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Interaction of lupin and sunflower secondary plant metabolites with lactic acid- and bifidobacteria

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Peer-reviewed journals

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Oral presentations

C. Fritsch, R.F. Vogel, S. Toelstede (2014) Growth behavior of different lactic acid bacteria in lupin flour and lupin protein isolate. *Microbiology and Infection. 4. Gemeinsamer Kongress von DGHM und VAAM.* Dresden 5. – 8. Oktober 2014

C. Fritsch, R.F. Vogel, S. Toelstede (2015) Einfluss sekundärer Pflanzeninhaltsstoffe in Lupinenprodukten auf die Fermentationsleistung von Milchsäurebakterien. *15. Fachsymposium Lebensmittelmikrobiologie.* Freising 15. – 17. April 2015

C. Fritsch, V. Heinrich, R.F. Vogel, S. Toelstede (2015) Phenolic acid degradation ability of lactic acid bacteria in sunflower substrates. *9th World Congress on Polyphenols Application*. St. Julian's, Malta 3. – 5. Juni 2015

C. Fritsch, R.F. Vogel, S. Toelstede (2015) Fermentation pflanzlicher Rohstoffe zur Verbesserung der nutritiven Wertigkeit - mikrobieller Abbau von antinutritiven Inhaltsstoffen. *Jahrestagung Trend Vegan – Stand der Forschung*. Freising 27. – 28. Oktober 2015

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Abbreviations

APS	ammonium persulfate
ATCC	American Type Culture Collection
Bif.	*Bifidobacterium*
BLAST	Basic Local Alignment Tool
bp	base pair
CA	coumaric acid
CFA	caffeic acid
CGA	chlorogenic acid
CD	conductivity detection
cfu	colony forming units
CO_2	carbon dioxide
d	layer thickness
Da	Dalton
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E	absorbance
ε	extinction coefficient
E.	*Escherichia*
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
GC	guanine and cytosine
g (centrifugation)	relative centrifugal force
g (weight)	gram
GC-MS	gas chromatography with mass spectrometry
GMO	genetically modified organism
h	hour
H	hydrogen
His	histidin
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High Performance Liquid Chromatography
K_m	Michaelis-Menten constant
L.	*Lactococcus*
Lb.	*Lactobacillus*
LB	lysogeny broth
µ	micro

MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MC	methyl caffeate
MIC	minimum inhibitory concentration
min	minute
M*p*C	methyl trans-*p*-coumarate
MRS	de Man Rogosa Sharp medium
MV	methyl vanillate
N	cell count
N_0	initial cell count
Na	sodium
NaOH	sodium hydroxide
NCBI	national center for biotechnology information
Ω	ohm
OD	optical density
orf	open reading frame
P.	*Pediococcus*
PAD	pulsed amperometric detection
PAGE	polyacrylamide gel electrophoresis
*p*CA	*p*-coumaric acid
PCR	polymerase chain reaction
QA	quinic acid
s	second
S	Substrate concentration
SDS	sodium dodecyl sulfate
SPM	secondary plant metabolites
SOC	super optimal broth with catabolite repression
subsp.	Subspecies
t	time
TBE	tris-borate-EDTA
TEMED	tetramethylethylendiamine
TMW	Technische Mikrobiologie Weihenstephan
UV	ultra violet
V	volt
V_0	initial enzyme reaction velocity
V_{max}	maximum enzyme velocity
VA	vanillic acid
v/v	volume / volume
w/v	weight / volume

1 Introduction

1.1 Lactic acid- and bifidobacteria

Lactic acid bacteria are Gram-positive, non-spore forming, catalase negative, acid- and aerotolerant rods or cocci with a GC content below 55 mol%, belonging to the *Firmicutes.* The cells are nonmotile and require complex growth factors, such as vitamins, salts or amino acids. Under anaerobic conditions they produce lactic acid as a major end product of glucose (Krämer, 2007; Stiles and Holzapfel, 1997). The genus *Lactobacillus* can be divided into three groups with regard to their carbohydrate metabolism (Hammes and Vogel, 1995; Weber, 2010).

Group A: obligately homofermentative lactic acid bacteria metabolize hexoses almost exclusively to lactic acid via the Embden-Meyerhof-Pathway. Due to the absence of required enzymes, they cannot ferment pentoses.

Group B: facultatively heterofermentative bacteria are able to utilize pentoses as well as hexoses. Depending on the substrates, the metabolites are lactic acid or lactic and acetic acid.

Group C: obligately heterofermentative ferment hexoses and pentoses via the phosphogluconate pathway to lactic acid, CO_2, ethanol and/or acetic acid.

Lactic acid bacteria are found in plants, milk, meat, human tissue, gastrointestinal tract and other nutrient-rich habitats (Hammes and Vogel, 1995).

Bifidobacteria belong to the *Actinomycetes* and are Gram-positive, catalase negative, non-spore forming, nonmotile, anaerobic rods with a high GC content in the DNA (55 – 67 mol%). During fermentation of hexoses they produce lactic and acetic acid via the Bifidus pathway, also termed as fructose-6 shunt due to the characteristic enzyme fructose 6-phosphate phosphoketolase. Due to their origin in the human and animal gastrointestinal tract, they can utilize a broad range of indigestible carbohydrates, like oligo- and polysaccharides (Biavatti et al., 1991; Schleifer and Ludwig, 1995).

Certain strains of lactic acid- and bifidobacteria have probiotic activity. Probiotic bacteria are defined as “viable microorganisms that exhibit a beneficial effect on the health of the host upon ingestion by improving the properties of its indigenous microbiota”. Therefore, they are commonly used for the production of probiotic products with health-promoting properties (Gomes and Malcata, 1999; Klein et al., 1998; Prasad et al., 1998).

In the present work, four different lactic acid bacteria and one bifidobacterial strain were examined in detail for their fermentation performance, degradation ability of antinutritives and

tolerance against secondary plant metabolites in lupin and sunflower. These microorganisms were characterized more detailed below.

Lactobacillus plantarum

This strain was firstly described by Orla-Jensen in 1919 as *Streptobacterium plantarum.* The cells (3 – 8 µm x 0.9 – 1.2 µm) are facultatively heterofermentative with a GC content of 44 – 46 mol% and occur as single cells, in pairs or in short chains. The optimum growth conditions are in a temperature range between 30 and 40 °C and a pH value of 5 – 6. *Lactobacillus plantarum* can be found in plants and fermented food, like sauerkraut or olives (De Vos et al., 2009; Fuchs, 2007; Ulmer et al., 2000).

Lactobacillus gasseri

This obligately homofermentative lactic acid bacterial strain was discovered by Lauer and Kandler (1980). The rods (3.0 – 5.0 x 0.6 – 0.8 µm) have rounded ends, occur in chains or single and have a GC content of 33 – 35 mol%. They were isolated from human and animal intestinal tract, wounds, urine and blood. *Lactobacillus gasseri* is phylogenetically positioned near to the *Lactobacillus delbrueckii* group and displays probiotic activity (De Vos et al., 2009; Pedrosa et al., 1995).

Lactococcus lactis subsp. *lactis*

This microaerophilic, homofermentative bacterium was first mentioned in 1873 by Lister. It grows best at a neutral pH value under mesophilic conditions (10 – 40 °C) and has a GC content of 34.4 – 36.3 mol%. Typical habitats of *Lactococcus lactis* subsp. *lactis* are dairy products, like milk, butter milk, gouda or kefir. Synonyms for *Lactococcus lactis* subsp. *lactis* are ‘*Streptococcus diacetilactis’* or *‘Streptococcus lactis* subsp. d*iacetilactis’* (De Vos et al., 2009).

Pediococcus pentosaceus

The description of this strain by Mees dates back to 1934. *Pediococcus pentosaceus* is a homofermentative, facultatively anaerobic coccus (0.6 – 1.0 µm) with a GC content of 35 – 39 mol% in the DNA and an optimal growth temperature of 28 – 32 °C, at pH 6.0 – 6.5. It can be naturally found in plants, fruits and also in fermented plants, like silages, cucumbers or olives. Some strains were also isolated from the gastrointestinal tract of poultry and ducks (Back, 1978; De Vos et al., 2009).

Bifidobacterium animalis subsp. *lactis*

This heterofermentative bifidobacterium was first mentioned in 1997 by Meile et al. (1997). The irregularly shaped rods (0.9 – 1.2 x 0.4 – 0.6 µm) grow optimal under anaerobic conditions at 39 – 42 °C and have a CG content of 61 mol%. The strains were isolated from human and animal feces, fermented milk products and sewage. Due to its probiotic characteristics, *Bifidobacterium animalis* subsp. *lactis* is supplemented to several food products (Goodfellow et al., 2012; Meile et al., 1997).

1.2 Lupin

Lupin (*Lupinus* L.), a legume belonging to the *Fabaceae*, is cultivated since ancient times all over the world, predominantly for animal feed (van Barneveld 1999; Pisarikova and Zraly 2009). In the 20th century, the German botanist von Sengbusch made the use of lupin for human nutrition accessible due to the breeding of a variety which contained low levels of bitter and toxic alkaloids (Hanelt, 1960; von Sengbusch, 1942). According to the FAO, the highest cultivation area of lupin is located in Australia with 450,200 ha, followed by Poland (64,265 ha), Russia (27,058 ha), Chile (19,605 ha) and Germany with 17,400 ha (FAO, 2013). The most important groups of the several hundred lupin species are *Lupinus mutabilis* L. which is cultivated in the Andean region and *Lupinus angustifolius* L., *Lupinus luteus* L. and *Lupinus albus* L. which are originated in the Mediterranean area. The latter three species are mainly cultivated in Germany. Their cultivation is advantageous for the soil quality during intensive cultivation because lupin, in symbiosis with rhizobia, can assimilate nitrogen which is desired in crop rotation. The application of lupin seeds for food production is very promising due to the simple, GMO-free cultivation and its valuable composition. The seeds are low in fat (4 – 11% dry matter), high in protein (28 – 45% dry matter) and dietary fiber with a high proportion of the essential amino acid lysine (Martínez-Villaluenga et al., 2006; Petterson, 2004; Sujak et al., 2006; Wink, 1992, 2006). In the Mediterranean region, the whole lupin kernels are often consumed pickled as appetizer or snack. Due to their useful techno-functional properties, lupin protein isolates have been applied so far as food ingredients in pasta, crisps, ice cream and bakery products (D'Agostina et al., 2006; Petterson and Crosbie, 1990; Wäsche et al., 2001).

1.3 Sunflower

The cultivated sunflower (*Helianthus annuus* L.) belongs to the genus *Helianthus* which is part of the composite plants (*Asteraceae*) (Heiser, 1976). The first cultivation was reported in Arizona and New Mexico 3000 years before Christ. In 1569, sunflower was brought by the Spanish explorer Monardes to Europe, first as ornamental plant and then for food production

and medical application (Fick, 1989). Sunflower seeds constitute a basic plant material for oil products, foods and pet food. The main use of cultivated sunflower seeds is for oil production. The greatest areas can be found in Europe (16.9 million ha), the Russian Federation (6.8 million ha), Ukraine (5.1 million ha) and Argentina (1.6 million ha) (FAO, 2013). This leads to high amounts of by-products, mainly sunflower flour. Due to the high protein content which varies from 40 – 66% the sunflower flour is regarded as a promising alternative protein source for animal protein. The main applications are for infant formula, meat, milk, pasta and bakery products (Bau et al., 1983; Fick, 1989; González-Pérez and Vereijken, 2007; Kausar et al., 2004). Further advantages of sunflower seeds include their widespread availability, low concentrations of antinutritional factors and valuable amino acid composition (Gassmann, 1983; González-Pérez and Vereijken, 2007).

1.4 Secondary plant metabolites and antinutritives

'Secondary plant metabolites' (SPM) is a collective term for more than 100,000 different substances that are produced by plants during their secondary metabolism. In contrast to primary substances (carbohydrates, protein, fat), these substances are not essential for plants but act as antibodies or growth regulators (Leitzmann et al., 2009; Watzl, 2008). Humans have been using SPM for food or medicine since hundreds of years even though their biological and chemical background remained unknown for a long time. In 1806, Friedrich Wilhelm Sertürner was the first who successfully isolated a natural pure substance. He showed that the active principle of certain plants can be ascribed to single compounds (Croteau et al., 2000; Fang, 2012). Later in 1888, Ernst Stahl revealed that the SPM can have protective effects against herbivores. Since then, many research studies were placed in this field. Today it is known that plants synthesize them for adaption to their environment, e.g. as repellents, fragrance or attractant (Fang, 2012; Hartmann, 2007; Wink, 2003).

Due to their chemical structure and functional properties they are divided into the main groups: polyphenols, carotenoids, phytoestrogens, glucosinolates, sulfides, monoterpene, saponins, protease-inhibitors, phytosterols and lectins (DGE, 2014). For a long time, SPM were classified as harmful to health and were therefore termed as antinutritives. In numerous studies of the last decades several health-promoting effects were described. For example, it is assumed that flavonoids reduce the risk for cardiovascular diseases and colon cancer (Jin et al., 2012; Kay et al., 2012; Woo and Kim, 2013). Moreover, a relation between an increased uptake of carotenoids and the reduced occurrence of cancer and vascular changes was observed (Eliassen et al., 2012). However, the advantageous effects could not be ascribed reliably to SPM because their intake is influenced by individual lifestyle factors and nutritional diseases. Some substances are still regarded as antinutritives due to their

harmful effects on health, like the toxic solanin in potatoes or hydrocyanic acid in bitter almonds (DGE, 2014; Leitzmann et al., 2009). Besides, they can have disadvantageous effects on sensory, nutritional value and product quality of foods. Due to their role as natural repellents, many substances have a bitter taste which is rejected by consumers (Streit et al., 2007). Their interaction with food ingredients can lead to a reduced bioavailability of proteins, vitamins or minerals. These complexes can also reduce the protein solubility or alter the color of the products (Bau et al., 1983; Cater et al., 1972; Graf et al., 1987).

1.4.1 Secondary plant metabolites in lupin

The direct application of lupin raw materials for human consumption is limited due to the content of several SPM with antinutritives effects, e.g. oligosaccharides or phytic acid (Macrae and Zand-Moghaddam, 1978; Trugo et al., 1988). Oligosaccharides, also known as α-galactosides or raffinose family oligosaccharides are carbohydrates with three to ten monosaccharides, linked by glycosidic bonds. In spite of their prebiotic properties with beneficial health effects, some oligosaccharides (e.g. raffinose, stachyose) are regarded as antinutritives because they are fermented by the microbiota in the large intestine, causing abdominal discomfort and flatulence. This is due to the absence of α-galactosidase in human body (Gibson and Roberfroid, 1995; Gitzelmann and Auricchio, 1965; Rackis et al., 1970). Since in lupin more than half of the total carbohydrates are oligosaccharides, this is of special interest (Camacho et al., 1991). The main oligosaccharides in lupin are raffinose (α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside), stachyose (α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) and verbascose (α-D-galactopyranosyl-(1→6)-[α-D-galactopyranosyl-(1→6)]$_2$-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside). The chemical structures are shown in Figure 1.

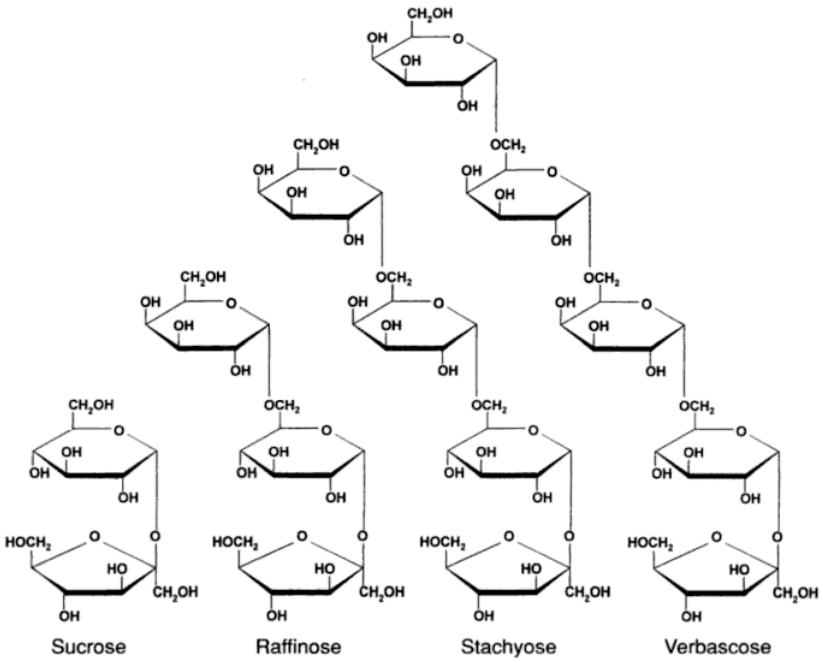


Figure 1: Structure of sucrose and oligosaccharides (Hedley, 2001)

Phytic acid (*myo*-inositol hexakisphosphate) is the major storage form of phosphorus in plants. Depending on the cultivar, lupin seeds contain 0.4 – 4.9% phytic acid (Camacho et al., 1991; Fritsch, Vogel, et al., 2015; Trugo et al., 1993). Its intake leads to a reduced bioavailability of proteins, vitamins and minerals like Ca^{2+}, Fe^{2+}, Mg^{2+} and Zn^{2+} due to its complex-forming ability. Possible interactions with different food ingredients are shown in Figure 2. Phytic acid is enzymatically degraded by phytases (*myo*-inositol hexaphosphate hydrolase) to lower *myo*-inositol-phosphates or *myo*-inositol which can have beneficial effects on health, like anticarcinogenic or antioxidant activity (Dvořáková, 1998; Graf et al., 1987; Shamsuddin, 1995; Wodzinski and Ullah, 1996). Beside the enzymatic pathway, degradation might occur during processing, like cooking, soaking, germination, fermentation or extraction. During cooking in boiling water for 30 – 45 min a slight phytate reduction can be achieved (Egounlety and Aworh, 2003; Reddy et al., 1978). More effective is an extraction in an alkaline milieu, followed by precipitation with calcium and barium ions and centrifugation (McKinney et al., 1949).

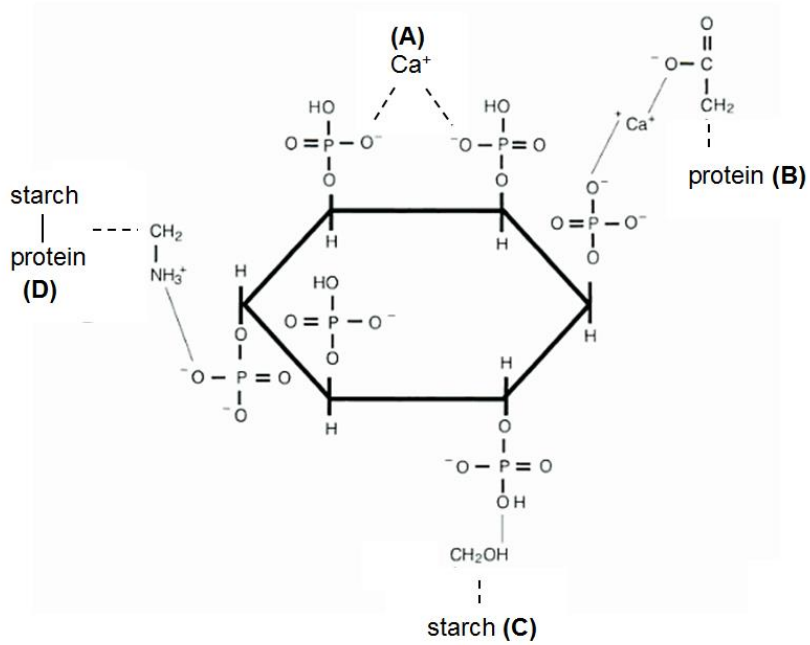
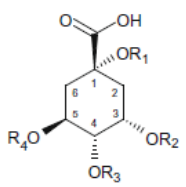


Figure 2: Possible interactions of phytic acid with calcium (A), protein (B), starch (C) and protein-starch-complex (D). Modified according to Thompson (1988)

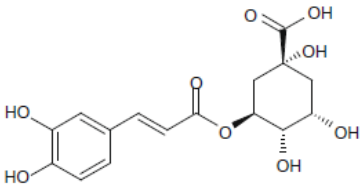
Another class of SPM in lupin is represented by quinolizidine alkaloids. Due to their toxic effects, alkaloid rich cultivars are not suitable for human nutrition. Meißner and Wink (1992) identified more than 200 different alkaloids in North American lupin varieties. The most common alkaloids are lupanin, lupinine, sparteine, α-isolupanine and 13-hydroxylupanin. However, concentrations in sweet lupin cultivars reach maximum levels of up to 0.02%. Therefore, they can be consumed without additional processing (Muzquiz et al., 1994; Wink, 1984). Further SPM in lupin are protease-inhibitors, especially trypsin-inhibitors which are typical for legumes. They consist of 100 – 200 amino acids and decrease the activity of proteases. As a consequence the resorption of nutrients is reduced and a deficiency of amino acids can occur. Therefore, they are regarded as antinutritives (Leitzmann et al., 2009). Saponins and tannins constitute SPM of lupin with antinutritional but also healthy action, depending on the dose. Saponins can have hemolytic and toxic effects but they are also described to show anticarcinogenic, antimicrobial, anti-inflammatory and cholesterol-lowering properties (Watzl and Leitzmann, 2005). Petterson and Fairbrother (1996) analyzed 442 – 740 mg/kg saponins in the Australian sweet lupin what is less than found in other legumes, like pea or horse bean. Similarly, tannin uptake leads on the one hand to an inhibition of digestive enzymes but on the other to a lowering of the blood glucose level. As sweet lupin varieties contain only 0.01% of condensed tannins, this can be neglected (Champ, 2002; Petterson, 1998). The isoflavones in lupin (genistein and daidzein) are described to function as anticarcinogens and antioxidants (DGE, 2014).

1.4.2 Secondary plant metabolites in sunflower

Sunflower seeds contain only low amounts of antinutritives like protease inhibitors, cyanogens or glucosinolates. They consist of approximately 3.2% indigestible oligosaccharides, especially raffinose and stachyose (Canella et al., 1984; Fritsch, Heinrich, et al., 2016; Kuo et al., 1988). Sunflower substrates are rich in phenolic compounds which amount up to 4% (Leung et al., 1981; Pedrosa et al., 2000; Weisz et al., 2009). All phenolic compounds are based on the structure of phenol and can be divided into seven subclasses: simple phenols, hydroxybenzoic acids, hydroxycinnamic acids, coumarins, flavonoids, lignans and lignins (Watzl and Leitzmann, 2005). The predominant compound in sunflower with 70% of the total phenolic compounds is chlorogenic acid (5-*O*-caffeoylquinic acid), an ester of caffeic and quinic acid which has a widespread occurrence in fruits and vegetables. The concentration of chlorogenic acid with 23 – 33 g/kg dry matter in sunflower kernels is distinctly higher than the amounts in apple (0.2 g/kg), potato (1.2 g/kg) or other vegetables (Clifford, 1999; Sabir, Sosulski and Kernan, 1974; Weisz et al., 2009). This high content can negatively affect the protein solubility and color of the sunflower substrates due to the formation of orthoquinones. These are converted into brown polymers under alkaline conditions. Besides, the irreversible linkage of chlorogenic acid to proteins can lead to dark-colored products (Bau et al., 1983; Cater et al., 1972; Gassmann, 1983; Prigent, 2005; Rawel et al., 2002; Saeed and Cheryan, 1989). Further phenolic compounds in sunflower are represented by non-esterified phenolic acids (quinic acid, caffeic acid), coumaric and ferulic acid derivates (5-*O*-*p*-coumaroylquinic acid, 5-*O*-feruloylquinic acid) and dicaffeoylquinic acids (3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid) which are depicted in Figure 3 (Weisz et al., 2009).



R1, R2, R3 and R4 = H: quinic acid



5-*O*-caffeoylquinic acid

Name and abbreviation	R_1	R_2	R_3	R_4
3-*O*-caffeoylquinic acid (3-CQA)	H	C	H	H
5-*O*-caffeoylquinic acid (5-CQA)	H	H	H	C
4-*O*-caffeoylquinic acid (4-CQA)	H	H	C	H
5-*O*-*p*-coumaroylquinic acid	H	H	H	*p*Co
5-*O*-feruloylquinic acid	H	H	H	F
3,4-di-*O*-caffeoylquinic acid (3,4-diCQA)	H	C	C	H
3,5-di-*O*-caffeoylquinic acid (3,5-diCQA)	H	C	H	C
4,5-di-*O*-caffeoylquinic acid (4,5-diCQA)	H	H	C	C

Figure 3: Structures of phenolic acids in sunflower seeds (Weisz et al., 2009)

Recent studies focused on the bioavailability of phenolic compounds and the health effects of free phenolic acids. Bioavailability is defined as the amount of a nutrient that is digested, resorbed and metabolized by physiological metabolic pathways (Forbes and Erdman, 1983). It was reported that free phenolic acids can be easily absorbed in the upper digestive tract of humans while esterified phenolic acids, like chlorogenic acid, are not absorbed efficiently. These substances are hydrolyzed in the colon by the microbiota, followed by the resorption of the respective metabolites (Konishi and Kobayashi, 2005; Olthof et al., 2001; Stalmach et al., 2010). Free caffeic acid was described to have antioxidant and anticarcinogenic effects and might stimulate the insulin secretion (Clifford, 2000; Oboh et al., 2015; Ong et al., 2013; Srinivasan et al., 2007). Sunflower seeds are also a source of tocopherol which has antioxidant properties (Franke et al., 2010; Watzl and Leitzmann, 2005).

1.5 Fermentation

Fermented foods are defined as products with characteristic properties that result from the microbial metabolism (Hammes, 1990). Fermentation can be traced back to prehistoric times and was mainly used for preservation (alcoholic, acetic acid and lactic acid fermentation). With the knowledge of Pasteur, that different microorganisms are responsible for fermentation, it was desired to find suitable microorganisms for food fermentation. In 1883, Christian Hansen was the first who successfully isolated special yeasts for brewing

processes. The use of starter cultures for fermentation processes can be divided into the following groups: ‘spontaneous/natural fermentation’ that comprise of the microbiota of the raw material or environment, ‘single-strain cultures’ that contain one defined microorganism and ‘multiple-strain cultures’ which consist of several defined strains. ‘Back-slopping’ is also a procedure which is used in food fermentation and is characterized by inserting a previous fermented food to a new batch (Buckenhüskes, 1993). In Europe, typical foods that derived from fermentation are sauerkraut, coffee, cacao, wine, beer or soy sauce. Milk or milk products are frequently fermented to curdled milk, yoghurt, butter or cheese. Bacteria, especially lactic acid bacteria and yeasts are most commonly used for fermentation in Europe, whereas fungi are more often applied in Asia or Africa. Fermented food plays an important role in the human diet as 25% of nutrition in Europe and 60% in developing countries are represented by fermented foods (Holzapfel et al., 1995; Steinkraus, 1983).

Fermentation of vegetables is mostly applied to enhance the sensory, change the texture or increase the digestibility and nutritional value of the product (Buckenhüskes, 1993; Gomes and Malcata, 1999; Hammes, 1990; Reddy and Pierson, 1994). Cereals like rye and wheat are often fermented by lactic acid bacteria and yeasts to sourdough to improve the flavor, rheology and nutritional characteristics of the breads. In the last decades, an increasing number of plants were used for sourdough production. Especially gluten-free cereals, like buckwheat, sorghum or amaranth are of great interest for people with celiac disease and for consumers following a gluten-free diet (Arendt et al., 2011; Moroni et al., 2011). Legumes represent another promising fermentation substrate as most varieties contain high amounts of protein and thus may represent an alternative source for animal or milk protein. The demand for new plant protein sources is increasing due to a rising number of people adopting a vegetarian, vegan or lactose-free diet (Jiménez-Martínez et al., 2003). Fermentation of lupin substrates has been mostly carried out with the purified protein isolate which contains low concentrations of SPM (Lampart-Szczapa et al., 2006; Schindler et al., 2011), whereas little information is available on the fermentation of lupin seed flour (Bartkiene et al., 2015; Camacho et al., 1991; Jul et al., 2003). Most of these studies did not include control samples in their experiments in order to get a reliable correlation of changes in SPM concentration with the microbial metabolism. Therefore, only scarce research information exists about the interaction of SPM and antinutritives in lupin raw materials with microorganisms.

Similarly, only little information is available about sunflower fermentation. Canella et al. (1984) conducted a spontaneous fermentation of germinated sunflower flour. Another study used sunflower hull hydrolysates for fermentation with *Pichia stipites* for ethanol production (Telli-Okur and Eken-Saraçoğlu, 2008). No lactic fermentation of sunflower flour or sunflower protein has been published so far. In contrast, fermentation of other oilseed plants, like soy,

peanut or sesame was already described by several researchers (Beuchat, 1976; Elfaki et al., 1991; Liong et al., 2009; Mital et al., 1974). In case of soy, fermentation is traditionally used in Asia for a long time, e.g. for the production of tempeh, an Indonesian food product which is fermented by molds (Steinkraus et al., 1960).

1.5.1 Inhibitory effects of plant substances on microorganisms

As mentioned above, several SPM are described to have antimicrobial properties. Some of these compounds are contained in lupin and sunflower, complicating the fermentation of these raw materials. Likewise, a lack of required nutrients or unappropriated growth conditions (e.g. pH value, redox potential) can prevent the suitability of plant substances as fermentation substrates. In lupin the most effective SPM against bacteria and fungi are alkaloids. The whole alkaloid extract of *Lupinus angustifolius* showed antimicrobial potential against *Bacillus subtilis, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Total growth inhibition of six bacteria (*Serratia marcescens, Bacillus megaterium, Bacillus subtilis, Streptococcus viridis, Micrococcus luteus, Mycobacterium phlei*) was observed in presence of the alkaloid sparteine in a concentration of 20 mmol/l (Erdemoglu et al., 2007; Wink, 1984).

In sunflower substrates, the major compounds with inhibitory potential are represented by esterified hydroxycinnamic acids, mainly chlorogenic acid, as well as free phenolic acids (caffeic acid, quinic acid). Chlorogenic acid is active against a wide range of bacteria, including pathogens like *Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Bacillus subtilis* as well as lactic acid bacteria (*Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus hammesii*) (Lou et al., 2011; Parkar et al., 2008; Sanchez-Maldonado et al., 2011). Similar, several researches measured an antimicrobial effect of the hydroxycinnamic acids caffeic, coumaric and ferulic acid against *Lactobacillus brevis, Lactobacillus plantarum* and *Lactobacillus rhamnosus* (Parkar et al., 2008; Sanchez-Maldonado et al., 2011; Stead, 1993). The mechanism of action was elucidated furthest for chlorogenic acid, indicating that an increased permeability of the plasma membrane is responsible for the loss of barrier functions. As a consequence, the membrane potential cannot be maintained and several cytoplasm macromolecules, including nucleotides, get lost (Lou et al., 2011).

Other studies examined influencing factors on the antimicrobial activity, like chemical structure (number and position of substitution in the benzene ring, double bonds, length of alkyl chain), pH value or esterification degree. It was reported that phenolic acids are less effective than their methyl or butyl esters and that the antimicrobial potential increased at lower pH values. Moreover, a higher inhibitory potential was measured with an increasing

length of the alkyl chain. The replacement of hydroxyl groups by methoxy groups leads to an enhanced antimicrobial activity of hydroxybenzoic acids. Whereas this effect could not be observed for hydroxycinnamic acids (Cueva et al., 2010; Merkl et al., 2010; Sanchez-Maldonado et al., 2011).

1.5.2 Microbial degradation of secondary plant metabolites

In spite of the antimicrobial effects of some SPM, several microorganisms possess the ability to degrade these substances. Of special interest has been the microbial degradation of oligosaccharides due to their antinutrional nature and their ubiquitous occurrence in plant based food. For lactobacilli that are used as fermentation strains, this ability is of high importance because oligosaccharides are the main carbohydrates in milk, cereals, legumes and fruits. The degradation ability is depending on the presence of specific enzymes, either intracellular or extracellular, hydrolyzing the glycosidic bonds in oligosaccharides, e.g. α-galactosidases, β-galactosidases or levansucrase. It was reported that strains of *Lactobacillus plantarum, Lactobacillus cellobiosis, Lactobacillus buchneri, Lactobacillus reuteri, Leuconostoc mesenteriodes* or *Bifidobacterium lactis* are potential microorganisms for an oligosaccharide degradation (Gänzle and Follador, 2012; Martínez-Villaluenga and Gómez, 2007; Mital et al., 1974; Teixeira et al., 2012; Yoon and Hwang, 2008). Two possible pathways of oligosaccharide metabolism in lactobacilli are shown in Figure 4. On the one hand, the extracellular levansucrase cleaves the glycosidic bond between fructose and glucose, resulting in α-galctooligosaccharides (e.g. melibiose). These molecules are transported into the cell and hydrolyzed by the intracellular α-galactosidase to monosaccharides. The other pathway starts with an oligosaccharides uptake, followed by α-galactosidase activity, leading to sucrose which is degraded by sucrose phosphorylase. This metabolism was also described in bifidobacteria (Kullin et al., 2006; Teixeira et al., 2012; Trindade et al., 2003).

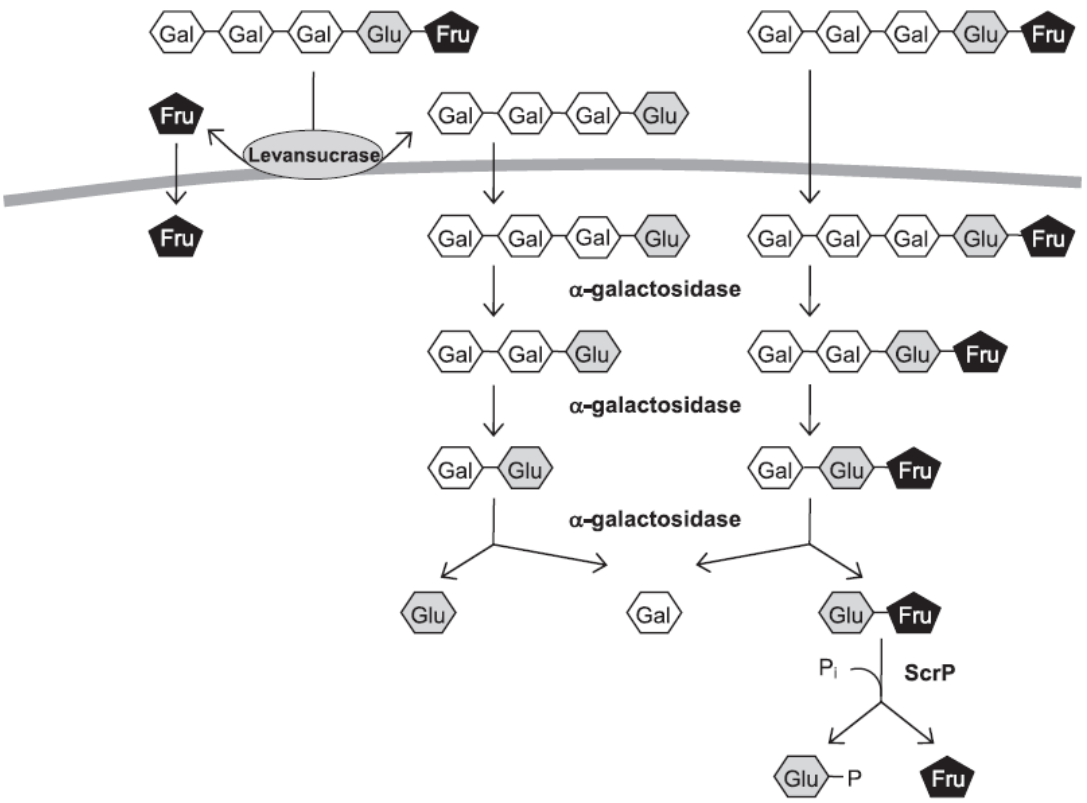


Figure 4: Metabolic pathways of verbascose utilization in *Lactobacillus reuteri* LTH5448 (Teixeira et al., 2012)

The degradation of phytic acid is caused by an enzymatic activity, in particular by phytases (3-phytase, 6-phytase) which hydrolyze phytic acid to several lower phosphoric esters of *myo*-inositol and inorganic phosphorus. They are produced by a wide range of plants, yeasts, fungi and bacteria, whereas 3-phytase is characteristic for microorganisms and 6-phosphatase for plants. Microorganisms with phytase activity include *Lactobacillus reuteri, Lactobacillus salivarius, Lactobacillus buchneri, Pediococcus pentosaceus* and *Bifidobacterium dentium* (Fischer et al., 2014; Palacios et al., 2008; Reddy and Pierson, 1994; Silva and Trugo, 1996).

A microbial cleavage of phenolic compounds was also ascribed to endogenous enzymes: phenolic acid decarboxylases were reported in different *Lactobacillus plantarum* strains which are able to cleave free phenolic acids like caffeic acid, ferulic acid or *p*-coumaric acid (Barthelmebs et al., 2000; Cavin et al., 1997; Rodríguez, Landete, Curiel, et al., 2008; Rodríguez, Landete, Rivas, et al., 2008). Quinic acid is converted by the quinate dehydrogenase to 3-dehydroquinate, found in *Gluconobacter oxydans* or *Lactobacillus pastorianus* (Adachi, Tanasupawat, et al., 2003; Carr et al., 1957). For the degradation of esterified phenolic acids cinnamoyl esterases, also termed as feruloyl esterases or ferulic acid esterases, were identified as the responsible enzymes. They hydrolyze ester bonds,

leading to free phenolic acids and were found in different bacteria and fungi, e.g. *Bacillus subtilis, Lactobacillus fermentum, Lactobacillus plantarum, Penicillium pinophilum* or *Aspergillus niger* (Castanares et al., 1992; Donaghy et al., 1998; Faulds and Williamson, 1991; Faulds and Williamson, 1993; Mackenzie et al., 1987). The presence of cinnamoyl esterases in bifidobacteria was so far only examined by Raimondi et al. (2015). However, the researchers did not further characterize the purified enzyme with respect to the substrate specificity or the biochemical properties. The hydrolysis of chlorogenic acid into caffeic acid and quinic acid is shown in Figure 5.

It is assumed that a relationship between the antimicrobial potential of phenolic acids and their metabolism by lactobacilli exist. Sanchez-Maldonado et al. (2011) suggested that the metabolism of phenolic compounds contributes to the detoxification of noxious substances because the cleavage products had lower antimicrobial activities. The researchers also observed that the sensitivity to phenolic substances correlated with the degradation ability. The metabolic pathways to utilize phenolic compounds provide another benefit for the microorganisms, because substances for energy generation are released (Gänzle, 2014).

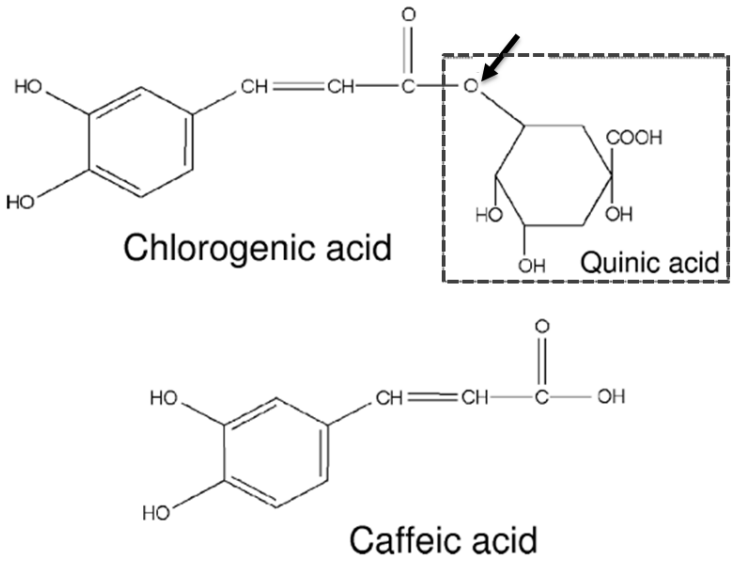


Figure 5: Structure of chlorogenic acid and the cleavage products quinic acid and caffeic acid. The arrow indicates the esterase bond hydrolyzed by cinnamoyl esterases (Guglielmetti et al., 2008)

1.6 Objectives of the study

The general objectives of this thesis were to investigate the suitability of lupin and sunflower raw materials for lactic fermentation and to examine if the nutritional value and product quality can be improved by fermentation. On the one hand, it should be evaluated if the secondary plant metabolites in lupin and sunflower have inhibitory effects on the selected fermentation strains. On the other, it should be investigated if the microorganisms have the competence to degrade undesired secondary plant metabolites in the different plant materials. The microbial degradation mechanism of chlorogenic acid should be elucidated using molecular biological methods, including heterologous gene expression, purification and biochemical characterization of the responsible enzyme.

The results obtained in this study are meant to broaden the knowledge about the fermentation performance of lactic acid- and bifidobacteria in plant substrates. With this, the application of suitable fermentation strains and the fermentation design will be facilitated, leading to improved products.

2 Materials and Methods

2.1 Research material and chemicals

Seeds of the lupin cultivar *Lupinus angustifolius* cv. *Boregine* were obtained from Saatzucht Steinach (Steinach, Germany). Due to the trace amounts of alkaloids, it was named 'sweet lupin' throughout this study. The alkaloid rich cultivar *Lupinus angustifolius* cv. *Azuro* ('bitter lupin') was provided by Feldsaaten Freudenberger (Krefeld, Germany). Both seeds were dehulled and milled to a particle size of 0.5 mm using a Retsch ZM-100 ultra centrifugal mill (Düsseldorf, Germany). The protein isolate was produced according to Wäsche et al. (2001). For the fermentation study of sunflower substrates, sunflower seeds were purchased from Goldene Mühle (Garrel, Germany). The seeds were processed in a 1.5 m^3 pilot plant percolator (e&e Verfahrenstechnik, Warendorf, Germany) according to Pickardt et al. (2009) to obtain a defatted sunflower flour. For the production of a sunflower protein concentrate, press cake from dehulled sunflower seeds was gently defatted with hexane and the derived defatted flour was extracted with an aqueous-alcoholic solution (70% ethanol).

All chemicals used in this study are listed in Table 1.

Table 1: Chemicals

Chemical	Purity	Supplier
Acetic acid	p.a.	Th. Geyer, Renningen, Germany
Acetonitrile	HPLC grade	Th. Geyer, Renningen, Germany
Acrylamide	30% (w/v)	Serva, Heidelberg, Germany
Ammonium persulfate (APS)	analytical grade	Serva, Heidelberg, Germany
Ampicillin sodium salt	93.3%	Gerbu Biotechnik GmbH, Heidelberg, Germany
L-Arabinose	> 99%	Roth, Karlsruhe, Germany
Barium chloride dihydrate	≥ 99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
BioRadX	-	BioRad, Hercules, CA, USA
Boric acid	≥ 99.5%	Roth, Karlsruhe, Germany
Bovine serum albumin	≥ 96%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Bromphenol blue	-	Merck, Darmstadt, Germany
Caffeic acid	≥ 98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Casein peptone	for microbiology	Merck, Darmstadt, Germany
Chlorogenic acid	≥ 95%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Citric acid	≥ 99.5%	Roth, Karlsruhe, Germany
Coomassie brilliant blue	-	Roth, Karlsruhe, Germany
L-Cysteine hydrochloride	≥ 98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
DMSO	≥ 99%	Merck, Darmstadt, Germany

EDTA	for molecular biology	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Ethanol	p.a.	Th. Geyer, Renningen, Germany
Ethidium bromide	1% in H_2O	Merck, Darmstadt, Germany
Ethyl acetate	p.a.	Th. Geyer, Renningen, Germany
Ethyl ferulate	98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Ferulic acid	> 99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Formic acid	99 – 100% p.a.	Th. Geyer, Renningen, Germany
Galactose	> 98%	Roth, Karlsruhe, Germany
Glucose	anhydrous	Th. Geyer, Renningen, Germany
Glycerin	87%	Gerbu Biotechnik GmbH, Heidelberg, Germany
Hexane	p.a.	Th. Geyer, Renningen, Germany
H_3PO_4	85%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Hydrochloric acid	37%	Merck, Darmstadt, Germany
Imidazole	≥ 99.5%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
K_2HPO_4	> 99%	Merck, Darmstadt, Germany
Meat extract	for microbiology	Merck, Darmstadt, Germany
2-Mercaptoethanol	99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Methanol	HPLC grade	Th. Geyer, Renningen, Germany
Methyl caffeate	analytical standard	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Methyl trans-*p*-coumarate	> 98%	TCI Deutschland, Eschborn Germany
Methyl vanillate	99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
$MgSO_4$ x 7 H_2O	≥ 99%, p.a.	Roth, Karlsruhe, Germany
$MnSO_4$ x H_2O	≥ 99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
NaH_2PO_4	≥ 98%	Roth, Karlsruhe, Germany
Na_2HPO_4	p.a.	Merck, Darmstadt, Germany
$(NH_4)_2$ citrate	≥ 99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Perchloric acid	70%	Th. Geyer, Renningen, Germany
Phytic acid sodium salt hydrate	-	Sigma-Aldrich Inc., St. Louis, Missouri, USA
p-coumaric acid	≥ 98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
p-Nitrophenol	analytical standard	Sigma-Aldrich Inc., St. Louis, Missouri, USA
p-Nitrophenol acetate	-	Sigma-Aldrich Inc., St. Louis, Missouri, USA
p-nitrophenyl butyrate	≥ 98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
p-nitrophenyl caprylate	≥ 94%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Quinic acid	98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Raffinose	> 98%	Roth, Karlsruhe, Germany
Ringer solution	-	Thermo Fisher Scientific, Waltham, USA
Sodium acetate	99.995%	Roth, Karlsruhe, Germany
Sodium chloride	≥ 99.5%	VWR, Darmstadt, Germany
Sodium DL-lactate	≥ 99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Sodium dodecyl sulfate (SDS)	research grade	Serva, Heidelberg, Germany

Sodium hydroxide	50%	Thermo Fisher Scientific, Waltham, USA
Sucrose	> 99.5%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Sulfuric acid	65%	Th. Geyer, Renningen, Germany
Stachyose	> 98%	TCI Europe, Zwijndrecht, Belgium
Tetramethylethylendiamine (TEMED)	99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Trisodium citrate	> 99%	Roth, Karlsruhe, Germany
TrisX (Tris-hydroxymethyl-aminomethane)	≥ 99.8%	Gerbu Biotechnik, Heidelberg, Germany
Tween 80	for synthesis	Merck, Darmstadt, Germany
Perchloric acid	p.a.	Th. Geyer, Renningen, Germany
Vanillic acid	≥ 97%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Verbascose	≥ 97%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Yeast extract	for microbiology	Merck, Darmstadt, Germany
Zinc sulfate	p.a.	Merck, Darmstadt, Germany

2.2 Microorganisms, media and culture conditions

For screening of the microbial utilization of oligosaccharides which are contained in lupin and sunflower 25 microorganisms were selected (Table 2). Most of the strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Other bacterial isolates were taken from the collection of the Lehrstuhl für Technische Mikrobiologie Weihenstephan (TMW) or provided by the Brau- und Getränketechnologie, Technische Universität München. Strains were cultivated under their respective optimal conditions on their recommended media and stored as cryo cultures or on angular agar tubes at 4 °C. For anaerobic incubations, anaerobic jars with AnaeroGen® sachets were used and controlled by an indicator strip (Oxoid, Thermo Fisher Scientific).

Table 2: Used microorganisms and their cultivation conditions

Strain	**Number**	**Cultivation conditions**
Lactobacillus plantarum	TMW 1.460	MRS 30 °C, anaerobic
Lactobacillus brevis	TMW 1.1326	MRS 30 °C, anaerobic
Lactobacillus plantarum	TMW 1.1468	MRS 30 °C, anaerobic
Lactobacillus curvatus	TMW 1.624	MRS 30 °C, anaerobic
Weissella cibaria	TMW 2.1333	MRS 30 °C, anaerobic
Lactobacillus acidophilus	DSM 20079^T	MRS+cysteine 37 °C, anaerobic
Lactobacillus casei	DSM 20011^T	MRS 30 °C, aerobic
Lactobacillus fermentum	DSM 20391	MRS 37 °C, aerobic
Lactobacillus perolens	DSM 12744^T	MRS 28 °C, aerobic

Lactococcus lactis subsp. *lactis*	DSM 20384	MRS 30 °C, aerobic
Lactococcus lactis subsp. *cremoris*	DSM 20069^T	MRS 30 °C, aerobic
Lactobacillus reuteri	DSM 20016^T	MRS 37 °C, aerobic
Lactobacillus sanfranciscensis	DSM 20451^T	MRS 30 °C, aerobic
Leuconostoc mesenteroides subsp. *cremoris*	DSM 20200	MRS 30 °C, aerobic
Lactobacillus helveticus	DSM 20075^T	MRS 37 °C, anaerobic
Pediococcus pentosaceus	DSM 20336^T	MRS 37 °C, anaerobic
Lactobacillus amylolyticus	TL 3	MRS 47 °C, aerobic
Lactobacillus amylolyticus	TL 5	MRS 47 °C, aerobic
Lactobacillus spec.	TL 11	MRS 47 °C, aerobic
Lactobacillus spec.	TL 13	MRS 47 °C, aerobic
Lactobacillus rossiae	L 1202	MRS 25 °C, anaerobic
Lactobacillus parabuchneri	L 150	MRS 30 °C, anaerobic
Lactobacillus buchneri	DSM 20057^T	MRS 37 °C, aerobic
Lactobacillus gasseri	DSM 20243^T	MRS+cysteine 37 °C, anaerobic
Bifidobacterium animalis subsp. *lactis*	DSM 10140^T	MRS+cysteine 37 °C, anaerobic

The composition of de Man Rogosa Shape medium (MRS) used within this study for cultivation of lactobacilli and bifidobacteria is listed in Table 3. Lysogeny broth (LB) was used for cultivation of *Escherichia coli* (Table 4).

Table 3: Composition of MRS medium, pH 6.2 – 6.5

Component	**Amount (g)**
Casein peptone, tryptic digest	10.00
Meat extract	10.00
Yeast extract	5.00
Glucose	20.00
Tween 80	1.00
K_2HPO_4	2.00
Sodium-acetate	5.00
$(NH_4)_2$ citrate	2.00
$MgSO_4$ x 7 H_2O	0.20
$MnSO_4$ x H_2O	0.05

The components were dissolved in 1000 ml distilled water and autoclaved 15 minutes at 118°C. The cultivation of some strains required addition of 0.05% cysteine which was added before autoclaving. For use as agar plates, 15 g agar was supplemented.

Table 4: Composition of LB-medium, pH 7.0

Component	Amount (g)
Peptone from casein	10.00
Yeast extract	5.00
Sodium chloride	5.00

The components were dissolved in 1000 ml distilled water and autoclaved 15 minutes at 121°C. For agar plates, 15 g agar was added.

2.3 Screening of microorganism activity against secondary plant metabolites

2.3.1 Oligosaccharide utilization

Modifying a method of Teixeira et al. (2012), growth of microorganisms in media containing various carbohydrates (2% raffinose or stachyose) as sole carbon source was analyzed in duplicate. Media with 2% glucose served as positive control. 10 ml of modified MRS bouillon (glucose replaced) was used for bacteria. The metabolism of the selected carbohydrates stachyose and raffinose was indirectly measured by the pH decrease which was ascribed to microbial metabolism with acid production as previously described (Martínez-Villaluenga and Gómez, 2007; Mital et al., 1974). Strong growth was correlated to a pH drop of minimum 1.5 or more after 48 hours. The pH was determined using a disinfected pH electrode (WTW, Weilheim, Germany) calibrated at pH 7 and 4.

2.3.2 Phytic acid degradation

The microbial ability to degrade phytic acid in media was determined by High Performance Anion Exchange Chromatography (HPAEC) according to chapter 2.5.3. Therefore, 10 ml modified MRS broth with 0.2% sodium phytate as sole source of phosphate (K_2HPO_4 replaced) was used for inoculation. The phytic acid content was analyzed at the beginning of fermentation and after 48 h. Experiments were performed in duplicate.

2.3.3 Phenolic acid degradation

The ability of selected microorganism to utilize various phenolic acids as sole carbon source for their metabolism was examined in modified MRS broth. The glucose content was thereby replaced by 0.05% chlorogenic acid, caffeic acid or quinic acid. Stock solutions (20 mg/ml) were prepared by dissolving chlorogenic and quinic acid in hot water, whereas caffeic acid was solubilized in 50% ethanol.

For degradation studies, MRS broth with glucose was used and 0.05% chlorogenic acid was added. The nutrient broth was inoculated in duplicate and cell counts as well as chlorogenic and caffeic acid concentrations were determined as described in chapter 2.4.1 and 2.5.4, respectively.

2.4 Fermentation experiments

2.4.1 Determination of cell counts

Microbial growth during fermentation was determined in duplicate using a spiral plater (Eddy Jet, IUL Instruments, Barcelona, Spain). Appropriate dilutions of lupin or sunflower suspensions in sterile ringer solution (Oxoid, Hampshire, UK) were plated on MRS (for *Lb. plantarum, P. pentosaceus* and *L. lactis* subsp*. lactis*) or MRS with cysteine (for *Bif. animalis* subsp*. lactis* and *Lb. gasseri*) agar plates and incubated two days under the optimal growth conditions for each strain. For comparative analyses the rise of bacterial cell count was calculated over fermentation time.

2.4.2 Experimental set-up

Fermentations of the different lupin substrates (sweet lupin flour, bitter lupin flour and lupin protein isolate) were performed in 10% (w/v) concentration with the four selected strains *Bifidobacterium (Bif.) animalis* subsp. *lactis*, *Pediococcus (P.) pentosaceus*, *Lactobacillus* (*Lb.) plantarum*, *Lactococcus* (*L.) lactis* subsp. *lactis* under their respective optimal growth conditions. Similarly, sunflower substrates (flour and protein concentrate) were used in a 10% (w/v) concentration and inoculated with *Bif. animalis* subsp. *lactis*, *P. pentosaceus*, *Lb. plantarum* and *Lb. gasseri*. To exclude an inhibitory growth effect of the lower carbohydrate content of the protein substrates, the carbohydrate content of lupin protein isolate and sunflower protein concentrate was adjusted to that of lupin flour and sunflower flour, respectively. Thereby, the amount of verbascose was converted into the molar mass of stachyose and added to the protein isolate. To reduce the endogenous microbiota of the raw materials, samples were pasteurized for 10 min at 85 °C. Three replicates of each suspension (50 ml) were inoculated with bacterial strains from precultures to obtain a concentration of about 10^8 colony-forming units per milliliter (cfu/ml). Therefore, colonies of angular agar tubes were precultured in 200 ml MRS or MRS with cysteine broth for 24 – 48 h under the respective optimal temperature. After cell enumeration, a calculated aliquot of the preculture was centrifuged (9,055 *g*, 10 min), resuspended in 1 ml sterile distilled water and used for inoculation.

Chemical acidified samples without bacteria were adjusted with hydrochloric acid to a pH of 4.5 in order to detect endogenous enzymatic activity under comparable circumstances in the substrates. The suspensions were stored for 48 h under the same conditions like the inoculated samples.

2.4.3 Verification of fermentation strains

In order to verify the competitiveness of the inoculated bacteria, colony morphology and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) measurements were conducted according to Kern et al. (2013). For analyses, Microflex LT MALDI-TOF MS equipped with a nitrogen laser (λ = 337 nm, Bruker Daltonics) operating in linear positive mode was used. Mass spectra of 240 laser shots for each sample were recorded in manual and automatic mode and used for analysis with MALDI Biotyper 3.0 Software (Bruker Daltonics, Bremen, Germany). Therefore, single colonies of fresh MRS agar plates were smeared on a stainless steel target and overlaid with 1 µl formic acid to enhance the protein release. After drying, 1 µl of matrix solution (α-cyano-4-hydroxy-cinnamic acid, 10 mg/ml in acetonitrile, water, trifluoroacetic acid 50:47.5:2.5, Bruker Daltonics) was added. For external mass calibration, a bacterial standard of Bruker Daltonics was applied.

2.5 Chemical analyses

2.5.1 Dry matter, protein, fat and alkaloids

Dry matter was determined according to AOAC methods (AOAC, 2005a). The protein concentration of the samples was assayed by the calculation of the nitrogen content (N x 6.25) based on the Dumas method (AOAC, 2005b) and fat was measured according to standard method (DGF, 2004). Quantitative and qualitative alkaloid analyses were performed with GC-MS by the Institute of Pharmacy and Molecular Biotechnology (IPMB, Heidelberg, Germany).

2.5.2 Oligosaccharides

Mono-, di- and oligosaccharides were analyzed by High Performance Anion Exchange Chromatography with pulsed amperometric detection (HPAEC-PAD) at 30 °C. CarboPac PA10 column and CarboPac PA10 guard column (Thermo Fisher Scientific) were used with ultrapure water (A), 100 mmol/l NaOH (B) and 250 mmol/l NaOH (C) as eluents at a flow rate of 0.25 ml/min with the following gradient: 0 min: 15% B, 0% C; 30 min: 100% B; 38 min:

100% C, followed by regeneration. The injection volume was 10 µl. For determination of carbohydrates, 100 µl of the sample was mixed with 450 µl zinc sulfate (10%, Merck) and 450 µl NaOH (5 mmol/l). After 20 minutes at room temperature, the sample was centrifuged (12,000 *g*, 10 min) prior dilution and filtration (0.45 µm nylon, Berrytec GmbH, Grünwald, Germany) of the supernatant. A calibration curve of galactose, sucrose, raffinose, stachyose and verbascose was measured in a range of 5 – 33 mg/l.

2.5.3 Phytic acid

Quantification of phytic acid (*myo*-inositol-6-phosphate) was carried out using an IonPac AS11 column (Thermo Fisher Scientific) in a HPAEC system with conductivity detection. Sodium hydroxide (12 mmol/l) was used for isocratic elution at a flow rate of 0.25 ml/min (30 °C). The calibration curve was recorded from 2 – 50 mg/l. 1 g of lupin raw materials was mixed with 10 ml hydrochloric acid (3 mol/l) and incubated in boiling water for 10 minutes. Afterwards, the sample was transferred into a 50 ml volumetric flask, filled up with ultrapure water (18.2 MΩ/cm) and filtered using ash-free filter paper (Schleicher and Schuell, Dassel, Germany). An appropriate dilution to match the calibration range was injected. This method was modified on basis of an application of Thermo Fisher Scientific (Phescatcha et al., 2012). In case of fermented suspensions, 1 g of sample and 0.5 ml hydrochloric acid (6 mol/l) were incubated for 10 minutes. After dilution, the sample was filtered (0.45 µm nylon) and used for analysis. Phytic acid quantification in nutrient media was performed after dilution and filtration.

2.5.4 Phenolic compounds

Quantification of chlorogenic acid and caffeic acid was carried out using a Synergy 4u Hydro-RP column with a security guard cartridge AQ C18 (Phenomenex, Aschaffenburg, Germany) at 25 °C in a High Performance Liquid Chromatography system with UV-detection (HPLC-UV, Thermo Fisher Scientific) operated by Chromeleon software (Dionex Softron, Germering, Germany). Separation was performed using HPLC water with 0.25% formic acid (A) and acetonitrile with 0.25% formic acid (B) as eluents at a flow rate of 0.5 ml/min with the following gradient: 0 – 5 min: 10% B; 5 – 35 min: 70% B; 35 – 45 min: 100% B. The detector was set to 280 nm. For extraction of phenolic compounds, 600 µl of liquid sample were mixed with 1400 µl methanol (70% with 0.7% acetic acid) and filtered through 0.2 µm nylon filters (Berrytec GmbH). Solid samples (sunflower flour and protein concentrate) were diluted 1:10 with 70% methanol and stirred on a magnetic stirrer for 30 minutes. After filtration (595 Schleicher & Schuell) the sediment was extracted twice and the combined filtrates were filled

up to 25 ml with methanol. The sample was filtered (0.2 µm nylon) and an appropriate dilution within the calibration range (2 – 50 mg/l) was injected.

Hydroxycinnamoyl esters (ethyl ferulate, methyl tans-*p*-coumarate, methyl caffeate, methyl vanillate) and free phenolic acids (ferulic acid, *p*-coumaric acid, caffeic acid, vanillic acid) were determined using a Nucleosil C18 RP column (125 x 3 mm, 5 µm, Macherey-Nagel, Düren, Germany) at 30 °C and UV-detection at 280 and 320 nm. An HPLC system of Agilent Technologies (Santa Clara, CA, USA) was used and operated by ChemStations software for LC and 3 D systems (Agilent Technologies). The following eluents and gradients were used: 0.25% formic acid in water (A) and 0.25% formic acid in acetonitrile (B), 0 – 5 min: 10% B; 5 – 35 min: 70% B; 35 – 45 min: 100% B at 0.5 ml/min. Quantifications were performed with an external standard calibration ranging from 0.1 – 5 mmol/l. Phenolic acids were extracted by means of liquid-liquid extraction. Therefore, the supernatant of centrifuged broth was acidified with hydrochloric acid to a pH of 1.5 and the free phenolic acids were extracted twice with ethyl acetate. The entire extract was evaporated to dryness and the residue was dissolved in 1.5 ml methanol. The filtered sample was analyzed in the HPLC-UV system.

2.5.5 Metabolic products

To determine the metabolic activity of the microorganisms during fermentation, the concentration of organic acids (lactate and acetate) was analyzed by HPLC-UV. An Aminex HPX-87 column (BioRad Laboratories) equipped with a security guard cartridge Carbo-H (Phenomenex) was used with 13 mmol/l H_2SO_4 as solvent at a flow rate of 0.7 ml/min at 50 °C. The UV detector was set to 210 nm. For sample preparation 400 µl of lupin or sunflower suspension was mixed with 790 µl water and 10 µl 15% perchloric acid and stored overnight at 7 °C. After centrifugation (12,000 *g*, 10 min), the supernatant was filtered through a 0.45 µm nylon filter. This procedure was performed according to Capuani et al. (2012).

2.6 Minimum inhibitory concentration assay and kinetic measurement

The antimicrobial effects of the main phenolic acids in sunflower (chlorogenic acid as well as its cleavage products caffeic and quinic acid) were analyzed by determination of the minimum inhibitory concentration (MIC). Therefore the broth micro-dilution method from Wiegand et al. (2008) was modified. Briefly, polyphenols were dissolved in hot water to obtain stock solutions of 40.96 mg/ml. Dilution series with MRS or MRS with cysteine bouillon were conducted in triplicate in 96 well microtiter plates (Greiner Bio-One International GmbH, Kremsmünster, Austria) to cover a concentration range between 20.48 – 0.32 mg/ml. To solubilize caffeic acid, 50% ethanol was used instead of water. Caffeic acid

was evaluated in a concentration range from 5.12 to 0.08 mg/ml as preliminary experiments revealed a higher sensitivity of the lactic acid bacteria to this substance. As controls, media without polyphenols was inoculated (growth control) and media without inoculum (sterility control) was used. Media with phenolic compounds but without bacteria served as blank samples. Bacterial suspensions of precultures were adjusted to a cell density of 10^8 cfu/ml using the McFarland 0.5 standard ($BaCl_2$ x 2 H_2O, H_2SO_4) as reference. After dilution to 10^6 cfu/ml, the suspension was applied for inoculation. All plates were incubated anaerobically for 48 h under the respective optimal growth temperature. After 24 and 48 h, the absorption of each well was measured spectrophotometrically at 595 nm after agitating for 10 s in linear mode and 10 s in orbital mode (Tecan Group Ltd., Männedorf, Switzerland). Every substance was tested in triplicate and analyzed by i-control 1.10 software (Tecan Group). MIC_{90} was defined as the lowest concentration of phenolic acids inhibiting microbial growth by ≥ 90% compared with the growth control.

To monitor the microbial growth in presence of chlorogenic acid (0.32 – 20.48 mg/ml) and thus get a deeper insight into the inhibitory mechanism, growth kinetics were recorded by spectrophotometric measurements every 15 minutes for 48 h under the respective optimal growth conditions. The absorption at 595 nm of each well was measured nine fold after agitating (3 s in orbital mode) and compared with the growth control. For anaerobic strains, the detection chamber was flushed with nitrogen by a gas control module (Tecan Group) to create an approximately anaerobic atmosphere (0.4 – 0.6% oxygen). As this oxygen concentration was too high for some sensible strains, the microtiter plates of *Bif. animalis* subsp. *lactis* and *Lb. gasseri* were incubated in anaerobic jars and measured manually after 0, 5, 21, 27, 33, 45, 51 and 70 h with the same parameters.

2.7 Molecular techniques

2.7.1 Isolation of genomic DNA and PCR amplification

Bif. animalis subsp*. lactis* was used for genetic investigations to elucidate the chlorogenic acid degradation. The strain was cultivated as described in chapter 2.2. Chromosomal DNA was isolated according to Lewington et al. (1987). The responsible genes encoding the predicted cinnamoyl esterase were amplified by PCR with the forward primer Bif_For_Nco 5'-TATACCATGGTTATGACGACGAGCACACATAC-3', the reverse primer Bif_rev_Sal 5'-TATAGTCGACCGCCACCTCATGATGCGTC-3' (Eurofins Genomics, Ebersberg, Germany) and Phusion® polymerase (BioLabs Inc., Frankfurt am Main, Germany). All components were mixed according to the recipe in Table 5. DNA amplification was performed for 32 cycles consisting of a denaturation step (94 °C, 1 min), primer annealing (melting temperature of

primer minus 3 °C, 45 seconds) and elongation (72 °C) with an automated thermocycler (Mastercycler gradient Eppendorf AG, Hamburg, Germany). Afterwards, the fragments were purified with E.Z.N.A. Cycle Pure Kit (Omega, BioTek Inc., Norcross, USA). For verification of the expected amplicon size, 1% agarose gel electrophoresis with 0.5 x TBE buffer was conducted (stock solution 5 x TBE buffer: 54 g/L Tris base, 27.5 g/L boric acid, 20 ml 0.5 mol/l EDTA, pH 8.0). PCR products were mixed with a loading dye (2:1) and separation was achieved at 100 V. For analysis, 5 µl of DNA ladder were applied, the gel was stained for approximately 30 min in ethidium bromide and the bands were visualized with UV light. Sequencing of PCR products was performed by GATC Biotech AG (Konstanz, Germany).

Table 5: Composition of the mastermix for PCR

Component	**Volume (µl)**
PCR H_2O	37.0
PCR buffer	10.0
dNTP	1.0
revers primer	0.5
forward primer	0.5
DNA	0.5
Phusion polymerase	0.5

2.7.2 Cloning, heterologous expression and enzyme purification

For the heterologous enzyme expression the pBAD/*Myc*-His-A plasmid (Figure 6, Invitrogen, Burlington, Canada) was used which is designed for regulated, dose-dependent protein expression in *E. coli*. In order to regulate the protein expression, araC is provided which turns the P_{BAD} promotor in presence of L-arabinose on while in absence the transcription runs only to a low level. For selection of cells harboring the plasmid, an ampicillin resistance gene is available (Lee, 1980; Lee et al., 1987). The amplified fragments were cloned into the expression vector using general techniques according to Sambrook (2001). Restriction enzymes *Nco*I and *Sal*I as well as T4-DNA ligase were purchased from BioLabs Inc. Digestion was performed as described by manufacturer's instruction. For ligation the components were mixed according to Table 6 and stored overnight at 4 °C. Subsequently, the sample was incubated at 65 °C for 15 minutes to inactivate T4-DNA ligase. Transformation was performed by mixing 200 µl of chemical competent *E. coli* TOP10 cells with 20 µl of ligation preparation and storing for 20 minutes on ice. After a heat shock (42 °C for 90 seconds) cells were chilled for 2 minutes on ice and incubated in SOC-broth (yeast extract 5 g/l, tryptone 20 g/l, sodium chloride, 0.6 g/l, potassium chloride 0.2 g/l, magnesium

chloride 10 mmol/l, magnesium sulphate 10 mmol/l and glucose 20 mmol/l) at 37 °C for 30 minutes. Positive clones were identified by a modified ethyl ferulate plate assay according to Donaghy et al. (1998). Therefore, 20, 100 and 200 µl of inoculated SOC-broth were plated on LB-agar plates, supplemented with 100 µg/ml ampicillin, 0.1 mmol/l L-arabinose as inducer for the expression from *ara*BAD promoter and 0.15% ethyl ferulate (dissolved in ethanol) leading to a turbidity of the agar. Due to the hydrolysis of ethyl ferulate, a clear area was formed around bacterial cells that were cinnamoyl esterase positive. The plasmids of positive clones were isolated, purified with the QIAprep Spin Minprep Kit (Qiagen, Hilden, Germany) and sequenced to examine the transformation success. Single colonies of positive clones were incubated overnight in 30 ml LB-broth with ampicillin, harvested and cryo-cultured in glycerin.

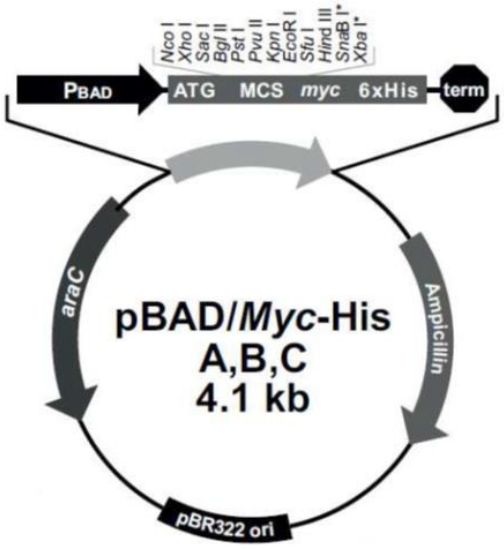


Figure 6: pBAD/*Myc*-His vector with P_{BAD} promoter, araC regulatory protein and ampicillin resistance (Invitrogen)

Table 6: Components for ligation approach

Component	**Volume (µl)**
PCR H_2O	8.0
insert DNA	6.0
vector DNA	3.0
buffer	2.0
T4-DNA ligase	1.0

For the expression of His_6-tagged proteins in *E. coli* TOP10, L-arabinose was used as inducer. Cells were grown aerobically in LB-broth at 37 °C until an optical density at 600 nm

of 0.7 was reached. After supplementation of 0.1 mmol/l L-arabinose, the strain was incubated overnight at 30 °C. The cells were harvested by centrifugation (6000 rpm, 10 min, 4 °C), washed and suspended in binding buffer (20 mmol/l NaH_2PO_4, 500 mmol/l NaCl, 20 mmol/l imidazole, pH 7.4). The cells were disrupted in five cycles (2 s at 90%) with breaks of 2 minutes on ice by use of an ultrasound homogenizer (Bandelin, Berlin, Germany). The lysate was centrifuged (12,000 rpm, 15 min, 4 °C) and proteins were purified to homogeneity using HisTrap HP columns (GE Healthcare, Freiburg, Germany). Before application of 1 ml sample, the column was washed with 5 ml distilled water and equilibrated with 5 ml binding buffer. The elution of proteins was performed with 5 x 1 ml elution buffer (20 mmol/l NaH_2PO_4, 500 mmol/l NaCl, 250 mmol/l imidazole, pH 7.4), collecting every fraction (1 ml).

2.7.3 Verification of heterologous expression using SDS-PAGE

To evaluate the protein size and purity of crude lysate as well as fractions after affinity chromatography, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Schägger and von Jagow (1987). For preparation of the SDS separating gel, 5.30 ml acrylamide (16%), 3.33 ml gel buffer, 1.26 ml deionized water, 40 µl SDS-solution (25%, w/v), 7 µl tetramethylethylendiamine (TEMED) and 50 µl ammonium persulfate (APS, 10%, w/v) were mixed. The stacking gel consisted of 0.68 acrylamide (4%), 1.29 ml gel buffer, 3.21 ml deionized water, 16 µl SDS-solution, 7 µl TEMED and 33 µl APS. Before loading on SDS gel, the sample was mixed with SDS buffer (250 mmol/l Tris-HCL, pH 8.5, 25% (v/v) glycerin, 12.5% (v/v) 2-mercaptoethanol, 7.5% (w/v) SDS, 0.25 mg/ml bromphenol blue) in equal ratios (7.5 µl : 7.5 µl) and incubated at 90 °C for 5 minutes. 10 µl of sample were applied to the gel cavities. Separation was started at 80 V for 10 minutes, followed by 100 V for 120 minutes. The proteins were visualized by staining with colloidal Coomassie brilliant blue overnight. Afterwards, the gel was shaken in destaining solution (20 ml methanol, 1 ml *o*-phosphoric acid (85%), 79 ml deionized water) for approximately 3 h and analyzed by scanning.

For protein quantification according to Bradford (1976), the BioRad standard assay was used with bovine serum albumin as standard in a calibration range of 125 – 1000 µg/ml.

2.8 Determination of cinnamoyl esterase activity

The activity of the purified cinnamoyl esterase was measured spectrophotometrically against different substrate concentration as described below. The velocity of the enzymatic reaction was calculated using the Beer-Lambert-law (1) according to Janes et al. (1998).

$$V = \frac{\Delta E}{\varepsilon * d * \Delta t} \quad (1)$$

V refers to the reaction velocity, E to the absorbance, ε to the extinction coefficient, d to the layer thickness and t to the time. In this assay $\varepsilon_{405\ nm}$ was 18,000 $M^{-1}*cm^{-1}$ (Zhang and VanEtten, 1991) and d = 0.52 cm. The calculated velocities were plotted against the substrate concentration in a curve chart. Commonly, this curve approaches the saturation area only asymptotically and solubility problem can occur at high substrate concentrations. Therefore, a transformation of the Michaelis-Menten equation (2) was performed. The maximum velocity V_{max} and the Michaelis-Menten constant K_m (substrate concentration that is required to reach half of V_{max}) were calculated after linearization according to Lineweaver-Burk (3) in which the reciprocals are used:

$$V_0 = V_{max} * \frac{S}{S + K_m} \quad (2)$$

$$\frac{1}{V_0} = \frac{K_m}{V_{max}} * \frac{1}{S} + \frac{1}{V_{max}} \quad (3)$$

with V_0 representing the initial velocity and S the substrate concentration (Lineweaver and Burk, 1934).

Due to the simple photometric measurement, *p*-nitrophenyl acetate was used as substrate as previously described (Jänsch, 2013). After hydrolysis, *p*-nitrophenyl and acetate occur, resulting in a yellow color which can be measured spectrophotometrically at 405 nm (Goldstone et al., 2010; Janes et al., 1998; Lai et al., 2009). The stock solution of *p*-nitrophenyl acetate (40 mmol/l in methanol) was diluted with sodium phosphate buffer (100 mmol/l, pH 7, Table 8) to obtain a concentration range of 0.25 – 3 mmol/l. An aliquot of 2 µl purified enzyme extract was mixed with 200 µl substrate-sodium phosphate buffer with varying substrate concentrations. The liberated *p*-nitrophenyl was measured in 60 cycles every 20 seconds at 405 nm using a spectrophotometer (Tecan Group). Sodium phosphate buffer without substrate served as reference. All analyses were performed in triplicate.

2.8.1 Determination of pH-dependent activity

The pH-dependent activity was examined in a pH-range of 4.0 – 9.0 at ambient temperature using citrate buffer for pH 4.0 – 6.0, sodium phosphate buffer for pH 6.5 – 8.0 and tris-HCl-

buffer for pH 8.5 – 9.0 (Table 7 – 9) according to Jänsch (2013). *p*-nitrophenyl acetate was used as substrate in a concentration of 2 mmol/l. The samples were measured in triplicate as described above.

Table 7: Preparation of citrate buffers in the pH range of 4.0 – 6.0

pH	0.1 M citric acid (ml)	0.1 M trisodium citrate x 2 H_2O (ml)
4.0	59.0	41.0
4.5	49.5	50.5
5.0	35.0	65.0
5.5	25.5	74.5
6.0	11.5	88.5

Table 8: Preparation of sodium phosphate buffers in the pH range of 6.5 – 8.0

pH	1 M Na_2HPO_4 x 2 H_2O (ml)	1 M NaH_2PO_4 x H_2O (ml)
6.5	18.2	81.8
7.0	57.7	42.3
7.5	66.1	33.9
8.0	93.2	6.8

Table 9: Preparation of tris-HCl buffers in the pH range of 8.5 – 9.0

pH	1 M Tris (ml)	1 M HCl (ml)	Distilled water (ml)
8.5	50.0	14.7	35.3
9.0	50.0	5.7	44.3

2.8.2 Determination of temperature-dependent activity

To determine the temperature optimum of the purified cinnamoyl esterase, reactions were performed at 20 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C and 60 °C in triplicates. Ethyl ferulate was used as substrate (final concentration 10 mmol/l) in sodium phosphate buffer (pH 7). For stock solutions (1.8 mol/l), ethyl ferulate was dissolved in ethanol using an ultrasonic bath. The reaction mixture was pre-incubated for 5 minutes at the respective temperature and the pH value was, if necessary, corrected. The reaction was started with the addition of 10 µl enzyme extract. After 10 minutes the reaction was stopped using a heating block at 95 °C. 500 µl of sample were mixed with 500 µl methanol and the inactivation was continued for 3 minutes. Afterwards, the sample was filtered (0.2 µm) and HPLC analyses were performed according to chapter 2.5.4 to determine the amount of liberated ferulic acid.

2.8.3 Determination of substrate chain length-dependent activity

It was evaluated if the substrate chain length influences the cinnamoyl esterase activity. Therefore, *p*-nitrophenyl acetate (*p*-NP-C2), *p*-nitrophenyl butyrate (*p*-NP-C4) and *p*-nitrophenyl caprylate (*p*-NP-C8, 2 mmol/l) were used in triplicate as substrates because these substances exhibit sterically similarities to ferulic acid (Goldstone et al., 2010; Lai et al., 2009; Rashamuse et al., 2007). The spectrophotometric measurements were performed at ambient temperature as mentioned above.

2.8.4 Determination of enzyme substrate specificity

In order to examine if the heterologously expressed esterase of *Bif. animalis* subsp. *lactis* is able to hydrolyze chlorogenic acid, it was used as substrate. Chlorogenic acid was dissolved in hot water, added to LB-broth (final concentration 1.4 mmol/l) and inoculated with *E. coli* TOP10 hosting the insert of *Bif. animalis* subsp. *lactis.* For control, *E. coli* TOP10 without an insert was used. After 24 h, phenolic acids were extracted with methanol (70% with 0.7% acetic acid) as described above. Samples were analyzed by HPLC according to 2.5.4.

To investigate the substrate specificity of the cinnamoyl esterase, several cinnamic acid derivatives were studied. Methyl trans-*p*-coumarate, methyl caffeate and methyl vanillate were dissolved in DMSO, sterile filtered and added to nutrient broth in a concentration of 2 mmol/l. After 24 h incubation, cells were removed by centrifugation (7,000 rpm, 10 min, 20 °C) and the reaction products were extracted as described in chapter 2.5.4. For control, blank samples (media with cinnamic acid derivatives without inoculation) were stored under the same conditions and analyzed as well. The experiments were performed in triplicate.

2.9 Statistical and bioinformatics analyses

Data were analyzed using SPSS Software (version 20; IBM, Armonk, New York USA). All analyses were expressed as the mean standard deviation of three replicates *(n* = 3). One-way ANOVA with the Bonferroni or Games-Howell correction was performed to determine significant differences ($p < 0.05$) between the samples.

Sequence data for *in silico* analyses were obtained from the National Center of Biotechnology and Information (NCBI) and the BioCyc Database Collection. Nucleotide and amino acid sequence homology analysis were performed using the Basic Local Alignment Tool (BLAST) of the NCBI.

3 Results

3.1 Screening of suitable fermentation strains: degradation of oligosaccharides, phytic and chlorogenic acid in media

Oligosaccharides

In total, 25 microorganisms were screened for their oligosaccharide metabolism by indirect measurement of the pH value. MRS medium with glucose served as positive control (Table 10). From the selected strains, 11 microorganisms showed a measurable growth in media containing raffinose or stachyose. This indicates the activity of enzymes, hydrolyzing the glycosidic bonds between fructose, glucose and galactose, e.g. α-galactosidases, β-galactosidases or levansucrase (Martínez-Villaluenga and Gómez, 2007; Teixeira et al., 2012). *Lb. plantarum* TMW 1.460, *Lb. plantarum* TMW 1.1468, *Lb. casei* and *Lb. buchneri* showed a stronger or exclusive acidification in media with raffinose than in MRS broth with stachyose which was also observed for other *Lb. plantarum* and *Lb. buchneri* strains by Mital et al. (1974). The strongest decrease of pH was observed for *Lb. acidophilus*, *L. lactis* subsp. *lactis*, *Lb. reuteri*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lb. helveticus*, *P. pentosaceus* and *Bif. animalis* subsp. *lactis*.

Phytic acid

In literature, strains of *Lb. plantarum, P. pentosaceus, L. lactis* subsp. *lactis* as well as bifidobacteria are reported to possess phytase activity (De Angelis et al., 2003; Fischer et al., 2014; Palacios et al., 2008; Shirai et al., 1994). To verify the phytic acid degradation ability, these strains were tested in MRS media with 0.2% phytic acid. All four strains were able to metabolize phytic acid within 48 h (Table 11). The strongest decrease with 54.8% was observed in MRS broth inoculated with *Lb. plantarum*. Similar results were examined by Shirai et al. (1994) who observed that *Lb. plantarum* APG Eurozyme showed a phytate reduction of 61.4% in whey medium. The control, which was run in acidified MRS broth (pH 4.5) without bacteria showed a minor decline of phytic acid of 3.7% when stored aerobically at 30 °C (growth conditions of *L. lactis* subsp*. lactis*) and of 6.6% at 37 °C anaerobically (incubation conditions of *Bif. animalis* subsp*. lactis* and *P. pentosaceus*). These observations are in agreement with Shirai et al. (1994) who measured a phytate reduction of 8% in sterile media.

Table 10: Sugar utilization of microorganisms in MRS or MRS with cysteine, modified regarding the carbon source*

Microorganism	**Carbon source**		
	Stachyose	**Raffinose**	**Glucose**
Lactobacillus plantarum TMW 1.460	+	++	++
Lactobacillus brevis TMW 1.1326	-	-	++
Lactobacillus plantarum TMW 1.1468	-	++	++
Lactobacillus curvatus TMW 1.624	-	-	++
Weissella cibaria TMW 2.1333	-	-	++
Lactobacillus acidophilus DSM 20079	++	++	++
Lactobacillus casei DSM 20011	-	+	++
Lactobacillus fermentum DSM 20391	+	+	++
Lactobacillus perolens DSM 12744	+	+	++
Lactococcus lactis subsp. *lactis* DSM 20384	++	++	++
Lactococcus lactis subsp. *cremoris* DSM 20069	-	-	++
Lactobacillus reuteri DSM 20016	++	++	++
Lactobacillus sanfranciscensis DSM 20451	-	-	++
Leuconostoc mesenteroides subsp. *cremoris* DSM 20200	++	++	++
Lactobacillus helveticus DSM 20057	++	++	++
Pediococcus pentosaceus DSM 20336	++	++	++
Lactobacillus amylolyticus TL 3	-	-	++
Lactobacillus amylolyticus TL 5	-	-	++
Lactobacillus spec. TL 11	-	-	++
Lactobacillus spec. TL 13	-	-	++
Lactobacillus rossiae L 1202	-	-	++
Lactobacillus parabuchneri L 150	-	-	++
Lactobacillus buchneri DSM 20057	-	++	++
Lactobacillus gasseri DSM 20243	+	+	++
Bifidobacterium animalis subsp. *lactis* DSM 10140	++	++	++

* Decrease of pH value was ascribed to the microbial metabolism with acid production; - = no microbial growth (Δ pH = ≤ 0.4); + = weak microbial growth (Δ pH = 0.5 – 1.4); ++ = strong microbial growth (Δ pH = ≥ 1.5)

Table 11: Changes in phytic acid content after 48 h in inoculated or sterile, acidified MRS broth

Media	**Phytic acid (%)**
MRS with *Bifidobacterium animalis* subsp. *lactis*	-23.2
MRS with *Pediococcus pentosaceus*	-22.9
MRS with *Lactobacillus plantarum*	-54.8
MRS with *Lactococcus lactis* subsp. *lactis*	-15.5
Sterile MRS broth pH 4,0 30 °C aerobic	-3.7
Sterile MRS broth pH 4,0 37 °C anaerobic	-6.6

Chlorogenic acid

It was described that strains of *Bif. lactis* and *Lb. gasseri* show the desired ability to degrade chlorogenic acid (Couteau et al., 2001; Raimondi et al., 2015). In order to examine if the type strains which were used in the present study are able to utilize phenolic acids for their metabolism, growth of *Bif. animalis* subsp. *lactis* and *Lb. gasseri* was monitored in modified MRS broth with 0.05% chlorogenic acid, caffeic acid and quinic acid as sole carbon source (Figure 7). A comparison of the growth curves with the positive control (media with glucose) showed that *Bif. animalis* subsp. *lactis* was not able to grow in media with phenolic acids (Figure 7 A). On the contrary, *Lb. gasseri* displayed a measurable growth in all samples, except in the blank sample which contained no carbon source (Figure 7 B). It was remarkable that this strain showed a stronger growth in MRS broth with caffeic acid than in media with glucose.

To verify the proclaimed ability of these strains to degrade chlorogenic acid, the concentration was analyzed during fermentation of unmodified MRS broth (with glucose) added with 0.05% (1.41 mmol/l) chlorogenic acid (Table 12). Both strains were able to reduce the chlorogenic acid content during 46 h of fermentation. *Lb. gasseri* was more effective (-78.4%) than *Bif. animalis* subsp. *lactis* (-69.1%). Due to enzymatic cleavage, the caffeic acid content increased.

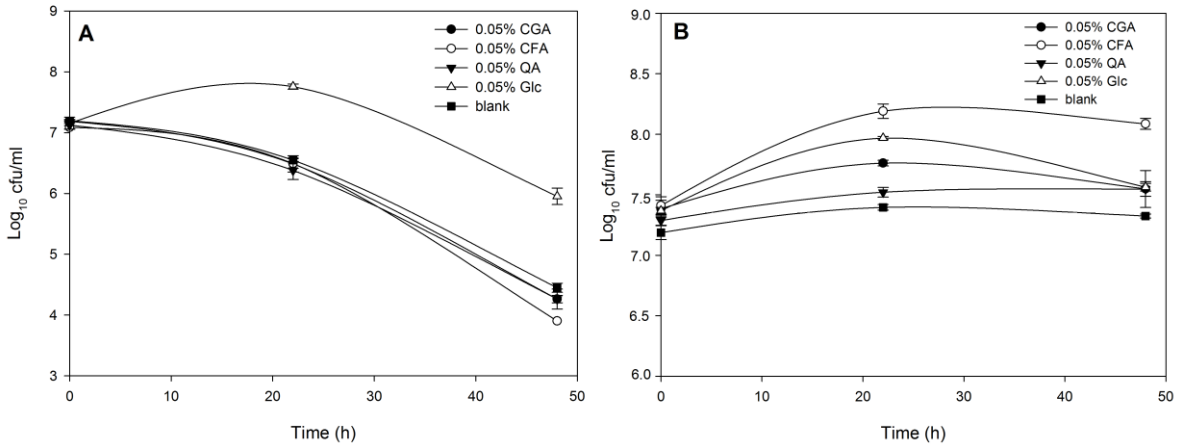


Figure 7: Growth of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 (A) and *Lactobacillus gasseri* DSM 20243 (B) in media with chlorogenic acid (CGA), caffeic acid (CFA), quinic acid (QA) and glucose (Glc) as sole carbon source or without any carbon source (blank)

Table 12: Changes in chlorogenic and caffeic acid content in MRS broth with 1.41 mmol/l chlorogenic acid after 46 h of fermentation

Microorganism	**Chlorogenic acid (mmol/l)**	**Caffeic acid (mmol/l)**
Bifidobacterium animalis subsp. *lactis*	-0.97	+0.04
Lactobacillus gasseri	-1.11	+0.08

3.2 Fermentation of lupin

3.2.1 Composition of lupin substrates

The proximate composition of the different lupin substrates is shown in Table 13. Sweet and bitter lupin flour revealed similar amounts of dry matter, carbohydrates, protein and phytic acid. The fat content was slightly different with 6.8% in sweet lupin flour and 8.5% in bitter lupin flour. The alkaloid content, which was mainly composed of lupanine, 13-hydroxylupanine and angustifoline was determined to be 0.5% in bitter lupin cultivar, 0.02% in sweet lupin and 0.0009% in lupin protein isolate. Due to the production process of lupin protein isolate which includes deoiling with carbon dioxide and acidic pre-extraction to separate oligosaccharides (Wäsche et al. 2001), significantly lower sugar and fat contents were analyzed. The detailed carbohydrate composition of the three substrates is given in Table 14. An exemplary HPAEC-PAD chromatogram is shown in Appendix A1. Sweet lupin flour of *Lupinus angustifolius* cv. *Boregine* mainly consisted of the oligosaccharides stachyose (4%), verbascose (1.8%) and the disaccharide sucrose (3%). The lupin seeds of *Lupinus angustifolius* cv. *Azuro* contained higher concentrations of raffinose (0.8%) but

significantly lower amounts of galactose (0.05%) than the sweet lupin flour. Phytic acid was quantified in comparable amounts in sweet and bitter lupin flour (4.7 – 4.9%) but a slightly lower concentration was analyzed in lupin protein isolate with 3.2%. The HPAEC-CD chromatogram of a phytic acid analysis is shown in Appendix A2.

Figure 8: Sweet lupin flour (A), bitter lupin flour (B), lupin protein isolate (C)

Table 13: Proximate composition of lupin substrates

Ingredients (%)	**Sweet lupin flour** *Lupinus angustifolius* cv. *Boregine*	**Bitter lupin flour** *Lupinus angustifolius* cv. *Azuro*	**Lupin protein isolate** *Lupinus angustifolius* cv. *Boregine*
Dry matter	88.7	90.5	92.7
Carbohydrates	10.0	9.7	0.1
Oligosaccharides	6.3	6.0	0.1
Protein	38.1	37.4	94.2
Fat	6.8	8.5	1.4
Alkaloids	$2.0\ 10^{-2}$	0.5	$9.0\ 10^{-4}$
Phytic acid	4.9	4.7	3.2

Table 14: Carbohydrate composition of lupin substrates

Carbohydrates (g/kg)	**Sweet lupin flour**	**Bitter lupin flour**	**Lupin protein isolate**
Galactose	7.7	0.5	0.1
Sucrose	29.6	37.1	0.4
Raffinose	4.2	8.0	0.2
Stachyose	40.4	34.4	0.3
Verbascose	18.4	17.3	0.3

3.2.2 Fermentation performance of selected strains and effects of secondary plant metabolites

For evaluation if lupin flour is suitable for lactic fermentation and to verify the results from the model experiments in media, four strains were selected (*Lb. plantarum* TMW 1.460, *P. pentosaceus* DSM 20336*, L. lactis* subsp. *lactis* DSM 20384, *Bif. animalis* subsp. *lactis* DSM 10140*)* and used for fermentation of lupin substrates. The strains were chosen due to their ability to utilize oligosaccharides and phytic acid (Tables 10 and 11). Sweet and bitter lupin flour as well as lupin protein isolate were used in a 10% (w/v) concentration. Preliminary experiments with 5% (w/v) lupin substrate suspensions revealed no sufficient growth of the tested strains (data not shown). The carbohydrate content of lupin protein isolate sample was adjusted to that of sweet lupin flour according to Table 14.

Figure 9 illustrates the microbial growth curves in the different lupin substrates. For comparison, Table 15 summarizes the calculated rises of bacterial cell counts over fermentation time. All tested strains were able to considerably grow on lupin protein isolate, sweet lupin flour as well as bitter lupin flour. The curve shapes indicate similar growth patterns of the strains in the diverse substrates. Minor differences were recorded for *P. pentosaceus* which showed a more pronounced lag-phase in the protein isolate suspension than in lupin flours (Figure 9 B). Nevertheless, this strain exhibited a higher rise of cell numbers in this substrate compared to the flours. A weaker growth curve in the protein suspension was monitored for *Lb. plantarum* (Figure 9 C) which is confirmed by the calculated increase of cell counts. An exception is *L. lactis* subsp. *lactis* which was only competitive in the lupin flour suspensions. No distinct difference could be observed between sweet and bitter lupin flour. After 24 h a mixed culture of *L. lactis* subsp. *lactis* and *Bacillus cereus* was detected in the protein isolate suspension. According to MALDI-TOF MS measurements, the other inoculated microorganisms were dominant in lupin samples. In chemical acidified blank samples, cell counts of aerobic, mesophilic bacteria did not exceed 8.1×10^3 cfu/ml.

The metabolic activity measured by the amounts of lactate and acetate, formed via the metabolic pathways is depicted in Table 16 and 17. The least amount of lactate was analyzed for *L. lactis* subsp. *lactis* with only 1.0 – 3.2 mmol/l, followed by *Bif. animalis* subsp. *lactis* with values between 14.4 and 32.1 mmol/l. *P. pentosaceus* and *Lb. plantarum* showed equal concentrations after 48 h of around 34 to 45 mmol/l. The highest concentration of lactic acid with 45.4 mmol/l was produced by *P. pentosaceus* in lupin protein isolate which underlined the homofermentative metabolic pattern. Acetic acid concentration was determined in a range between 1.4 to 65.3 mmol/l. The highest amount was produced by heterofermentative *Bif. animalis* subsp. *lactis* in lupin protein isolate suspension. According to the statistical evaluation, no significant difference in the metabolite content between sweet

and bitter lupin flour was observed for any fermentation after 48 h. A comparison of the two lupin flours with the lupin protein isolate revealed that *Bif. animalis* subsp. *lactis* produced significantly higher amounts of organic acids in the protein isolate suspension (Table 16). On the contrary, the metabolic activity of *Lb. plantarum* was higher in both lupin flours than in the protein isolate, but the difference was only statistically significant for acetate concentrations between sweet lupin flour and lupin protein isolate. *P. pentosaceus* formed similar amounts of acids in all substrates (Table 17). The pH value significantly decreased during fermentation of lupin substrates. In lupin flours, the pH dropped from 5.7 to 4.2, except in fermentation with *L. lactis* subsp. *lactis* where the pH remained nearly unchanged. The fastest decrease in pH value with 1.2 during the first 6 h of fermentation was monitored for *Lb. plantarum* in bitter lupin flour. In lupin protein isolate the fastest and strongest pH decrease was recorded for *Bif. animalis* subsp. *lactis.*

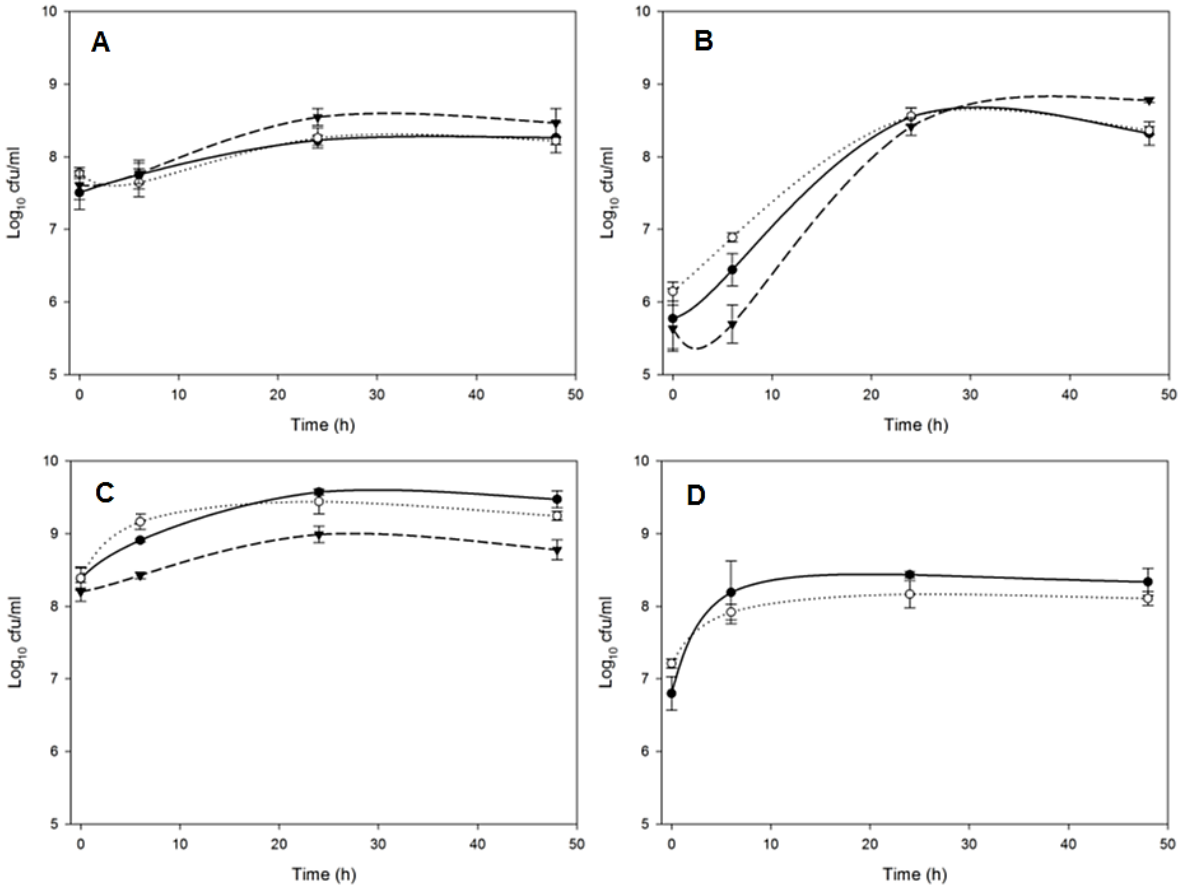


Figure 9: Microbial growth in sweet lupin flour (●), bitter lupin flour (○) and lupin protein isolate (▼). A = *Bifidobacterium animalis* subsp. *lactis* DSM 10140, B = *Pediococcus pentosaceus* DSM 20336, C = *Lactobacillus plantarum* TMW 1.460, D = *Lactococcus lactis* subsp. *lactis* DSM 20384

Table 15: Rise of bacterial count as $\Delta(N - N_0)$ in cfu/ml during 48 h of fermentation

Strain	Sweet lupin flour	Bitter lupin flour	Lupin protein isolate
Bifidobacterium animalis subsp. *lactis*	1.7×10^8	1.1×10^8	3.0×10^8
Pediococcus pentosaceus	2.2×10^8	2.3×10^8	6.0×10^8
Lactobacillus plantarum	2.8×10^9	1.5×10^9	4.6×10^8
Lactococcus lactis subsp. *lactis*	3.0×10^8	1.1×10^8	-

Table 16: Metabolite formation and pH values of heterofermentative *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and *Lactobacillus plantarum* TMW 1.460 in lupin substrates

	Lactate (mmol/l)*			Acetate (mmol/l)*			pH		
	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Bifidobacterium animalis* subsp. *lactis									
Sweet lupin flour	0.5^a ± 0.1	16.0^a ± 0.0	22.8^a ± 0.1	5.7^a ± 0.4	35.8^a ± 1.1	48.3^a ± 0.9	5.4	4.4	4.2
Bitter lupin flour	0.7^a ± 0.3	8.1^b ± 0.5	14.4^a ± 2.0	4.3^a ± 0.5	16.3^b ± 1.0	29.9^a ± 4.2	5.4	4.4	4.2
Lupin protein isolate	0.8^a ± 0.1	31.1^c ± 0.3	32.1^b ± 0.1	4.7^a ± 0.4	61.5^c ± 1.2	65.3^b ± 0.8	6.4	4.6	4.9
Lactobacillus plantarum									
Sweet lupin flour	13.4^a ± 1.6	33.7^a ± 6.1	41.1^a ± 5.2	1.7^a ± 0.2	6.8^a ± 1.3	7.8^a ± 0.9	4.9	4.3	4.3
Bitter lupin flour	$24.7^{a, b}$ ± 3.1	34.8^a ± 7.4	35.6^a ± 7.3	2.7^a ± 0.4	9.8^a ± 2.0	$9.7^{a, b}$ ± 1.8	4.5	4.3	4.3
Lupin protein isolate	$4.6^{a, c}$ ± 1.2	32.2^a ± 3.7	33.5^a ± 6.1	0.6^b ± 0.2	1.3^a ± 0.1	1.4^b ± 0.3	6.3	5.1	5.1

* Mean values were calculated from three replicates ± standard deviation. Values within one column of each microorganism with different superscript letter are significant different ($p < 0.05$).

Table 17: Metabolite formation and pH values of homofermentative *Pediococcus pentosaceus* DSM 20336 and *Lactococcus lactis* subsp. *lactis* DSM 20384 in lupin substrates

	Lactate (mmol/l)*			**pH**		
	6 h	24 h	48 h	6 h	24 h	48 h
Pediococcus pentosaceus						
Sweet lupin flour	0.8^{a} ± 0.7	39.3^{a} ± 0.5	39.4^{a} ± 5.9	5.7	4.3	4.2
Bitter lupin flour	0.5^{a} ± 0.2	39.6^{a} ± 6.0	36.0^{a} ± 9.3	5.7	4.2	4.3
Lupin protein isolate	0.7^{a} ± 0.0	25.2^{a} ± 2.8	45.4^{a} ± 5.3	6.5	5.1	4.6
Lactococcus lactis* subsp. *lactis						
Sweet lupin flour	1.2^{a} ± 0.5	2.0^{a} ± 0.5	1.0^{a} ± 0.9	5.7	5.6	5.7
Bitter lupin flour	3.2^{b} ± 0.4	2.1^{a} ± 0.1	1.7^{a} ± 0.1	5.6	5.7	5.8
Lupin protein isolate	-	-	-	-	-	-

* Mean values were calculated from three replicates ± standard deviation. Values within one column of each microorganism with different superscript letter are significant different ($p < 0.05$).

3.2.3 Microbial degradation of secondary plant metabolites in lupin

3.2.3.1 Oligosaccharides

The carbohydrate content in lupin samples was analyzed during fermentation to examine the microbial sugar metabolism, especially their ability to utilize raffinose and stachyose (Figures 10 – 13). Within 48 h, *Bif. animalis* subsp. *lactis, Lb. plantarum* and *P. pentosaceus* assimilated the total sucrose content of all lupin substrates. Similarly, most strains degraded the entire amount of galactose in the different suspensions. In contrast, the galactose content remained approximately constant in lupin flour samples inoculated with *Bif. animalis* subsp. *lactis* and in lupin protein samples the concentration increased by 23%.

The oligosaccharide content was considerably decreased during fermentation with *Bif. animalis* subsp. *lactis* and *P. pentosaceus.* The highest oligosaccharide degradation was observed for *Bif. animalis* subsp. *lactis* in lupin protein isolate with a utilization degree of 97.8% for raffinose and 100% for stachyose. During fermentation of sweet lupin flour with the same strain, oligosaccharides were decreased to a lower extent (-92.3% raffinose, -22.5% stachyose). In the bitter lupin flour a degradation in the same order of magnitude was analyzed (-100% raffinose and -23.4% stachyose, Figure 10).

Similarly, *P. pentosaceus* assimilated higher concentrations of raffinose and stachyose in the lupin protein isolate (-59.8% and -58.4%) than in the two kinds of lupin flour suspensions (Figure 11).

The fermentation of bitter lupin flour with *Lb. plantarum* led to a slight raffinose decrease (-13.2%). Stachyose was not degraded at all (Figure 12). *L. lactis* subsp. *lactis* degraded 19% of the raffinose content and 11.9% of stachyose in sweet lupin flour. No significant decreases were observed in bitter lupin flour (Figure 13).

In the corresponding blank samples of sweet lupin flour, a maximum reduction of raffinose with 24.3% and stachyose with 5.2% was determined, whereas in bitter lupin blank samples the carbohydrate content did not decrease (Table 18). A slight increase was monitored instead. In lupin protein isolate a reduction was only observed for raffinose (max. -21.2%).

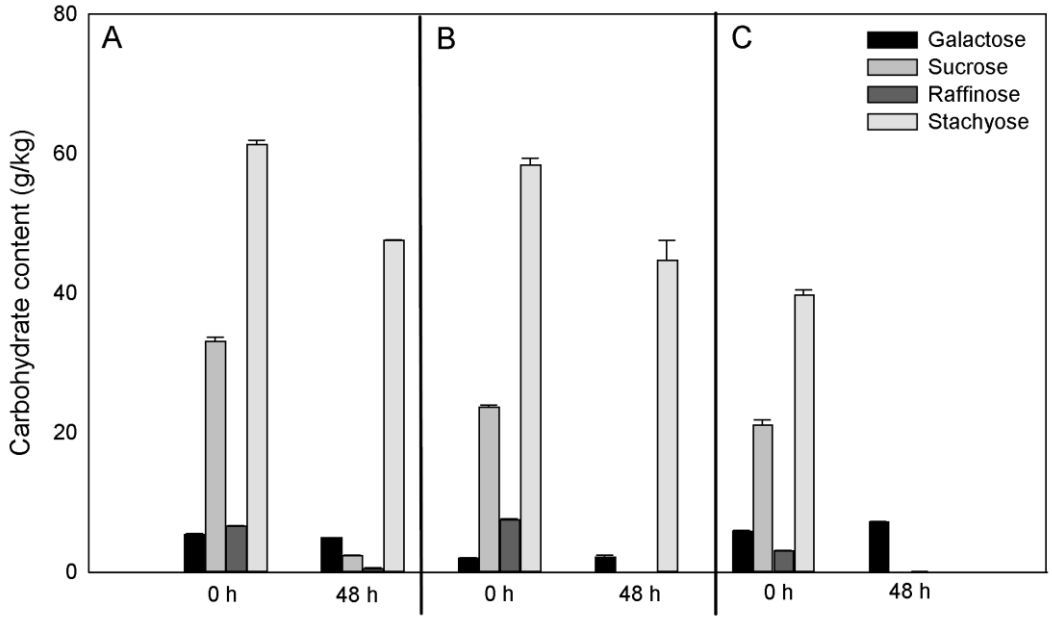


Figure 10: Carbohydrate content in sweet lupin flour (A), bitter lupin flour (B) and lupin protein isolate (B) during fermentation with *Bifidobacterium animalis* subsp. *lactis* DSM 10140

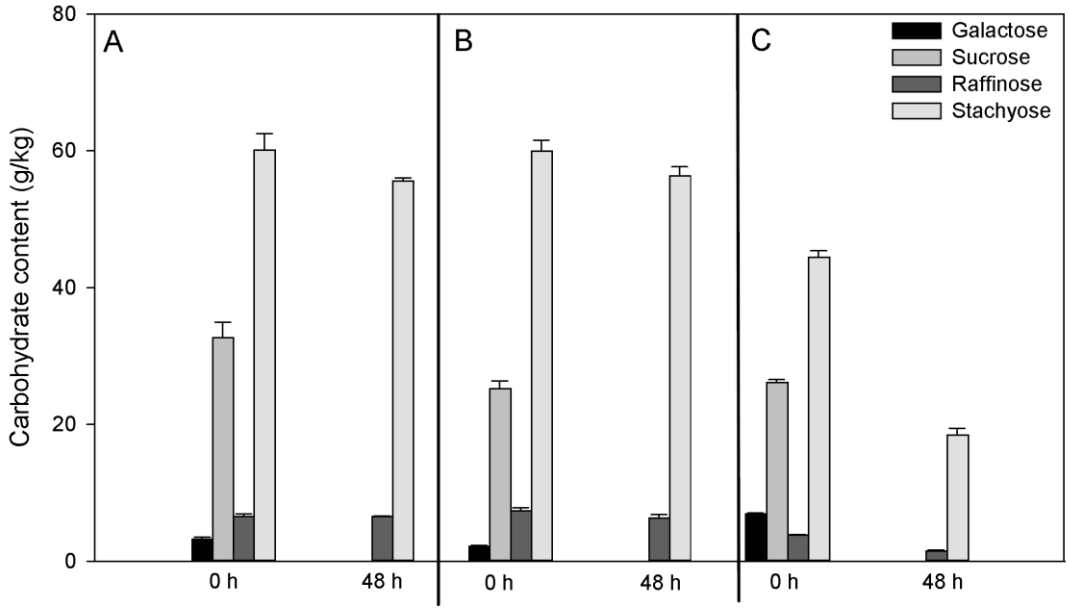


Figure 11: Carbohydrate content in sweet lupin flour (A), bitter lupin flour (B) and lupin protein isolate (C) during fermentation with *Pediococcus pentosaceus* DSM 20336

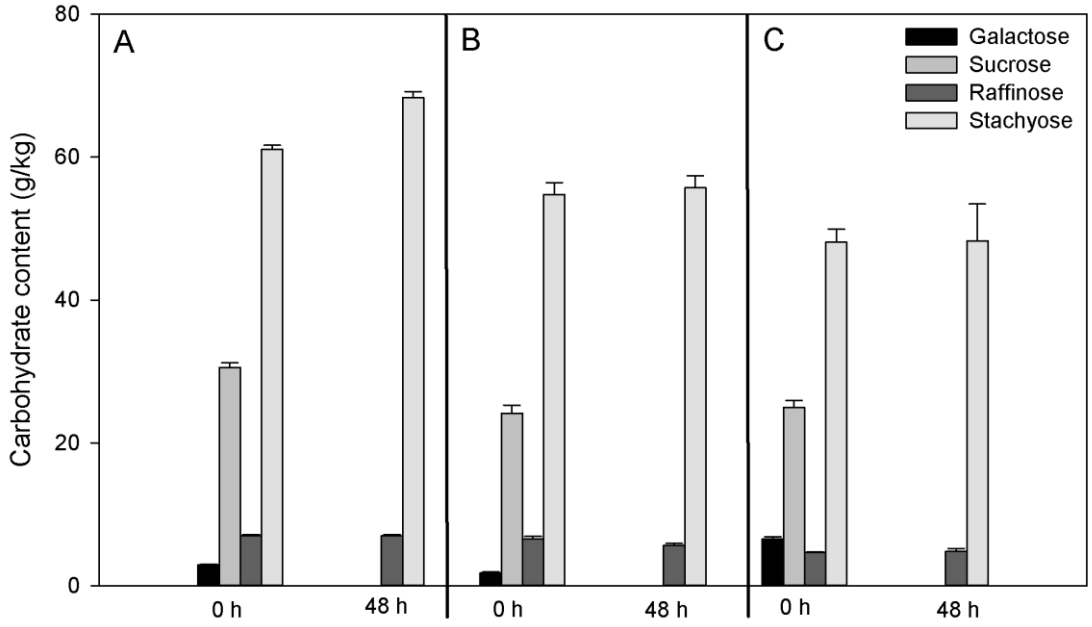


Figure 12: Carbohydrate content in sweet lupin flour (A), bitter lupin flour (B) and lupin protein isolate (C) during fermentation with *Lactobacillus plantarum* TMW 1.460

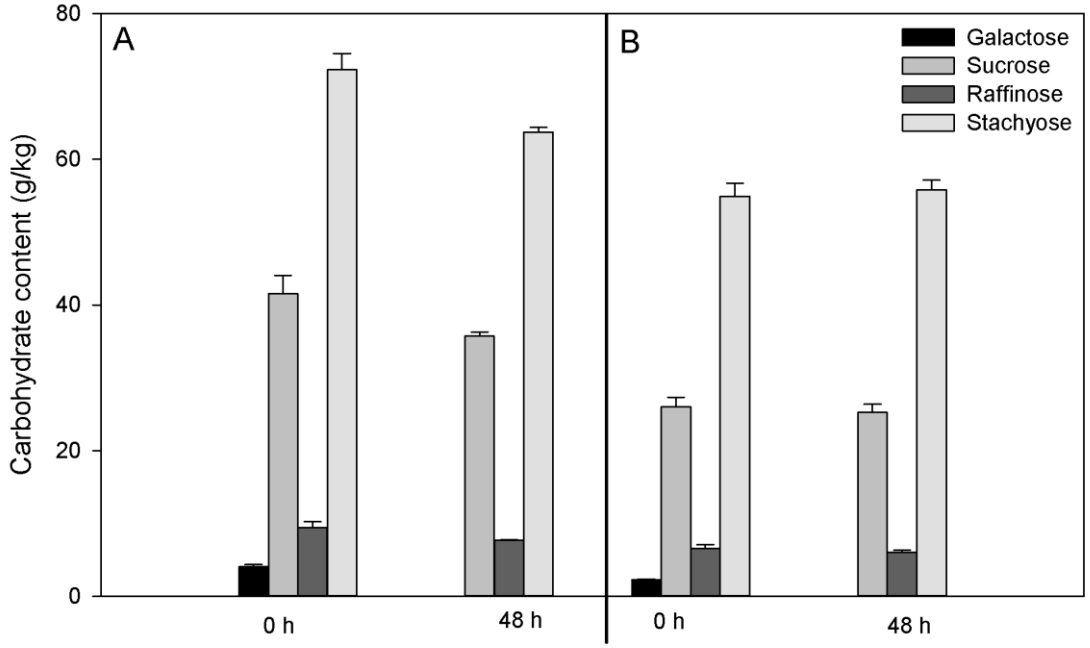


Figure 13: Carbohydrate content in sweet lupin flour (A) and bitter lupin flour (B) during fermentation with *Lactococcus lactis* subsp. *lactis* DSM 20384

Table 18: Maximum carbohydrate changes in lupin blank samples after 48 h incubation

	Galactose (%)	**Sucrose (%)**	**Raffinose (%)**	**Stachyose (%)**
Sweet lupin flour	-1.4	-1.9	-24.3	-5.2
Bitter lupin flour	+8.5	+7.0	+13.7	+8.5
Lupin protein	+13.0	+14.6	-21.2	-0.0

3.2.3.2 Phytic acid

The initial phytic acid concentration of the three suspensions was slightly different with 0.5% phytic acid in sweet and bitter lupin suspensions and 0.3% in lupin protein isolate suspension (Table 13). Table 19 illustrates the mean values of phytic acid reduction during fermentation and for blank samples. An obvious degradation around 10 to 14% was monitored in all tested lupin substrates using *Lb. plantarum* for fermentation. The highest phytic acid reduction with 23.5% occurred within the fermentation of lupin protein isolate with *Bif. animalis* subsp. *lactis,* whereas in lupin flour suspensions the content remained unchanged. Limited ability to degrade phytic acid was shown for *P. pentosaceus* in bitter lupin flour (-9.3%) and for *L. lactis* subsp. *lactis* in sweet lupin flour (-6.9%). The degradation of phytic acid in blank samples was different in the diverse substrates: 0.1% reduction in sweet lupin flour, 6.2% in bitter lupin flour and 3.9% in lupin protein isolate.

Table 19: Changes in phytic acid content in inoculated and lupin blank samples after 48 h incubation

Microorganism	Phytic acid (%)		
	Sweet lupin flour	**Bitter lupin flour**	**Lupin protein isolate**
Bifidobacterium animalis subsp. *lactis*	0.0	0.0	-23.5
Pediococcus pentosaceus	-0.7	-9.3	0.0
Lactobacillus plantarum	-13.9	-14.0	-10.8
Lactococcus lactis subsp. *lactis*	-6.9	0.0	-
Blank	-0.1	-6.2	-3.9

3.3 Fermentation of sunflower

3.3.1 Composition of sunflower substrates

The proximate composition of sunflower flour and protein concentrate is given in Table 20. Almost half of sunflower flour consisted of protein (49.8%), around 8% carbohydrates in total and only marginal amounts of fat (1.8%). With 2.9% of chlorogenic acid and 0.06% of caffeic acid similar concentrations were measured as reported by Weisz et al. (2009). An exemplary HPLC-UV chromatogram of phenolic acids in sunflower flour is shown in Appendix A3. The protein concentrate had an expected higher protein content (60.0%) and reduced amounts of carbohydrates (0.1%), fat (0.7%) and phenolic acids (0.08%) due to the applied aqueous-alcoholic extraction. The main carbohydrates in sunflower flour were sucrose (4.1%) and raffinose (2.8%), followed by galactose (0.6%) and stachyose (0.2%). Only minor amounts of carbohydrates were analyzed in the protein concentrate (Table 21).

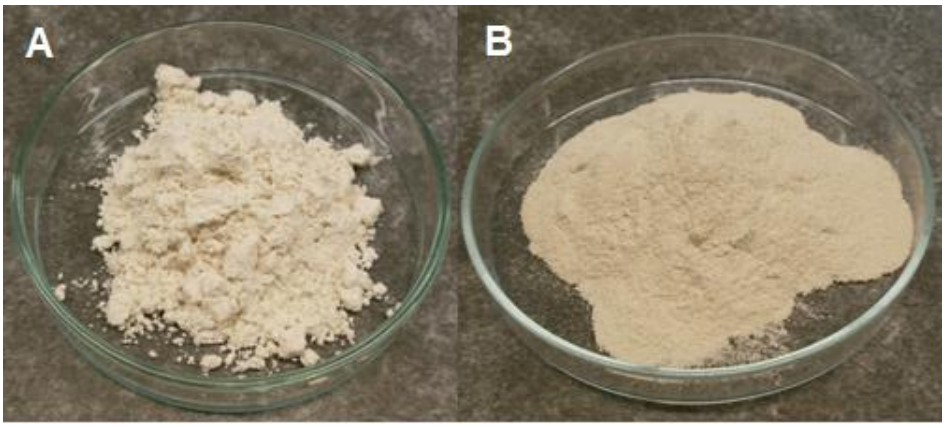


Figure 14: Sunflower flour (A) and sunflower protein concentrate (B)

Table 20: Proximate composition of sunflower substrates

Ingredients (%)	Sunflower flour	Sunflower protein concentrate
Dry matter	90.5	89.0
Carbohydrates	7.6	0.1
Protein	49.8	60.0
Fat	1.8	0.7
Chlorogenic acid	2.9	0.1
Caffeic acid	0.1	2.0×10^{-3}

Table 21: Carbohydrate composition of sunflower substrates

Carbohydrates (g/kg)	Sunflower flour	Sunflower protein concentrate
Galactose	5.7	0.3
Sucrose	40.7	0.3
Raffinose	27.5	0.2
Stachyose	2.2	0.0
Verbascose	0.2	0.0

3.3.2 Fermentation performance of selected strains and effects of secondary plant metabolites

In order to evaluate the influence of SPM in sunflower substrates on the microbial fermentation performance, growth behavior and metabolism of four different bacteria was examined. As sunflower flour contains considerably amounts of oligosaccharides (Canella et al., 1984; Kuo et al., 1988, Table 21), strains capable of metabolizing raffinose and stachyose were selected (*Lb. plantarum* TMW 1.460, *P. pentosaceus* DSM 20336 and *Bif. animalis* subsp. *lactis* DSM 10140). According to studies of Couteau et al. (2001) and Raimondi et al. (2015), strains of *Bif. lactis* and *Lb. gasseri* degrade chlorogenic acid which is in line with the screening in media (Table 12). Therefore, *Lb. gasseri* DSM 20243 was additionally selected for fermentation experiments of sunflower substrates. Cell counts and organic acid concentrations were analyzed during fermentation of sunflower flour suspension and compared to those in sunflower protein suspension. The protein sample contained after addition the same carbohydrate composition like the flour but only trace amounts of SPM.

All tested strains exhibited a stronger growth in sunflower flour suspensions than in protein suspensions during 48 h of fermentation, except for *P. pentosaceus* (Figure 15). This strain reached higher cell counts in sunflower protein suspensions due to an earlier occurrence of

death-phase in sunflower flour. Similar to the fermentation of lupin protein isolate, this strain demonstrated a more pronounced lag-phase in the sunflower protein sample (Figure 15 B). Table 22 summarizes the rise of bacterial cell numbers. According to MALDI-TOF MS measurements the inoculated bacteria were dominant in all sunflower suspensions. Due to the pH-adjustment and pasteurization step of the sunflower blank samples, bacterial strains were below the detection limit (< 200 cfu/ml).

The analyses of lactate and acetate (Tables 23 and 24) confirmed the results of the microbial growth measurements. All strains produced significantly higher quantities of organic acids in sunflower flour suspensions indicating a stronger metabolic activity. The highest concentration of lactic acid with 90.2 mmol/l was produced by *Lb. plantarum* after 48 h in sunflower flour. This strain also exhibited the strongest rise of bacterial count with 2.4 x 10^9 cfu/ml during 48 h. The highest concentration of acetate was measured in sunflower flour fermented with *Bif. animalis* subsp. *lactis* (52.5 mmol/l). In line with these results, a pH drop from 6.3 to 4.2 – 4.5 in sunflower flour and from 6.5 to 4.5 – 4.7 in sunflower protein suspension was measured.

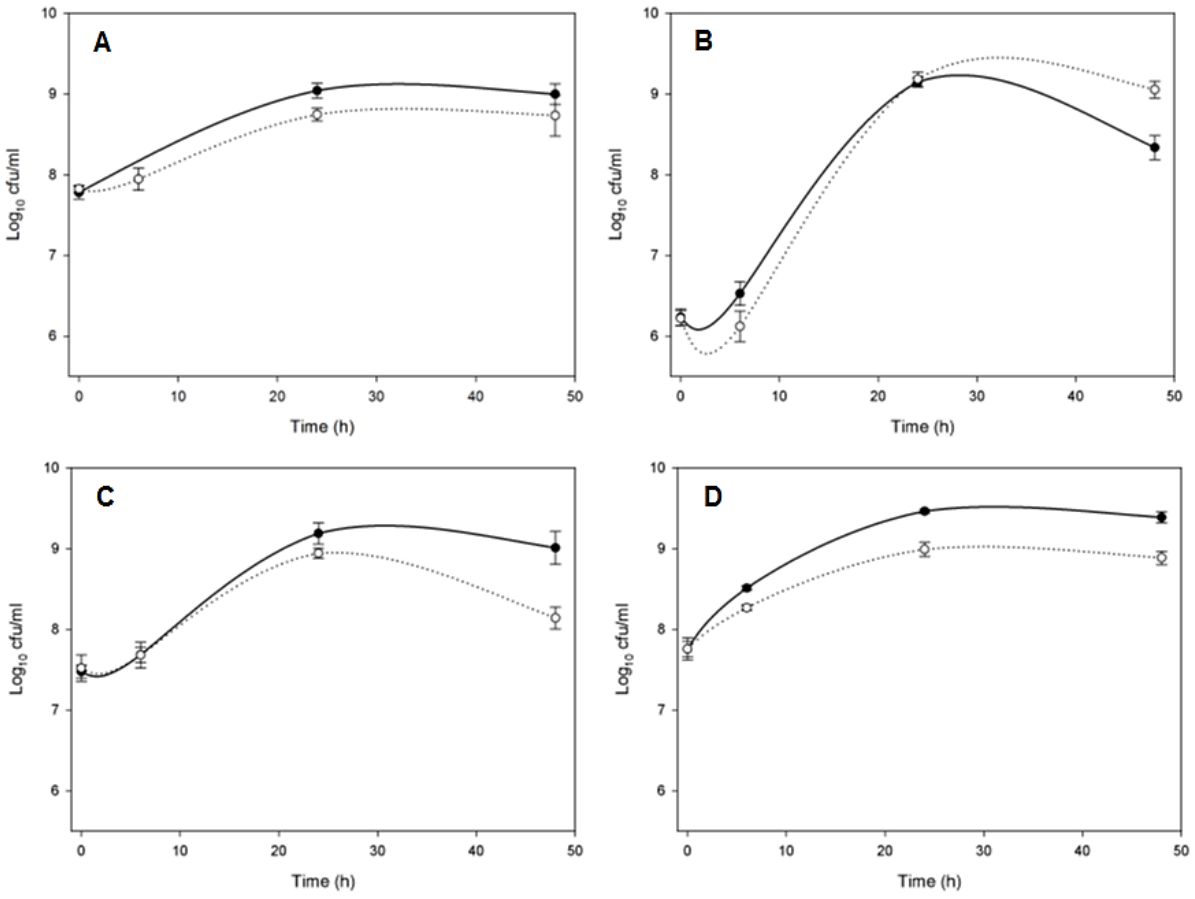


Figure 15: Microbial growth in sunflower flour (●) and sunflower protein concentrate (○). A = *Bifidobacterium animalis* subsp. *lactis* DSM 10140. Cell counts in sunflower flour after 6 h could not be analyzed due to a defect in the anaerobe jar, B = *Pediococcus pentosaceus* DSM 20336, C = *Lactobacillus gasseri* DSM 20243, D = *Lactobacillus plantarum* TMW 1.460

Table 22: Rise of bacterial count as $\Delta(N - N_0)$ in cfu/ml during 48 h of fermentation

Strain	Sunflower flour	Sunflower protein concentrate
Bifidobacterium animalis subsp. *lactis*	9.7×10^8	5.5×10^8
Pediococcus pentosaceus	2.3×10^8	1.2×10^9
Lactobacillus plantarum	2.4×10^9	$7{,}3 \times 10^8$
Lactobacillus gasseri	1.1×10^9	1.1×10^8

Table 23: Metabolite formation and pH values of heterofermentative *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and *Lactobacillus plantarum* TMW 1.460 in sunflower substrates

	Lactate (mmol/l)*			Acetate (mmol/l)*			pH		
	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Bifidobacterium animalis* subsp. *lactis									
Sunflower flour	2.1^a ± 0.1	25.7^a ± 0.1	28.5^a ± 0.7	6.1^a ± 0.3	47.3^a ± 0.6	52.5^a ± 1.7	5.9	4.6	4.5
Sunflower protein concentrate	1.8^a ± 0.3	19.1^b ± 0.5	19.6^b ± 0.1	6.0^a ± 0.7	42.5^b ± 0.2	48.9^a ± 2.0	6.0	4.8	4.7
Lactobacillus plantarum									
Sunflower flour	15.7^a ± 0.4	86.5^a ± 6.6	90.2^a ± 0.8	1.0^a ± 0.2	3.4^a ± 0.3	5.3^a ± 0.7	5.6	4.2	4.2
Sunflower protein concentrate	9.7^b ± 0.5	39.3^b ± 2.7	69.1^b ± 0.5	0.9^a ± 0.1	1.0^b ± 0.1	2.3^b ± 0.1	6.1	5.1	4.5

* Mean values were calculated from three replicates ± standard deviation. Values within one column of each microorganism with different superscript letter are significant different ($p < 0.05$).

Table 24: Metabolite formation and pH values of homofermentative *Pediococcus pentosaceus* DSM 20336 and *Lactobacillus gasseri* DSM 20243 in sunflower substrates

	Lactate (mmol/l)*			pH		
	6 h	24 h	48 h	6 h	24 h	48 h
Pediococcus pentosaceus						
Sunflower flour	4.0^a ± 0.5	85.5^a ± 1.7	88.6^a ± 0.8	6.2	4.2	4.2
Sunflower protein concentrate	1.2^a ± 0.2	65.0^b ± 1.4	69.5^b ± 2.4	6.4	4.6	4.5
Lactobacillus gasseri						
Sunflower flour	8.6^a ± 0.6	72.2^a ± 2.3	69.3^a ± 1.1	5.9	4.3	4.4
Sunflower protein concentrate	6.9^a ± 1.0	43.9^b ± 2.6	44.9^b ± 1.8	6.0	4.6	4.7

* Mean values were calculated from three replicates ± standard deviation. Values within one column of each microorganism with different superscript letter are significant different ($p < 0.05$).

3.3.3 Minimum inhibitory concentration assay and kinetic measurements

The antimicrobial activities of the main phenolic acids in sunflower seeds, chlorogenic acid and its cleavage products caffeic and quinic acid were measured by the broth micro-dilution method. The concentrations were chosen according to the initial amount contained in 10% (w/v) sunflower flour suspensions. The results are given as the MIC_{90} of each phenolic acid in Table 25. Sensitivity to the compounds differed between the strains. *Bif. animalis* subsp. *lactis* exhibited the strongest sensitivity against all tested phenolic acids, whereas *Lb. gasseri, Lb. plantarum* and *P. pentosaceus* demonstrated similar tolerances. The MICs of chlorogenic acid after 48 h against these strains were higher than the highest concentration used in this assay (20.48 mg/ml). This is in accordance to Sanchez-Maldonado et al. (2011) who measured a MIC of >6.7 mg/ml chlorogenic against *Lb. plantarum* TMW 1.460 and *Lb. hammesii* DSM 16381 after 24 h. The authors reported a MIC of 1.52 mg/ml caffeic acid for *Lb. plantarum* TMW 1.460. This strain showed in the present study a slightly higher tolerance against caffeic acid with 2.56 mg/ml after 24 h. In all cases MICs of chlorogenic, caffeic and quinic acid were significantly higher than the concentrations found in sunflower substrates (Table 20). The MICs of most compounds increased from 24 to 48 h.

Table 25: Antibacterial activity of chlorogenic acid (CGA), caffeic acid (CFA) and quinic acid (QA)

	Minimum inhibitory concentration (mg/ml)*					
	CGA		**CFA**		**QA**	
	24 h	48 h	24 h	48 h	24 h	48 h
Bifidobacterium animalis subsp. *lactis*	2.56	10.24	0.32	0.64	2.56	5.12
Lactobacillus gasseri	20.48	>20.48	2.56	2.56	20.48	20.48
Lactobacillus plantarum	>20.48	>20.48	2.56	5.12	20.48	>20.48
Pediococcus pentosaceus	20.48	>20.48	1.28	2.56	10.24	20.48

* Minimum inhibitory concentration was defined as the lowest concentration that inhibited the microbial growth by ≥90% compared with the growth control

The microbial growth kinetics in presence of 0.32 – 20.48 mg/ml chlorogenic acid as well as in media without addition (growth control) were measured spectrophotometrically at 595 nm. Kinetic measurements of *Lb. plantarum* and *P. pentosaceus* were recorded in the nitrogen flushed detection chamber of the photometer (Figures 16 and 17).

The growth curves of *Lb. plantarum* confirmed the results of the MIC assay because this strain showed a distinct growth in MRS broth with all tested chlorogenic acid concentrations. Although a significant longer lag-phase was observed in presence of 20.48 mg/ml chlorogenic acid, *Lb. plantarum* approximately reached the same optical density as the growth control after 48 h. *P. pentosaceus* was more sensitive, showing a measurable growth behavior up to a maximum of 2.56 mg/ml chlorogenic acid. Concentrations of ≥5.12 mg/ml

totally inhibited the growth, whereas the MIC-assay revealed a MIC_{90} of >20.48 mg/ml after 48 h (Table 25). The different incubation conditions (anaerobic jar versus detection chamber of the photometer) might explain this difference.

Measurements with *Bif. animalis* subsp. *lactis* and *Lb*. *gasseri* were performed manually because these strains showed no growth if the microtiter plates were flushed with nitrogen inside the photometer. Probably, the remaining oxygen concentration of 0.4 – 0.6% was too high for these anaerobic strains. During the first 33 h no significant growth of *Bif. animalis* subsp. *lactis* was observed neither in any media with chlorogenic acid, nor in growth control (Figure 18). It can be assumed that no sufficient anaerobic milieu was reached for this strain due to the regularly opening of the anaerobic jar for measurement. After 44 h of incubation the optical density increased, revealing comparable growth patterns in media with 0.32 mg/ml as the growth control. In presence of 10.24 and 20.48 mg/ml chlorogenic acid no growth was detected within 70 h. Growth inhibition of *Lb. gasseri* was only given during the first 20 h in presence of the highest concentration tested (20.48 mg/ml), whereas in the other samples a comparable growth behavior like the growth control was observed (Figure 19).

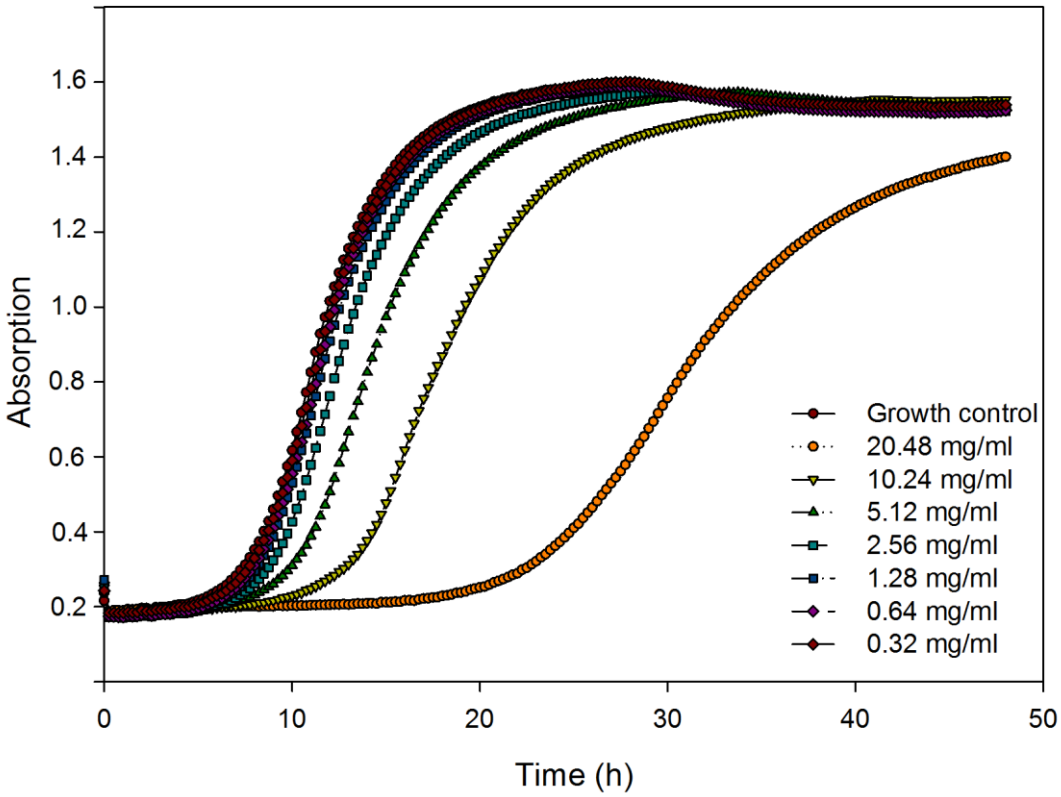


Figure 16: Growth kinetics of *Lactobacillus plantarum* TMW 1.460 in MRS broth with different concentrations of chlorogenic acid. The absorption was measured at 595 nm

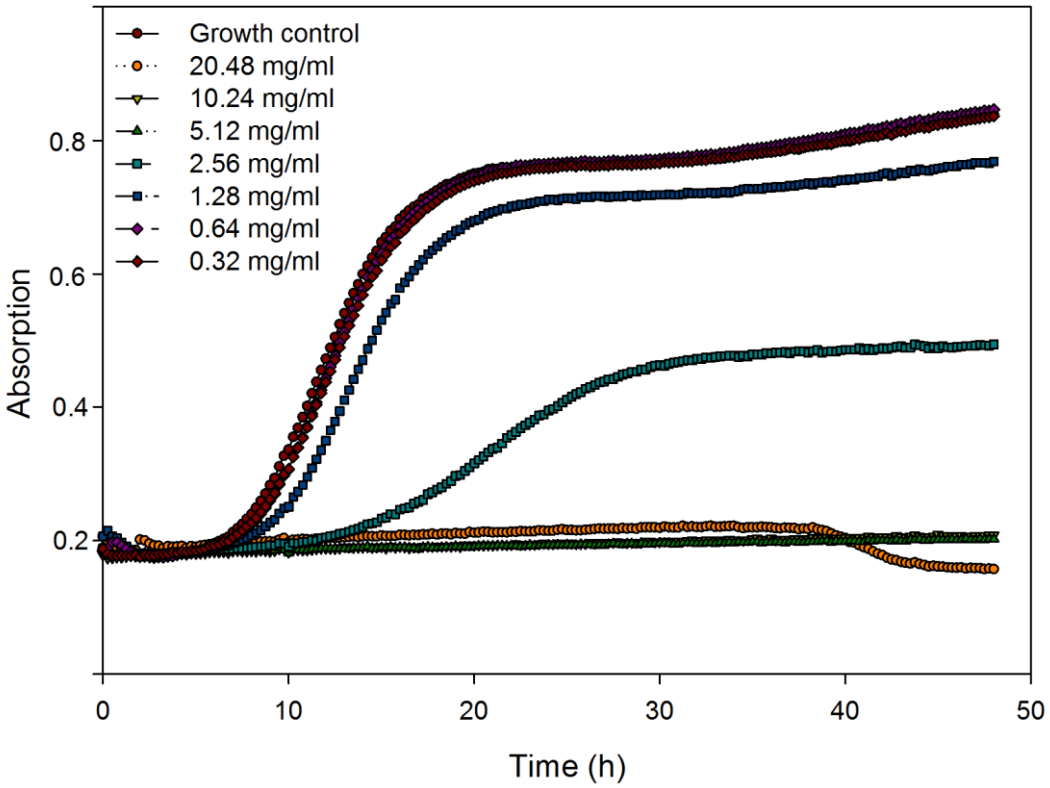


Figure 17: Growth kinetics of *Pediococcus pentosaceus* DSM 20336 in MRS broth with different concentrations of chlorogenic acid. The absorption was measured at 595 nm

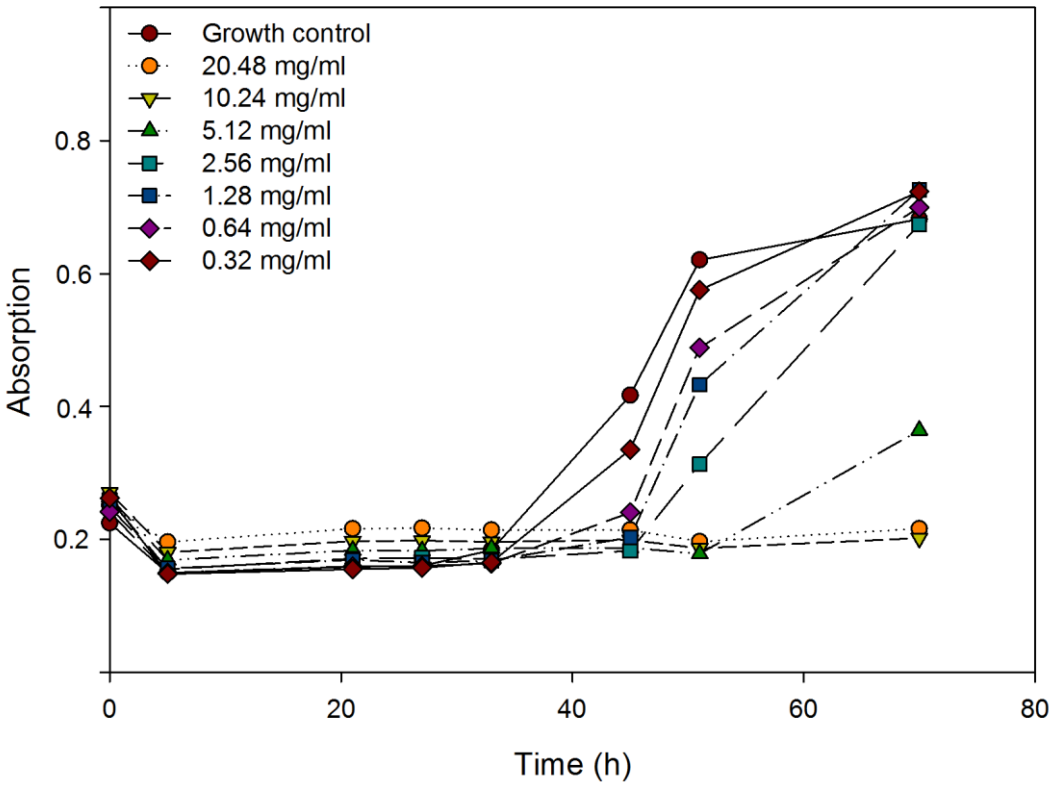


Figure 18: Growth kinetics of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 in MRS with cysteine broth with different concentrations of chlorogenic acid. The absorption was measured at 595 nm

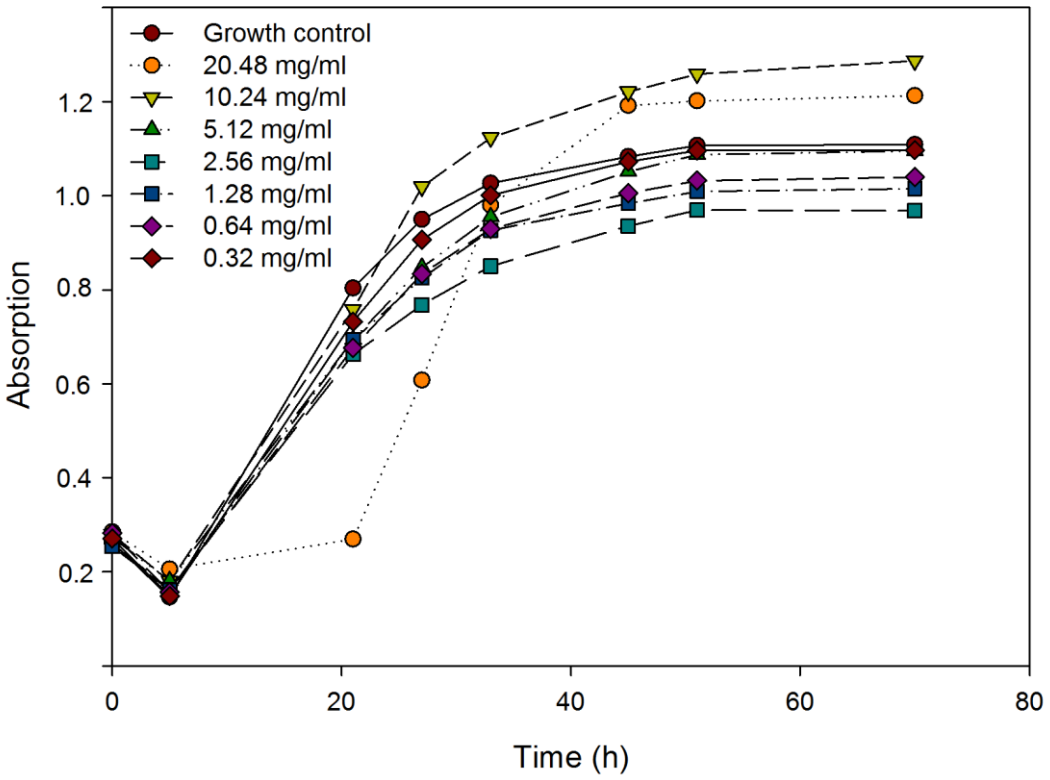


Figure 19: Growth kinetics of *Lactobacillus gasseri* DSM 20243 in MRS with cysteine broth with different concentrations of chlorogenic acid. The absorption was measured at 595 nm

3.3.4 Microbial degradation of secondary plant metabolites in sunflower

3.3.4.1 Oligosaccharides

The carbohydrate content during sunflower fermentation is shown in Figures 20 – 23. Similar to the fermentation of lupin, all strains, except *Bif. animalis* subsp. *lactis* assimilated the entire amount of galactose and sucrose within 48 h. In sunflower flour with *Bif. animalis* subsp. *lactis* the galactose amount increased by 38% and in sunflower protein concentrate by 25%. The highest oligosaccharide reduction in sunflower flour was measured for *P. pentosaceus* and *Lb. plantarum* (-98.6% raffinose, -82.4% stachyose and -100% raffinose and stachyose, respectively), followed by *Bif. animalis* subsp. *lactis* (-100% raffinose, -53.8% stachyose). *Lb. gasseri* degraded only minor amounts of oligosaccharides in sunflower flour as well as in the protein concentrate (Figure 21). A total utilization of raffinose and stachyose in sunflower protein concentrate was shown by *Bif. animalis* subsp. *lactis* (Figure 20). *P. pentosaceus* also reduced the entire amount of raffinose and 68.5% stachyose (Figure 22), whereas *Lb. plantarum* only significantly degraded raffinose (75.2%, Figure 23).

Carbohydrate reductions in chemical acidified blank samples were significantly lower than in inoculated substrates. The maximal decrease of raffinose and stachyose in sunflower flour

was 13.5% and 18.9%, respectively. In sunflower protein, the reductions were even lower (-8.8% raffinose and -5.0% stachyose, Table 26). Therefore, oligosaccharide degradation was mainly ascribed to microbial metabolism.

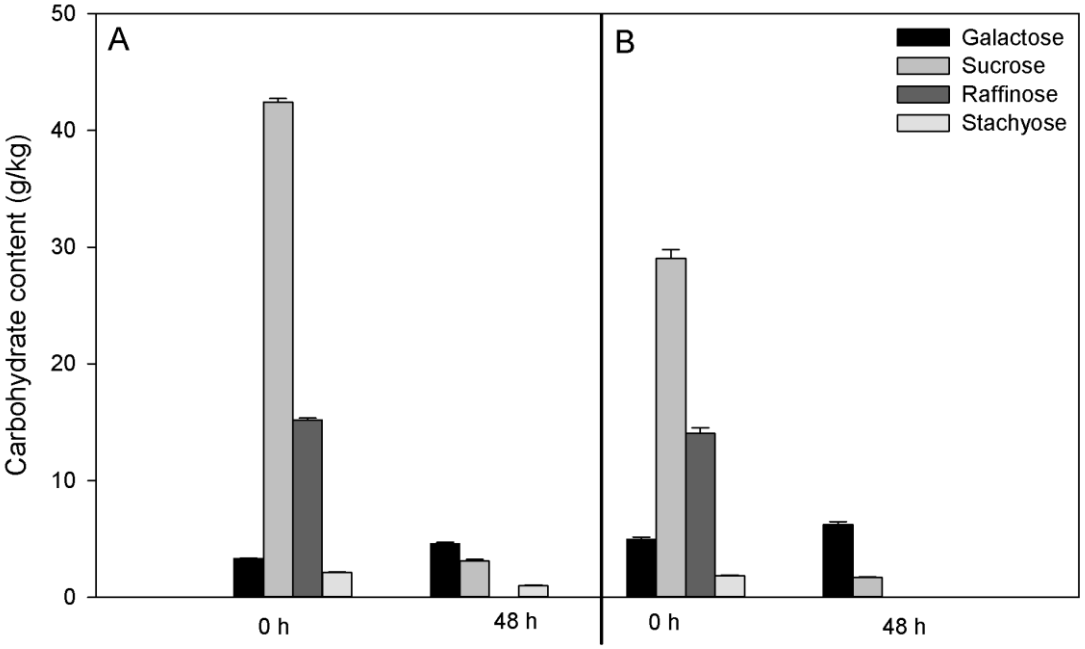


Figure 20: Carbohydrate content in sunflower flour (A) and protein concentrate (B) during fermentation with *Bifidobacterium animalis* subsp. *lactis* DSM 10140

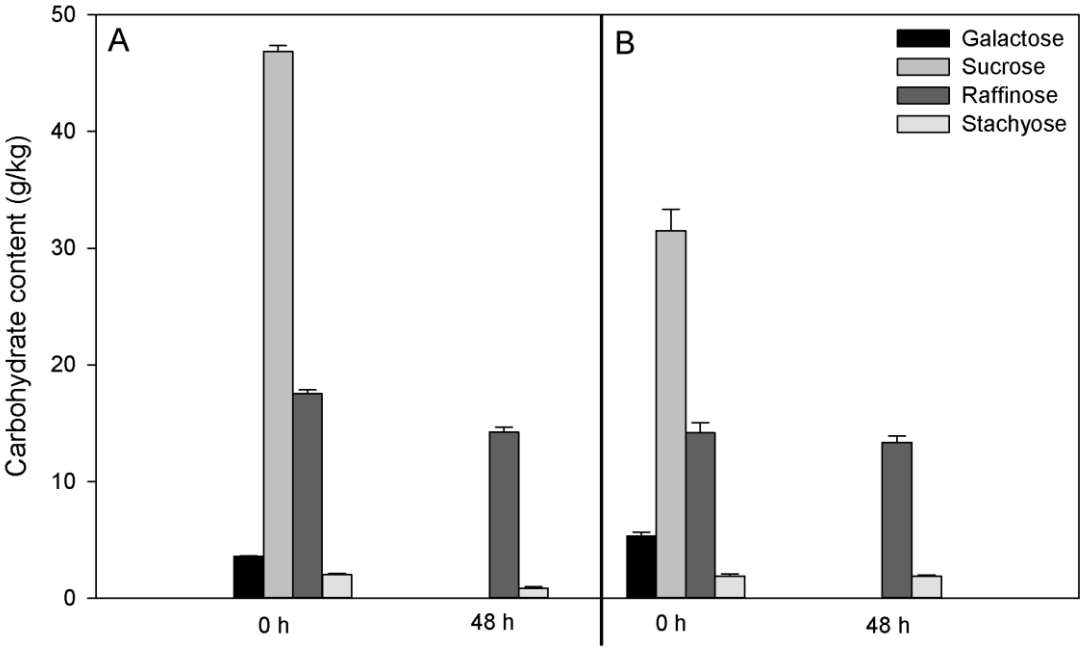


Figure 21: Carbohydrate content in sunflower flour (A) and protein concentrate (B) during fermentation with *Lactobacillus gasseri* DSM 20243

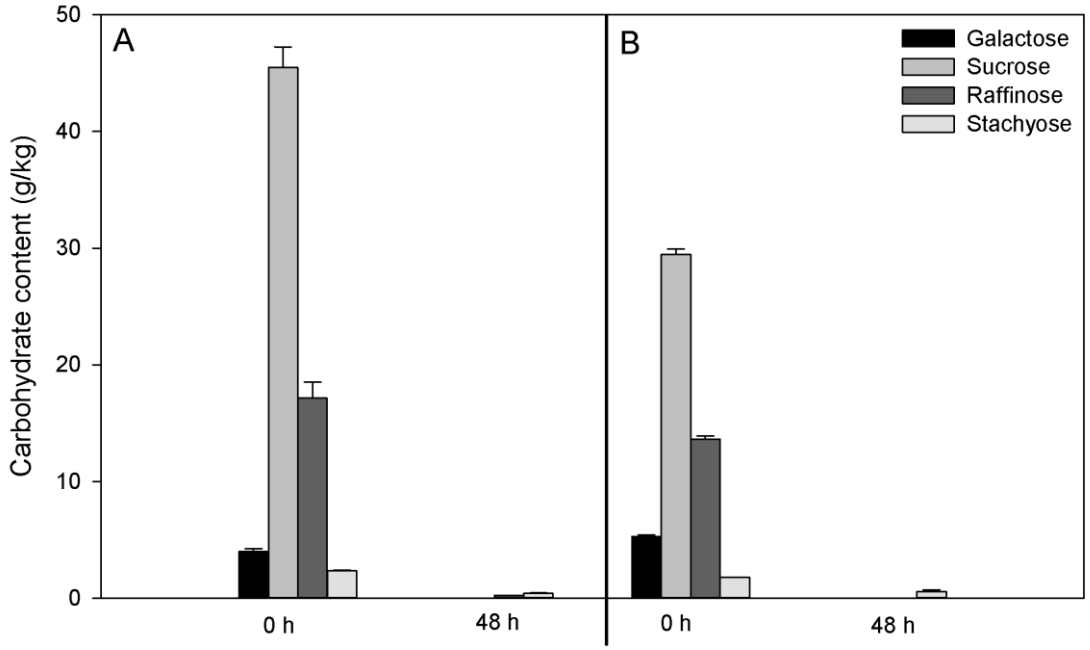


Figure 22: Carbohydrate content in sunflower flour (A) and protein concentrate (B) during fermentation with *Pediococcus pentosaceus* DSM 20336

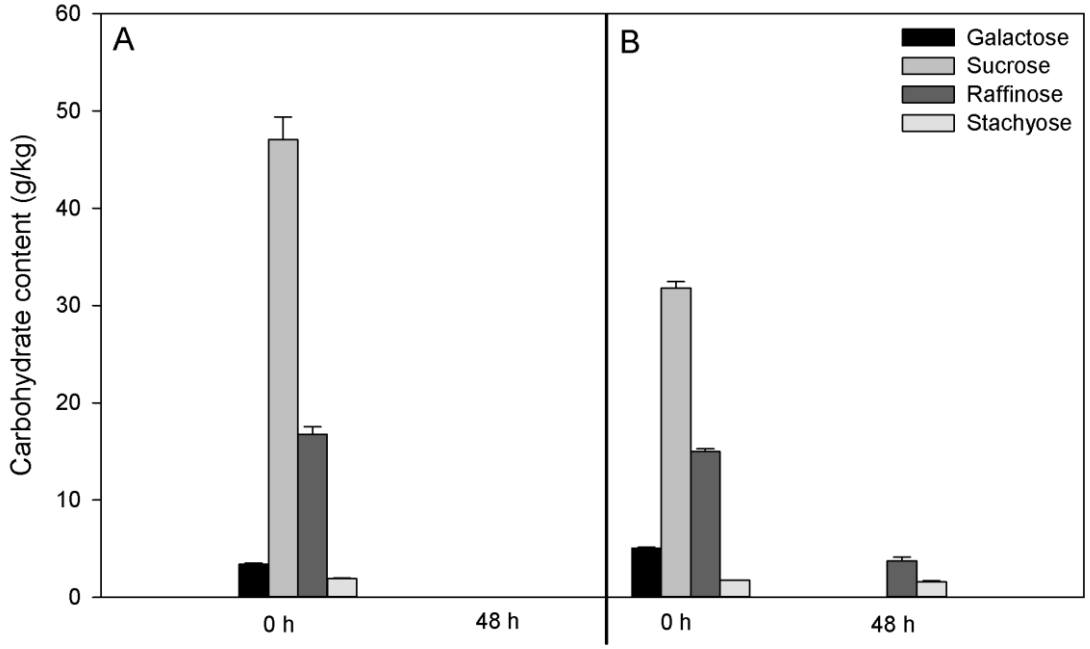


Figure 23: Carbohydrate content in sunflower flour (A) and protein concentrate (B) during fermentation with *Lactobacillus plantarum* TMW 1.460

Table 26: Maximum carbohydrate changes in sunflower blank samples after 48 h incubation

	Galactose (%)	**Sucrose (%)**	**Raffinose (%)**	**Stachyose (%)**
Sunflower flour	-11.2	-11.5	-13.5	-18.9
Sunflower protein	-8.0	-10.7	-8.8	-5.0

3.3.4.2 Chlorogenic acid

The microbial metabolism of phenolic compounds in sunflower substrates was examined by determination of the chlorogenic acid concentration during fermentation. In order to confirm the microbial degradation of chlorogenic acid, the amount of its metabolite, caffeic acid, was analyzed as well.

A reduction of chlorogenic acid was monitored in sunflower substrates fermented with *Bif. animalis* subsp. *lactis* (Figure 24). The decrease in sunflower flour accounted for 355.6 µg/ml (-11.4%) and in sunflower protein suspension for 44.6 µg/ml (-50.7%). Simultaneously, the caffeic acid content increased (+94.7 µg/ml and +19.6 µg/ml, respectively) indicating that *Bif. animalis* subsp. *lactis* possessed the enzymatic equipment to hydrolyze chlorogenic acid liberating caffeic acid. This is in accordance to previous studies (Couteau et al., 2001; Raimondi et al., 2015).

A stronger reduction of chlorogenic acid with 499.7 µg/ml (-19.8%) in sunflower flour and 50.9 µg/ml (-95.6%) in sunflower protein was analyzed during fermentation with *Lb. gasseri* (Figure 25). Similarly, Couteau et al. (2001) observed a higher esterase activity of *Lb. gasseri* compared to *Bif. lactis*. The caffeic acid concentration increased within the first 24 h in sunflower flour by 55.4 µg/ml and within the first 6 h in sunflower protein by 23.7 µg/ml followed by a decline.

During fermentation of sunflower flour with *P. pentosaceus* a reduction of caffeic acid (-33.5 µg/ml, -55.9%) was analyzed, whereas the chlorogenic acid concentration did not decreased (Figure 26). In sunflower protein concentrate the chlorogenic acid amount dropped by 29.5 µg/ml (-37.9%) and the caffeic acid content slightly increased (+0.23 µg/ml, 12.6%).

The chlorogenic acid content was not markedly changed in fermentation with *Lb. plantarum* (Figure 27) which was confirmed by a study of Sanchez-Maldonado et al. (2011) with the same strain. The increase of chlorogenic acid after 24 h with the subsequent decrease in sunflower protein suspension was probably not a result of microbial enzyme activity because the concentrations in the blank samples showed the same trend.

Only slight decreases of chlorogenic acid in sunflower flour blank samples (-88.9 µg/ml, -2.9%) and sunflower protein blank samples (-7.9 µg/ml, -10%) were recorded. The caffeic acid concentration increased maximally by 10.8 µg/ml (+15.6%) and 0.7 µg/ml (38.3%) in sunflower flour and protein blank samples, respectively (Table 27).

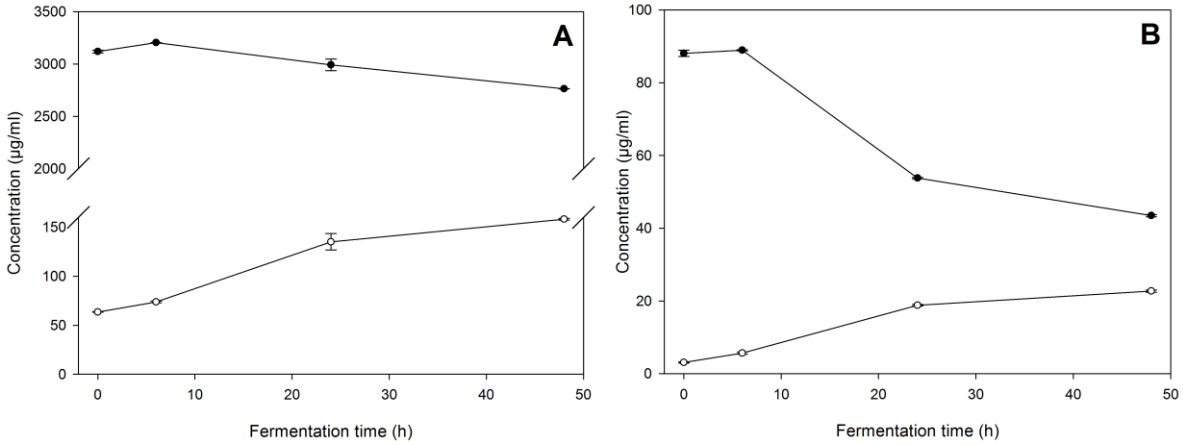


Figure 24: Chlorogenic acid (●) and caffeic acid (○) concentration during fermentation of sunflower flour (A) and sunflower protein concentrate (B) with *Bifidobacterium animalis* subsp*. lactis* DSM 10140

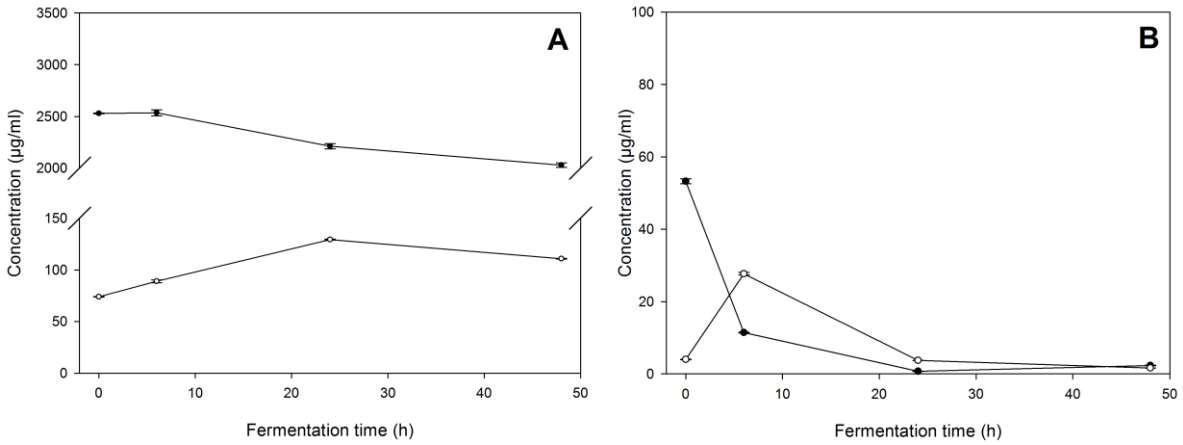


Figure 25: Chlorogenic acid (●) and caffeic acid (○) concentration during fermentation of sunflower flour (A) and sunflower protein concentrate (B) with *Lactobacillus gasseri* DSM 20243

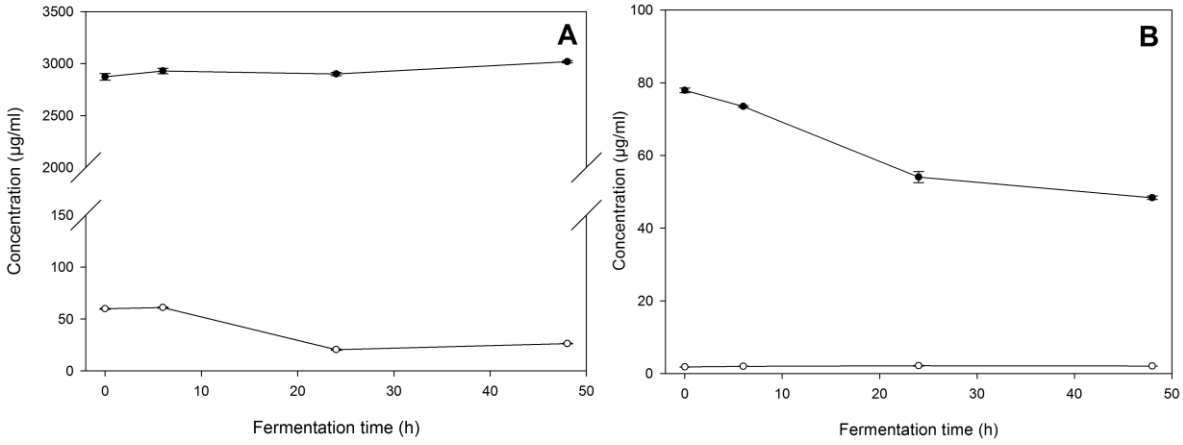


Figure 26: Chlorogenic acid (●) and caffeic acid (○) concentration during fermentation of sunflower flour (A) and sunflower protein concentrate (B) with *Pediococcus pentosaceus* DSM 20336

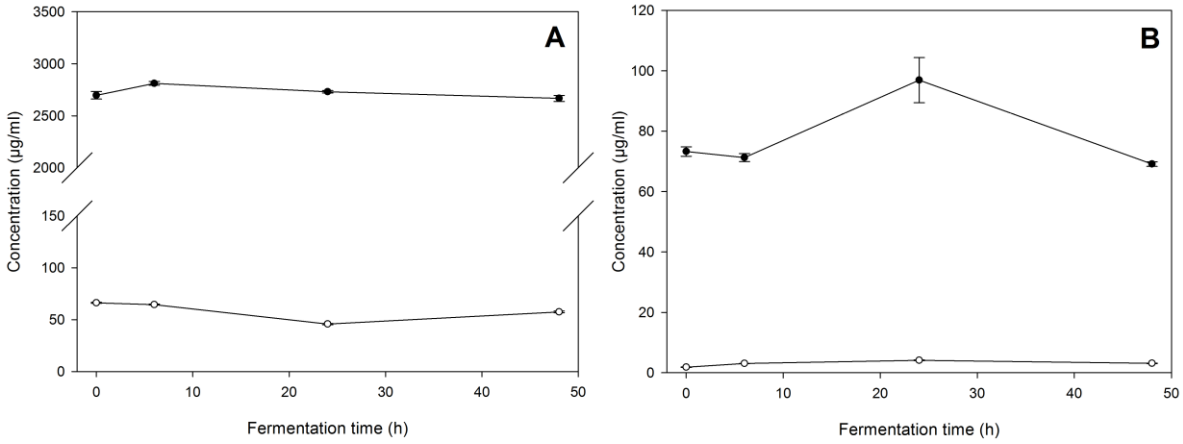


Figure 27: Chlorogenic acid (●) and caffeic acid (○) concentration during fermentation of sunflower flour (A) and sunflower protein concentrate (B) with *Lactobacillus plantarum* TMW 1.460

Table 27: Maximum changes of chlorogenic and caffeic acid in sunflower blank samples after 48 h incubation

	Chlorogenic acid (%)	**Caffeic acid (%)**
Sunflower flour	-2.9	+15.6
Sunflower protein	-10.0	+38.3

3.3.5 Microbial growth and metabolism in chlorogenic acid-spiked sunflower flour

It was investigated if chlorogenic acid maintains its antimicrobial effect in sunflower flour matrix. Therefore, microbial growth, sugar utilization and phenolic acid metabolism of the four selected bacteria were monitored in sunflower flour which was spiked with the respective MIC of chlorogenic acid (10.24 mg/ml for *Bif. animalis* subsp. *lactis* and 20.48 mg/ml for *Lb. plantarum, Lb. gasseri* and *P. pentosaceus*). The growth curves in chlorogenic acid-spiked flour were compared to those in sunflower flour without addition (Figure 28). Three strains (*Bif. animalis* subsp*. lactis*, *P. pentosaceus and Lb. gasseri*) demonstrated a slightly weaker growth. During fermentation of the spiked flour with *Bif. animalis* subsp*. lactis,* a prolonged lag-phase was observed (Figure 28 A). Cell counts after 6 h of fermentation with *Bif. animalis* subsp*. lactis* could not be evaluated due to a defect in the anaerobic jar. Therefore, the prolonged lag phase in the spiked sample might have been detected in the unspiked sample as well.

Initial cell counts of *P. pentosaceus* in the original sunflower flour were distinctly lower than in the spiked flour (Figure 28 B). A reliable comparison of the growth curves was therefore not possible. However, it can be recognized that *P. pentosaceus* grew faster and without a lag-phase in the spiked sunflower flour than in the unspiked flour. On the other hand, the cell counts already decreased drastically after 6 h and were below the detection limit after 48 h,

indicating a premature death phase. Similarly, *Lb. plantarum* revealed a faster growth in chlorogenic acid-spiked sunflower flour and reached the maximum cell counts already after 6 h of fermentation. Unlike *P. pentosaceus,* this strain displayed a constant stationary phase.

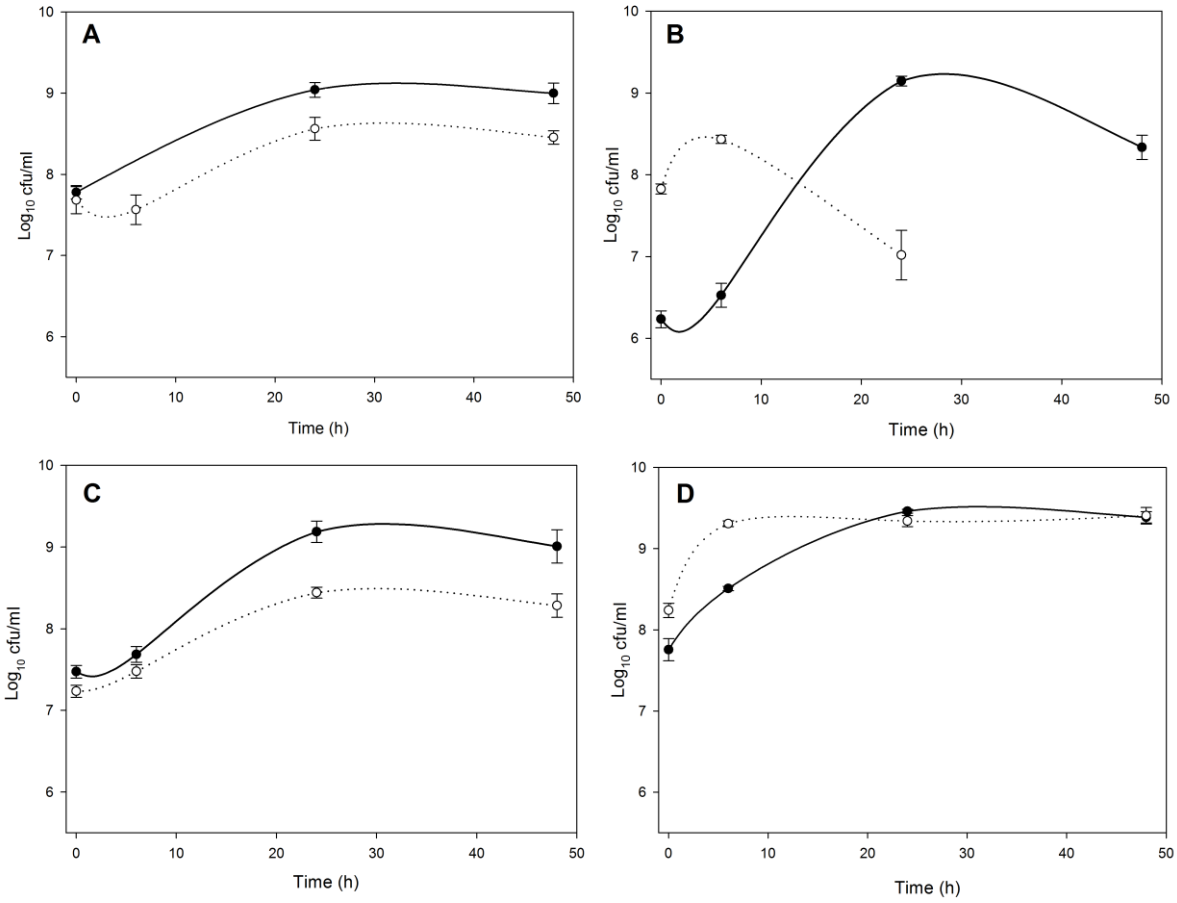


Figure 28: Microbial growth in sunflower flour (●) and sunflower flour spiked with chlorogenic acid (○). A = *Bifidobacterium animalis* subsp*. lactis* DSM 10140. Cell counts in sunflower flour after 6 h could not be analyzed due to a defect in the anaerobe jar. B = *Pediococcus pentosaceus* DSM 20336, C = *Lactobacillus gasseri* DSM 20243, D = *Lactobacillus plantarum* TMW 1.460

The carbohydrate metabolism is summarized in Table 28. The utilization of galactose and sucrose was in the same order of magnitude as in unspiked sunflower flour (chapter 3.3.4.1). An exception was the fermentation with *Bif. animalis* subsp. *lactis* in which galactose decreased, whereas it increased in the original sunflower flour.

The oligosaccharides were assimilated to a lower extend in the spiked flour. The strongest difference was measured for *Lb. plantarum* and *P. pentosaceus* which degraded only 9.3% and 16.5% of raffinose in the chlorogenic acid-spiked sunflower flour. In contrast, degradation rates around 100% were observed in the untreated flour. The stachyose metabolism of these strains in spiked sunflower flour was only half as high as in unspiked flour.

Table 28: Carbohydrate changes during 48 h fermentation of chlorogenic acid-spiked sunflower flour

	Bifidobacterium animalis* subsp. *lactis	***Pediococcus pentosaceus***	***Lactobacillus gasseri***	***Lactobacillus plantarum***
Galactose (%)	-14.4 ± 5.3	-100.0 ± 0.0	-100.0 ± 0.0	-100.0 ± 0.0
Sucrose (%)	-94.9 ± 0.6	-100.0 ± 0.0	-100.0 ± 0.0	-100.0 ± 0.0
Raffinose (%)	-100.0 ± 0.0	-9.3 ± 9.1	+1.8 ± 4.1	-16.5 ± 5.1
Stachyose (%)	-46.4 ± 5.3	-38.3 ± 10.2	-49.8 ± 1.6	-36.2 ± 3.9

Figure 29 shows the changes in chlorogenic acid and caffeic acid concentrations during fermentation of the spiked flour. Similar to the fermentation of original sunflower flour, significant decreases were observed for *Bif. animalis* subsp. *lactis* and *Lb. gasseri* with 605.3 µg/ml (6.6%) and 1290.0 µg/ml (6.7%) chlorogenic acid, respectively. The chlorogenic acid degradation by *Bif. animalis* subsp. *lactis* was accompanied by a rise of the caffeic acid concentration (Figure 29 A). During fermentation with *Lb. gasseri* the caffeic acid amount decreased by 9.3% which also occurred in fermentation of unspiked flour. Only small amounts of chlorogenic acid were metabolized by *Lb. plantarum* (-3.9%) and no degradation of chlorogenic acid was observed for *P. pentosaceus.*

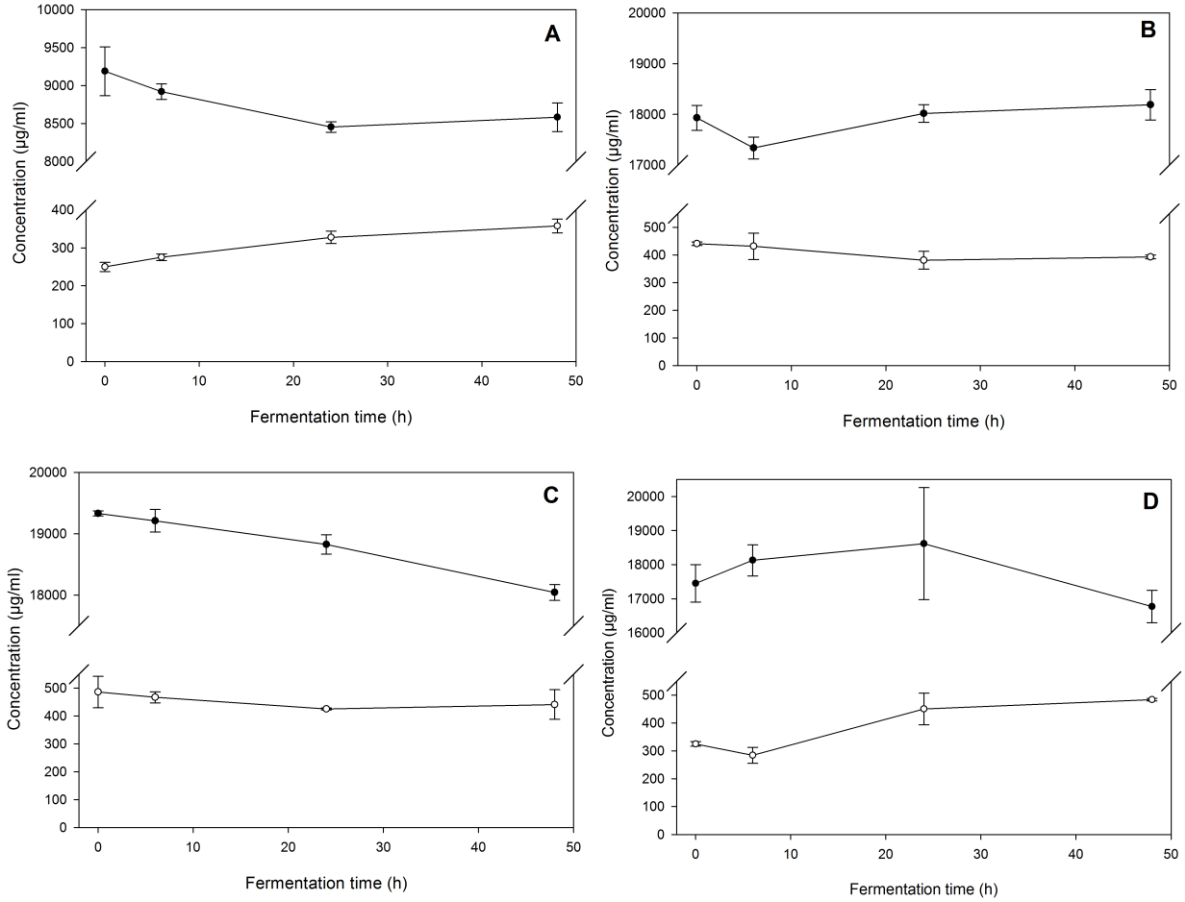


Figure 29: Chlorogenic acid (●) and caffeic acid (○) concentration during fermentation of chlorogenic acid-spiked sunflower flour. A = *Bifidobacterium animalis* subsp*. lactis* DSM 10140. B = *Pediococcus pentosaceus* DSM 20336, C = *Lactobacillus gasseri* DSM 20243, D = *Lactobacillus plantarum* TMW 1.460

3.4 Characterization of cinnamoyl esterase activity in *Bifidobacterium animalis* subsp. *lactis*

In silico analyses of the *Bif. animalis* subsp*. lactis* DSM 10140 genome revealed 94% sequence identity of balat_0669 (Accession YP_002969671.1) with a cinnamoyl esterase. The orf balat_0669 is annotated as 'alpha/beta hydrolase' and consists of 789 bp (262 amino acids). The designed primer pair *Bif_for* and *Bif_rev* was used to amplify the fragment. The sequences can be retrieved from appendices A4 and A5. Gel electrophoresis verified the expected amplicon size of 811 bp (Figure 31). As this work was in progress, Raimondi et al. (2015) purified balat_0669 of *Bif. animalis* subsp. *lactis* WC 0432 and observed a chlorogenic acid degradation ability of the expressed protein. However, further biochemical properties of the purified enzyme were not determined.

3.4.1 Cloning and heterologous expression of cinnamoyl esterase

The purified fragments were expressed in *E. coli* TOP10 with the expression vector pBAD/Myc-His-A. After induction with L-arabinose, the cell extract was examined for overproduced proteins by SDS-PAGE analysis (Figure 30). SDS-PAGE revealed an overexpressed protein with a molecular mass around 27 – 28 kDa what is consistent with the estimated molecular weight of 28.25 kDa (BioCyc) and with the results of Raimondi et al. (2015). Due to the His_6-tagged sequence, the proteins could be purified close to homogeneity using HisTrap columns. The purified fraction after 3 ml elution buffer (Figure 30 lane 6) resulted in a protein yield of 0.147 mg/ml. For control, the plasmids were isolated and sequenced. The results proved the transformation success (data not shown). The heterologous expression of cinnamoyl esterase was additionally verified with an ethyl ferulate plate assay (Figure 32). The clearing zones around the colonies resulted from the enzymatic release of ferulic acid, indicating functional expression.

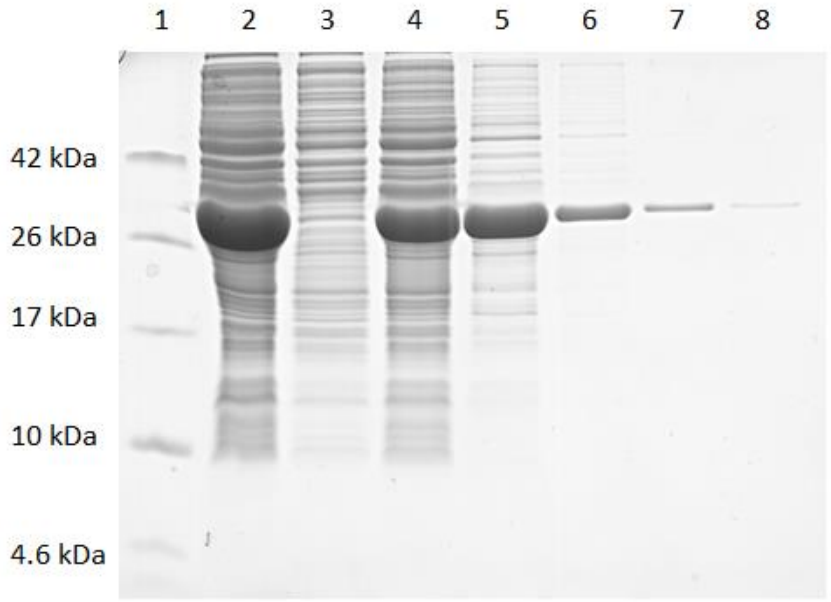


Figure 30: SDS-PAGE gel of the heterologously expressed esterase of *Bif. animalis* subsp. *lactis* DSM 10140. 1: molecular weight marker, 2: crude lysate, 3: unbound fractions, 4 - 8: extract after elution with 1, 2, 3, 4, 5 ml elution buffer

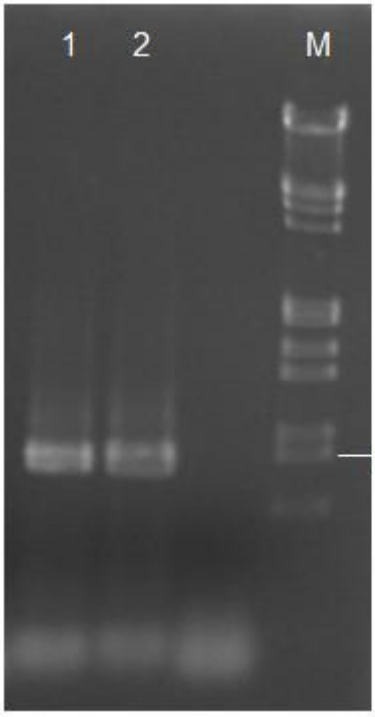


Figure 31: Gel electrophoresis of the amplified fragments of *Bifidobacterium animalis* subsp. *lactis* DSM 10140. Lane 1 and 2 = amplicon in dual approach, M = molecular weight marker

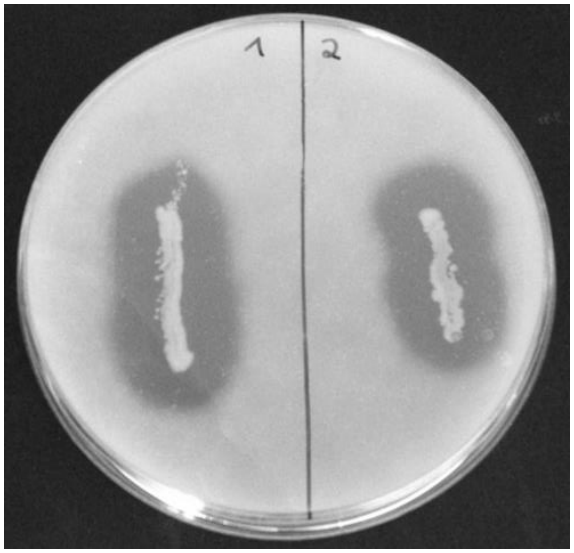


Figure 32: Plate-assay on LB-plates with ethyl-ferulate. Shown are the clear zones after a two day-incubation of *E. coli* TOP10 hosting vector pBAD (insert: predicted cinnamoyl esterase from *Bif. animalis* subsp*. lactis* DSM 10140 in dual approach)

3.4.2 Enzyme kinetics of the heterologously expressed cinnamoyl esterase

The reaction kinetics of the cinnamoyl esterase of *Bif. animalis* subsp*. lactis* were analyzed using *p*-nitrophenyl acetate in a concentration range of 0.25 – 3 mmol/l as substrate. The enzymatic parameters obtained by spectrophotometric measurements and calculation according to Lambert-Beer and Michaelis-Menten are shown in Figure 33. It is obvious that the curve only approximates its saturation value. As in higher substrate concentrations no reliable absorption measurements could be conducted, a transformation according to Lineweaver and Burk was performed (Figure 34). In this linear equation, also known as 'double-reciprocal method' the ordinate intercept is defined as $1/V_{max}$, the slope as K_m/V_{max} and the absissa intercept as $-1/K_m$ (Lineweaver and Burk, 1934). The equotation was y = 11.7467 x + 3.9307 and a K_m of 2.99 mmol/l and a maximum velocity of 0.25 mmol/mg/min was calculated.

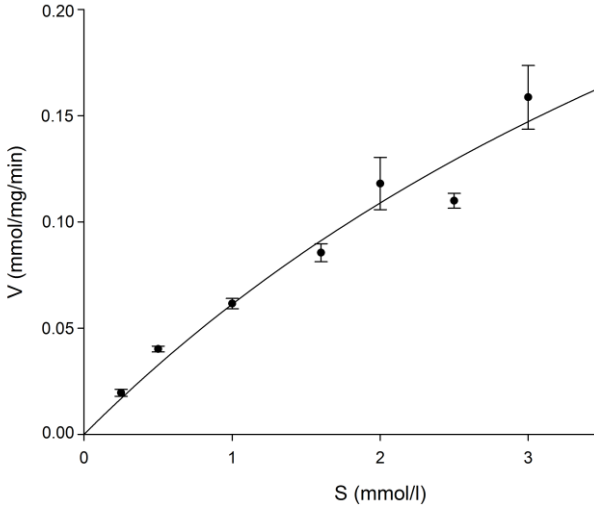


Figure 33: Michaelis-Menten-plot of the cinnamoyl esterase activity of *Bif. animalis* subsp. *lactis* with *p*-nitrophenyl acetate as substrate

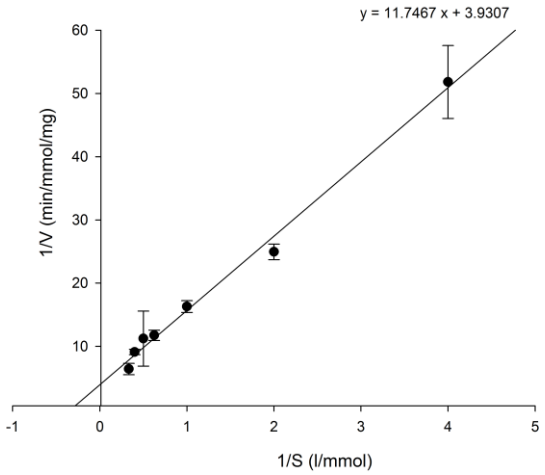


Figure 34: Lineweaver-Burk-plot of the cinnamoyl esterase activity of *Bif. animalis* subsp. *lactis* with *p*-nitrophenyl acetate as substrat

3.4.2.1 pH-dependent activity

Figure 35 shows the effect of the pH value on the activity of the heterologously expressed cinnamoyl esterase. The highest activity with 94.97 µmol/mg/min was measured at pH 7.0, followed by pH 7.5 (83.17 µmol/mg/min). The esterase activity decreased continuously with increasing pH value. In an acidic milieu, the activity diminished rapidly. At pH 4.0 – 5.0 no considerably activity was observed. Cinnamoyl esterases of lactobacilli (*Lb. plantarum, Lb. reuteri, Lb. gasseri, Lb. acidophilus* and *Lb. fermentum*) demonstrated maximum activities at similar pH values of 7.0 to 8.0 (Esteban-Torres et al., 2013; Inmaculada et al., 2013; Jänsch, 2013), whereas *Lb. acidophilus* IFO 13951 showed the highest activity at pH 5.6 (Wang et al., 2004). So far, no data about the biochemical properties of cinnamoyl esterases of other bifidobacteria is available.

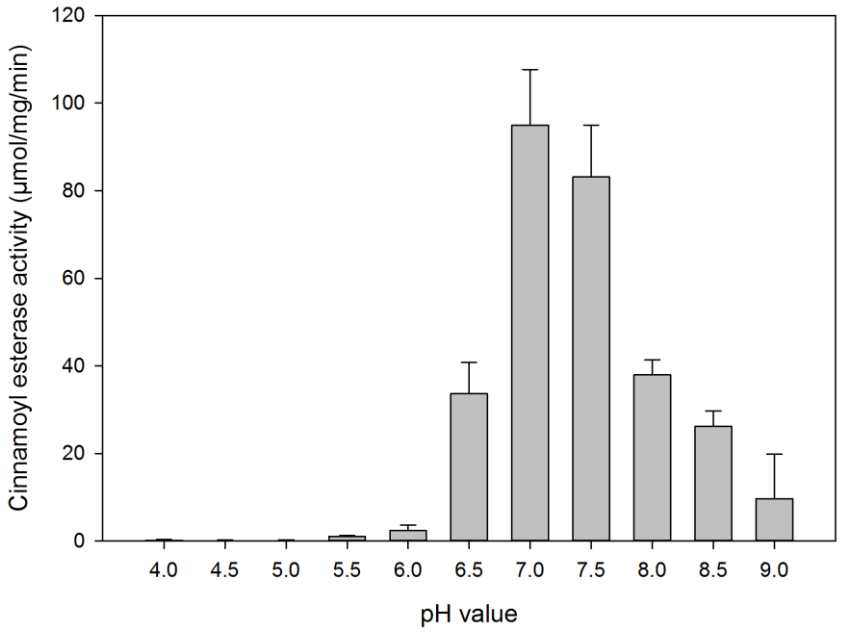


Figure 35: pH-dependent activity of the recombinant cinnamoyl esterase of *Bif. animalis* subsp. *lactis*

3.4.2.2 Temperature-dependent activity

As the reaction temperature plays an important factor in enzymatic activity, the temperature-dependent activity of the purified cinnamoyl esterase activity was determined in a range of 20 – 60 °C (Figure 36). Therefore, ethyl ferulate was used as substrate and the liberated ferulic acid was quantified by HPLC-UV. The esterase showed a maximum activity at 40 and 45 °C, which drastically decreased at higher temperatures. However, at 50 °C a relative activity of 57.4% was measured. This is significantly higher than the activity of *Lb. plantarum* WCFS1, having approximately 10% remaining activity at 50 °C (Esteban-Torres et al., 2013). In a temperature range of 30 – 35 °C, the purified esterase demonstrated a moderate activity with 79.2 – 97.1% relative activity. In conclusion, the cinnamoyl esterase of *Bif. animalis* subsp. *lactis* can be classified as a moderate heat-stabile enzyme as it still had a relative activity of 37% at 60 °C. In comparison, esterases of lactobacilli (*Lb. plantarum, Lb. acidophilus, Lb. reuteri*) showed activities below 5% at this temperature (Esteban-Torres et al., 2013; Fritsch, Jänsch et al., 2016, Wang et al., 2004).

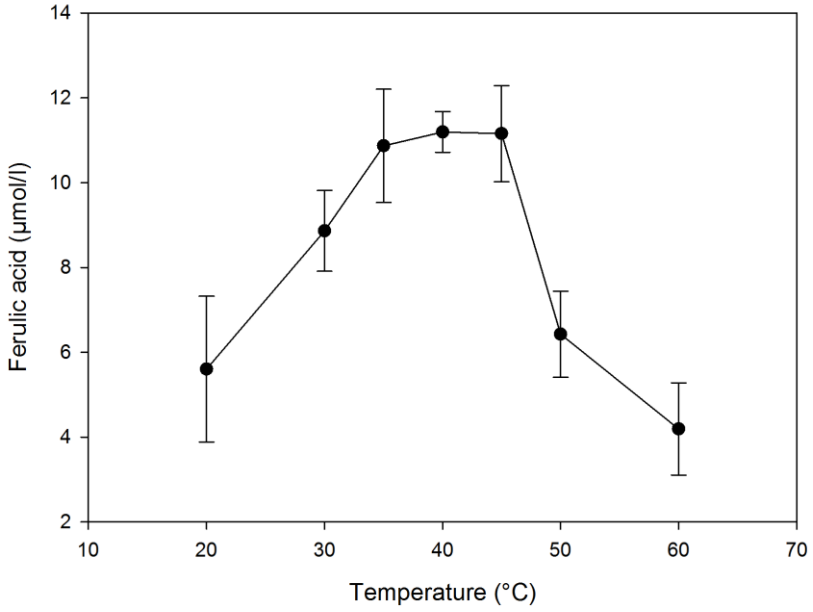


Figure 36: Temperature-dependent activity of the recombinant cinnamoyl esterase of *Bif. animalis* subsp. *lactis*

3.4.2.3 Substrate chain length-dependant activity

Three different *p*-nitrophenyl esters with diverse side chain lengths were applied to determine the substrate chain length-dependant activity (Figure 37). The heterologously expressed cinnamoyl esterase showed the maximum activity with 131.26 µmol/mg/min against the short acyl chain ester *p*-nitrophenyl acetate (*p*-NP-C2). The activity against *p*-nitrophenyl butyrate (*p*-NP-C4) was lower (36.95 µmol/mg/min), demonstrating only 28.2% relative activity. The substrate with the longest acyl chain assayed, *p*-nitrophenyl caprylate (*p*-NP-C8), was not degraded at all. This is in accordance to the substrate chain length-dependent activities of esterases in *Lb. plantarum* NCIMB8826 and *Lb. plantarum* ATCC 8014 (Brod et al., 2010; Inmaculada et al., 2013). In contrast to these results, the feruloyl esterase of *Lb. plantarum* WCFS1 showed the highest relative activity against p-nitrophenyl butyrate, followed by *p*-nitrophenyl acetate and *p*-nitrophenyl caprylate (Esteban-Torres et al., 2013).

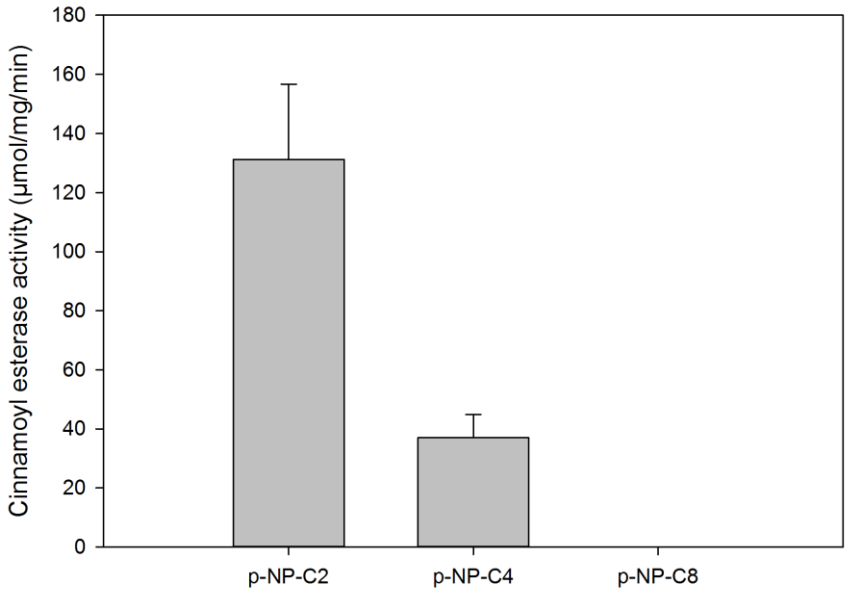


Figure 37: Substrate chain length-dependent activity of the recombinant cinnamoyl esterase of *Bif. animalis* subsp. *lactis* against *p*-nitrophenyl acetate (*p*-NP-C2), *p*-nitrophenyl butyrate (*p*-NP-C4) and *p*-nitrophenyl caprylate (*p*-NP-C8).

3.4.2.4 Substrate specificity

In order to verify the participation of the purified esterase of *Bif. animalis* subsp. *lactis* in the observed chlorogenic acid degradation, the cloned strain as well as the control (*E. coli* TOP10 without insert) were incubated in LB-broth with 1.46 mmol/l chlorogenic acid. The results are summarized in Table 29. *E. coli* with the insert of *Bif. animalis* subsp. *lactis* almost completely reduced the chologenic acid content (-99.1%) and the concentration of the cleavage product caffeic acid significantly rised. The chlorogenic acid concentration in media with the transformation strain without genetically modification decreased only slightly (-3.9%) and the caffeic acid content remained unchanged.

To examine the substrate specificity of the heterologously expressed cinnamoyl esterase, several hydroxycinnamoyl esters were added to nutrient broth and inoculated with the cloned bacterial strain. To check wheather the substances decay during incubation, blank samples (media with hydroxycinnamoyl esters without bacteria) were analyzed as well. The chromatogramms of the inoculated samples clearly demonstrated the ability of the cloned strain to hydrolyze methyl trans-*p*-coumarate, methyl vanillate and methyl caffeate because the cleavage products *p*-coumaric acid, vanillic acid and caffeic acid were detected after incubation (Figure 38 column B). In comparison, the content of the hydroxycinnamoyl esters remained constant in blank samples (Figure 38 column A).

Table 29: Changes in chlorogenic and caffeic acid concentration during fermentation of LB-broth with 1.46 mmol/l chlorogenic acid

	Chlorogenic acid (mmol/l)	Caffeic acid (mmol/l)
E. coli TOP10 with the insert of *Bif. animalis* subsp. *lactis*	-1.45	+1.99
E. coli TOP10 without insert	-0.06	0.0

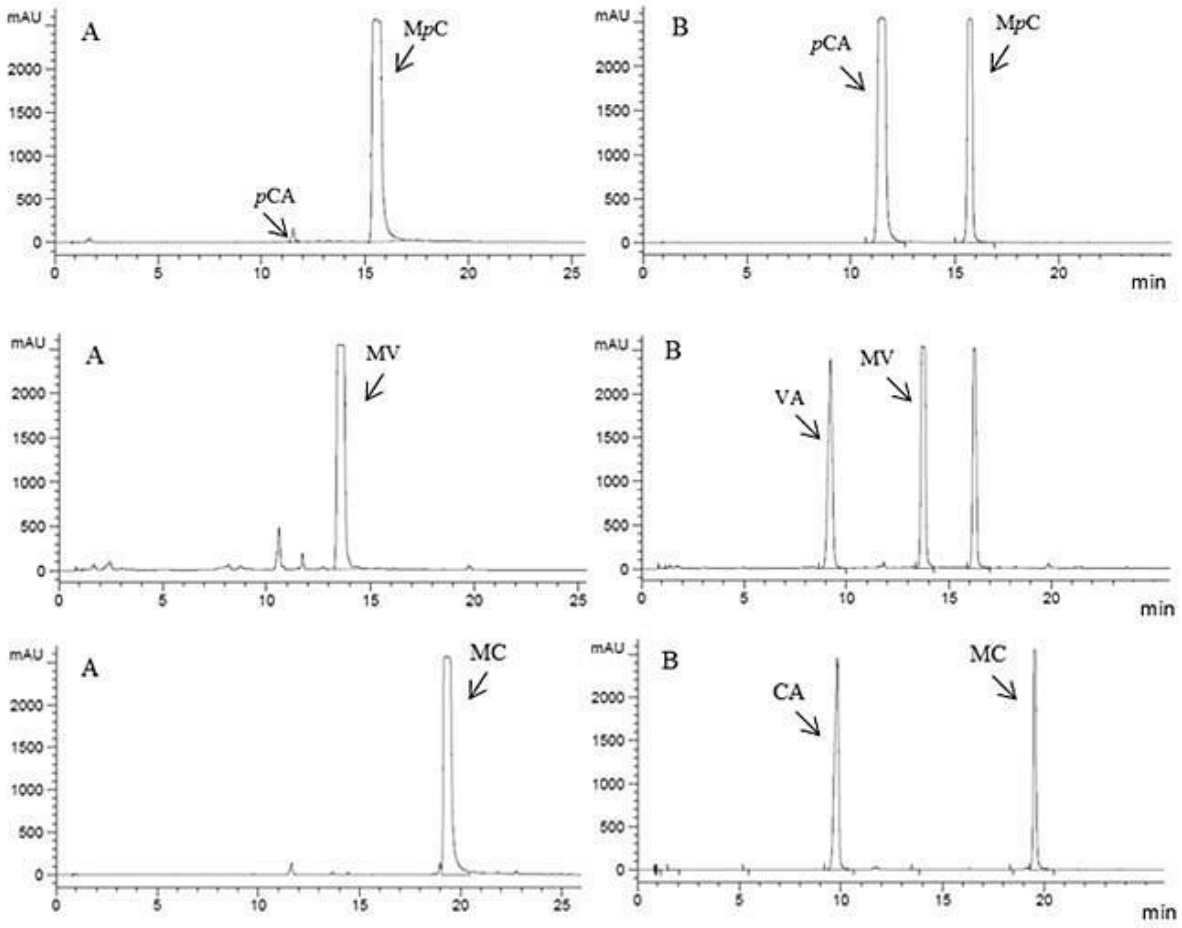


Figure 38: HPLC analysis of the hydroxy cinnamoyl esters in blank samples (A) with 2 mmol/l methyl trans-*p*-coumarate (M*p*C), methyl vanillate (MV), methyl caffeate (MC) and in inoculated media with *E. coli* TOP10 with the insert of *Bif. animalis* subsp*. lactis* DSM 10140 after 24 h at 37 °C (B). The cleavage products *p*-coumaric acid (*p*CA), vanillic acid (VA) and caffeic acid (CA) were detected.

4 Discussion

The present work successfully demonstrated the suitability of lupin and sunflower raw materials for lactic fermentation. Four out of five tested strains (*Bif. animalis* subsp. *lactis, Lb. plantarum, Lb. gasseri, P. pentosaceus*) showed a strong growth and high metabolic activity on protein isolates as well as on flours. A distinct inhibitory influence of SPM was not observed. Neither a lack of specific nutrients nor unsuitable fermentation conditions could be identified, except in fermentation with *L. lactis* sups. *lactis*. Depending on the selected strain, the nutritional quality of lupin substrates could be enhanced by a significant lowering of indigestible oligosaccharides and antinutritive phytic acid. During fermentation of sunflower substrates, the oligosaccharides raffinose and stachyose were degraded. Chlorogenic acid was partially metabolized, releasing free caffeic acid. Due to known beneficial health effects of caffeic acid, the substrates are further valorized by fermentation. Moreover, the product quality might be improved because undesired discolorations of sunflower substrates, caused by chlorogenic acid in alkaline milieu, and a reduced protein solubility can be diminished or prevented.

It was proven that the chlorogenic acid metabolism in *Bif. animalis* subsp. *lactis* is caused by the activity of a cinnamoyl esterase. By means of heterologous gene expression and enzyme purification, the contribution of cinnamoyl esterase to chlorogenic acid hydrolysis was verified. The biochemical properties of the purified esterase were described for the first time.

These results are discussed more detailed in the following sections to broaden the knowledge about the fermentation performance of lactic acid- and bifidobacteria in lupin and sunflower substrates, their interactions with SPM and the chlorogenic acid degradation mechanism in bifidobacteria.

4.1 Influence of lupin secondary plant metabolites on microbial growth and metabolism

In order to evaluate the inhibitory potential of SPM in lupin on the microbial fermentation performance, two different lupin seed flour suspensions were compared with a pure lupin protein isolate suspension which contained only trace amounts of phytochemicals (chapter 3.2.2). The results showed that the SPM and antinutritives in sweet and bitter lupin flour did not markedly influence the metabolism of the tested bacteria. Most strains showed similar growth patterns in the 10% (w/v) lupin flour suspensions to those in 10% lupin protein isolate suspension. Only *Bif. animalis* subsp. *lactis* exhibited a stronger growth behavior with significant higher amounts of organic acids and a higher oligosaccharide utilization in lupin protein isolate. This might be due to the increased protein content in the substrate. According to several researchers, free amino acids and nitrogen had stimulatory effects on different

bifidobacteria due to an easier assimilation (Gomes et al., 1998; Hansen, 1985; Klaver et al., 1993). On the contrary, growth and metabolism of *Lb. plantarum* was higher in both lupin flours than in the protein isolate. However, the difference was not significant. *L. lactis* subsp. *lactis* was only dominant in the lupin flour suspensions, whereas in lupin protein isolate suspensions a mixed culture was detected. Possibly, the protein suspensions lacked of specific nutrients which are required for the growth of this strain.

A comparison of the fermentation performance in sweet and bitter lupin flour revealed that the 20-fold increased alkaloid content in the bitter lupin cultivar *Lupinus angustifolius* cv. *Azuro* did not inhibit the microbial growth or metabolism. No significant differences in cell growth or the produced amounts of organic acids could be observed between these substrates. *Lb. plantarum* and *P. pentosaceus* were least influenced by the alkaloids, indicating the highest tolerances. Since some researchers had proven the antimicrobial effects of lupin alkaloids on *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Serratia marcescens, Streptococcus viridis, Micrococcus luteus* and some phytopathogenic fungi (Erdemoglu et al., 2007; Wink, 1984), these observations indicate that either the selected bacteria are not sensitive for the inhibitory mechanism or the respective inhibitory alkaloid concentration was not reached in *Lupinus angustifolius* cv. *Azuro*. Similarly, Camacho et al. (1991) measured no growth inhibition of different lactobacilli during fermentation of lupin flour. However, the alkaloid content of the applied lupin seeds (0.032 ppm in *Lupinus albus* cv. Multolupa) was significantly lower than in the lupin substrates used in this study which contained up to 0.5% alkaloids.

The study demonstrated that lupin flour can be used in a 10% (w/v) concentration as fermentation substrate. Preliminary experiments revealed that reduced concentrations (5% (w/v) lupin flour suspension) are not suitable probably due to the low concentration of fermentable sugars. With 1% total carbohydrate content in a 10% suspension it seems to be the limiting factor. Similarly, Charalampopoulos et al. (2002) attributed poor growth of *Lb. reuteri* and *Lb. acidophilus* in malt medium to the low sugar concentration of approximately 1.5%.

4.2 Influence of sunflower secondary plant metabolites on microbial growth and metabolism

Similar to the fermentation study with lupin, two different substrates were used for the experiments with sunflower: sunflower flour and sunflower protein concentrate. Main SPM in sunflower are phenolic compounds which are described to have an antimicrobial potential on different lactic acid bacteria. According to the results obtained in chapter 3.3.2, the increased concentration of SPM and other ingredients in sunflower flour did not prevent growth of the

selected lactic acid bacteria. Contrary to the expectations, three out of four strains showed a stronger growth and higher metabolic activity on sunflower flour than on sunflower protein. It was therefore assumed that the phenolic compounds in the flour (mainly chlorogenic acid) did not have an inhibitory effect on the selected strains as described for other lactobacilli (Parkar et al., 2008; Stead, 1993) but may have stimulating effects. To verify this suggestion, growth of *Lb. gasseri* and *Bif. animalis* subsp. *lactis* was monitored in MRS broth containing 500 mg/l chlorogenic, caffeic or quinic acid as sole carbon source and compared to growth in MRS with glucose. Bacterial growth curves indicated a stimulating effect of caffeic acid on *Lb. gasseri* because higher cell counts were reached in this media compared to the standard media with glucose (Figure 7). This strain was also able to grow in MRS broth with chlorogenic acid and quinic acid. Similarly, Stead (1994) observed a stronger growth of *Lb. collinoides* during early growth phase in presence of 100, 500 and 1000 mg/l chlorogenic acid in tomato broth. The here applied sunflower flour suspension (10% (w/v)) contained an even higher concentration of chlorogenic acid with approximately 2890 mg/l. Another study demonstrated the enhanced growth effect of caffeic acid in a concentration of 100 mg/l on *Lb. collinoides* and *Lb. brevis* (Stead, 1993). This caffeic acid concentration was nearly reached in the sunflower flour suspension (65 mg/l). According to these results, it can be assumed that stimulating effects of phenolic acids on lactic acid bacteria can also appear in sunflower substrates. This would explain the higher fermentation performance of most strains in sunflower flour. In contrast, cell counts of *Bif. animalis* subsp. *lactis* decreased in modified MRS broth with phenolic acids, revealing that this strain was not able to metabolize phenolic acids in absence of other carbon sources, especially carbohydrates.

The stronger microbial growth in sunflower flour might also be caused by a higher availability of required nutrients, like vitamins or minerals. Likewise, interactions of the phenolic acids with sunflower compounds may contribute to a decreased antimicrobial potential of phenolic acids. It was demonstrated by von Staszewski et al. (2011) that the food matrix can reduce the antibacterial activity of phenolic compounds. The authors observed a decreased inhibitory effect of polyphenols from green tea infusion in presence of whey proteins. Similarly, Salgado et al. (2012) reported that the phenolic compounds in sunflower did not maintain their bioactivity in sunflower protein concentrate films. It can be assumed that the proteins interact with phenolic compounds and reduced the antimicrobial activity. This is supported by the study from Saeed and Cheryan (1989). To investigate if the sunflower matrix decreased the antimicrobial activity of chlorogenic acid, an experimental set up with chlorogenic acid-spiked sunflower flour was conducted (chapter 3.3.5). The microbial growth in sunflower flour with the respective MIC of chlorogenic acid was compared to that in untreated flour. The collected data did not provide any indications of the bacteriostatic effect of chlorogenic acid that was observed in media (chapter 3.3.3). Three strains showed a

slightly less growth behavior, but no antibacterial effects could be identified. On the contrary, *Lb. plantarum* revealed a stronger growth in chlorogenic acid-spiked sunflower flour. The carbohydrate metabolism of all strains was lower in the spiked samples than in the unmodified ones, indicating a slightly inhibited metabolic activity.

As growth and carbohydrate metabolism was not totally inhibited, the hypothesis of a decreased antimicrobial activity of chlorogenic acid in sunflower flour matrix based on the interaction of chlorogenic acid with proteins might be supported. Although no verification was performed in sunflower protein matrix, an even stronger effect may be assumed due to the higher protein content of the sample. Several studies highlighted this strong interaction (Sabir, Sosulski and Finlayso, 1974; Saeed and Cheryan, 1989; Salgado et al., 2012; Sastry and Subramanian, 1984; von Staszewski et al., 2011).

Further, it has to be taken into consideration that the added chlorogenic acid might be in a different way available for reactions than chlorogenic acid which is naturally present in the substrate. Therefore, phenolic acid degradation rates in spiked flour cannot be compared to original sunflower substrates. However, this experiment provides indications about matrix effects of sunflower ingredients on the antibacterial activity of chlorogenic acid. To verify the results, an experimental set up with sunflower substrates lacking chlorogenic acid has to be conducted.

As several authors reported an antimicrobial activity of various phenolic compounds, including chlorogenic acid and caffeic acid, the minimum inhibitory concentration of the pure substances in media was assayed against the selected strains (chapter 3.3.3). In spite of the proclaimed antimicrobial effects, the studied lactic acid- and bifidobacteria exhibited high tolerances against the phenolic acids. The highest MICs were observed for chlorogenic and quinic acid against *Lb. plantarum, Lb. gasseri* and *P. pentosaceus* with 10.24 – 20.48 mg/ml after 24 h. *Bif. animalis* subsp. *lactis* was most sensitive against all tested phenolic compounds with a MIC of 2.56 mg/ml after 24 h. Similar MICs of bifidobacteria were reported by Raimondi et al. (2015). Compared to other bacteria like *Escherichia coli, Staphylococcus aureus* or *Salmonella enterica,* for which MIC values between 0.1 – 0.2 mg/ml chlorogenic acid were published, the phenolic compounds showed only low to moderate antimicrobial effects on the selected strains (Parkar et al., 2008; Xia et al., 2011). It was observed that the MICs of all phenolic acids increased from 24 to 48 h, indicating that the substances are not bactericidal for the tested strains but caused a prolonged lag-phase in which the bacteria probably get acclimated to the phenolic acids.

The inhibitory mechanism of phenolic acids includes the damage of cytoplasmic membrane, changes in permeabilization, enzyme inhibition and disruption of the membrane-associated

respiration chain. These changes have a greater influence on Gram-positive bacteria than on Gram-negative bacteria due to the different construction of the cell wall. Some researches proposed that the outer membrane of Gram-negative strains which is mainly composed of lipopolysaccharide, acts like a barrier and repulses phenolic compounds due to their charge (Ikigai et al., 1993; von Staszewski et al., 2011; Xia et al., 2011; Zhao et al., 2001). Since all strains in this study were Gram-positive, these high tolerances are particularly remarkable. Possibly, the origin of probiotic bifidobacteria and lactobacilli contributes to the high tolerances. These organisms may have adapted to common dietary phenolic acids due to the fact that they have residing in the intestine (Lee et al., 2006). The microbial growth kinetics in presence of varying concentrations of chlorogenic acid supported the observations of high tolerances. *Lb. plantarum* and *Lb. gasseri* showed approximately the same growth behavior in MRS broth with 10.24 mg/ml chlorogenic acid as in media without addition (Figures 16 and 19). In media with 20.48 mg/ml an extended lag-phase was recognized.

Another influencing factor on the antimicrobial activity of phenolic substances is the pH value. It was shown by several researchers that the antibacterial effectiveness of phenolic acids, hydroxycinnamic acids and benzoic acid increased with decreasing pH value (Almajano et al., 2007; Herald and Davidson, 1983; Rahn and Conn, 1944; Wen et al., 2003). At low pH, higher concentrations of the undissociated form of the phenolic acids are present which are better soluble in the cytoplasmic membrane (Ramos-Nino et al., 1996). As the fermentation of sunflower substrates started at pH 6.3 (initial pH value of 10% (w/v) sunflower suspension), the antimicrobial activity of phenolic compounds might be therefore reduced as most of the acids were apparently dissociated. In contrast, MRS broth spiked with chlorogenic acid, caffeic acid and quinic acid had pH values around 4.0 – 5.5 at the beginning of the MIC assays and growth kinetics measurements. Consequently, the antimicrobial activity should be higher in media than in sunflower substrates which was confirmed by the study of microbial growth and metabolism in chlorogenic acid-spiked sunflower flour (chapter 3.3.5).

Saeed and Cheryan (1989) investigated the influence of the pH value on the binding capacity of chlorogenic acid with sunflower proteins. They monitored a stronger complex formation at pH 7 and pH 3. The authors proposed that a hydrogen bond between the unionized hydroxyl group of phenolic compounds and the carbonyl group of the peptide bond in proteins is responsible for this effect at low pH values (Sabir, Sosulski and Kernan, 1974). The higher binding ratio at pH 7 was related to electrostatic interactions between the ionized carboxyl group of chlorogenic acid and positive charged side chains of proteins (Saeed and Cheryan, 1989). Consequently, with a starting pH of 6.3 in sunflower fermentations, higher amounts of chlorogenic acid-protein-complexes should be present leading to a reduced antibacterial

activity. At the end of fermentation (approximately pH 4.4) a higher proportion of chlorogenic acid should be present in an undissociated form so that their antimicrobial activity should be increased. However, with the mentioned complex formation at low pH this effect might be compensated. As von Staszewski et al. (2011) reported no significant differences in the antibacterial effect of polyphenols in a pH range of 4.0 – 7.0, it might be assumed that the dissociation of phenolic acids did not markedly changed until a pH of 4.0 or that it was not mainly responsible for antimicrobial activity.

Sanchez-Maldonado et al. (2011) analyzed higher antimicrobial potentials of caffeic acid, *p*-coumaric acid and protocatechuic acid than of their respective metabolites dihydrocaffeic acid, phloretic acid and catechol. Therefore, they suggested that the metabolism of phenolic compounds serve as a mechanism of detoxification. The researchers could not confirm this effect for chlorogenic acid. Instead, they found the exact opposite: chlorogenic acid exhibited a MIC higher than 6.7 g/l against *Lb. plantarum* and *Lb. hammesii,* whereas these strains were already inhibited by 1.52 and 0.63 g/l caffeic acid, respectively. Likewise, a mechanism of detoxification could not be observed in the present study, because caffeic acid revealed a distinct higher antimicrobial potential than chlorogenic acid. The second cleavage product, quinic acid, had comparable MICs like chlorogenic acid. It might be possible, that chlorogenic acid initially displays a low antimicrobial potential against lactic acid bacteria due to the fact that it is abundant in many beverages, fruits, vegetables and food products. As described earlier, the human gut microbiota is regularly exposed to phenolic compounds which might enhance their tolerance against these substances. It was shown that habitual coffee drinkers have a daily intake of around 1 g chlorogenic acid. Considering a colonic volume of approximately 1 kg and the fact that only small amounts (max. 30%) are absorbed in the small intestine, the analyzed MICs for chlorogenic acid were markedly higher than this concentration (Clifford, 1999; Lee et al., 2006; Manach et al., 2004; Raimondi et al., 2015; Scalbert and Williamson, 2000).

4.3 Microbial degradation of secondary plant metabolites and antinutritives

4.3.1 In lupin substrates

Lupin seeds contain several SPM with antinutritive properties. Therefore, the direct consumption has several disadvantages, like gastrointestinal disorders or a reduced bioavailability of nutrients. The most important antinutritives in lupin are oligosaccharides (raffinose and stachyose) and phytic acid. The results obtained in chapter 3.2.3 of this thesis showed that the nutritional value of lupin flour can be enhanced with the selection of the fermentation strain. Fermentation with *Bif. animalis* subsp. *lactis* led to a degradation of 92 –

100% of the raffinose content and a reduction of stachyose of about 23% in sweet and bitter lupin flour. The screening of oligosaccharide metabolism in media confirmed these results. Due to the lack of enzymes utilizing oligosaccharides in human gastrointestinal tract, this could lead to an improved digestibility of the fermented product (Gitzelmann and Auricchio, 1965; Rackis et al., 1970). In the corresponding blank samples of lupin flours raffinose and stachyose contents were reduced to a maximum of 24% for raffinose and 5% for stachyose. These changes might be caused by lupin endogenous enzymes like α-galactosidases which were found in lentils, beans and yellow lupin (Davis et al., 1997; Dey et al., 1983; Lahuta et al., 2000; Prakash, 1984). However, the degradations in lupin blank samples were significantly lower than in most of the inoculated samples. Therefore, the oligosaccharide decrease can be mainly ascribed to the microbial metabolism. These results were verified by *in silico* genome analyses of *Bif. animalis* subsp. *lactis* DSM 10140. Different genes which are responsible for the expression of oligosaccharides-hydrolyzing enzymes like α-galactosidase, β-galactosidase, β-fructosidase and sucrose phosphorylase were identified by BLAST. This is confirmed by a study of Gopal et al. (2001) who measured a raffinose reduction of more than 80% by *Bif. lactis* DR10 in purified raffinose fractions. The researchers also described a preference of this strain for tri- and tetrasaccharides while no utilization of monosaccharides was detected. This is in accordance with the observation of constant remaining galactose content during lupin flour fermentation with *Bif. animalis* subsp. *lactis.* The rise of galactose by 23% in lupin protein isolate suspensions probably occurred as a result of the stronger oligosaccharide cleavage in this substrate that released mono- and disaccharides like galactose, glucose or sucrose. Since *Bif. animalis* subsp. *lactis* is unable to utilize galactose, the concentration increased over fermentation time. The preferred utilization of oligosaccharides might be due to the induction of a specific permease that simplifies the oligosaccharide uptake and that is stimulated by oligosaccharides (Gopal et al., 2001). Similarly, a stimulated expression of sucrose phosphorylase in *Bif. animalis* subsp. *lactis* by sucrose and raffinose was suggested by other authors (Martínez-Villaluenga and Gómez, 2007; Trindade et al., 2003).

P. pentosaceus was identified as capable of metabolizing raffinose and stachyose in nutrient media as well as in lupin substrates. In accordance, Gonzalez and Kunka (1986) measured α-galactosidase and sucrose hydrolase activity in this species. The higher degradation in lupin protein isolate may be attributed to the stronger metabolic activity in this substrate (Table 17).

Although a distinct acid production of *Lb. plantarum* was measured in MRS broth containing oligosaccharides (more strongly for raffinose), the raffinose content of lupin substrates decreased only during fermentation of bitter lupin flour. The amount of stachyose remained constant. On the contrary, this strain degraded the total galactose and sucrose content within

24 h. These findings indicate that mono- and disaccharides are the preferred carbon source for *Lb. plantarum*. A similar trend was described by Mital et al. (1974) who measured a stronger drop of pH value in media containing glucose or sucrose than in media with raffinose or stachyose. These results are in agreement with further published studies (Charalampopoulos et al., 2002; Gobbetti et al., 1994; Samuel et al., 1980). Similarly, *L. lactis* subsp. *lactis* showed a measurable growth in MRS broth with raffinose or stachyose as sole carbon source while these carbohydrates were only slightly degraded in sweet lupin flour. In bitter lupin flour samples, no changes in oligosaccharide content were monitored but the entire amount of galactose was utilized which may also suggest a favored monosaccharide degradation due to the enzymatic equipment.

During fermentation with *Lb. plantarum* a phytate degradation of about 14% in sweet and bitter lupin flour was observed (Table 19). To a low extent, complex formation of proteins, minerals and vitamins with phytic acid might be reduced and thus nutritional value of the fermented lupin substrates is enhanced (Dvořáková, 1998; Wodzinski and Ullah, 1996). As the phytate reductions in acidified blank samples were lower (maximum -6%), it is suggested that that the observed degradation was not mainly caused by plant endogenous enzymes. Consequently, it can be assumed that *Lb. plantarum* exhibits phytase activity which catalyzes the hydrolysis of phytic acid to lower *myo*-inositol phosphates (Martinez et al., 1996). This is in accordance with several studies (Lopez et al., 2000; Shirai et al., 1994; Songré-Ouattara et al., 2008) and the screening of microbial ability to degrade phytic acid in modified MRS broth (Table 11). In this model experiment a phytic acid decrease by more than 50% was measured during fermentation with *Lb. plantarum*. In blank samples the concentration dropped by 3.7 – 6.6%. However, the observed phytate degradation by the selected strains was rather low compared to other lactic acid bacteria which showed degradation rates of 60 – 100% (Fischer et al., 2014; Palacios et al., 2008). Phytic acid was degraded by *Bif. animalis* subsp. *lactis, L. lactis* subsp. *lactis* and *P. pentosaceus* to varying extents in the different substrates. While partially no reduction was observed in lupin suspensions, the phytic acid content decreased in modified MRS media with all of the three strains by 15 – 23%. As described by Bohn et al. (2007) the surrounding matrix influences the access of phytase to the substrate resulting in a higher enzyme activity if the pure substance is provided. This could explain the higher phytate degradation of the strains in MRS broth where a purified phytic acid solution was added to the media. On the contrary, in lupin substrates a complex mixture of several plant substances is contained in which phytic acid might be embedded, hindering the phytase activity.

4.3.2 In sunflower substrates

Sunflower substrates contain high amounts of chlorogenic acid, an ester of caffeic and quinic acid. Although several studies described antioxidant and anticarcinogenic effects of chlorogenic acid (Clifford, 2000; Kono et al., 1995; Tanaka and Mori, 1995), it was also proven that the majority of digested chlorogenic acid is not absorbed in the upper digestive tract of humans (Olthof et al., 2001; Stalmach et al., 2010). The disadvantages of high concentrations of chlorogenic acid in food products include an undesired discoloration and decreased protein solubility. As its cleavage products caffeic acid and quinic acid provide several health benefits, different microorganisms were examined for their chlorogenic acid degradation ability. Therefore, screenings in modified MRS broth with chlorogenic acid and in sunflower substrates which naturally contained chlorogenic acid were conducted (chapter 3.1 and 3.3.4). The results showed that *Bif. animalis* subsp. *lactis* and *Lb. gasseri* markedly decreased the content of chlorogenic acid in media (69 – 78%) as well as in sunflower substrates (flour: 11 – 20%, protein: 51 – 96%). Due to enzymatic hydrolysis, the caffeic acid content increased in the samples. The chlorogenic acid reductions in blank samples were significantly lower. Therefore, the decrease was ascribed to the microbial metabolism. These results are in agreement with a study of Couteau et al. (2001) who figured out that *Bif. lactis* and *Lb. gasseri* exhibited a chlorogenic acid degradation ability in nutrient media.

The responsible enzymes for a chlorogenic acid cleavage are cinnamoyl esterases [E.C. 3.1.1.73], also termed as feruloyl esterases or ferulic acid esterases (Kroon and Williamson, 1999; Mackenzie et al., 1987). These enzymes were described to hydrolyze ester bonds in hydroxycinnamates and are produced by a wide range of microorganisms found in the digestive tract or fermented foods. The metabolism of phenolic compounds contributes to the microbial adaption in adverse environments due to the widespread occurrence of phenolic acids in plants and food products (Donaghy et al., 1998; Filannino et al., 2015; Lai et al., 2009; Raimondi et al., 2015). Couteau et al. (2001) proved that the active enzymes in *Bif. lactis* and *Lb. gasseri* are essentially located intracellular because the activity against chlorogenic acid was 14-fold lower in intact cells compared to sonicated cultures. This was supported for cinnamoyl esterases of *Lb. plantarum, Lb. johnosnii* and *Bif. animalis* subsp. *lactis* by several researchers (Bel-Rhlid et al., 2012; Donaghy et al., 1998; Raimondi et al., 2015).

In silico analyses of the amino acid sequence of *Bif. animalis* subsp. *lactis* DSM 10140 showed 94% sequence identity with a cinnamoyl esterase. The contribution of this enzyme to the observed chlorogenic acid reduction was examined in chapter 3.4 and is discussed in section 4.5. Alignment scores of the amino acid sequences of a cinnamoyl esterase with the genome of *Lb. gasseri* DSM 20243 displayed a 98% identity, indicating that this strain is able

to hydrolyze chlorogenic acid. This is also supported by a study of Couteau et al. (2001) in which *Lb. gasseri* Ro-2 and Ro-8 showed high esterase activity against chlorogenic acid. Moreover, a genetically modified transformation strain with the insert of a predicted cinnamoyl esterase of *Lb. gasseri* DSM 20243^{T} (Accession YP_815563) showed a chlorogenic acid reduction of 99% in media, whereas the transformation strain itself was not able to degrade chlorogenic acid (Fritsch, Jänsch, et al., 2016). These observations were strengthened by numerous reports about the metabolism of chlorogenic acid in human and animal gastrointestinal tract. The studies showed that chlorogenic acid was not degraded in the upper digestive tract or by extracts of human liver, small intestine or human plasma but its cleavage product caffeic acid occurred in human fecal samples. Subsequently, the fecal microflora was identified for this reduction (Booth et al., 1957; Goodwin et al., 1994; Olthof et al., 2001; Plumb et al., 1999). Since *Bif animalis* subsp. *lactis* and *Lb. gasseri* belong to the human gut microflora, the capability of chlorogenic acid metabolism can be related to the origin of the microorganisms.

The metabolic pathways to utilize phenolic compounds provide several benefits for the bacteria, including the release of substances for energy generation and the detoxification of compounds with antimicrobial properties (Gänzle, 2014; Sanchez-Maldonado et al., 2011). As mentioned above, a detoxification of chlorogenic acid could not be verified in this study. However, it can be suggested that the cleavage of chlorogenic acid served for energy generation. In case of *Lb. gasseri* this hypothesis might be appropriate due to the observation that the initially increased concentration of caffeic acid subsequently decreased (Figure 25), whereas the caffeic acid content remained unchanged in blank samples. Thus, it can be assumed that this strain is able to metabolize caffeic acid. Several researchers examined the degradation of hydroxycinnamic acids like *p*-coumaric, caffeic and ferulic acid by lactic acid bacteria and figured out that the phenolic acid decarboxylase splits hydroxycinnamic acids up into their vinyl derivates. This ability was reported for *Lb. plantarum, Lb. hammesii, Lb. fermentum, Lb. brevis* and *P. pentosaceus* strains (Cavin et al., 1997; De Las Rivas et al., 2009; Rodríguez, Landete, Curiel, et al., 2008; Sanchez-Maldonado et al., 2011). The competence might therefore be also available for other lactobacilli like *Lb. gasseri*. However, in a study of Couteau et al. (2001) *Lb. gasseri* Ro-8 and *Bif. lactis* Be-3 did not metabolize caffeic acid. A caffeic acid utilization by *Bif. animalis* subsp. *lactis* could not be observed in the present study. However, it might be possible that the increased quinic acid content was utilized by this strain. Quinic acid or its metabolites could not be measured because the required equipment for this analysis was not available. Indications on quinic acid metabolism by different microorganisms can be found in literature: Tomas-Barberan et al. (2014) observed a degradation of quinic acid released from chlorogenic acid by the human gut microbiota, resulting in caffeoyl-glycerol metabolites.

Adachi, Yoshihara, et al. (2003) identified *Gluconobacter melanogenes, Gluconobacter oxydans* and *Acinetobacter calcoaceticus* as capable of converting quinic acid into 3-dehydroquinate through the activity of the quinate dehydrogenase and Carr et al. (1957) described a conversion of quinic acid into dihydroshikimic acid (which was most likely 3-dehydroquinate) by *Lb. pastorianus* var. *quinicus*. Similarly, Filannino et al. (2015) observed a quinic acid metabolism in *Lb. plantarum* and *Lb. fermentum*. Based on these data, it can be suggested that *Bif. animalis* subsp. *lactis* utilizes quinic acid. Nevertheless, there are no specific reports about a quinic acid degradation by bifidobacteria which could support this hypothesis.

Another energetic benefit for microorganisms metabolizing phenolic acids might be the regeneration of NAD^+. As the hydrolysis of phenolic compounds requires a hydrogen donor, reduced NADH may be reoxidized which subsequently provides an energy advantage (Filannino et al., 2015; Filannino et al., 2014). This is supported by Silva et al. (2011), who observed a preferred degradation of *p*-coumaric acid to 4-vinylphenol and a subsequent reduction to 4-ethylphenol in *Lb. plantarum* and *Lb. collinoides* under anaerobic conditions. Since under anaerobiosis a lack of NAD+ can occur, this may be solved by the described metabolic pathway due to the increase of NAD^+ concentration.

According to the results of chapter 3.3.4 it can be assumed that *P. pentosaceus,* similar to *Lb. gasseri* is able to metabolize caffeic acid. During fermentation of sunflower flour with *P. pentosaceus* the caffeic acid concentration decreased, whereas the chlorogenic acid content remained constant (Figure 26). The concentration ratio of chlorogenic acid and caffeic acid in sunflower protein samples strengthened this suggestion because the chlorogenic acid content was reduced by 0.08 mmol/l, while caffeic acid increased only by 0.001 mmol/l. Possibly, the concentration of caffeic acid increased but was simultaneously metabolized by *P. pentosaceus*. This assumption is supported by a study of De Las Rivas et al. (2009) in which *P. pentosaceus* CECT 4695^T completely decarboxylated caffeic acid into its vinyl derivate.

As mentioned above, *Bif. animalis* subsp. *lactis* seems not to degrade caffeic acid. Therefore, the caffeic acid concentration increased during fermentation. This led to an enhanced nutritional value of the fermented substrates because caffeic acid is easily absorbed in the upper digestive tract of humans, provides antioxidant and anticarcinogenic effects and might stimulate the insulin secretion (Clifford, 2000; Konishi and Kobayashi, 2005; Oboh et al., 2015; Olthof et al., 2001; Ong et al., 2013; Srinivasan et al., 2007). The anticarcinogenic potential was related to chlorogenic and caffeic acid which act as inhibitors of the N-nitrosation reaction in vitro. This prevents the formation of mutagenic and carcinogenic compounds. Similarly, the activity of methylazoxymethanol, a trigger of colon

carcinogenesis, was lower when caffeic acid was ingested (Clifford, 2000; Kono et al., 1995; Tanaka and Mori, 1995). The knowledge about health benefits of caffeic acid was already implemented in food development, e.g. for the manufacturing of a novel food product with antioxidant properties due to an increased amount of free caffeic acid (Guglielmetti et al., 2008). The researchers used *Lb. helveticus* as fermentation strain which is capable of hydrolyzing chlorogenic acid.

Depending on the fermentation strain, the nutritional value of the fermented sunflower substrates was further increased due to a reduced content of the indigestible oligosaccharides raffinose and stachyose. The highest oligosaccharide reduction in both substrates (flour and protein concentrate) was measured for *P. pentosaceus* with a degradation of 98.6% raffinose and 82.4% stachyose in the flour and a total reduction of the raffinose content and a degradation of 68.5% stachyose in the protein concentrate. A high utilization was also shown by *Lb. plantarum* with a total degradation of both oligosaccharides in sunflower flour. This is in accordance with the preliminary screening in media (chapter 3.1) and a study of Mital et al. (1974) who observed the ability of *Lb. plantarum* B-246 to metabolize raffinose and stachyose. On the contrary, *Lb. gasseri* reduced raffinose and stachyose only slightly in both substrates. *Bif. animalis* subsp. *lactis* and *P. pentosaceus* demonstrated the strongest oligosaccharide utilization in sunflower protein concentrate. Equally, the latter two strains significantly decreased raffinose and stachyose during fermentation of lupin flour and protein isolate. These bacteria might possess the enzymatic equipment for oligosaccharide degradation which was confirmed by several researchers (Gonzalez and Kunka, 1986; Gopal et al., 2001; Martínez-Villaluenga and Gómez, 2007). The capability of metabolizing oligosaccharides enhances the ecological fitness of bacteria derived from fermented foods or human intestinal tract. Since oligosaccharides are contained in various plants like cereals, legumes and fruits, strains with the ability to utilize these carbohydrates for their metabolism exhibit growth benefits in those substrates. Due to the lack of oligosaccharide cleaving enzymes in humans, they attain in the digestive tract where strains with the required enzymatic equipment display this growth advantage as well (Gänzle and Follador, 2012).

4.4 Comparison of the microbial fermentation performance on lupin and sunflower

It was shown that lupin and sunflower raw materials as well as protein isolates are suitable substrates for lactic fermentation with the selected strains. For comparison of the different plant materials, the metabolic activity in terms of organic acid production and rise of bacterial cell counts as well as the substrate uptake were considered. All strains showed a higher

metabolic activity in sunflower flour and sunflower protein concentrate than in lupin substrates because higher amounts of lactate and acetate were analyzed. Likewise, cell counts of most bacteria rose to a higher extent in sunflower substrates. An exception was *Bif animalis* subsp. *lactis* which produced higher concentrations of organic acids in lupin protein suspension compared to sunflower protein suspension. Similarly, *Lb. plantarum* grew better in lupin flour than in sunflower flour. The substrate uptake, especially the oligosaccharide utilization of all strains was significantly higher in sunflower samples. This could be ascribed to the nutrient composition of sunflower substrates which might stimulate microbial growth. The stimulating influence of phenolic compounds was discussed earlier (chapter 4.2). It can be suggested, that other ingredients had the same effect. Conversely, the results may indicate that lupin samples contained higher amounts of substances with inhibitory potential.

The overall highest degradation of raffinose and stachyose was shown by *Bif animalis* subsp. *lactis* and *P. pentosaceus.* It can be concluded that these strains might be suitable for fermentation of other plant substrates with high oligosaccharide contents, like soy, peas or beans. However, it has to be noted that every vegetable substrate contain different SPM in varying amounts. The bacterial strains of this study showed high tolerances against the secondary plant compounds and antinutritives contained in lupin and sunflower, with *Lb. plantarum* and *P. pentosaceus* being least affected by lupin alkaloids. The lowest sensitivity against phenolic compounds in sunflower was demonstrated by *Lb. plantarum* and *Lb. gasseri*.
However, the microbial degradation ability of phenolic compounds, phytic acid and oligosaccharides is influenced by the fermentation substrate due to a modulated enzyme activity through inducer substances or a modified access to the substrate (Barthelmebs et al., 2000; Bohn et al., 2007; Filannino et al., 2015; Trindade et al., 2003). These results show once again that the microbial metabolism is complex and depends on many environmental factors so that transferability of results from one plant fermentation substrate to another is not feasible.

4.5 Detection of chlorogenic acid degrading enzymes in *Bifidobacterium animalis* subsp. *lactis*

Several cinnamoyl esterases capable of hydrolyzing chlorogenic acid were purified of various lactobacilli but at the present moment only one study performed molecular biological studies about these enzymes in bifidobacteria. This is surprising because the beneficial health effects of bifidobacteria have been ascribed to the biotransformation of plant compounds into substances with positive effects on health, like caffeic or ferulic acid (Couteau et al., 2001; Raimondi et al., 2015; Yuan et al., 2007). The results in chapter 3.1 and 3.3.4 of this thesis

demonstrated that *Lb. gasseri* and *Bif animalis* subsp. *lactis* were obviously capable of metabolizing chlorogenic acid in media and in sunflower substrates. It was reported for *Lb. gasseri* DSM 20243^T that the degradation can be ascribed to cinnamoyl esterase activity (Fritsch, Jänsch, et al., 2016). To examine if the same degradation mechanism exists in *Bif animalis* subsp. *lactis* DSM 10140, genetic investigations were performed. It was hypothesized that balat_0669 is responsible for the expression of predicted cinnamoyl esterase. Therefore, this orf was heterologously expressed in *E. coli* TOP10 and the protein was characterized. The experiments verified the hypothesis because the cloned strain significantly degraded chlorogenic acid and the cleavage product caffeic acid occurred, whereas the transformation strain with an empty vector did not show this ability (Table 29). These results are supported by a study of Raimondi et al. (2015) who purified a cinnamoyl esterase of *Bif animalis* subsp. *lactis* WC 0432 and verified the activity against chlorogenic acid. The researchers also demonstrated that bifidobacteria species without this enzyme activity were not able to degrade chlorogenic acid, e.g. *Bif. bifidum, Bif. breve, Bif. catenulatum* and *Bif. pseudocatenulatum.*

To broaden the knowledge about the cinnamoyl esterase of *Bif animalis* subsp. *lactis,* several investigations were conducted. The purified esterase revealed a relatively low affinity towards the substrate *p*-nitrophenyl acetate with a K_m of 2.99 mmol/l, compared to cinnamoyl esterases of *Lb. johnsonii* strains which displayed K_m of 0.47 and 0.95 mmol/l (Lai et al., 2009). On the contrary, Jänsch (2013) examined cinnamoyl esterases of six different lactobacilli and measured significantly higher K_m values (5.25 – 28.21 mmol/l) towards the same substrate. The maximum reaction velocity of the purified cinnamoyl esterase of *Bif. animalis* subsp. *lactis* was reached at 0.25 mmol/mg/min. In comparison to lactobacilli (4.57 – 134.35 mmol/mg/min), this was distinctly lower. Nevertheless, it was higher than those of *Lb. johnsonii* with 0.64 – 8.4 µmol/mg/min (Jänsch, 2013; Lai et al., 2009). It can be assumed that the substrate was not ideal for the purified enzyme of this bifidobacterium although it has structural similarity to ferulic acid and is commonly used for assays with bacterial esterases (Chen et al., 1995; Goldstone et al., 2010; Lai et al., 2009; Rashamuse et al., 2007). Likewise, the use of different calculation formulae for enzyme activities and reaction velocities might explain the deviations. A broad variety of evaluation methods exist, e.g. nonlinear regression analysis, linear transformation of the Michaelis-Menten equotation according to Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf or Eisenthal & Cornish-Bowden. Each one apply different calculations and make diverse assumptions about the error distribution. This leads to divergent results which can barely be compared to each other (Atkins and Nimmo, 1975; Dowd and Riggs, 1965). Therefore, the most common Lineweaver-Burk plot was used for evaluation in this study.

In chapters 3.4.2.1 – 3.4.2.3 factors affecting the enzyme activity, like pH, temperature or substrate chain length were examined. The heterologously expressed cinnamoyl esterase of *Bif. animalis* subsp. *lactis* showed an optimum at pH 7.0 and 40 – 45 °C. The pH-dependant activity with a moderate action in an alkaline milieu but a significantly lower activity in an acid pH range is similar to those of lactobacilli (Esteban-Torres et al., 2013; Jänsch, 2013; Kim and Baik, 2015). A distinct difference was observed with regard to the temperature dependent activity: esterase activity of *Bif. animalis* subsp. *lactis* was highest at 40 °C and 45 °C. At 60 °C, a relative activity of 37.4% was measured, demonstrating a moderate heat tolerance. In contrast to that, the maximum activity of cinnamoyl esterases of *Lb. plantarum* and *Lb. acidophilus* was measured between 30 °C and 37 °C. The activity was significantly reduced to 14 – 32% of the maximal activity at 45 °C (Esteban-Torres et al., 2013; Wang et al., 2004). This can be attributed to the optimum growth conditions of the bacteria which correlate with their habitats: *Bif. animalis* subsp. *lactis* grows best at 39 – 42 °C, whereas many lactic acid bacteria have their optimal growth at 30 – 40 °C. Since *Bif. animalis* subsp. *lactis* can be found in human gastrointestinal tract, its natural habitat exhibits temperatures around 37 °C (De Vos et al., 2009; Goodfellow et al., 2012). The optimum at pH 7.0 further underlines the gastrointestinal origin, because pH values of 5.0 – 7.0 are found in the duodenum, jejunum, ileum and colon of humans (Kararli, 1995).

The moderate heat stability provides several benefits for the industrial application of this enzyme. As chlorogenic acid causes several disadvantages in food products, the use of enzyme extracts hydroylzing esterified phenolic acids attracts much attention in food industry and technology. In the last decades several processes were developed to utilize microbial enzyme activities for technological purposes. Hydroxycinnamates are applied as precursors for flavors, e.g. the biotransformation of ferulic acid to natural vanillin by microbial cinnamoyl esterases (Falconnier et al., 1994; Mathew and Abraham, 2004). Besides, it is known that certain bacteria are able to modulate the bioavailability of hydroxycinnamic acids in food and in the human gut. Therefore, their use as probiotics is of major interest (Couteau et al., 2001; Kroon et al., 1997).

The purified enzyme of *Bif. animalis* subsp. *lactis* exhibited a pH optimum of 7.0 and a moderate activity at pH 7.5 – 8.0. This facilitates the application in bakery products, plant flours or other substrates with a neutral and alkaline pH value. On the contrary, the use of this enzyme in acidic products, like the clarification of fruit juices is complicated. For industrial enzyme application, a broad substrate range is important due to the fact that food plants contain a high variety of hydroxycinnamic acids. The cinnamoyl esterase of *Bif. animalis* subsp. *lactis* demonstrated activity against all tested substrates (ethyl ferulate, methyl trans-*p*-coumarate, methyl vanillate, methyl caffeate) which was not described earlier for this strain. It might be assumed that this enzyme possess an even broader substrate spectrum as

it was described for other cinnamoyl esterases of lactic acid bacteria (Esteban-Torres et al., 2013; Lai et al., 2009). Contrary to the high substrate activity, the purified esterase demonstrated only a narrow substrate range with regard to varying chain lengths because only the short chain ester *p*-nitrophenyl acetate was metabolized (Figure 37). The longer acyl chains in *p*-nitrophenyl butyrate and *p*-nitrophenyl caprylate seemed to disturb the enzyme activity. This is supported by Wang et al. (2004) who observed a relationship between the feruloyl esterase activity of *Lb. acidophilus* IFO 13951 and the size of sugar moieties which are linked to the ester bonds. The esterase activity decreased with increasing polymerization degree. This might be due to sterical difficulties of substrate interaction with the binding capacity of enzymes. However, it was described that esterases preferentially hydrolyze esters with short side chains (Bornscheuer, 2002).

5 Summary

Lactic acid- and bifidobacteria are commonly used for fermentation of food products, like cheese, beer or sauerkraut. Recently, their application in new plant substrates is of great interest due to an increased demand for plant-based foods. Besides, the microbial ability to degrade undesired secondary plant metabolites and antinutritives is highly desired in order to enhance the nutritional value and digestibility of plant products. For the application of bacterial strains in novel fermentation substrates, a profound knowledge about their growth and metabolism is required since many plants contain secondary plant compounds with antimicrobial effects or lack specific nutrients for microbial growth. Therefore, the aim of the present work was to examine the fermentation performance of different lactic acid- and bifidobacteria in lupin and sunflower substrates and to characterize interactions of the microorganisms with secondary plant metabolites. For a deeper insight into these interactions, the antimicrobial activity as well as the microbial metabolism of selected compounds was examined.

Lupin (*Lupinus angustifolius*) and sunflower (*Helianthus annuus* L.) were selected for this study, because they are promising substrates for human nutrition due to their widespread availability, GMO-free cultivation and high protein content. Protein rich plants are favored for food product development in order to create alternatives for milk and animal protein. However, the concentration of secondary plant metabolites with antinutritive potential limits the direct application of lupin and sunflower for human consumption or negatively affects the product quality. The most important compounds with antinutritive effects in lupin are represented by phytic acid and the oligosaccharides raffinose, stachyose and verbascose. Phytic acid forms complexes with proteins, minerals and vitamins and thus reduces the nutritional value of food products. Oligosaccharides are not absorbed by humans due to the absence of required enzymes. After ingestion they are fermented by the intestinal microbiota which might cause gastrointestinal disorders. Sunflower seeds contain the indigestible oligosaccharides raffinose and stachyose and high amounts of phenolic compounds, mainly chlorogenic acid. Several studies reported an antimicrobial activity of chlorogenic acid against different microorganisms. Besides, chlorogenic acid can oxidize under alkaline conditions to dark-colored reaction products which are undesired in food products.

According to literature, several microorganisms possess the ability to degrade phytic acid, phenolic acids and are capable of utilizing oligosaccharides for their metabolism. These properties depend on the microbial enzymatic equipment. For example, α- and β-galactosidases, β-fructosidase or levansucrase cleave the glycosidic bonds in carbohydrates and are required for the metabolism of oligosaccharides. Phytic acid is degraded by phytases

to lower *myo*-inositol phosphates and chlorogenic acid is hydrolyzed by cinnamoyl esterases to caffeic acid and quinic acid.

In order to identify suitable microorganisms for the fermentation of lupin and sunflower, 25 microorganisms were screened for their ability to metabolize raffinose and stachyose in media, because these carbohydrates were analyzed in considerable amounts in both substrates. The microbial ability to metabolize the main carbohydrate fraction in fermentation substrates represents a fundamental requirement for a high fermentation performance. Out of 11 competent strains, four lactic acid bacteria (*Lactobacillus (Lb.) plantarum* TMW 1.460*, Lb. gasseri* DSM 20243^{T}*, Pediococcus (P.) pentosaceus* DSM 20336^{T}*, Lactococcus* (*L*.) *lactis* subsp. *lactis* DSM 20384) and one bifidobacterium (*Bifidobacterium (Bif.) animalis* subsp. *lactis* DSM 10140^{T}) were selected for further investigations.

Fermentations were carried out in pasteurized 10% (w/v) suspensions of lupin and sunflower raw materials (flours) and compared to suspensions of protein concentrates (10%) which contained only trace amounts of secondary plant compounds. In order to examine the influence of lupin alkaloids which are described to have antibacterial effects, two different lupin flours were applied. The so called ‘sweet’ lupin flour was obtained from a lupin variety with low concentrations of alkaloids (*Lupinus angustifolius* cv. *Boregine*) and ‘bitter’ lupin flour was produced of lupin seeds from an alkaloid rich cultivar (*Lupinus angustifolius* cv. *Azuro*). To evaluate the microbial fermentation performance, growth behavior and acid production were monitored during 48 h of fermentation. For determination of the microbial ability to degrade oligosaccharides, phytic acid and chlorogenic acid, the concentrations were quantified over fermentation time.

Generally, it can be stated that lupin and sunflower raw materials are suitable substrates for fermentation with the selected strains because high viable cell counts and distinct amounts of metabolic products were analyzed. An exception was *L. lactis* subsp. *lactis* which was not competitive in lupin protein suspensions.

The fermentation performance of *Bif. animalis* subsp. *lactis, P. pentosaceus* and *Lb. plantarum* in purified lupin protein isolate was not significantly different to that in lupin flours. Similarly, no distinct differences were observed between sweet and bitter lupin flour. It was concluded that the secondary plant metabolites in *Lupinus angustifolius* have no significant inhibitory effect on the tested strains. During fermentation with *Bif. animalis* subsp. *lactis* and *P. pentosaceus*, a considerable reduction of oligosaccharides was analyzed, indicating that these strains possess oligosaccharide hydrolyzing enzymes. This was confirmed by *in silico* analyses, screenings in nutrient broth and reference analyses of blank samples. All tested microorganisms showed the ability to metabolize phytic acid, with *Lb. plantarum* being most

effective in all lupin substrates and nutrient media. As a consequence, the nutritional value and digestibility of fermented lupin products are enhanced due to an improved bioavailability of nutrients and a decreased content of indigestible oligosaccharides.

A comparison between sunflower flour and sunflower protein concentrate revealed stronger growth and significantly higher metabolic activity of all strains in the flour. It was therefore assumed that the secondary plant metabolites in sunflower seeds did not inhibit the bacteria of this study as described for other lactic acid bacteria. The antimicrobial activity of chlorogenic acid and its cleavage products caffeic acid and quinic acid was determined in media by means of the minimum inhibitory concentration. For evaluation if the antibacterial effect is maintained in sunflower flour matrix, microbial growth and metabolism was monitored in sunflower flour which was spiked with the respective minimum inhibitory concentration. It was demonstrated that the strains exhibited high tolerances against the phenolic compounds with inhibitory concentrations far above the amounts found in sunflower substrates. Caffeic acid displayed a higher antimicrobial potential than chlorogenic acid and quinic acid. Within the tested strains, *Bif. animalis* subsp. *lactis* was most sensitive against all phenolic compounds. It was suggested that the inhibitory effect of chlorogenic acid decreased in sunflower flour matrix due to interactions with proteins. This may also explain the stronger microbial growth in sunflower flour.

During fermentation with most bacteria the oligosaccharide content was markedly reduced leading to an increased digestibility. *Lb. gasseri* revealed only slight degradation activities. Significantly decreased amounts of chlorogenic acid were analyzed in sunflower products fermented with *Bif. animalis* subsp. *lactis* and *Lb. gasseri*, indicating that these strains are capable of metabolizing chlorogenic acid. As chlorogenic acid causes undesired color changes and decreased protein solubility, these degradations might result in an improved food product. The results further suggested that *Lb. gasseri* is able to utilize caffeic acid for energy generation. Screenings in media confirmed this assumption.

The degradation mechanism in *Bif. animalis* subsp. *lactis* was examined by means of heterologous gene expression, protein purification and characterization. It was verified that the open reading frame balat_0669 expressed a cinnamoyl esterase which hydrolyzes chlorogenic acid into caffeic acid and quinic acid. The optimal enzyme activity was measured at pH 7.0 and 40 – 45 °C, clearly indicating the gastrointestinal origin of this bifidobacterium. The purified cinnamoyl esterase displayed activity against further esterified phenolic acids, like ethyl ferulate, methyl trans-*p*-coumarate, methyl vanillate and methyl caffeate, underlining the specificity for ester bonds. Due to these characteristics, the enzyme might be applied in food industry to degrade chlorogenic acid or other esterified phenolic compounds with undesired effects in plant substrates. Besides, the enzyme activity can be used to enrich

food products with free phenolic acids which exhibit a high bioavailability and beneficial health activities.

In conclusion, the knowledge obtained in this thesis about interactions of secondary plant metabolites with lactic acid- and bifidobacteria facilitates the selection of starter cultures for the fermentation of lupin and sunflower substrates. Possible applications are the production of fermented milk alternatives or the enrichment of food and beverages with plant proteins. The flours can be used as valuable food ingredients or for sourdough production. However, the aroma of the fermented substrates was not examined in the present work and should be studied in future to ensure that the products are accepted by consumers. It was evidenced that the tested strains are tolerant against the secondary plant metabolites of lupin and sunflower substrates and that some bacteria are capable of metabolizing antinutritives. Moreover, the mechanism of chlorogenic acid metabolism in *Bif. animalis* subsp. *lactis* was elucidated and the purified enzyme was biochemically characterized for the first time. This provides important knowledge about the metabolism of phenolic compounds in human gastrointestinal tract or probiotic products.

6 Zusammenfassung

Milchsäure- und Bifidobakterien werden häufig zur Fermentation von Lebensmitteln wie beispielsweise Käse, Bier oder Sauerkraut verwendet. Aufgrund einer steigenden Nachfrage nach pflanzlichen Lebensmitteln ist ihr Einsatz in neuen Pflanzensubstraten seit einiger Zeit von großem Interesse. Zudem ist die mikrobielle Fähigkeit, unerwünschte sekundäre Pflanzenstoffe und antinutritive Stoffe abzubauen sehr begehrt, um den Gesundheitswert sowie die Bekömmlichkeit von pflanzlichen Produkten zu erhöhen. Viele Pflanzen enthalten sekundäre Pflanzenstoffe mit antimikrobieller Wirkung oder weisen einen Mangel an spezifischen Nährstoffen für mikrobielles Wachstum auf. Für den Einsatz von Bakterienstämmen in neuartigen Fermentationssubstraten ist daher ein fundiertes Wissen über das mikrobielle Wachstum und ihren Stoffwechsel notwendig. Es war das Ziel dieser Arbeit, die Fermentationsleistung von verschiedenen Milchsäure- und Bifidobakterien in Lupinen- und Sonnenblumensubstraten zu untersuchen und Wechselwirkungen zwischen den Mikroorganismen und sekundären Pflanzenstoffen zu charakterisieren. Für einen tieferen Einblick in diese Wechselwirkungen wurde die antimikrobielle Aktivität wie auch der mikrobielle Stoffwechsel von ausgewählten Substanzen untersucht.

Für diese Studie wurden Lupinen (*Lupinus angustifolius*) und Sonnenblumen (*Helianthus annuus* L.) ausgewählt, da sie aufgrund ihrer weiten Verfügbarkeit, ihres gentechnikfreien Anbaus und hohen Proteingehalts vielversprechende Substrate für die menschliche Ernährung darstellen. Proteinreiche Pflanzen werden für die Produktentwicklung bevorzugt, um Alternativen zu Milch- und Tierprotein zu schaffen. Der Gehalt an sekundären Pflanzeninhaltsstoffen mit antinutritiven Eigenschaften begrenzt jedoch den direkten Einsatz von Lupinen und Sonnenblumen für den menschlichen Verzehr. Ebenso kann die Produktqualität negativ beeinflusst werden. Die wichtigsten Inhaltsstoffe mit antinutritiven Effekten in der Lupine werden durch Phytinsäure und die Oligosaccharide Raffinose, Stachyose und Verbascose repräsentiert. Phytinsäure bildet mit Proteinen, Mineralstoffen und Vitaminen Komplexe, wodurch der nutritive Wert der Lebensmittel herabgesetzt wird. Oligosaccharide können aufgrund fehlender Enzyme nicht vom Menschen absorbiert werden. Nach Aufnahme werden sie von der Darmmikrobiota fermentiert, was zu Magen- und Darmbeschwerden führen kann. Sonnenblumenkerne enthalten ebenfalls die unverdaulichen Oligosaccharide Raffinose und Stachyose sowie hohe Mengen an phenolischen Substanzen, hauptsächlich Chlorogensäure. Etliche Studien berichten, dass Chlorogensäure antimikrobielle Eigenschaften gegenüber verschiedenen Mikroorganismen besitzt. Zudem kann Chlorogensäure unter alkalischen Bedingungen zu dunkel gefärbten Verbindungen oxidieren, was in Lebensmitteln unerwünscht ist.

Laut Literatur besitzen einige Mikroorganismen die Fähigkeit, Phytinsäure und Phenolsäuren abzubauen sowie Oligosaccharide für ihren Stoffwechsel zu nutzen. Diese Eigenschaften

sind von der Enzymausstattung der Mikroorganismen abhängig. Zum Beispiel spalten α- und β-Galactosidasen, β-Fructosidasen oder Levansucrasen die glycosidische Bindung in Kohlenhydraten auf und sind für den Stoffwechsel von Oligosacchariden notwendig. Phytinsäure wird durch Phytasen zu kleineren *myo*-Inositolphosphaten abgebaut und Chlorogensäure wird durch Zimtsäureesterasen in Kaffee- und Chinasäure hydrolysiert.

Um geeignete Mikroorganismen für die Fermentation von Lupinen und Sonnenblumen zu identifizieren, wurden 25 Mikroorganismen auf ihre Fähigkeit untersucht, Raffinose und Stachyose im Medium zu verstoffwechseln, da diese Kohlenhydrate in erheblichen Mengen in beiden Substraten analysiert wurden. Die mikrobielle Fähigkeit, die größte Zuckerfraktion im Fermentationssubstrat zu verstoffwechseln, stellt eine grundlegende Voraussetzung für eine hohe Fermentationsleistung dar. Von den elf kompetenten Stämmen wurden vier Milchsäurebakterien (*Lactobacillus (Lb.) plantarum* TMW 1.460*, Lb. gasseri* DSM 20243^T*, Pediococcus (P.) pentosaceus* DSM 20336^T*, Lactococcus* (*L*.) *lactis* subsp. *lactis* DSM 20384) und ein Bifidobakterium (*Bifidobacterium (Bif.) animalis* subsp. *lactis* DSM 10140^T) für weitere Untersuchungen ausgewählt.

Die Fermentationen wurden in pasteurisierten, 10%igen (w/v) Suspensionen von Lupinen- und Sonnenblumenrohstoffen (Mehle) durchgeführt und mit Proteinkonzentraten gleicher Konzentration (10% w/v) verglichen, die nur Spuren von sekundären Pflanzenstoffen enthalten. Um den Einfluss von Lupinenalkaloiden, denen antibakterielle Eigenschaften zugeschrieben werden, zu untersuchen, wurden verschiedene Lupinenmehle eingesetzt. Das sogenannte „Süßlupinenmehl“ wurde aus einer Lupinensorte mit geringen Alkaloidkonzentrationen (*Lupinus angustifolius* cv. *Boregine*) hergestellt und das „Bitterlupinenmehl“ aus einer alkaloidreichen Kultursorte (*Lupinus angustifolius* cv. *Azuro*). Um die Fermentationsleistung der Mikroorganismen zu beurteilen, wurden das Wachstumsverhalten und die Säurebildung während der 48-stündigen Fermentation beobachtet. Zur Bestimmung der mikrobiellen Abbaufähigkeit von Oligosacchariden, Phytinsäure und Chlorogensäure wurden die Konzentrationen während der Fermentation quantifiziert.

Im Allgemeinen kann gesagt werden, dass Lupinen- und Sonnenblumenrohstoffe geeignete Substrate für die Fermentation mit den ausgewählten Stämmen sind, da hohe Zelldichten und deutliche Mengen an Stoffwechselprodukten analysiert wurden. Eine Ausnahme bildete *L. lactis* subsp. *lactis*, der im Lupinenproteinisolat nicht durchsetzungsfähig war.

Die Fermentationsleistung von *Bif. animalis* subsp. *lactis*, *P. pentosaceus* und *Lb. plantarum* in dem aufgereinigten Proteinisolat war nicht signifikant unterschiedlich zu der in den Lupinenmehlen. Ebenso wurden keine deutlichen Unterschiede zwischen dem Süß- und

Bitterlupinenmehl beobachtet. Es wurde geschlussfolgert, dass die sekundären Pflanzenstoffe in *Lupinus angustifolius* keine signifikant hemmenden Effekte auf die untersuchten Stämme haben. Während der Fermentation mit *Bif. animalis* subsp. *lactis* und *P. pentosaceus* wurde eine deutliche Verringerung des Oligosaccharidgehaltes analysiert, die darauf hinweist, dass diese Stämme oligosaccharid-spaltende Enzyme besitzen. Dies wurde durch *in silico* Analysen, Untersuchungen in Nährbouillon und Referenzanalysen von Blindproben bestätigt. Alle getesteten Mikroorganismen zeigten die Fähigkeit Phytinsäure zu metabolisieren, wobei *Lb. plantarum* in allen Lupinensubstraten und dem Nährmedium am effektivsten war. Aufgrund einer erhöhten Nährstoffverfügbarkeit und verringerten Konzentration an unverdaulichen Oligosacchariden wurden der Nährwert und die Verdaulichkeit der fermentierten Lupinensubstrate verbessert.

Ein Vergleich von Sonnenblumenmehl mit Sonnenblumen-Proteinkonzentrat zeigte ein stärkeres Wachstum und eine signifikant höhere Stoffwechselaktivität aller Stämme im Mehl. Es wurde daher vermutet, dass die sekundären Pflanzenstoffe in Sonnenblumenkernen keinen hemmenden Einfluss auf die Bakterien dieser Studie haben, wie es für andere Milchsäurebakterien beschrieben wurde. Die antimikrobielle Aktivität von Chlorogensäure und den Spaltprodukten Kaffeesäure und Chinasäure wurde mit Hilfe der minimalen Hemmkonzentration im Medium bestimmt. Um zu überprüfen, ob der antibakterielle Effekt in der Sonnenblumenmehlmatrix aufrechterhalten wird, wurde das mikrobielle Wachstum und der Stoffwechsel in Sonnenblumenmehl, dem die jeweilige minimale Hemmkonzentration zugesetzt wurde, untersucht. Es wurde gezeigt, dass alle Bakterienstämme hohe Toleranzen gegenüber den phenolischen Stoffen besitzen. Die minimalen Hemmkonzentrationen lagen weit über den Mengen, die in Sonnenblumensubstraten enthalten sind. Kaffeesäure besaß ein höheres antimikrobielles Potential als Chlorogensäure und Chinasäure. Unter den getesteten Stämmen war *Bif. animalis* subsp. *lactis* am empfindlichsten gegenüber allen Stoffen. Es wurde vermutet, dass sich der hemmende Effekt von Chlorogensäure in der Sonnenblumenmehlmatrix aufgrund von Wechselwirkungen mit Proteinen verringerte. Dadurch könnte auch das stärkere mikrobielle Wachstum im Mehl erklärt werden.

Während der Fermentation mit den meisten Bakterien wurde der Oligosaccharidgehalt deutlich reduziert, was zu einer erhöhten Bekömmlichkeit der Produkte führt. *Lb. gasseri* zeigte dagegen nur geringe Abbauraten. In den Fermentationen mit *Bif. animalis* subsp. *lactis* und *Lb. gasseri* wurden signifikant reduzierte Mengen an Chlorogensäure analysiert, die darauf hindeuten, dass diese Stämme imstande sind, Chlorogensäure zu verstoffwechseln. Da Chlorogensäure unerwünschte Farbveränderungen und eine herabgesetzte Proteinlöslichkeit bewirkt, kann dieser Abbau zu einem verbesserten Lebensmittelprodukt führen. Die Ergebnisse geben weiter Hinweise darauf, dass *Lb. gasseri*

fähig ist Kaffeesäure zur Energiegewinnung zu nutzen. Screenings im Medium bestätigten diese Vermutung.

Der Abbaumechanismus in *Bif. animalis* subsp. *lactis* wurde mit Hilfe von heterologer Genexpression, Proteinaufreinigung und –charakterisierung untersucht. Es wurde bewiesen, dass der offene Leserahmen balat_0669 eine Zimtsäureesterase exprimiert, die Chlorogensäure in Kaffee- und Chinasäure hydrolysiert. Die optimale Enzymaktivität wurde bei pH 7,0 und 40 – 45 °C gemessen, was deutlich auf die gastrointestinale Herkunft dieses Bifidobakteriums hinweist. Die gereinigte Zimtsäureesterase besaß Aktivitäten gegenüber weiteren veresterten Phenolsäuren, wie Ethylferulat, Methyl-trans-*p*-Coumarat, Methylvanillat und Methylkaffeat, was die Spezifität für Esterbindungen hervorhebt. Aufgrund dieser Eigenschaften könnte das Enzym in der Lebensmittelindustrie eingesetzt werden, um Chlorogensäure oder andere veresterte phenolische Substanzen mit unerwünschten Auswirkungen in pflanzlichen Substraten abzubauen. Darüber hinaus kann die Enzymaktivität dazu genutzt werden, um Lebensmittelprodukte mit freien Phenolsäuren anzureichern, die eine hohe Bioverfügbarkeit und gesundheitsfördernde Wirkungen aufweisen.

Das Wissen über Wechselwirkungen von sekundären Pflanzeninhaltsstoffen mit Milchsäure- und Bifidobakterien, das in dieser Arbeit erlangt wurde, vereinfacht die Auswahl von Starterkulturen für die Fermentation von Lupinen- und Sonnenblumensubstraten. Mögliche Anwendungen sind die Herstellung von fermentierten Milchalternativen oder die Anreicherung von Nahrungsmitteln und Getränken mit pflanzlichen Proteinen. Die Mehle können als wertvolle Lebensmittelzutaten oder für die Sauerteigproduktion genutzt werden. Das Aroma der fermentierten Substrate wurde in der vorliegenden Arbeit jedoch nicht untersucht und sollte in Zukunft erforscht werden, um zu sicherzustellen, dass die Produkte von den Konsumenten akzeptiert werden. Es wurde bewiesen, dass die getesteten Stämme gegenüber den sekundären Pflanzenstoffen in Lupine und Sonnenblume tolerant sind und einige Bakterien die Fähigkeit besitzen, antinutritive Stoffe zu verstoffwechseln. Zudem wurde der Mechanismus der Chlorogensäureverstoffwechselung in *Bif. animalis* subsp. *lactis* aufgeklärt und das gereinigte Enzym zum ersten Mal biochemisch charakterisiert. Dies liefert bedeutende Erkenntnisse über den Stoffwechsel von phenolischen Stoffen im menschlichen Magen-Darm-Trakt oder in probiotischen Produkten.

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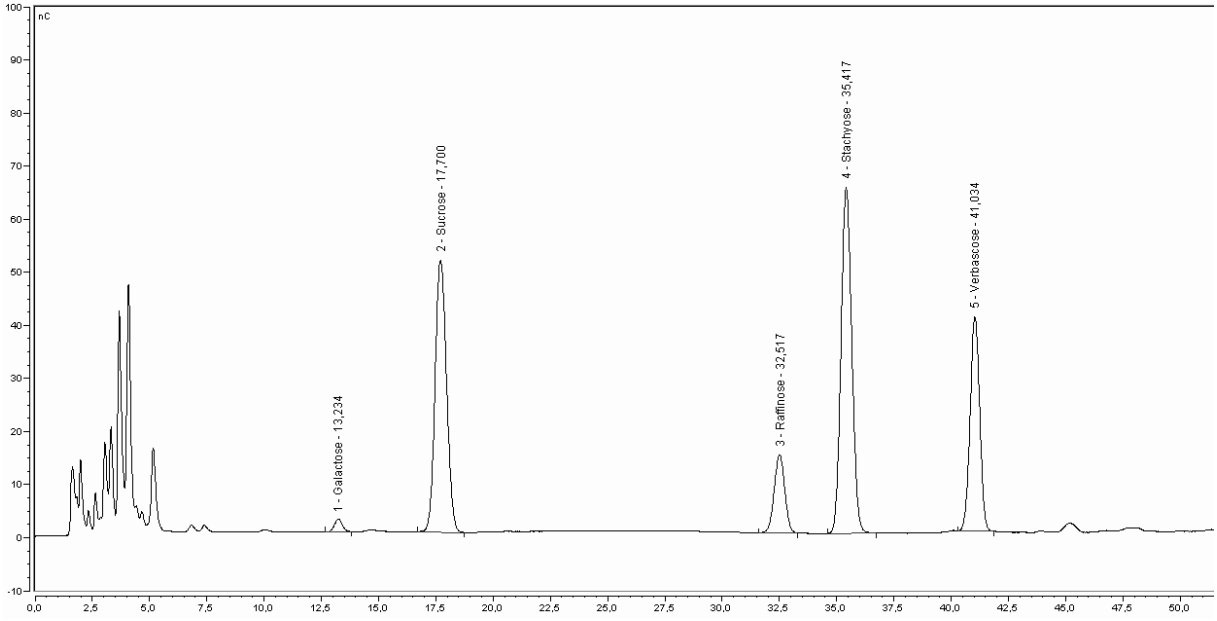
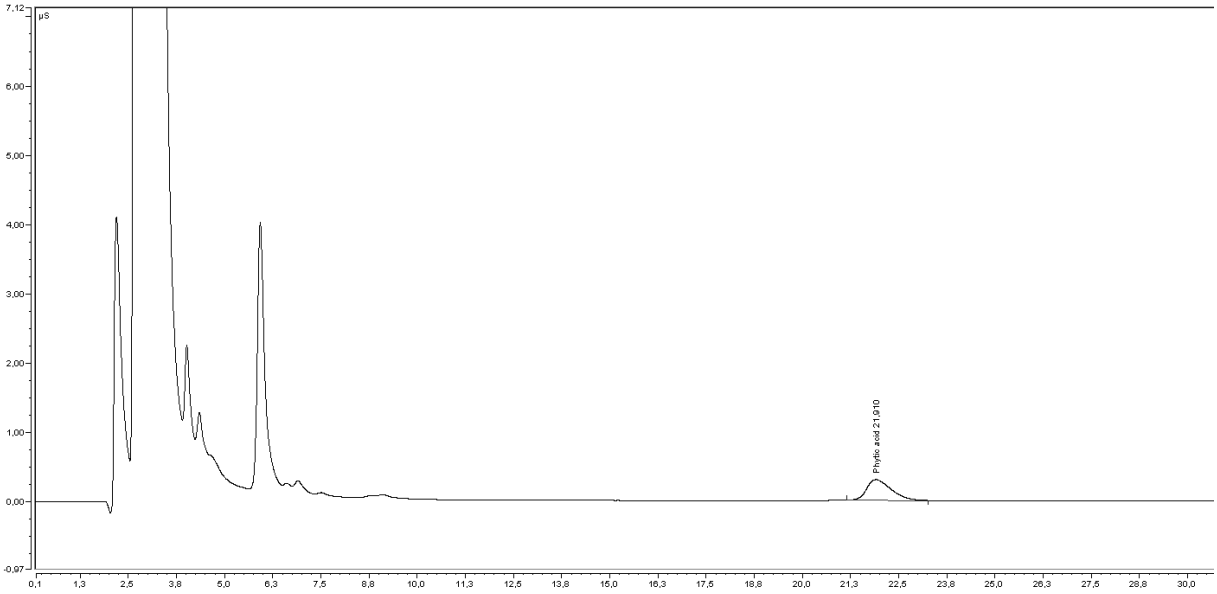
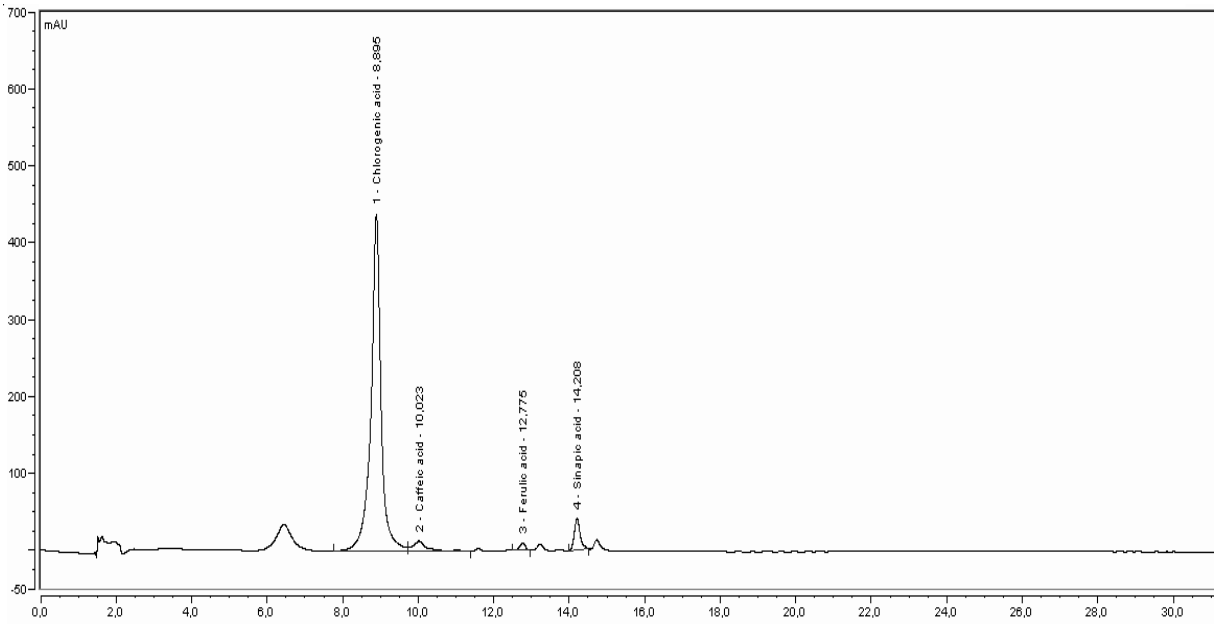
8 Appendix



Appendix 1: HPAEC-PAD chromatogram of carbohydrates in lupin flour



Appendix 2: HPAEC-CD chromatogram of phytic acid in lupin flour



Appendix 3: HPLC-UV chromatogram of phenolic acids in sunflower flour

MTTSTHTEEI TVMRDGLRLH GRIDAPQGEP KGPVVILMHG FMADLGYEPG SLLQQVSDQL
VEAGFTSVRF DFNGRGNSDG SFANSDVCNQ VEDAIAVLNF VRDRFEPAEI SLLGHSQGGV
IAGMTAGMYA DVVHSLVLLS PAASIKDDAL RGRVLGVPFD PYHIPRRIAL ADGKHEVAGK
YSRIAKTIPV YEAAAMFKGP ALAIQGEQDK VIDPSCAHNY GNAMANCTVS LYTNLDHKFN
GDDRMRAIGE AVAFLQTHHE VA

Appendix 4: Protein sequence of balat_0669, putative cinnamoyl esterase in *Bif. animalis* subsp. *lactis* DSM 10140 (www.biocyc.org)

atgACGACGA GCACACATAC CGAGGAAATC ACCGTGATGC GTGATGGGCT GAGGCTTCAT
GGACGCATCG ACGCACCACA GGGCGAACCG AAAGGGCCGG TGGTGATTCT GATGCACGGG
TTCATGGCCG ATCTGGGGTA TGAACCCGGC AGCCTGCTTC AGCAGGTGAG CGACCAGCTC
GTCGAAGCGG GATTCACGTC CGTGAGGTTC GATTTCAACG GTCGTGGCAA CAGTGACGGA
TCATTTGCGA ATTCCGACGT CTGCAATCAG GTGGAGGATG CCATCGCGGT ACTCAATTTT
GTACGTGATC GTTTCGAGCC AGCGGAGATC TCATTGCTCG GACATTCACA AGGCGGTGTG
ATTGCAGGCA TGACGGCGGG TATGTATGCC GATGTGGTCC ATTCCCTAGT CCTGCTTTCG
CCTGCGGCCT CGATCAAGGA TGACGCATTG CGTGGGCGTG TGCTTGGCGT CCCATTCGAC
CCCTATCACA TTCCACGGCG CATCGCGCTC GCGGATGGCA AGCATGAGGT CGCAGGCAAA
TACTCTCGCA TCGCCAAGAC GATTCCGGTG TATGAGGCGG CTGCGATGTT CAAGGGACCC
GCTCTGGCGA TCCAGGGTGA GCAGGACAAG GTGATCGACC CCAGCTGCGC GCACAACTAC
GGCAACGCTA TGGCGAACTG CACCGTGAGC CTGTACACGA ACCTCGACCA CAAGTTCAAC
GGCGATGACC GCATGCGTGC GATAGGCGAG GCGGTGGCAT TCCTTCAGAC GCATCATGAG
GTGGCGtga

Appendix 5: Complete nucleotide sequence of *Bif. animalis* subsp. *lactis* DSM 10140 cinnamoyl esterase (www.biocyc.org)