.

	Phytic acid (mg/ g dm)	
Sample	native	irradiated
Small scale series [a]		
Spontaneous fermentation	4.25 ± 0.31	5.87 ± 0.56
E76	4.29 ± 0.27	4.34 ± 0.18
E2298_EPS	4.23 ± 0.10	4.47 ± 0.40
E2298_Control	3.09 ± 0.32	5.19 ± 0.16
E3403_EPS	4.87 ± 0.51	5.96 ± 0.90
E3403_Control	5.69 ± 0.55	4.76 ± 0.60
Large scale series [b]		
E76 #3	9.31 ± 0.40	
E2298_EPS #1	6.75 ± 0.16	
E2298_Control #1	8.45 ± 0.07	
E3403_EPS #2	8.03 ± 0.08	
E3403_EPS #1	5.09 ± 0.18	
E3403_Control	8.62 ± 0.05	
Phytase treated large scale	1.27 ± 0.12	

a results are average ± SD of duplicate analysis of three individual fermentation replicates

b results are average ± SD of triplicate analysis.

E76: L. plantarum; E2298: Lc. lactis; E3403: W. confusa.

EPS: sucrose added in the medium; Control: no sucrose added.

APPENDIX 6

Free amino acids (FAA) results

Table 1. Free amino acid composition (mg/kg) of fermented samples from large scale series and 6 h phytase treated faba bean flour.

	Native faba	***Lc. lactis* E2298 (EPS)**			***Lc. lactis* E2298 (Control)**			***L. plantarum* E76**	***W. confusa* E-3403 (EPS)**	***W. confusa* E-3403 (Control)**	**Phytase treated 6h, 25°C**
	Native faba	E2298_EPS #1	E2298_EPS #2	Average	E2298_Control #1	E2298_Control #2	Average	E76	E3403_EPS	E3403_Control	Phytase 6h
Asp	438	167	271	219	191	286	238	351	455	420	0
Thr	220	177	238	208	319	343	331	201	389	438	140
Ser	36	309	411	360	543	556	550	308	386	769	437
Glu	1577	1534	1486	1510	1847	1648	1748	1721	1391	1571	1260
Gly	159	253	307	280	377	380	379	339	328	493	298
Ala	174	213	430	321	384	559	471	117	496	481	386
Cys	92	174	306	240	292	441	367	111	343	427	239
Val	214	373	494	434	560	720	640	346	548	450	443
Met	16	69	115	92	87	153	120	15	103	78	67
Ile	21	108	172	140	235	285	260	100	259	296	121
Leu	29	255	400	328	413	740	577	227	406	427	165
Tyr	65	71	113	92	136	207	171	28	273	142	192
Phe	108	256	373	314	421	530	475	166	350	393	245
GABA	105	899	1108	1004	1097	1227	1162	1164	1082	1156	933
His	126	204	264	234	333	345	339	295	6	594	235
Trp	52	279	404	341	336	473	404	249	4	58	85
Orn	30	254	8	131	24	14	19	98	1074	1182	60
Lys	103	324	452	388	519	603	561	382	579	680	357
Amm	45	395	301	348	279	346	313	416	1315	1371	119
Arg	1994	1550	2096	1823	2273	2364	2318	2190	12	0	2197
Pro	205	301	361	331	461	499	480	373	597	872	366
Total	**5811**	**8166**	**10111**	**9138**	**11125**	**12718**	**11922**	**9198**	**10394**	**12300**	**8346**
Change		2355	4300	3328	5315	6907	6111	3388	4583	6489	2535
%		41	74	57	91	119	105	58	79	112	44

Extrusion parameters

Table 1. Summary of extrusion parameters within the trials.

Sample	Feed speeding	Water rate (ml/min)	Temperature	Screw speed (rpm)	Torque
Native 100 %	1.3 ± 0.1	2.25 ± 0.75	125-110-90-80	452-454	50-70
Native 50%	1.4	3.0	125-110-90-80	450	70-74
Native 25 %	1.4	2.5	125-110-90-80	449-450	76-83
E76 100 %	1.4	7.25 ± 1.75	125-110-90-80		
E76 50%	1.2 ± 0.2	4.5	125-110-90-80	450-452	70-81
E76 25%	1.4	5 ± 1	125-110-90-80	450-452	74-78
E2298_EPS 100%	1.4	3.75 ± 2.25	125-110-90-80	452-454	55-70
E2298_EPS 50%	1.3	5.5	125-110-90-80	450-456	63-70
E2298_EPS 25%	1.3	5.0	125-110-90-80	450-451	73-74
E2298_Control 100%	1.2	7.0	125-110-90-80	450-453	75-80
E2298_Control 50%	1.0	7.0	120-110-90-80	456	73
E2298_Control 25%					
E3403_EPS 100%	1.4	5.5	125-110-90-80	452-456	65-67
E403_EPS 50%	1.3	5.0	130-110-90-80	451-456	71-78
E3403_EPS 25%	1.3	5.0	130-120-90-80	450-452	73-79
E3403_Control 100%	1.4	6.0	130-110-90-80	450	68
E3403_Control 50%	1.4	5.5 ± 0.5	130-110-90-80	450-456	74-84
E3403_Control 25%	1.4	5.0	130-120-90-80	450	80

E76*: L. plantarum*; E2298: *Lc. lactis*; E3403: *W. confusa*.

EPS: sucrose added in the medium; Control: no sucrose added.

Statistical reports

Table 1. Post hoc test for PA analyses. Significant differences between enzyme activity units

Multiple Comparisons

Dependent Variable: PA

Tukey HSD

(I) Units		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	2	3,5158*	0,14458	0,000	3,1342	3,8973
	10	6,2395*	0,14669	0,000	5,8523	6,6266
	20	6,8903*	0,14903	0,000	6,4970	7,2835
2	0	-3,5158*	0,14458	0,000	-3,8973	-3,1342
	10	2,7237*	0,14669	0,000	2,3366	3,1108
	20	3,3745*	0,14903	0,000	2,9812	3,7678
10	0	-6,2395*	0,14669	0,000	-6,6266	-5,8523
	2	-2,7237*	0,14669	0,000	-3,1108	-2,3366
	20	,6508*	0,15108	0,000	0,2521	1,0495
20	0	-6,8903*	0,14903	0,000	-7,2835	-6,4970
	2	-3,3745*	0,14903	0,000	-3,7678	-2,9812
	10	-,6508*	0,15108	0,000	-1,0495	-0,2521

Table 2. Post hoc test for PA analyses. Significant differences between incubation time

Multiple Comparisons

Dependent Variable: PA

Tukey HSD

(I) Time		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	0,2600	0,12656	0,108	-0,0438	0,5637
	4	,5561*	0,12935	0,000	0,2457	0,8666
2	1	-0,2600	0,12656	0,108	-0,5637	0,0438
	4	0,2962	0,12802	0,061	-0,0111	0,6035
4	1	-,5561*	0,12935	0,000	-0,8666	-0,2457
	2	-0,2962	0,12802	0,061	-0,6035	0,0111

Table 3. Significant differences in macrostructure, type of fermentation as independent variable.

Multiple Comparisons

Tukey HSD

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
ER	Native	E76	28,8025*	4,64948	0,000	15,4916	42,1133
		E2298_EPS	51,5343*	5,10169	0,000	36,9288	66,1397
		E2298_Control	-42,4511*	6,42063	0,000	-60,8325	-24,0697
		E3403_EPS	83,2227*	4,25449	0,000	71,0427	95,4027
		E3403_Control	41,2304*	5,10169	0,000	26,6249	55,8358
	E76	Native	-28,8025*	4,64948	0,000	-42,1133	-15,4916
		E2298_EPS	22,7318*	5,19930	0,000	7,8469	37,6167
		E2298_Control	-71,2536*	6,49845	0,000	-89,8578	-52,6494
		E3403_EPS	54,4203*	4,37105	0,000	41,9065	66,9340
		E3403_Control	12,4279	5,19930	0,162	-2,4570	27,3128
	E2298_EPS	Native	-51,5343*	5,10169	0,000	-66,1397	-36,9288
		E76	-22,7318*	5,19930	0,000	-37,6167	-7,8469
		E2298_Control	-93,9854*	6,82931	0,000	-113,5368	-74,4340
		E3403_EPS	31,6885*	4,84930	0,000	17,8056	45,5714
		E3403_Control	-10,3039	5,60735	0,443	-26,3570	5,7492
	E2298_Control	Native	42,4511*	6,42063	0,000	24,0697	60,8325
		E76	71,2536*	6,49845	0,000	52,6494	89,8578
		E2298_EPS	93,9854*	6,82931	0,000	74,4340	113,5368
		E3403_EPS	125,6738*	6,22197	0,000	107,8612	143,4865
		E3403_Control	83,6815*	6,82931	0,000	64,1301	103,2329
	E3403_EPS	Native	-83,2227*	4,25449	0,000	-95,4027	-71,0427
		E76	-54,4203*	4,37105	0,000	-66,9340	-41,9065
		E2298_EPS	-31,6885*	4,84930	0,000	-45,5714	-17,8056
		E2298_Control	-125,6738*	6,22197	0,000	-143,4865	-107,8612
		E3403_Control	-41,9924*	4,84930	0,000	-55,8753	-28,1095
	E3403_Control	Native	-41,2304*	5,10169	0,000	-55,8358	-26,6249
		E76	-12,4279	5,19930	0,162	-27,3128	2,4570
		E2298_EPS	10,3039	5,60735	0,443	-5,7492	26,3570
		E2298_Control	-83,6815*	6,82931	0,000	-103,2329	-64,1301
		E3403_EPS	41,9924*	4,84930	0,000	28,1095	55,8753
Lsp	Native	E76	-26,8583*	2,24034	0,000	-33,2720	-20,4445
		E2298_EPS	-19,0651*	2,45824	0,000	-26,1027	-12,0275
		E2298_Control	-6,2844	3,09376	0,326	-15,1415	2,5726
		E3403_EPS	-33,0388*	2,05001	0,000	-38,9077	-27,1699
		E3403_Control	-36,7752*	2,45824	0,000	-43,8128	-29,7376
	E76	Native	26,8583*	2,24034	0,000	20,4445	33,2720
		E2298_EPS	7,7932*	2,50527	0,024	0,6210	14,9654
		E2298_Control	20,5738*	3,13126	0,000	11,6095	29,5382
		E3403_EPS	-6,1806*	2,10618	0,041	-12,2103	-0,1508
		E3403_Control	-9,9170*	2,50527	0,001	-17,0892	-2,7447
	E2298_EPS	Native	19,0651*	2,45824	0,000	12,0275	26,1027
		E76	-7,7932*	2,50527	0,024	-14,9654	-0,6210
		E2298_Control	12,7806*	3,29068	0,002	3,3598	22,2014
		E3403_EPS	-13,9738*	2,33662	0,000	-20,6632	-7,2843
		E3403_Control	-17,7102*	2,70188	0,000	-25,4453	-9,9750
	E2298_Control	Native	6,2844	3,09376	0,326	-2,5726	15,1415
		E76	-20,5738*	3,13126	0,000	-29,5382	-11,6095

		E2298_EPS	-12,7806*	3,29068	0,002	-22,2014	-3,3598
		E3403_EPS	-26,7544*	2,99803	0,000	-35,3373	-18,1714
		E3403_Control	-30,4908*	3,29068	0,000	-39,9116	-21,0700
	E3403_EPS	Native	33,0388*	2,05001	0,000	27,1699	38,9077
		E76	6,1806*	2,10618	0,041	0,1508	12,2103
		E2298_EPS	13,9738*	2,33662	0,000	7,2843	20,6632
		E2298_Control	26,7544*	2,99803	0,000	18,1714	35,3373
		E3403_Control	-3,7364	2,33662	0,600	-10,4258	2,9530
	E3403_Control	Native	36,7752*	2,45824	0,000	29,7376	43,8128
		E76	9,9170*	2,50527	0,001	2,7447	17,0892
		E2298_EPS	17,7102*	2,70188	0,000	9,9750	25,4453
		E2298_Control	30,4908*	3,29068	0,000	21,0700	39,9116
		E3403_EPS	3,7364	2,33662	0,600	-2,9530	10,4258
PD	Native	E76	6,4626*	1,65835	0,002	1,7150	11,2102
		E2298_EPS	0,7867	1,81964	0,998	-4,4227	5,9961
		E2298_Control	19,6600*	2,29007	0,000	13,1038	26,2162
		E3403_EPS	-3,4868	1,51747	0,197	-7,8311	0,8575
		E3403_Control	12,7799*	1,81964	0,000	7,5706	17,9893
	E76	Native	-6,4626*	1,65835	0,002	-11,2102	-1,7150
		E2298_EPS	-5,6759*	1,85446	0,028	-10,9849	-0,3668
		E2298_Control	13,1974*	2,31783	0,000	6,5618	19,8330
		E3403_EPS	-9,9494*	1,55904	0,000	-14,4127	-5,4861
		E3403_Control	6,3173*	1,85446	0,009	1,0083	11,6264
	E2298_EPS	Native	-0,7867	1,81964	0,998	-5,9961	4,4227
		E76	5,6759*	1,85446	0,028	0,3668	10,9849
		E2298_Control	18,8733*	2,43584	0,000	11,8998	25,8468
		E3403_EPS	-4,2735	1,72962	0,135	-9,2252	0,6782
		E3403_Control	11,9932*	2,00000	0,000	6,2675	17,7189
	E2298_Control	Native	-19,6600*	2,29007	0,000	-26,2162	-13,1038
		E76	-13,1974*	2,31783	0,000	-19,8330	-6,5618
		E2298_EPS	-18,8733*	2,43584	0,000	-25,8468	-11,8998
		E3403_EPS	-23,1468*	2,21922	0,000	-29,5001	-16,7935
		E3403_Control	-6,8801	2,43584	0,056	-13,8535	0,0934
	E3403_EPS	Native	3,4868	1,51747	0,197	-0,8575	7,8311
		E76	9,9494*	1,55904	0,000	5,4861	14,4127
		E2298_EPS	4,2735	1,72962	0,135	-0,6782	9,2252
		E2298_Control	23,1468*	2,21922	0,000	16,7935	29,5001
		E3403_Control	16,2667*	1,72962	0,000	11,3151	21,2184
	E3403_Control	Native	-12,7799*	1,81964	0,000	-17,9893	-7,5706
		E76	-6,3173*	1,85446	0,009	-11,6264	-1,0083
		E2298_EPS	-11,9932*	2,00000	0,000	-17,7189	-6,2675
		E2298_Control	6,8801	2,43584	0,056	-0,0934	13,8535
		E3403_EPS	-16,2667*	1,72962	0,000	-21,2184	-11,3151

Based on observed means.

The error term is Mean Square(Error) = 118,000.

*. The mean difference is significant at the ,05 level.

Table 4. Significant differences of macrostructure, addition level as independent variable.

Multiple Comparisons

Tukey HSD

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
ER	100 %	50 %	-58,7284*	3,54577	0,000	-67,0681	-50,3887
		25 %	-11,9309*	3,77256	0,005	-20,8040	-3,0578
	50 %	100 %	58,7284*	3,54577	0,000	50,3887	67,0681
		25 %	46,7975*	3,47587	0,000	38,6222	54,9728
	25 %	100 %	11,9309*	3,77256	0,005	3,0578	20,8040
		50 %	-46,7975*	3,47587	0,000	-54,9728	-38,6222
Lsp	100 %	50 %	3,5308	1,70852	0,098	-0,4876	7,5493
		25 %	23,9747*	1,81780	0,000	19,6992	28,2502
	50 %	100 %	-3,5308	1,70852	0,098	-7,5493	0,4876
		25 %	20,4439*	1,67484	0,000	16,5047	24,3831
	25 %	100 %	-23,9747*	1,81780	0,000	-28,2502	-19,6992
		50 %	-20,4439*	1,67484	0,000	-24,3831	-16,5047
PD	100 %	50 %	17,6129*	1,26468	0,000	14,6384	20,5875
		25 %	-9,0277*	1,34558	0,000	-12,1925	-5,8628
	50 %	100 %	-17,6129*	1,26468	0,000	-20,5875	-14,6384
		25 %	-26,6406*	1,23975	0,000	-29,5565	-23,7247
	25 %	100 %	9,0277*	1,34558	0,000	5,8628	12,1925
		50 %	26,6406*	1,23975	0,000	23,7247	29,5565

Based on observed means.
The error term is Mean Square(Error) = 118,000.
*. The mean difference is significant at the ,05 level.

Table 5. Significant differences of mechanical properties, addition level as independent variable.

Multiple Comparisons

Tukey HSD

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Ci	100 %	50 %	-0,0178	0,00967	0,158	-0,0405	0,0049
		25 %	,1076*	0,01011	0,000	0,0839	0,1314
	50 %	100 %	0,0178	0,00967	0,158	-0,0049	0,0405
		25 %	,1254*	0,00959	0,000	0,1029	0,1479
	25 %	100 %	-,1076*	0,01011	0,000	-0,1314	-0,0839
		50 %	-,1254*	0,00959	0,000	-0,1479	-0,1029
Fcr	100 %	50 %	-0,0096	0,16607	0,998	-0,3998	0,3806
		25 %	-3,8404*	0,17366	0,000	-4,2484	-3,4324
	50 %	100 %	0,0096	0,16607	0,998	-0,3806	0,3998
		25 %	-3,8308*	0,16464	0,000	-4,2176	-3,4440
	25 %	100 %	3,8404*	0,17366	0,000	3,4324	4,2484
		50 %	3,8308*	0,16464	0,000	3,4440	4,2176

Table 6. Significant differences in mechanical properties, type of fermentation as independent variable.

Multiple Comparisons

Tukey HSD

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Ci	Native	E76	-,0661*	0,01411	0,000	-0,1065	-0,0258
		E2298_EPS	-0,0370	0,01374	0,078	-0,0763	0,0023
		E2298_Control	-,2913*	0,01691	0,000	-0,3396	-0,2429
		E3403_EPS	,0855*	0,01205	0,000	0,0511	0,1200
		E3403_Control	,1181*	0,01481	0,000	0,0757	0,1604
	E76	Native	,0661*	0,01411	0,000	0,0258	0,1065
		E2298_EPS	0,0291	0,01417	0,312	-0,0114	0,0697
		E2298_Control	-,2251*	0,01726	0,000	-0,2745	-0,1758
		E3403_EPS	,1517*	0,01254	0,000	0,1158	0,1875
		E3403_Control	,1842*	0,01521	0,000	0,1407	0,2277
	E2298_EPS	Native	0,0370	0,01374	0,078	-0,0023	0,0763
		E76	-0,0291	0,01417	0,312	-0,0697	0,0114
		E2298_Control	-,2543*	0,01697	0,000	-0,3028	-0,2058
		E3403_EPS	,1225*	0,01212	0,000	0,0879	0,1572
		E3403_Control	,1551*	0,01487	0,000	0,1126	0,1976
	E2298_Control	Native	,2913*	0,01691	0,000	0,2429	0,3396
		E76	,2251*	0,01726	0,000	0,1758	0,2745
		E2298_EPS	,2543*	0,01697	0,000	0,2058	0,3028
		E3403_EPS	,3768*	0,01563	0,000	0,3321	0,4215
		E3403_Control	,4094*	0,01784	0,000	0,3584	0,4604
	E3403_EPS	Native	-,0855*	0,01205	0,000	-0,1200	-0,0511
		E76	-,1517*	0,01254	0,000	-0,1875	-0,1158
		E2298_EPS	-,1225*	0,01212	0,000	-0,1572	-0,0879
		E2298_Control	-,3768*	0,01563	0,000	-0,4215	-0,3321
		E3403_Control	0,0326	0,01332	0,143	-0,0055	0,0707
	E3403_Control	Native	-,1181*	0,01481	0,000	-0,1604	-0,0757
		E76	-,1842*	0,01521	0,000	-0,2277	-0,1407
		E2298_EPS	-,1551*	0,01487	0,000	-0,1976	-0,1126
		E2298_Control	-,4094*	0,01784	0,000	-0,4604	-0,3584
		E3403_EPS	-0,0326	0,01332	0,143	-0,0707	0,0055
Fcr	Native	E76	3,1460*	0,24225	0,000	2,4534	3,8385
		E2298_EPS	2,8626*	0,23593	0,000	2,1881	3,5372
		E2298_Control	3,9216*	0,29037	0,000	3,0915	4,7518
		E3403_EPS	0,2665	0,20688	0,792	-0,3249	0,8579
		E3403_Control	-0,5367	0,25431	0,283	-1,2637	0,1904
	E76	Native	-3,1460*	0,24225	0,000	-3,8385	-2,4534

	E2298_EPS	-0,2833	0,24336	0,854	-0,9791	0,4124
	E2298_Control	0,7757	0,29643	0,095	-0,0718	1,6232
	E3403_EPS	-2,8795*	0,21530	0,000	-3,4950	-2,2639
	E3403_Control	-3,6826*	0,26121	0,000	-4,4294	-2,9358
E2298_EPS	Native	-2,8626*	0,23593	0,000	-3,5372	-2,1881
	E76	0,2833	0,24336	0,854	-0,4124	0,9791
	E2298_Control	1,0590*	0,29129	0,004	0,2262	1,8918
	E3403_EPS	-2,5961*	0,20817	0,000	-3,1913	-2,0010
	E3403_Control	-3,3993*	0,25536	0,000	-4,1293	-2,6692
E2298_Control	Native	-3,9216*	0,29037	0,000	-4,7518	-3,0915
	E76	-0,7757	0,29643	0,095	-1,6232	0,0718
	E2298_EPS	-1,0590*	0,29129	0,004	-1,8918	-0,2262
	E3403_EPS	-3,6551*	0,26830	0,000	-4,4222	-2,8881
	E3403_Control	-4,4583*	0,30636	0,000	-5,3342	-3,5824
E3403_EPS	Native	-0,2665	0,20688	0,792	-0,8579	0,3249
	E76	2,8795*	0,21530	0,000	2,2639	3,4950
	E2298_EPS	2,5961*	0,20817	0,000	2,0010	3,1913
	E2298_Control	3,6551*	0,26830	0,000	2,8881	4,4222
	E3403_Control	-,8031*	0,22878	0,006	-1,4572	-0,1491
E3403_Control	Native	0,5367	0,25431	0,283	-0,1904	1,2637
	E76	3,6826*	0,26121	0,000	2,9358	4,4294
	E2298_EPS	3,3993*	0,25536	0,000	2,6692	4,1293
	E2298_Control	4,4583*	0,30636	0,000	3,5824	5,3342
	E3403_EPS	,8031*	0,22878	0,006	0,1491	1,4572