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Impact of lactic fermentation and thermal treatments on the texture of a lupin-based yogurt alternative

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PRELIMINARY REMARKS

Parts of the work presented in this thesis were published as papers in international peer-reviewed journals, as conference presentations or posters, which are listed below. The permission of the respective journal was given.

Peer-reviewed journals

Hickisch, A., Beer, R., Vogel, R.F., Toelstede, S. (2016): Influence of lupin-based milk alternative heat treatment and exopolysaccharide-producing lactic acid bacteria on the physical characteristics of lupin-based yogurt alternatives. Food Research International, 84, 180–188.

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Oral presentations

Toelstede, S., Jacobs, D., Hickisch, A.: Fermentation of lupin protein for the development of dairy alternatives. 1st International Scientific Congress: Advances in Sustainable Food Processing: Challenges for the Future, Campinas, Sao Paulo, Brazil 5. – 7. November 2014.

Hickisch, A., Vogel, R.F., Toelstede, S.: Exopolysaccharide von Milchsäurebakterien: Einfluss auf die Textur von Lupinenjoghurt. 16. Fachsymposium Lebensmittelmikrobiologie, Stuttgart Hohenheim 30. März – 1. April 2016.

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Hickisch, A.: Gelbildung pflanzlicher Lupinenproteine – Einfluss auf die Herstellung von Joghurtalternativen. Vortrag: Freisinger Tage – Pflanzliche Lebensmittelproteine, Freising 12. – 13. April 2018.

Hickisch, A., Schweiggert-Weisz, U.: Development of lupin-based yogurt alternatives – Challenges regarding processing and fermentation. XV International Lupin Conference, Cochabamba, Bolivia 18. – 21. March 2019.

Hickisch, A., Schweiggert-Weisz, U.: Lupine – ein vielversprechender Rohstoff für die Herstellung von Milchproduktalternativen. GDL Symposium: Pflanzliche Proteine – Eigenschaften, Potential, Anwendungstechnologie, Gerlingen bei Stuttgart 24. – 25. September 2019.

Poster

Hickisch, A., Beer, R., Vogel, R.F., Toelstede, S.: Development of a lupin yogurt – optimization of textural properties. 28th EFFoST International Conference, Uppsala, Sweden 25. – 28. November 2014.

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Patent application

Eisner, P., Fischl, R., Mittermaier, S., Zacherl, C., Toelstede, S., Jacobs, D., Schreiber, K., Hickisch, A.: Emulsion mit Lupinenprotein. WO 2016000939 A1.

For all publications listed above that constitute the basis for this thesis the author of this thesis is the main author. This means that Dipl.-Ing. Andrea Hickisch has basically been responsible for the design and execution of all scientific experiments. The intellectual processing of experimental information gathered and the setup of the scientific context as well as the actual writing of the papers are practically exclusive her product.

The co-authors' contributions to the papers presented in this thesis are specified as follows:

The work presented in this thesis was carried out under the supervision of Professor Rudi F. Vogel, Technical University Munich. Dr. Simone Toelstede, Fraunhofer Institute for Process Engineering and Packaging (IVV), was the instructor of this work and substantially contributed to the conception of this work. Moreover, she functioned as an advisor with regard to content of the work and publication results. She has also proofread all manuscripts. Dr. Ute Schweiggert-Weisz and Prof. Dr. Peter Eisner (IVV), have also proofread this thesis.

The following student thesis was supervised. The resulting raw data were partially incorporated into this thesis with permission by the respective students: Regina Beer, Master Thesis 2014: Rheological and textural properties of yoghurt fermented with UHT and pasteurized lupin milk and different EPS – producing LAB.

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ABBREVIATIONS AND SYMBOLS

a*	color coordinate: red-green
A_H	hysteresis loop area
ANOVA	Analysis of Variance
b*	color coordinate: yellow-blue
B.C.	Before Christ
BGT	Lehrstuhl für Brau- und Getränketechnologie
cf.	compare
CFU/ml	colony-forming units per ml
CLSM	confocal laser scanning microscopy
cryo-SEM	cryo-scanning electron microscopy
cv.	cultivar
D	dilution factor
Da	Dalton
DSC	differential scanning calorimetry
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
EPS	exopolysaccharide
g (centrifugation)	relative centrifugal force
g (weight)	gram
G*	complex modulus
G’	storage or elastic modulus
G’’	loss or viscous modulus
GMO	genetically modified organism
h	hour
ha	hectare
HePS	heteropolysaccharides
HMW	high relative molecular weight
homog.	homogenized
HoPS	homopolysaccharides
HPLC-MS	high-performance liquid chromatography-mass spectrometry
IMW	intermediate relative molecular weight
IP	isoelectric point
l	liter

L*	color coordinate: lightness to darkness
L.	*Lactobacillus*
LAB	lactic acid bacteria
LBMA	lupin-based milk alternative
LBYA	lupin-based yogurt alternative
LMW	low relative molecular weight
log_{10}	decadic logarithm
LOX	lipoxygenase
LPI	lupin protein isolate
LPI_2%	2% (w/v) LPI solution
m	meter
M	molar
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
M_i	molecular weight
mMRS	modified De Man, Rogosa and Sharpe
MRS	De Man, Rogosa and Sharpe
MRS+CYS	De Man, Rogosa and Sharpe supplemented with cysteine
n	number of samples
n.d.	not determined
n.m.	not mentioned
NaOH	sodium hydroxide
P	pressure
P	significance level
P.	*Pediococcus*
p.a.	pro analysi
Pa	pascal
pasteur.	pasteurized
PCA	Plate Count Agar
PR	Propionibacterium (medium)
rpm	rounds per minute
S	Svedberg (sedimentation coefficient)
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SH-group	sulfhydryl group
SS-bonding	disulfide bonding
ssp.	subspecies
S.	*Streptococcus*

t	time
T	temperature
TCA	trichloroacetic acid
T_d	peak denaturation temperature
TMW	Technische Mikrobiologie Weihenstephan
T_{onset}	onset temperature
Tris	tris(hydroxymethyl)-aminomethane
TSYE	Trypticase Soy Yeast Extract
UHT	ultra-high temperature
UK	United Kingdom
USA	United States of America
V	volt
v	speed
v/v	volume per volume
W	watt
w/v	weight per volume
ʎ	lambda
YGC	Yeast Glucose Chloramphenicol (medium)
z	path length
α	alpha
β	beta
γ (protein fraction)	gamma
γ (rheology)	shear strain
γ_f	flow point
γ_y	yield point
$\dot{\gamma}$	shear rate
δ (protein fraction)	delta
δ (rheology)	phase angle
ΔA_{412}	absorbance at 412 nm
ΔE	color difference
ΔH	enthalpy of denaturation (joule/gram)
ε	extinction coefficient
η_a	apparent viscosity
τ	shear stress
ω	angular frequency

1 Introduction

1.1 Lupin

Beside soy beans, peanuts, peas, chickpeas and lentils, lupins (*Lupinus* L.) are another species belonging to the genus of legumes, from the family of *Fabaceae*. Several hundred different species are known, the most common are *Lupinus mutabilis* L. originated in the Andean region, whereas *Lupinus albus* L. (white lupin), *Lupinus angustifolius* L. (narrow-leafed lupin) and *Lupinus luteus* L. (yellow lupin) are predominantly cultivated in the Mediterranean area (Gladstones, 1970). The wild type lupin varieties are called 'bitter lupins', as they may contain up to 5% of bitter-tasting and toxic alkaloids (Wink, 1988). Only in the 20th century the breeding by Reinhold von Sengbusch of an alkaloid-poor variety made lupin accessible for human nutrition. So-called 'sweet lupins' contain alkaloid levels lower than 0.02% (von Sengbusch, 1942). However, the worldwide production of lupin seeds in 2017 amounted to 930,717 ha, which is only 0.75% of the worldwide soy bean production (FAO, 2017). Thereby, the main producer of lupin seeds is Australia with a worldwide share of 68%, followed by the European Union with 20% (particularly Poland and Germany with 14% and 4%, respectively) and finally the Russian Federation with 14% (FAO, 2017). The cultivation of lupins is simple as lupin tolerates nutritionally poor soils and requires low demands on climate (Trinick, 1977). Cooperating with rhizobia, which are located in the roots of lupins, lupins as all other legumes fix and assimilate atmospheric nitrogen. After harvest considerable amounts of nitrogen remain in the soil and enhance the soil quality for other plants (Lodwig et al., 2003; Manhart and Wong, 1980; Peoples et al., 1995). However, a crucial advantage concerning cultivation is addressed to *L. angustifolius* L.: A considerable higher resistance to the plant disease anthracnose was recorded for *L. angustifolius* L. compared to *L. albus* L. and *L. luteus* L.. Anthracnose is caused by a fungus of the genus *Colletotrichum lupini*, spreading over the whole plant destroying it thereby (Tschöpe and Racca, 2011). In contrast to soy, genetically modified varieties of lupins are not commercially available up to now (Eapen, 2008), which is of high relevance for European consumers due to their strong movement of opposition to the agricultural application of genetic engineering.

1.1.1 Composition of lupin seeds

The composition of lupin seeds varies due to different annual climate and soil conditions (Bhardwaj et al., 1998; Cowling and Tarr, 2004). Lupin seeds are rich in proteins, almost as high as in soy, which can be attributed to nitrogen assimilation. The protein content of the seeds of different varieties of *L. angustifolius* L. range between 29 – 35% in dry matter,

whereas *L. albus* L. and *L. luteus* L. varieties amount to 32 – 39% and 38 – 48% in dry matter, respectively (Pisarikova and Zraly, 2009; Sujak et al., 2006). The composition of essential amino acids is almost balanced according to the requirements of the FAO amino acid scoring pattern (FAO, 2011) except for the sulfur-containing amino acids cysteine and methionine, as well as lysine, tryptophan and valine, which are underrepresented (Lqari et al., 2002; Sujak et al., 2006). Moreover, seeds are characterized by a high content of dietary fiber of about 12 – 18% in dry matter, as well as of a low content of oil of 5 – 13% in dry matter and starch of 4 – 10% in dry matter (Pisarikova and Zraly, 2009; Sujak et al., 2006). Beside fiber and starch, other carbohydrates are present in lupins such as mono-, di-, oligo- and polysaccharides. In lupin seeds, mono- and disaccharides make up 5 – 7% in dry matter. Thereby, sucrose exhibit with ~ 4% the highest share followed by galactose with~ 0.4%, glucose with ~ 0.4%, ribose with ~ 0.3%, maltose with ~ 0.3%, fructose with ~ 0.2%, and traces of xylose (Erbaş et al., 2005)). Polysaccharides like e.g. galactans and arabinogalactans are known to occur in lupin and are often glycosylated with proteins (al-Kaisey and Wilkie, 1992; Duranti et al., 1981). Beside these major nutritive compounds, lupin seeds contain several secondary plant metabolites with antinutritive effects, e.g. flatulence-causing oligosaccharides such as raffinose, stachyose and verbascose, which account for ~ 5.8 – 8.6% in dry matter and phytic acid with ~ 0.7 – 4.9% in dry matter (Camacho et al., 1991; Fritsch et al., 2015; Macrae and Zand-Moghaddam, 1978; Trugo et al., 1988; Trugo et al., 1993). As human's gastrointestinal tract is lacking of the oligosaccharide utilizing enzyme α-galactosidase, the reduction of oligosaccharides is advantageous in terms of digestibility (Gitzelmann and Auricchio, 1965; Rackis, 1975). Otherwise, oligosaccharides are discussed as presumable prebiotics (Martínez-Villaluenga et al., 2006; Martínez-Villaluenga and Gómez, 2007). Indeed, phytic acid was recognized as anticarcinogenic (Shamsuddin, 1995) and antioxidant substance (Graf et al., 1987), but nevertheless its reduction is useful as its intake leads to a reduced bioavailability of minerals, vitamins and proteins due to its complex-forming ability (Dvořáková, 1998; Graf et al., 1987). Further antinutritives with relative low contents are quinolizidine alkaloids (Muzquiz et al., 1994; Wink, 1988), as well as trypsin-inhibitors (Scarafoni et al., 2008), saponins, tannins (Dupont et al., 1994) and isoflavones (Sirtori et al., 2004).

Beneficial health-effects of lupin proteins on the lipid metabolism were observed in human and animal studies compared to casein (Bähr et al., 2013; Bettzieche et al., 2008a; Bettzieche et al., 2008b; Pilvi et al., 2006; Sirtori et al., 2012; Spielmann et al., 2007). Furthermore, glucose-lowering activity of lupin protein fraction conglutin γ was reported (Bertoglio et al., 2011; Lovati et al., 2012). However, lupin protein fraction conglutin β has

been designated as allergen by the International Union of Immunological Societies (IUIS) allergen nomenclature subcommittee (Goggin et al., 2008; Hieta et al., 2009).

1.1.2 Lupin protein fractions

According to Osborne and Campbell (1898) and thus in relation to their solubility in different solvents, lupin seeds contain mainly two classes of proteins, namely albumins and globulins, whereas other fractions, such as prolamins and glutelins, are only present as minorities (Cerletti et al., 1978; Duranti et al., 1981; Gulewicz et al., 2008; Plant and Moore, 1983). The mass ratio of albumins to globulins is around 1 to 9 (Duranti et al., 2008). Due to their electrophoretic mobility, the globulins can be further divided into conglutin α, β and γ, whereas albumin is also referred as conglutin δ (Blagrove and Gillespie, 1975). An overview of the main properties of these fractions is presented in Table 1 and is described more thoroughly below.

Table 1: Summary of the main properties of the conglutin proteins from *Lupinus albus* L. (Berghout et al., 2015; Duranti et al., 2008).

Conglutin	Protein family	Native proteins			Monomer composition				
		M_i [kDa]	IP	Quarternary structure	Subunit	M [kDa]	IP	Amount of SS-bondings	Glyco-sylation
α	11S (legumin)	330-430	5.1-5.8	Hexamer	Acidic Basic	42-52 20-22	4.5-4.7 6.7-8.6	6	Yes/No* No
β	7S (vicilin)	143-260	5.0-6.0	Trimer	HMW IMW LMW	53-64 25-46 17-20	5.1-5.7 5.3-8.4 4.2-5.0	0	Yes Yes/No* Yes/No*
γ	7S	200	7.9	Tetramer	Large Small	29 17	8.2-8.9 5.8-6.6	2	Yes No
δ	2S	13	Acidic	Monomer	Large Small	9 4	4.1-4.3 n.d.	4	No No

M_i: molecular weight
IP: isoelectric point
SS-bondings: disulfide bondings
S: sedimentation coefficient
HMW, IMW, LMW: high, intermediate and low relative molecular weight, respectively
n.d.: not determined
*not all polypeptides are glycosylated

Conglutin α

Conglutin α is a globular, legumin-like lupin protein with a sedimentation coefficient of 11S (S = Svedberg). The proteins in the seeds of *Lupinus angustifolius* L. are composed of around 40% conglutin α and thereby represent the main protein fraction (Plant and Moore, 1983). The seeds of *Lupinus albus* L. contain about 35 to 37% conglutin α, which is the second largest protein fraction (Duranti et al., 1990). The quaternary structure of conglutin α is hexameric with the largest molecular weight of 330 to 430 kDa among the conglutins. Each monomer of conglutin α is composed of two subunits, an acidic and a basic chain with 42 to 52 kDa and 20 to 22 kDa, respectively, which are linked by a single disulfide (SS) bonding (Berghout et al., 2015; Duranti et al., 2008; Duranti et al., 1988). Duranti et al. (1988) showed that transitions from hexameric to trimeric structures can occur in accordance to a concentration- and pH-dependent equilibrium. Furthermore, legumins can be glycosylated with mannose and galactose (Eaton-Mordas and Moore, 1978). The IP is between 5.1 and 5.8 (Duranti et al., 2008).

Conglutin β

Conglutin β is a 7S vicilin-like storage protein and represent the second largest fraction with around 33% of total proteins in the seeds of *Lupinus angustifolius* L. (Plant and Moore, 1983); in *L. albus* L. conglutin β amounts to the largest protein fraction with 44 to 45% (Duranti et al., 1990). The molecular weight of the native conglutin β varies between 143 to 260 kDa (Duranti et al., 1990). It is composed of a trimeric quaternary structure with subunits ranging from 17 to 20 kDa (low molecular weight subunit), from 25 to 46 kDa (intermediate molecular weight subunit) and from 53 to 64 kDa for the high molecular weight fraction. Furthermore, all subunits can be glycosylated with mannose and galactose (Duranti et al., 1990; Eaton-Mordas and Moore, 1978), but covalently linkages such as SS-bonding upon the different side chains do not occur. The IP of conglutin β is similar to conglutin α (between 5.0 and 6.0) (Duranti et al., 2008). However, as mentioned before conglutin β was identified as allergenic in seeds of *Lupinus angustifolius* L. and *Lupinus albus* L. (Goggin et al., 2008).

Conglutin γ

Conglutin γ is a rather unusual 7S protein: it is soluble in both, demineralized water and salt solutions. At neutral pH the oligomeric protein conglutin γ is a tetramer (Duranti et al., 2000) or according to Blagrove et al. (1980) a hexamer. At acidic pH, the oligomer dissociates to its monomers composed of a large subunit with 29 kDa and a small subunit with 17 kDa

(Blagrove and Gillespie, 1975; Restani et al., 1981). Both subunits are linked by SS-bondings and the large subunit is additionally glycosylated with mannose and galactose (Duranti et al., 2008; Eaton-Mordas and Moore, 1978). Conglutin γ represents only 5% of total proteins in the seeds of *Lupinus angustifolius* L. (Plant and Moore, 1983). The molecular weight of the native conglutin γ is 200 kDa and its IP is at pH 7.9 (Duranti et al., 2008; Duranti et al., 1990).

Conglutin δ

Conglutin δ is a sulfur-rich 2S albumin (Blagrove and Gillespie, 1975). It contains about 70% of the sulfur present in lupin seeds, which is incorporated in the amino acids methionine and cysteine and is provided upon germination for the developing cotyledon (Lilley, 1986; Lilley and Inglis, 1986). The presence of albumins in the seeds of *Lupinus angustifolius* L. varies between 5 to 11% (Gulewicz et al., 2008; Plant and Moore, 1983). Conglutin δ is a monomeric protein of low molecular weight of 13 kDa. It is composed of a large and small subunit, with 9 and 4 kDa, respectively, linked by a SS-bonding without glycosylation. Further, the native protein has an acidic IP of 4.3. Covalent dimerization of two monomeric units can occur due to exposed and reactive cysteine residues via SS-bonding resulting in dimers with molecular weights of 23 to 25 kDa (Duranti et al., 2008).

1.1.3 Lupin protein isolation techniques

Proteins are the most important components of lupins for human consumption. Therefore, lupin flour is often further processed to obtain protein concentrates or isolates, which are nutritionally advantageous as lupin raw materials contain antinutritives (cf. Section 1.1.1). Depending on the protein content, these preparations are classified into protein concentrates with 45 – 80% protein content in dry matter and protein isolates with at least 90% protein content in dry matter (Cheftel et al., 1992). Various protein isolation and purification processes are applicable for lupin protein. Usually, the seeds get dehulled, separated, flaked, defatted and milled. For the preparation of protein concentrates, the defatted flakes are soaked in acidified water. The majority of the protein fractions is insoluble and remains in the solid residue, whereas components such as oligosaccharides and alkaloids are solved in the supernatant and removed by centrifugation (Belitz et al., 2009). Protein isolates with high functional behavior are obtained mainly by two techniques: the salt-induced extraction followed by dilutive precipitation (Muranyi et al., 2016; Murray et al., 1979; Murray et al., 1981) and the alkaline extraction with subsequent isoelectric precipitation (Chew et al., 2003; Eisner, 2013; Lqari et al., 2002; Wäsche et al., 2001).

The extraction and precipitation of proteins out of complex food systems such as legume seeds is determined by their solubility behavior. Protein solubility is affected by environmental factors such as ionic strength, type of solvent, pH, temperature and processing conditions (Zayas, 1997). All of these factors can cause various reversible or irreversible conformational transitions (Englard and Seifter, 1990). Taking the alkaline extraction and isoelectric precipitation as well as the salt-induced extraction and dilutive precipitation into account, the solubility behavior of proteins is affected by the ionic strength and pH, respectively, which is described in more detail in the following paragraph.

The ionic strength influences the protein solubility, dependent on the type of ion, its concentration and its valency. Low concentrations of salts, for example 0.5 – 1 M sodium chloride, lead to interactions of sodium and chloride ions to the charged counterions of the peptide chains. Thereby, the electrostatic protein-protein interactions are decreased, which destabilize the protein and lead to protein solubility. This increase in protein solubility was named the ‘salting-in’ effect (Cohn and Edsall, 1943; Maurer et al., 2011; Zayas, 1997). However, further increases of the salt concentration in water above 1 M lead to the ‘salting-out’ effect. The protein solubility drops until protein precipitation, as the salt ions and the charged proteins compete with free water molecules. Consequently, the proteins can bind less water until the protein aggregates (Cohn and Edsall, 1943). Indeed, the ionic strength can also be modified in the reverse way: salt-solubilized proteins in solutions with low ionic strength can be precipitated by a drastic reduction of the ionic strength. Thereby, the amphiphilic character of the proteins is exploited, as these amphiphiles associate into thermodynamically stable aggregates known as micelles, where the polar residues are oriented outwards to the water and the nonpolar residues are buried into the hydrophobic core of the micelles, leading to precipitation. This effect is referred as ‘hydrophobic-out’ effect. The ‘salting-in’ and ‘hydrophobic-out’ effect are utilized by the above mentioned salt-induced extraction followed by dilutive precipitation (Murray et al., 1979; Murray et al., 1981).

Beside the ionic strength, the solubility of proteins can be modified by pH-shifts. At pH-values above the IP the total net charge of globular proteins is negative, as the negative charges of the acidic amino acids, glutamic and aspartic acids, predominates in contrast to the alkaline amino acids, which are neutral (histidine) or positively charged (arginine, lysine). In this state, proteins are dissolved, as these charged protein side chains interact with the water molecules. Protein solubility is mainly driven by electrostatic repulsion and hydrophobic interactions. When the electrostatic repulsion between proteins is higher than the hydrophobic interactions of the proteins, proteins are soluble. However, when the pH is shifted towards the IP, the alkaline amino acids get gradually compensated, leading to a neutral net charge of the protein. Hereby, attractive forces predominate and molecules tend

to associate, resulting in insolubility (Zayas, 1997). The before mentioned alkaline extraction with subsequent isoelectric precipitation is followed by these mechanisms (Chew et al., 2003; Lqari et al., 2002; Wäsche et al., 2001). It is one of the most often applied isolation techniques, as it can be easily and economically incorporated into industrial processes (Mittermaier, 2013). Furthermore, antinutritives get considerably separated: oligosaccharides such as raffinose, stachyose and verbascose were reduced from 6.3% to 0.1%, whereas phytic acid was depleted from 4.9% to at least 3.2% following the study of Fritsch et al. (2015). This isolation technique was used throughout this thesis.

1.1.4 Techno-functional properties of lupin proteins with focus on the gelation behavior of globular proteins

The selection of a suitable lupin species has to be considered in accordance to the respective application and is addressed to a variety of characteristics. Beside the protein content of the seeds, the techno-functional properties of the proteins are of considerable importance. For the production of dairy alternatives, the gelling properties are absolutely essential, followed by the protein solubility and the emulsifying capacity. LPI exhibit promising techno-functional properties (Lqari et al., 2002; Wäsche et al., 2001): Mittermaier (2013) for example showed excellent protein solubility at pH 7 amounting 85 – 100% for all lupin species investigated, which is quite high compared to soy protein isolates, that exhibited only a solubility of around 44% (Meinlschmidt et al., 2016a). However, the emulsifying capacity was strongly species dependent: high emulsifying capacities of 620 – 720 ml/g were found for *L. angustifolius* L. (Mittermaier, 2013), which is similar to soy protein isolate with around 660 ml/g (Meinlschmidt et al., 2016a), followed by moderate emulsifying capacities of 580 ml/g for *L. albus* L. and lower capacities of 530 ml/g for *L. luteus* L.. Gelling properties were rather low in lupins: only protein isolated from *L. albus* L. showed gelation behavior at concentrations of 15% (w/w), the other isolates did not show gelling behavior at all (Mittermaier, 2013). Poor gelling behavior of LPI was also seen by other authors (Batista et al., 2005; Berghout et al., 2015), whereas comparative analysis subscribed soy protein isolates strong gelling abilities.

Globular proteins undergo three successive steps during thermal gel formation: (i) molecular unfolding (denaturation) of proteins exposing residues previously masked in the core, (ii) dissociation–association of the exposed residues to form aggregates and (iii) arrangement of aggregates into a continuous network (Batista et al., 2005; Hermansson, 1986). In more detail, denaturation is any modification in conformation involving changes in the secondary, tertiary or quaternary structure – without cleaving peptide bonds (primary structure). Various

inter- and intramolecular bonds, that stabilize the native protein conformation, can be disrupted and rearranged resulting in the conversion from a native into a more unfolded state (Tanford, 1968). The stepwise unfolding of the proteins can lead to the exposure and activation of functional groups e.g. sulfhydryl (SH) groups or hydrophobic groups (Boye et al., 1996). These functional and reactive groups, which were previously hidden in the interior of the protein, can now interact with each other and SS-bondings and hydrophobic reactions occur, which facilitate protein aggregation (Wang and Damodaran, 1991). Consequently, a sufficient content of sulfurous and hydrophobic amino acids within the proteins is essential for gelation (Cheftel et al., 1992). Finally, aggregated proteins are arranged to a network. In comparison to soy proteins, lupin proteins are associated with relatively weak gelling properties (Berghout et al., 2015). Thermal denaturation of conglutin γ begins at ~ 70 °C, followed by conglutin β at ~ 81 °C, whereas conglutin α is thermally more stable with ~ 96 °C (Bader et al., 2011a).

Beside thermal gelation, which is most commonly studied for globular proteins, gelation can also be induced by hydrostatic pressure, enzymes, salt and also by acid, as it happens in yogurt (Totosaus et al., 2002). Acid-induced gelation is mainly driven by changes in the net charge of the proteins. The acidification of a protein solution leads to an increase of positive residues due to the protonation of the amino group in e.g. lysine and arginine. These additional positive residues facilitate electrostatic interactions with the present negative residues of the proteins. Due to these interactions, a network embedded with water is formed. Above the IP, some negative residues are not bound and can interact with water molecules keeping the proteins in solution. When the pH approaches the IP upon acidification, the negative net charge gets gradually compensated and the protein tends to coagulate due to a minimum of interaction with water molecules (Cheftel et al., 1992). The acid-induced gelation can be favored by implementing a heating step before acidification, in which the protein molecule denatures and forms soluble protein aggregates by heat. After cooling, the pH is lowered by acid, leading to precipitation of the proteins (Alting, 2003). In 1.3, gelation is described more detailed on the basis of casein micelles.

1.2 Plant-based dairy alternatives

Interest in vegetarian or vegan nutrition has been rising in recent years. Lifestyle choices lead to diets free from animal-based products. According to an estimate, 14% of European consumers avoid dairy products (Jago, 2011; Mäkinen et al., 2016). Ethical and sustainability worries are mentioned due to raising consumer consciousness; according to FAO, livestock is responsible of 18% of the global greenhouse gases, while dairy production thereof

amounts to 3% (Steinfeld et al., 2006). Besides, health-related reasons such as lactose intolerance or cow milk allergy play an important role. Whereas lactose intolerant consumers can replace dairy products with lactose-free equivalents, for cow milk allergic consumers only complete avoidance remains (Mäkinen et al., 2016). Furthermore, calorie intake and prevalence of hypercholesterolemia leads to a further increased demand for dairy-free alternatives (Sethi et al., 2016). Moreover, consumer awareness is rising due to reports about growth hormones and antibiotic residues in cow milk (Jago, 2011; Mäkinen et al., 2016).

Among dairy alternatives, soy-based products such as milk and yogurt alternatives or tofu are dominating the market in the Western World. However, other plant sources should be considered to broaden the variety of plant derived dairy alternatives. Indeed, nowadays the share of soy-products has decreased, as comparable products from other plant raw materials such as coconut, oat and almond emerged (Jago, 2011; Mäkinen et al., 2016). This development is due to increased worries of consumers about soy products regarding genetically modified varieties and allergenicity (Mäkinen et al., 2016).

According to article 2 of Council Regulation No. 1898/87 of the European Economic Community, “the term 'milk' shall mean exclusively the normal mammary secretion obtained from one or more milking without either addition thereto or extraction therefrom” (Anonymous, 1987). Thereby, the origin of the milk must be stated, when it is not bovine (e.g. goat milk). Beside the term ‘milk’, every milk derived product is also legally protected such as ‘yogurt’. Consequently plant-based milk-like products shall not be designated as ‘milk’ or ‘yogurt’; in terms of milk-like products, producers often call them ‘drinks’. Therefore, throughout this thesis products are designated as lupin-based milk alternatives (LBMA) and lupin-based yogurt alternatives (LBYA); with this terminology, products are clearly differentiated to the original cow milk-based products, but it is still expressed that products mimic these products.

1.2.1 Production of dairy alternatives

According to Mäkinen et al. (2016) “plant-based milk alternatives are colloidal suspensions or emulsions consisting of dissolved and disintegrated plant material, resembling cow milk in appearance”. The first plant-based milk alternative was manufactured out of soy and was probably invented in China in the second century B.C.. Since ancient times, it is produced especially throughout China, also in Japan and other parts of East Asia (Shurtleff and Aoyagi, 1979).

The traditional way to produce soy-based milk alternatives involves soaking of the whole seeds usually over night at room temperature, followed by wet grinding, filtering to remove the solids and cooking the slurry for preservation (Debruyne, 2006; Kwok and Niranjan, 1995; Morse and Piper, 1923). Essentially, soy-based milk alternatives are the water extract of soy beans (Kwok and Niranjan, 1995). Numerous variations of this process have been reported, as the resulting products comprise 'green' and 'beany' flavors, which are highly appreciated by Chinese and other Asian consumers, but not preferred by the majority of consumers of the rest of the world (Debruyne, 2006; Kwok and Niranjan, 1995; Nelson et al., 1976). For example implementing a washing step of soaked beans can be advantageous to separate water soluble antinutritives (Debruyne, 2006). Some further modifications are hot water soaking (Debruyne, 2006; Wilkens et al., 1967) with minimal air contact (Debruyne, 2006), alkali soaking (Badenhop and Hackler, 1970), including a blanching step after soaking, e.g. hot blanching (Debruyne, 2006) or blanching in sodium hydrogen carbonate (Nelson et al., 1976) and acid grinding (Kon et al., 1970). All these process variations lead to flavor improvement, as they are addressed to lipoxygenase (LOX) inactivation or at least the reduction of LOX activity. LOX catalyzes the autoxidation of polyunsaturated fatty acids to hydroperoxides in presence of water and oxygen. These hydroperoxides then decompose to volatile compounds such as hexanal, 1-octen-3-one and (*E,E*)-2,4-nonadienal during processing noticeable as 'green' and 'beany' off-flavors. Autoxidation takes place instantly whenever the beans are exposed to water and oxygen (Kwok and Niranjan, 1995). Therefore, optimizations should be addressed to the reduction or blocking access to oxygen or the denaturation of LOX by thermal treatment such as toasting, as LOX is heat labile (Debruyne, 2006). Beside whole soy beans, soy flour, concentrate or isolate can be reconstituted and used as a base for a flavor improved soy-based milk alternative. The different processed soy-based milk alternatives can be refined with the addition of sugar, oil, flavorings and stabilizers. For physical stability, homogenization and preservation, heat treatment like pasteurization or ultra-high temperature heating (UHT) is followed (Mäkinen et al., 2016).

The nutritional properties of plant-based milk alternatives vary considerably among species: most of the plant-based milk alternatives cannot provide protein contents comparable to cow milk and offer only ≤ 1% protein, except for soy, which is comparable to cow milk. This may pose a safety risk especially concerning protein malnutrition of young children when replacing cow milk completely with low protein plant-based milk alternatives (Carvalho et al., 2001; Mäkinen et al., 2016). Moreover dairy products provide 30 – 40% of the dietary calcium, iodine, vitamin B_{12} and riboflavin (Black et al., 2002; Mäkinen et al., 2016). Therefore, many plant-based milk alternatives are fortified with vitamins and minerals

(vitamin B_2, B_{12}, D, E and calcium) to be competitive as alternative to cow milk (Debruyne, 2006; Mäkinen et al., 2016).

Soy-based milk alternatives are not only used as drinks, they are also a suitable basis for lactic acid fermentation, receiving e.g. yogurt-like alternatives. So far, main research on dairy alternatives has been focused on soy-based products like milk and yogurt alternatives (Ferragut et al., 2009; Li et al., 2014; Peng and Guo, 2014; Yang et al., 2012; Yang and Li, 2010). But in recent years, yogurt alternatives out of other plants are emerging: For example, Mårtensson et al. (2001) developed an oat-based yogurt alternative and characterized the products in terms of texture, syneresis, color and sensory parameters. Products showed viscosities comparable to cow milk yogurt and exhibited less syneresis. Besides, survival of probiotic lactic acid bacteria (LAB) was determined (Mårtensson et al., 2002). Further, Yaakob et al. (2012) studied the ingredient and processing parameters of a coconut-based yogurt alternative, whereas Park et al. (2005) investigated yogurt alternatives based on brown rice (Park and Oh, 2005). Among legumes, peanut-based (Diarra et al., 2005; Lee and Beuchat, 1992) and pea-based (Denkova et al., 2014) yogurt alternatives were also studied.

Lactic acid fermentation of plant-based milk alternatives has positive effects regarding digestibility, as some LAB exhibit α-galactosidase activity and can degrade antinutritives upon fermentation such as flatulence-causing oligosaccharides; shown e.g. in a soy-based yogurt alternative (Donkor et al., 2007). Moreover, beany off-flavors were considerably reduced in a peanut-based milk alternative after fermentation, as levels of hexanal, which are responsible for these off-flavors, were efficiently degraded (Lee and Beuchat, 1992). Besides, through the release of lactic acid upon fermentation the texture of the yogurt is influenced, which is more thoroughly discussed in Section 1.3.

1.2.2 Dairy alternatives based on lupin

In contrast to soy products, lupin-based dairy alternatives were first described in 1985 (Han et al., 1985). Only preliminary investigations were performed regarding the development of LBMA and their derived products such as LBYA. Analogue to soy-based milk alternatives, LBMA can be prepared according to the traditional procedure (cf. Section 1.2.1) including soaking, grinding, filtering and boiling of the whole seeds (Camacho et al. (1988); Elsamani et al. (2014); Gugger et al. (2016); Jiménez-Martínez et al. (2003); cf. Table 2). But also the manufacture of protein enriched extracts beforehand such as protein concentrates or isolates was performed (Han et al. (1985); Kuznetsova et al. (2014); Snowden et al. (2007), cf. Table 2), which can be further fortified with e.g. fats or sugars afterwards.

Table 2: Literature overview of dairy alternatives such as LBMA and LBYA.

Species/ Varieties	Raw material	Products	Source
n.m.	Protein concentrate	Lupin-based yogurt drink	Han et al. (1985)
Lupinus albus cv. Multolupa	Seeds	LBMA	Camacho et al. (1988)
Lupinus campestris L.	Seeds	LBYA	Jiménez-Martínez et al. (2003)
Lupinus angustifolius L.	Protein-enriched extract	LBMA, LBYA	Snowden et al. (2007)
Lupinus albus L.	Seeds	LBMA, lupin-based cheese alternative (rennet-induced)	Elsamani et al. (2014)
Lupinus angustifolius L.	Enzymatic hydrolyzed concentrate	As ingredient for cow milk yogurt	Kuznetsova et al. (2014)
Legumes in general (including lupin)	Seeds	Legume-based milk, yogurt and cheese alternatives	Gugger et al. (2016)

n.m.: not mentioned

Some of the developed LBMA were further subjected to lactic acid fermentation. Han et al. (1985) developed a LBMA out of a protein concentrate. LBMA was partly hydrolyzed with carbohydrate composing enzymes to increase the content of free sugars or was left untreated. Different common LAB were tested alone or in combination gaining a lupin-based yogurt drink. The stability of the dispersion was significantly higher in respective products when prepared with enzymatically untreated LBMA. But, yogurt drinks prepared out of untreated LBMA yielded a poor sensory acceptance, while prepared from enzymatically treated milk product acceptance was increased (Han et al., 1985).

Jiménez-Martínez et al. (2003) developed a milk analogue out of *Lupinus campestris* seeds by alkaline thermal treatment. The LBMA was fermented to LBYA by adding traditional yogurt starters, as well as lactose and sucrose, which makes it not consumable for vegans or lactose intolerant people. The sensory acceptance was low and products were described as ‘tasteless’ and ‘unacceptable’. The structural investigation revealed pseudoplastic behavior and the gel structure of these LBYA were slightly weaker compared to cow milk yogurt, especially with higher temperatures than 4 °C (Jiménez-Martínez et al., 2003).

Snowden et al. (2007) published a patent application for a production process of lupin-based dairy substitutes. After protein enrichment, the slurry is further processed with vegetable oil, emulsifiers, skim milk powder or whitening ingredient to receive a LBMA; so again the

product is not vegan or lactose-free. After fermentation to manufacture LBYA, data about the textural properties of the received LBYA are not given (Snowden et al., 2007).

Kuznetsova et al. (2014) produced an enzymatic hydrolyzed lupin protein concentrate, which was afterwards dispersed in water, mixed with skimmed milk and was fermented with yogurt starter cultures. In this case, the products were obviously not vegan and the lupin protein concentrate acts only as ingredient for a cow milk yogurt. Consequently, no conclusion can be drawn about the suitability of the lupin protein concentrate for LBMA and LBYA (Kuznetsova et al., 2014).

Recently, Gugger et al. (2016) released a patent application for processing various legumes, including lupins, to dairy alternatives. The process involves soaking of the beans in water and the addition of amylases, to reduce the starch content. After heating and filtering, a liquefied legume material is received, functioning as plant-based milk alternative, which can be inoculated with bacterial cultures and can be fermented to produce dairy alternatives such as legume-based yogurt and cheese (Gugger et al., 2016).

To our knowledge no LBMA and its derived products such as LBYA was manufactured out of LPI so far. Therefore, process development is needed as well as detailed description of respective products.

1.3 Texture formation during cow milk fermentation

In traditional yogurt manufacture, cow milk is fermented with both, *Streptococcus* (*S.*) *thermophilus* and *Lactobacillus* (*L.*) *delbrueckii* ssp. *bulgaricus*. The main role of those microorganisms is the acidification of the milk by producing a large amount of lactic acid from lactose thereby lowering the pH of the milk. Besides, these strains are acting in a symbiotic relationship: In the beginning *S. thermophilus* dominates the process and produces as a heterofermentative LAB organic acids such as formic and folic acid as well as carbon dioxide. Thereby, *L. delbrueckii* ssp. *bulgaricus* is stimulated in growth, which is proteolytic active and can thereby maintain essential amino acids for *S. thermophilus* (Tamime and Robinson, 2007; Zourari et al., 1992).

The formation of a gel network is mainly addressed to the alteration of casein micelles, as they amount about 80% of the proteins in cow milk (Holt, 1992; Tamime and Robinson, 2007). Casein micelles are stabilized via colloidal calcium phosphate bridges (Horne, 1999; Lucey, 2002). Upon acidification the negative net charge of the casein micelles is gradually compensated, whereby the electrostatic repulsion among casein micelles is reduced and the internal structure of the casein micelles is weakened. The colloidal calcium phosphate is

progressively depleted and solubilized, further destabilizing the casein micelle through the diffusion of κ-casein and β-casein to the serum phase (Horne, 1999). When the pH of the milk approaches the IP of casein (pH 4.6), in addition to the reduced electrostatic repulsion, attractive forces between caseins emerge such as hydrophobic and electrostatic interactions (Lee and Lucey, 2010). Finally, the caseins aggregate and form a three dimensional network, entrapping serum, fat globules and LAB in the cavities (Tamime and Robinson, 2007).

In general, yogurt shows time-dependent shear-thinning, pseudoplastic behavior of a non-*Newtonian* fluid, as yogurt becomes continuously thinner with increasing shear rate $\dot{\gamma}$. With low shear rates the shear stress τ still increases proportionally, whereas with higher shear rates the curve flattens continuously (cf. Figure 1A). At the same time, the apparent viscosity η_a decreases with increasing shear rates, which is also a time-dependent phenomenon (cf. Figure 1B) (Mezger, 2006). However, when the shear rate is reduced thereafter, yogurt cannot retain its original gel-like consistency completely. Therefore, it is often described as an ‘incomplete’ or ‘false’ thixotropic material since structural breakdown due to shear is not completely reversible when the shear stops (Lee and Lucey, 2010). The degree of thixotropy can be expressed as hysteresis loop area A_H, namely the area between the upward and downward curve (cf. Figure 1C).

The elastic and viscous properties of a gelling system can be displayed with the storage (G’) and loss modulus (G’’), respectively. Yogurt can be recognized as a weak gel, with G’ higher than G’’, but with a rather low gap between them (cf. Figure 1D, E). G’ can also be seen as a measure for gel strength. With amplitude sweeps, the viscoelastic behavior of the sample can be displayed (cf. Figure 1D): at first, with smaller strain, yogurt can regain its original structure anytime, if the strain stops. This is called the linear viscoelastic region. The end of this region is marked with the yield point τ_y. Beyond the yield point, the sample cannot maintain its structure anymore once the strain stops and the reversible viscoelastic behavior is lost. When the flow point τ_f is reached, G’ is equal to G’’ and the yogurt cannot be recognized as a gel anymore. With increasing G’’ the yogurt passes through a transition from a predominantly elastic to a viscous structure (cf. Figure 1D). Moreover, yogurt shows frequency dependency in the linear viscoelastic region, as the moduli G’ and G’’ constantly increase with increasing frequency (cf. Figure 1E). Thereby, long-term storage stability of samples can be displayed with low frequencies (Mezger, 2006).

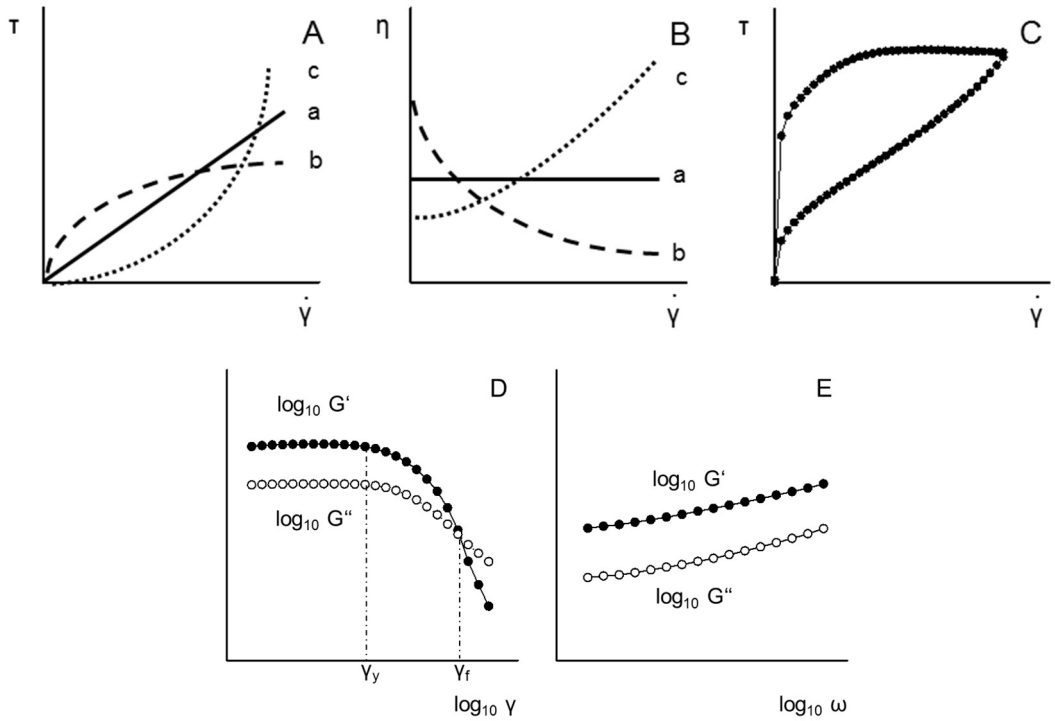


Figure 1: Flow (A) and viscosity (B) curves of ideal-viscous, *Newtonian* (a), shear-thinning, pseudoplastic, non-*Newtonian* (b) and shear-thickening, dilatant (c) samples, as well as a flow curve with hysteresis loop area (C), an amplitude sweep (D) and a frequency sweep (E) of yogurt (in accordance to and with permission of Mezger (2006)). $\dot{\gamma}$: shear rate; γ: shear strain; η_a: apparent viscosity; $\log_{10}$ G' and G'': decadic logarithm of the storage and loss modulus; $\log_{10}$ γ and ω: decadic logarithm of the shear strain and angular frequency; γ_y: yield point; γ_f: flow point.

Upon yogurt processing several defects can occur. One major defect is syneresis – the separation of serum water from the gel network, which occurs upon production or storage. Other well-known problems are associated with the viscosity and gel strength, which are mostly too weak (Lee and Lucey, 2003). These defects affect sensory properties of the products: e.g. viscosity of products is positively correlated with mouth thickness and creaminess (Folkenberg et al., 2006a). Therefore, an adequate processing is of crucial importance for the production of the characteristic yogurt texture. Factors that can cause prominent changes in yogurt texture are mainly addressed to the pretreatment of the milk, such as homogenization, heating and milk fortification e.g. addition of stabilizers, but also to the fermentation conditions (choice of starter cultures, temperature, inoculum size) and storage of the yogurt (Tamime and Robinson, 2007). These factors and the mentioned defects will be discussed more detailed in the following Sections 1.3.1 and 1.3.2.

1.3.1 Milk treatment prior to fermentation

The heating of milk is mainly addressed to preservation. Due to the inactivation of pathogenic and spoilage bacteria, as well as the inactivation of endogenous enzyme activities, the shelf life of the milk can be prolonged (Tamime and Robinson, 2007). Furthermore, stimulatory and inhibitory components can emerge upon milk heating, affecting yogurt starter cultures in their metabolism: the release of cysteine, glutathione or thioglycolate and the expulsion of oxygen upon milk heating resulted in a stimulatory effect on starter cultures, however, cysteine in excess was inhibitory (Greene and Jezbski, 1957). Potentially arising Maillard products can lead to growth retardation of LAB due to their antimicrobial potential (Lanciotti et al., 1999; Stecchini et al., 1991). Another effect of milk heating involves the physiochemical properties of the milk constituents: the thermal treatment leads to modification of the proteins and consequently affects yogurt texture, which is outlined more thoroughly in the following.

Adequate milk heating prior to fermentation can lead to increased conversion of calcium into a soluble form leading to a decreased coagulation time, as casein micelles are destabilized sooner (Lucey, 2004). Besides, whey proteins are denatured partially or completely by heating, which changes their functionality fundamentally: upon stepwise unfolding of the major whey protein β-lactoglobulin a higher amount of reactive SH-groups is exposed. Thereby β-lactoglobulin is more prone to association with κ-casein on the casein micelle surface in terms of covalent cross-linking like SS-bondings; finally β-lactoglobulin co-precipitates with caseins leading to higher output yields (Lee and Lucey, 2003, 2010).

It was found that physical characteristics of yogurt, namely susceptibility to syneresis and gel strength, are related to the microstructure and the amount of linkages within products. The network of yogurt shows a honeycomb-like and open net of aggregated casein particles and comprises interspaces of different sizes (Yang et al., 2012). This network is associated with the soft texture of milk gels and it is just dense enough to entrap water and to maintain gel stability (Folkenberg et al., 2006a). In contrast, when the network is too loose and open through a lack of colloidal linkages, e.g. by process failures, gel instability and syneresis is the consequence (Kovalenko and Briggs, 2002; Yang and Li, 2010). However, too compact and rigid networks are also not expedient. These dense and close-meshed networks have small interspaces, caused by a high number of physiochemical bonds. In consequence, they are more likely to entrap water, but this is resulting in a firm and brittle gel (Yang et al., 2012). Summed up, network density and hence capability of water-binding and gel strength is caused through physiochemical bonds and colloidal linkages, which can be achieved by protein denaturation.

Adequate homogenization prior to milk heating can enhance the effect of milk heating. Primarily, homogenization is applied to reduce the size and to stabilize the fat globules in the milk, which is indispensable for the stability of milk among storage. Further, the proteins are affected by homogenization: casein-whey protein interactions may occur, as a result of whey protein denaturation and the exposure unpaired SH-groups (Tamime and Robinson, 2007). Consequently, fat separation (creaming) during fermentation or storage is prevented, the whiteness of products is increased, the tendency for whey separation is reduced and the consistency is enhanced (Tamime and Robinson, 2007; Vedamuthu, 1991).

Dry matter fortification of milk is another approach to modify the texture of respective yogurts. Milk can be either evaporated to condense the substances, or different substances can be added, such as whey proteins, skimmed milk powder, caseins, coagulants (calcium citrate) or simply stabilizers (carboxymethyl cellulose, pectin, gelatine) (Tamime and Robinson, 2007). Several authors showed, that a higher content of dry matter strongly affected gel firmness of respective yogurts (Kristo et al., 2003; Lucey et al., 1999). In addition, Remeuf et al. (2003) studied caseinate and whey protein concentrate fortification in milk with different heat intensities. A more severe heat treatment of the whey protein concentrate enriched milk led to a denser network of respective yogurts and hence increasing yogurt viscosity, as the amount of cross-linkings was increased. In contrast for caseinate, the heating effect was negligible (Remeuf et al., 2003).

Although the gelation of globular plant-based proteins is fundamental different to cow milk proteins (cf. 1.1.4 and 1.3), the mentioned defects can also arise in plant-based yogurt alternatives (Li et al., 2014; Peng and Guo, 2014; Yang et al., 2012). So far, the influence of heat treatment on plant-based milk alternatives and their impact on respective yogurt alternatives was not studied thoroughly.

1.3.2 Fermentation of milk to yogurt and influence of storage on yogurt texture

Beside *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, nowadays also other types of strains are applied e.g. probiotic cultures such as *L. acidophilus* or *L. casei*, as well as exopolysaccharide (EPS)-producing bacteria; these strains are commonly used in the dairy industry, but their function is still not well understood. Certain strains of LAB are able to express EPS, which were recognized to affect yogurt texture regarding consistency and rheology (De Vuyst and Degeest, 1999). EPS are high molecular weight carbohydrates. Depending on their composition, EPS are divided into hetero- (HePS) and homopolysaccharides (HoPS). HoPS are composed of a single type of monosaccharide

(glucose or fructose), whereas HePS comprise repeating units of different sugar monomers, mainly D-galactose, D-glucose and L-rhamnose with different branchings (De Vuyst et al., 2001). The molecular mass of HePS range from 10^4 to 10^6 Da, while those of the HoPS can be even larger (up to ~ 2 x 10^7 Da) (Cerning, 1990). Beside to the structural differentiation, EPS can be further distinguished by their position (Hassan, 2008): if EPS are non-covalently bound onto cell surfaces after synthesis, they belong to the groups of *capsular* EPS; if they are released to the medium, they are called *free* EPS. Thereby some strains build only one form, others can build both (Mende et al., 2014). Another differentiation is addressed to the EPS phenotype: strains are referred as *ropy*, when they show strands when touched with a toothpick, or they show simply a slimy appearance (Ruas-Madiedo and de los Reyes-Gavilán, 2005). Often, *free* EPS express ropy strands (Hassan, 2008). In yogurt, especially HePS were found. Although they are only built in small amounts ranging from 50-600 mg/L, they are highly effective. Thereby, the effective concentrations are much smaller than for conventional stabilizers and they don't have to be declared, as EPS are produced *in situ* (Cerning, 1995; Mende et al., 2012a). The relationship between structure and function of EPS in yogurt is still unclear, due to a wide range of different HePS. Their functionality is based on their sugar monomers, chain length, degree of branching, molecular size, amount, charge and interaction with milk constituents (De Vuyst and Degeest, 1999). It was shown that some EPS improved the sensory appearance of products, regarding mouth feeling, creaminess, shiny surface or intense aroma (Duboc and Mollet, 2001; Folkenberg et al., 2006a; Folkenberg et al., 2006b; Marshall and Rawson, 1999). Regarding the texture of EPS-containing yogurts, some authors stated increased gel firmness (Gentès et al., 2011; Kristo et al., 2011), others found decreased gel firmness (Amatayakul et al., 2006a; Marshall and Rawson, 1999), which is due to the different nature of EPS and therefore the different interactions of the polymers with the proteins (Gentès et al., 2013; Girard and Schaffer-Lequart, 2008). In case of susceptibility to syneresis of yogurts, contradictory results were found: Several studies showed that EPS can limit syneresis, whereas some found no influence of EPS or even increased syneresis (Amatayakul et al., 2006b; Folkenberg et al., 2006a; Folkenberg et al., 2006b; Güler-Akın et al., 2009; Hassan et al., 1996).

So far, only few studies reported about the influence of the EPS-producing LAB on the texture in plant-based yogurt alternatives: Mårtensson et al. (2000) found that an EPS-producing strain of *L. delbrueckii* ssp. *bulgaricus* yielded viscosities in oat-based yogurt alternatives comparable to dairy yogurts. Li et al. (2014) described the texture-promoting ability of *L. plantarum* 70810 in soy-based yogurt alternatives.

Beside the type of strains, the fermentation conditions are also of particular importance for the texture of the respective yogurts. Usually, incubation temperatures of 40 – 45 °C are

considered for *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, as this is the optimum growth condition for this mixed culture (Tamime and Robinson, 2007). However, texture of respective yogurts can be enhanced, when the incubation temperature is lowered. It was shown, that slightly lower incubation temperatures led to firmer and more viscous gels, which were less prone to syneresis (Lucey, 2002). It is suggested that with lower incubation temperatures the gel network is less prone to structural rearrangements, causing texture-enhanced yogurt gels (Lee and Lucey, 2004; Purohit et al., 2009). Another influencing factor is the inoculum size. With higher inoculum sizes, gel firmness of yogurts was increased (Lee and Lucey, 2004). Furthermore, several authors found a relation between incubation temperature and inoculation sizes favoring a combination of lower incubation temperatures and higher inoculation sizes, as yogurt gels with higher gel firmness were observed (Kristo et al., 2003; Lee and Lucey, 2004). As described above, casein micelles are stabilized via colloidal calcium phosphate bridges, which get progressively depleted and solubilized upon acidification (Horne, 1999; Lucey, 2002). In yogurt with lower inoculation rates it was observed that the calcium phosphate was dissolved at higher a pH-value. At this higher pH, the caseins are more prone to rearrangement, causing gels with lower gel firmness (Lee and Lucey, 2004). To stop fermentation the temperature is lowered, as the metabolic activity of the strains decreases. Thereby, a distinct textural change occurs in yogurts upon storage: The level of syneresis decreases as a result of the hydration of casein micelles, whereas the gel strength increases (Lucey, 2004). Consequently, it is of high importance to store the yogurt at least overnight at chilled temperatures and avoid rough mechanical handling to prevent excessive syneresis (Donkor, 2007).

2 Objectives of the study

While a large number of research activities dealt with gel formation and gel properties of cow milk-based yogurts for many years, the insights of gelation behavior of plant-based proteins in yogurt accompanied by lactic fermentation have been scarcely investigated up to now. As plant-based milk and yogurt alternatives gain more and more importance also for European consumers, the knowledge of the structure formation within these products is a prerequisite for the development of well accepted products with similar properties to milk-based products. However, it seems that the direct transfer of the traditional yogurt production based on cow milk is unfeasible, as far as the structural composition of the globular lupin proteins vary widely to the micellar caseins of cow milk. In addition, another carbon source than lactose is usually used for the production of dairy alternatives, to provide those products also for lactose-intolerant people. Consequently, the nutrient basis for the microorganisms considerably changes, which might influence the growth behavior and the texture formation of the respective yogurts.

In this study, the impact factors for texture formation of plant-based yogurt alternatives taking lupin as example should be investigated. In particular, this work should give insights into:

(i) The screening for suitable strains for lupin protein fermentation regarding considerable acidification performance and yogurt-like aroma and texture perception.

(ii) The development of a process for LBYA production adapting traditional cow milk yogurt manufacture and thereby identifying key process parameters influencing yogurt texture.

(iii) The impact of LBMA heat treatment on the growth performance of selected strains, the *in situ* EPS-expression of those strains and the influence of EPS on texture of respective LBYA.

(iv) The impact of LBMA heat treatment on the alteration of lupin proteins in terms of the degree of denaturation, the content of SH-groups and SS-bondings, as well as particle size and molecular weight distribution.

(v) The impact of LBMA heat treatment on the growth performance of different strains and on the texture and network of respective LBYA.

The texture of a food is one of the major criteria for the acceptance. With the deeper understanding, how the texture of a plant-based yogurt alternative can be modified and optimized, the quality of these and further upcoming plant-based products can be greatly enhanced.

3 Material and Methods

3.1 Materials and chemicals

Seeds of the lupin cultivar *Lupinus angustifolius* cv. Boregine were obtained from Saatzucht Steinach (Steinach, Germany). The commercial cow milk as well as soy-based yogurt samples (*Berchtesgardener*, *Danone, Ehrmann, Weihenstephan, Alpro Soja*) were used as reference materials and were purchased from local supermarkets. All chemicals and ingredients used in this study are listed in Table 3.

Table 3: Chemicals and ingredients.

Chemical or Ingredient	Purity	Supplier
(−)-Riboflavin	98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
2-Mercapto ethanol	≥ 99.0%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
2-Propanol	p.a.	Merck KGaA, Darmstadt, Germany
5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB)	≥ 98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Acetone	p.a.	Merck KGaA, Darmstadt, Germany
Acetonitrile	HPLC grade	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Adenine	≥99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Agar	for microbiology	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Alcalase ® 2.4 L FG (endoprotease from *Bacillus licheniformis*)	–	Novozymes A/S, Bagsvaerd, Denmark
Ammonium citrate dibasic	≥ 99.0%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Ammonium sulfate	≥99.0%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Bromophenole blue	–	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Calcium chloride dihydrate	≥ 99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Casein peptone, tryptic digest	for microbiology	Merck KGaA, Darmstadt, Germany
Cobalamin (vitamin B_{12})	≥ 98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Coconut fat	food grade	Palmin, Peter Kölln, Germany
D(+)-Maltose monohydrate	≥ 95%, for biochemistry	Carl Roth GmbH & Co KG, Karlsruhe, Germany
D(+)-Sucrose	≥ 99.7%	Carl Roth GmbH & Co KG, Karlsruhe, Germany
DL-Dithiothreitol (DTT)	≥ 98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Ethanol absolute	p.a.	Merck KGaA, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	≥ 98.0%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Folic acid	≥ 97%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Formic acid	≥ 98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Galactose	> 98%	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Glucose monohydrate	food grade	Avebe, Netherlands
Glucose, anhydrous	p.a.	Carl Roth GmbH & Co KG, Karlsruhe, Germany

Glycerol, anhydrous	for synthesis	Merck KGaA, Darmstadt, Germany
Glycine	≥ 99%, for electrophoresis	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Guanidine hydrochloride	≥ 99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Guanine	98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
HPLC water	for chromatography	Merck KGaA, Darmstadt, Germany
Hydrochloric acid	37%, fuming	Merck KGaA, Darmstadt, Germany
L-Cysteine	≥ 97%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
L-Cysteine hydrochloride	≥ 98%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Lectin soybean agglutinin from Glycine max (soybean), Alexa Fluor® 488 Conjugate	–	Thermo Fisher Scientific, Waltham, USA
Magnesium sulfate heptahydrate	≥ 99%, p.a.	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Manganese sulfate monohydrate	≥ 99%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Meat extract	for microbiology	Merck KGaA, Darmstadt, Germany
n-Hexane	p.a.	Th. Geyer GmbH & Co. KG, Renningen, Germany
Nile red	for microscopy	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Potassium dihydrogen phosphate	> 99%	Merck KGaA, Darmstadt, Germany
Precision Plus Protein™ Unstained Protein Standards	–	Bio-Rad Laboratories Ltd., Hempstead, UK
Pronase E from *Streptomyces griseus* min. 6.0 DMC-U/mg	–	SERVA Electrophoresis GmbH, Heidelberg, Germany
Rhodamine B	≥ 95%, HPLC grade	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Ringer solution	–	Thermo Fisher Scientific, Waltham, USA
Skim milk powder	–	Merck KGaA, Darmstadt, Germany
Sodium acetate, anhydrous	≥ 99.0%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Sodium azide	≥ 99.5%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Sodium dihydrogen phosphate dehydrate	> 99%	Merck KGaA, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	for electrophoresis	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Sodium hydroxide (NaOH)	5 mol/l	Merck KGaA, Darmstadt, Germany
Sodium hydroxide pellets	40%	Merck KGaA, Darmstadt, Germany
Sodium L-lactate	≥ 99.0%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Trichloroacetic acid (TCA)	99.0%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Tris(hydroxymethyl)-aminomethane (Tris)	for 2-D electrophoresis	GE Healthcare Bio-Sciences, Uppsala, Sweden
Tryptone soya broth	–	Thermo Fisher Scientific, Waltham, USA
Tween 80	for synthesis	Merck KGaA, Darmstadt, Germany
Uracil	≥99.0%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Urea	–	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Xanthine	≥ 99.5%	Sigma-Aldrich Inc., St. Louis, Missouri, USA
Yeast extract	for microbiology	Merck KGaA , Darmstadt, Germany
α-Cyano-4-hydroxy-cinnamic acid	–	Bruker Daltonics, Bremen, Germany

3.2 Strains, media and cultivation

For the lupin protein fermentation, 35 strains were screened regarding their suitability for the production of lupin-based products (Table 4). Most of the strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Others were provided by *Lehrstuhl für Brau- und Getränketechnologie* (BGT, Technische Universität München, Germany) or taken from the collection of the *Lehrstuhl für Technische Mikrobiologie Weihenstephan* (TMW, Technische Universität, Germany). Strains were propagated under optimal growth conditions on their recommended media according to Table 4. Storage of stock cultures was maintained at −18 °C in 10% (w/v) sterile skim milk powder as cryo cultures for long term and for short term on angular tubes at 4 °C. For anaerobic incubations, anaerobic jars with AnaeroGen® sachets were used and controlled by an indicator strip (Thermo Fisher Scientific, Waltham, USA).

The composition of de Man, Rogosa and Sharpe broth (MRS), which was used for the cultivation of most lactobacilli, is listed in Table 5. The preparation of the broth was performed according to the information supplied by the manufacturer. The components were weighed in, were filled up to 1000 ml with distilled water and were autoclaved at 118 °C for 15 min after solving. The cultivation of some strains required addition of 0.05% cysteine hydrochloride, which was added before autoclaving (MRS+CYS).

Other bacterial isolates were grown in Trypticase Soy Yeast Extract broth (TSYE) (Table 6) and were autoclaved at 121 °C for 15 min. Solely *Propionibacterium freudenreichii* ssp. *shermanii* was propagated in Propionibacterium broth (PR, Table 7), which was autoclaved at 121 °C for 15 min. Yeasts were cultivated in Yeast Glucose Chloramphenicol broth (YGC, Table 8), which was autoclaved at the same conditions.

For the preparation of agar plates, 15 g/l agar was supplemented to any of the mentioned media before autoclaving. For the determination of total cell counts in LPI suspensions, LBMA or LBYA (cf. 3.4.2, 3.7.1) Plate Count Agar (PCA) was used (Table 9).

Table 4: Growth conditions of selected strains.

Strain	Origin	Growth conditions
L. delbrueckii ssp. *bulgaricus*	DSM 20080	TSYE, 37 °C, anaerobic
S. salivarius ssp. *thermophilus*	DSM 20259	TSYE, 37 °C, anaerobic
L. acidophilus	DSM 20079	MRS+CYS, 37 °C, anaerobic
L. casei	DSM 20011	MRS, 30 °C, aerobic
L. helveticus	DSM 20057	MRS, 37 °C, anaerobic
L. perolens	DSM 12744	MRS, 28 °C, aerobic
Lc. lactis ssp. *lactis*	DSM 20384	TSYE, 30 °C, aerobic
Lc. lactis ssp. *cremoris*	DSM 20069	TSYE, 30 °C, aerobic
Leuconostoc mesenteroides ssp. *cremoris*	DSM 20200	MRS, 30 °C, aerobic
Brevibacterium linens	DSM 1233	MRS, 30 °C, aerobic
Propionibacterium freudenreichii ssp. *shermanii*	DSM 4902	PR, 30 °C, anaerobic
Geotrichum candidum	DSM 10452	YGC, 25 °C, aerobic
Bifidobacterium bifidum	DSM 20239	MRS+CYS, 37 °C, anaerobic
Bifidobacterium animalis ssp. *lactis*	DSM 10140	MRS+CYS, 37 °C, anaerobic
L. plantarum	TMW 1.460	MRS, 30 °C, anaerobic
L. plantarum	TMW 1.1468	MRS, 30 °C, anaerobic
L. fermentum	DSM 20391	MRS, 37 °C, aerobic
L. pontis	TMW 1.1086	MRS, 37 °C, anaerobic
L. sanfranciscensis	DSM 20451	MRS, 30, aerobic
Weissella cibaria	TMW 2.1333	MRS, 30 °C, anaerobic
L. brevis	TMW 1.1326	MRS, 30 °C, anaerobic
L. brevis	BGT L150	MRS, 30 °C, anaerobic
L. amylolyticus	BGT TL3	MRS, 47 °C, aerobic
L. amylolyticus	BGT TL5	MRS, 47 °C, aerobic
*L. species**	BGT TL11	MRS, 47 °C, aerobic
*L. species**	BGT TL13	MRS, 47 °C, aerobic
L. rossiae	BGT L1202	MRS, 30 °C, aerobic
P. pentosaceus	BGT B34	MRS, 30 °C, aerobic
P. pentosaceus	DSM 20336	MRS, 30 °C, aerobic
L. curvatus	TMW 1.624	MRS, 30 °C, anaerobic
L. reuteri	DSM 20016	MRS, 37 °C, aerobic
L. buchneri	DSM 20057	MRS, 37 °C, aerobic
L. gasseri	DSM 20243	MRS+CYS, 37 °C, anaerobic
Saccharomyces cerevisiae	TMW 3.210	YGC, 25 °C, anaerobic
Kluyveromyces fragilis	TMW 3.188	YGC, 25 °C, anaerobic

L.: Lactobacillus, P.: Pediococcus, L.: Lactococcus
* Strains not yet identified

Table 5: Composition of MRS broth, pH 6.2 – 6.5 in g per 1000 ml distilled water.

Component	Amount [g]
Casein peptone, tryptic digest	10.00
Meat extract	10.00
Yeast extract	5.00
Glucose	20.00
Tween 80	1.00
Potassium dihydrogen phosphate	2.00
Sodium acetate	5.00
Ammonium citrate	2.00
Magnesium sulfate heptahydrate	0.20
Manganese sulfate monohydrate	0.05

Table 6: Composition of TSYE broth, pH 7.0 – 7.2 in g per 1000 ml distilled water.

Component	Amount [g]
Tryptone soya broth	30.00
Yeast extract	3.00

Table 7: Composition of PR broth, pH 7.0 – 7.2 in g per 1000 ml distilled water.

Component	Amount [g]
Casein peptone, tryptic digest	10.00
Yeast extract	5.00
Sodium lactate	10.00

Table 8: Composition of YGC broth, pH 6.6 ± 0.2 in g per 1000 ml distilled water.

Component	Amount [g]
Yeast extract	5.00
Glucose	20.00
Chloramphenicol	0.10

Table 9: Composition of PCA, pH 7.0 in g per 1000 ml distilled water.

Component	Amount [g]
Tryptone	5.00
Yeast extract	2.50
Glucose	1.00
Agar	15.00

3.3 Preparation of a lupin protein isolate

Lupin protein isolate manufacture was performed at a 2000 L pilot-scale according to the process by Wäsche et al. (2001). Therefore, the seeds of *L. angustifolius* cv. Boregine were dehulled with an underflow peeler (Streckel & Schrader GmbH, Hamburg, Germany) and were classified in an air-lift system (Alpine Hosokawa AG, Augsburg, Germany) (cf. Figure 2). Afterwards, the dehulled kernels were flaked using a roller mill (Streckel & Schrader) and were de-oiled with n-hexane. These flakes were then subjected to an acidic pre-extraction step at pH 4.5 and to a neutral extraction step at pH 7.2 and were separated with a decanter (GEA Westfalia Separator Group GmbH, Oelde, Germany). Then, the proteins were precipitated isoelectrically at pH 4.5, separated from the clarified extract by a separator (GEA Westfalia Separator Group) and were neutralized (pH 6.8) using 3 M NaOH. The neutralized protein isolate was pasteurized at 70 °C for 3 min and spray-dried (Anhydro Holding A/S, Soeborg, Denmark). The dry matter of the received isolates was determined according to AOAC (2005a). Therefore, samples were dried to weight constancy at 105 °C in a thermo-gravimetrical system (TGA 601, Leco Corporation, St. Joseph, USA). 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) using a nitrogen analyzer FP 528 (Leco Corporation).

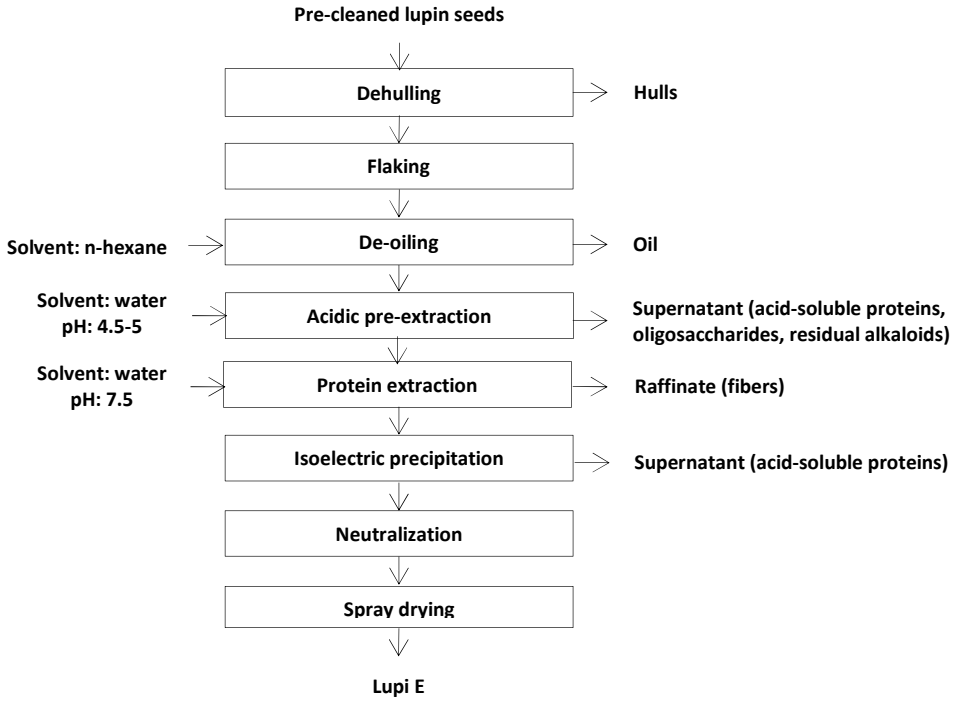


Figure 2: Process scheme for the preparation of lupin protein isolates (in accordance to Eisner et al. (2016)).

3.4 Preliminary fermentation experiments with lupin protein isolate

3.4.1 Propagation of the strains and determination of total cell counts

For the propagation of the different strains listed in Table 4, colonies of the angular agar tubes were picked and 200 ml of the recommended broth was inoculated and propagated for 24 – 48 h to prepare a preculture. After propagation, appropriate dilutions of the preculture were prepared with sterile ringer solution. Total cell counts of these bacterial dilutions were determined using a spiral plating method by means of an Eddy-Jet spiral plater (IUL Instruments, Königswinter, Germany). Therefore, agar plates with the recommended medium were prepared, on which the bacterial dilutions were spread. The plates were incubated at strain specific conditions and the number of colony-forming units per ml (CFU/ml) was determined with the Countermat Flash and Grow Software (IUL Instruments). After cell enumeration, a calculated aliquot of the preculture was centrifuged with 3330 *g* at 20 °C for 10 min (Universal 320 R, Hettich Lab Technology, Tuttlingen, Germany), resuspended in 1 ml sterile distilled water and used for inoculation.

For cell enumeration of the endogenous biota in LPI suspensions or LBMA, PCA medium was used for the preparation of agar plates. For the determination of total cell counts of inoculated LBMA or LBYA, the medium of the inoculated strain was used.

3.4.2 Fermentation of lupin protein isolate suspensions

Upon a preliminary screening 35 different strains which are listed in Table 4 were evaluated regarding their acidification rate, their yogurt-like texture and odor on LPI. Therefore, suspensions of 5% (w/v) LPI and 2% (w/v) glucose were prepared and selected experiments were also conducted with 2% (w/v) sucrose. Afterwards, samples were heat treated at 65 °C for 30 min in a water bath. Two technical replicates of these suspensions (50 ml) were inoculated with each bacterial strain from precultures with 7.0 ± 0.1 $\log_{10}$ CFU/ml (cf. Section 3.4.1). The pH-value of the inoculated LPI suspensions with the different sugars was measured and samples were then incubated under optimal conditions of the strains. Additionally a suspension without bacteria was cultivated concomitantly as blank sample. After 48 h, fermentation was stopped, the pH-value was measured and samples were examined with simple descriptive tests according to their yogurt-like odor with '–' (not yogurt-like), '+' (adequate yogurt-like) and '++' (strong yogurt-like). Besides, panelists established three categories for the texture, which are described in detail in Section 4.1.1 and evaluated the samples according to these categories.

3.5 Screening of exopolysaccharide-producing strains in MRS media

3.5.1 Determination of the exopolysaccharide phenotype

The strains listed in Table 4 were evaluated regarding their ability to produce EPS. Therefore, agar plates were prepared out of the recommended medium for each strain as described in 3.2, fortified with 80 g/l of glucose, maltose or sucrose instead of 20 g/l (van Geel-Schutten et al., 1998). Bacterial cultures were transferred on the prepared agar plates with an inoculation loop and were incubated under strain specific conditions for 24 h. Strains were referred as EPS-producing strains, when they showed ropy strands by touching with a toothpick or when they had a slimy appearance with '++' (strong EPS production), '+' (adequate EPS production) and '–' (no EPS production).

3.5.2 Propagation of exopolysaccharide-producing lactic acid bacteria

For the isolation and quantification of *free* and *capsular* EPS produced by *L. plantarum* TMW 1.460 and 1.1468 (cf. Section 3.5.3), a suitable growth medium had to be found. Therefore, the strains were propagated in modified MRS broth in comparison to common MRS broth (mMRS, cf. Table 10). This mMRS medium is a MRS broth without meat and yeast extract. To stimulate cell growth of the LAB, mMRS was spiked with a number of supplements such as nucleobases (adenine, guanine, xanthine and uracil), vitamins (folic acid, riboflavin, cobalamin), mineral salts (sodium dihydrogen phosphate, calcium chloride dihydrate) and cysteine in different combinations (Mende et al., 2012b). Glucose was autoclaved separately and vitamins were sterile filtered and were added after autoclaving. To monitor microbial growth of LAB, mMRS with different supplements were combined with different inoculum sizes of LAB (4.0, 5.0, 6.0, 7.0 or 8.0 ± 0.1 $\log_{10}$ CFU/ml) in 96 well microtiter plates (Greiner Bio-One International GmbH, Kremsmünster, Austria). Growth kinetics were recorded spectrophotometrically at 595 nm after agitating for 10 s in linear mode and 10 s in orbital mode for 40 h (Tecan Group Ltd., Männedorf, Switzerland). Tests were performed with three replicates for each combination and analyzed by i-control 1.10 software (Tecan Group). As blanks, sterile broth without inoculum was measured in each assay.

Table 10: Composition of mMRS medium, pH 6.2 – 6.5 per 1000 ml distilled water.

Component	Supplement	Amount	Unit
Casein peptone, tryptic digest		10.00	g
Glucose		20.00	g
Tween 80		1.00	g
Potassium dihydrogen phosphate		2.00	g
Sodium acetate		5.00	g
Ammonium citrate		2.00	g
Magnesium sulfate heptahydrate		0.20	g
Manganese sulfate monohydrate		0.05	g
Folic acid	Vitamins	0.06	mg
Riboflavin		0.40	mg
Cobalamin		0.02	mg
Adenine	Nucleobases	50	mg
Guanine		50	mg
Xanthine		50	mg
Uracil		50	mg
Sodium dihydrogen phosphate	Salts	0.80	g
Calcium chloride dihydrate		0.36	g
Cysteine	Cysteine	0.50	g

3.5.3 Isolation and quantification of *free* and *capsular* exopolysaccharides

For the isolation and quantification of *free* and *capsular* EPS, *L. plantarum* TMW 1.460 and TMW 1.1468 were propagated in a 5 L BIOSTAT® B fermenter (Sartorius, Göttingen, Germany) with two biological replicates. The fermentation was carried out with supplemented mMRS media that caused the highest cell density in the pretests. The decline of the pH was registered with BIOPAT® MFCS/DA software (Sartorius). Fermentation was stopped by cooling shortly before the end of the exponential growth phase was reached. Then, isolation of *free* and *capsular* EPS was performed according to Mende et al. (2013a) with some modifications (Figure 3). After neutralization of the supplemented mMRS medium with 10% (w/w) NaOH to pH 6.0, a centrifugation step followed by 17000 *g* at 4°C for 20 min (6K16, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The *free* EPS remained in the supernatant and the *capsular* EPS – bound to the cells – remained in the pellet. To detach them, cells were resuspended in distilled water and the solution was heated at 90 °C for 30 min. After cooling to room temperature, proteins were precipitated with an aqueous TCA solution 20% (w/w) in a ratio of 1:11 (v/v). This precipitation step was also performed with the supernatant.

The precipitated proteins were separated by centrifugation (17000 *g*, 4°C, 20 min with 6K16 Sigma Laborzentrifugen), the supernatant was then neutralized again with 10% (w/w) NaOH to pH 7.0. EPS were precipitated by adding two volumes of cold ethanol absolute to the total volume of the collected supernatant (ratio: 3:1 (v/v)). After storage at 4 °C for 24 h, EPS was harvested by centrifugation. The precipitate was redissolved in distilled water and dialyzed in tubes with a molecular mass cut-off of 3500 Da (Serva Electrophoresis, Heidelberg, Germany) at 4 °C for two days against distilled water with water exchange three times per day. For quantification, EPS was lyophilized and weighed.

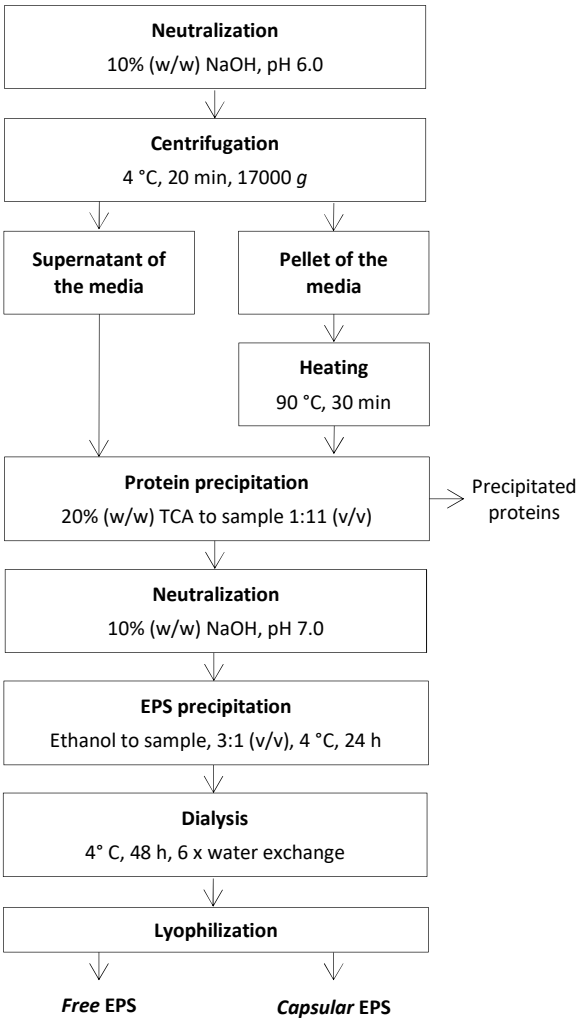


Figure 3: Isolation of *free* and *capsular* EPS produced of *L. plantarum* TMW 1.460 and 1.1468 in mMRS broth according to Mende et al. (2013a) with slight modifications.

3.6 Production of dairy alternatives

3.6.1 Production of lupin-based milk alternatives

Production of LBMA was performed according to Figure 4 in small scale (max. 800 ml, laboratory reactor, Janke & Kunkel IKA Labortechnik, Staufen, Germany) and selected experiments were conducted on large scale (max. 20 l, laboratory processing machine Type OS 362, Esco-Labor, Riehen, Switzerland). Therefore, a LPI solution (2.0% (w/v) with pH 7.0 was prepared using a stirring and an ultraturrax device (Janke & Kunkel IKA Labortechnik) for 2 min with 70 and 4500 rpm, respectively. The pH of 7.0 was maintained throughout the process. The solution was heated to 50 °C and dextrose monohydrate (4.0% (w/v)) was added. The solution was further stirred for 10 min with 70 rpm. Then, coconut fat (4.0% (w/v))

was emulsified with the suspension for 10 min using a stirrer and ultraturrax until further processing. To further reduce particle sizes, the emulsion was homogenized with a two-stage high pressure homogenizer at 250 bar and 50 bar at 50 °C (APV-2000, SPX FLOW Inc., Charlotte, USA). Then the emulsion was subjected to different heat treatments, namely pasteurization at 80 °C for 60 s and UHT treatment at 140 °C for 10 s with a tubular heat exchanger (HT 220 HTST/UHT System, Omwe, Schalkwijk, Netherlands) followed by storage at 1 °C. Cell enumeration of different LBMA was performed as described in Section 3.4.1.

3.6.2 Production of lupin-based yogurt alternatives

For the production of LBYA (cf. Figure 5), test strains were propagated in their respective media (cf. Table 4) and total cell counts were determined as described above using their specific media (cf. Section 3.2). Defined aliquots of culture broth were centrifuged for the inoculation of LBMA in order to obtain initial cell counts of 7.0 to 8.0 ± 0.1 $\log_{10}$ CFU/ml in LBMA. After resuspension of cell pellets in sterile distilled water, a second centrifugation step was conducted prior to LBMA inoculation.

For the production of set-type LBYA, aliquots of LBMA were inoculated and were filled in appropriate glass containers (mostly cylindrical glass containers, diameter 54 mm, height 70 mm, J. Weck, Wehr-Öflingen, Germany) and were fermented motionless at the recommended temperature. After a pH of 4.5 ± 0.1 was reached, LBYA were quickly cooled to 1 °C to stop fermentation and a storage at 1 °C followed. For the preparation of stirred LBYA, LBMA was inoculated and was incubated as described above for the set-type LBYA, but in one batch without filling aliquots into glass containers. When a pH of 4.5 ± 0.1 was reached, LBYA was cooled at 1 °C for 1 h to reach temperatures of 20 °C under gently stirring. Finally, LBYA were filled in appropriate containers followed by storage at 1 °C for 24 h.

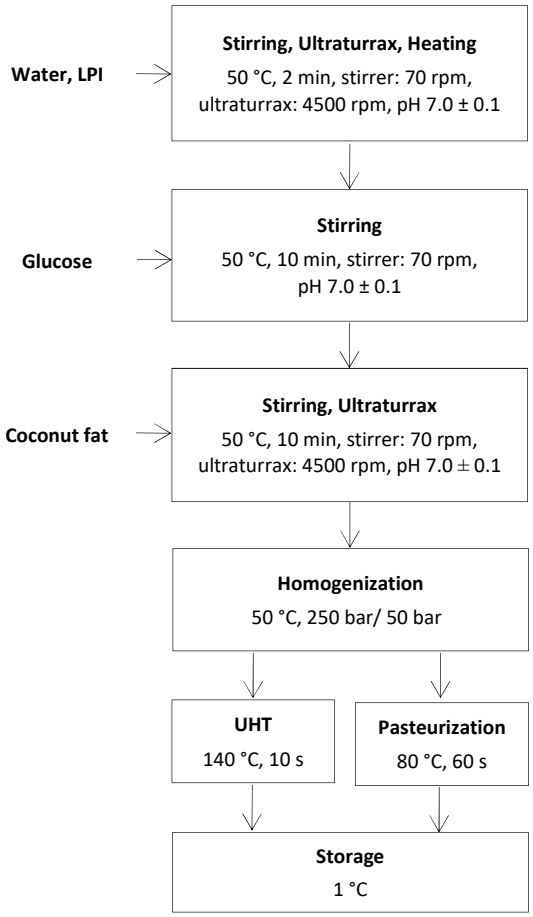


Figure 4: Process scheme for the preparation of LBMA.

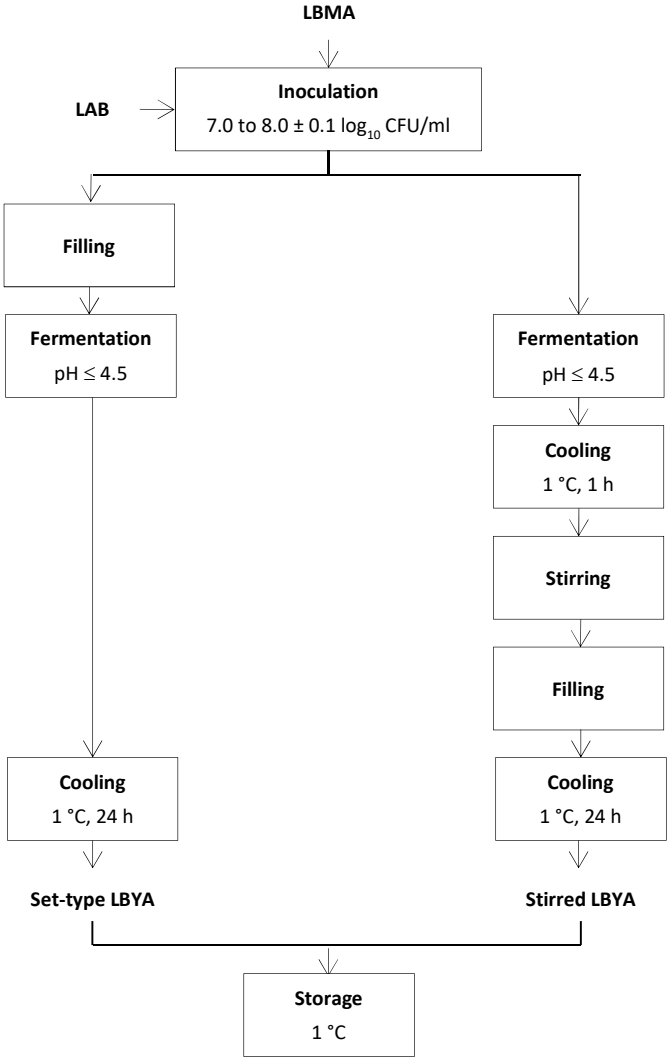


Figure 5: Process scheme for the preparation of set-type and stirred LBYA.

3.7 Growth pattern of lactic acid bacteria and exopolysaccharide production in lupin-based milk alternatives

3.7.1 Acidification rates, cell counts and back-slopping

During fermentation the decline of the pH of LBMA was monitored online with a BIOSTAT® B fermenter (Sartorius) and was registered with BIOPAT® MFCS/DA software (Sartorius). Therefore, 500 ml aliquots of LBMA in a threefold repetition were inoculated with 8.0 ± 0.1 $\log_{10}$ CFU/ml and were fermented until a pH of 4.5 was reached.

Colony-forming units were determined with appropriate diluted suspensions of the UHT and pasteurized LBMA samples by the pour-plating method (cf. 3.4.2) using plate count agar (cf. 3.2). Additionally, samples of the inoculated LBMA (t = 0 h), fermented LBMA after 10 h and

final LBYA with pH = 4.5 were spread onto MRS agar and total cell counts were determined with three technical replicates. Besides, *L. plantarum* TMW 1.460 was additionally evaluated on unheated LBMA.

Moreover, back-slopping experiments were performed with slow-growing *L. brevis* BGT L150 in pasteurized LBMA. Therefore, pasteurized LBMA was fermented with *L. brevis* and the decline of the pH was monitored as described above with BIOSTAT® B fermenter and BIOPAT® MFCS/DA software until pH 4.5 was reached. Then, fresh pasteurized LBMA was inoculated with 1% (w/v) of the recently fermented LBMA and was again fermented until pH 4.5 was reached (n = 3, first back-slopping). Back-slopping was then repeated a second time (n = 3, second back-slopping).

3.7.2 Verification of fermentation strains

The assertiveness of the inoculated microorganisms towards endogenous microbiota was verified by measurements of the colony morphology and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) according to Kern et al. (2013). First, single colonies of fresh MRS agar plates were picked and were smeared on a stainless steel target. Spots were 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 (Bremen, Germany) was applied. Targets were placed in a Microflex LT MALDI-TOF MS (Bruker Daltonics) equipped with a nitrogen laser (λ = 337 nm, Bruker Daltonics) operating in linear positive mode. Mass spectra of 240 laser shots for each sample were recorded in manual and automatic mode and were used for analysis with MALDI Biotyper 3.0 Software (Bruker Daltonics).

3.7.3 Exopolysaccharide isolation, purification and quantification

The isolation of EPS out of LBYA was performed as described for cow milk yogurt by Amatayakul et al. (2006b) with slight modifications (cf. Figure 6A). Each LBYA sample (threefold replicate) was diluted with distilled water (ratio 1:1, v/v) and proteins were precipitated by adding 20% (w/w) TCA to the diluted LBYA (ratio 1:13, v/v). After stirring for 5 min, precipitated proteins were separated by centrifugation at 3330 *g* at 4 °C for 30 min (Hettich Lab Technology). The supernatant was adjusted to a pH of 6.8 with 40% (w/w) NaOH. A second precipitation step followed by heating at 100 °C for 30 min and precipitated proteins were separated by centrifugation. EPS precipitation, dialysis and EPS quantification

was performed as described before (cf. Section 3.5.3). As reference, EPS was also extracted out of pasteurized and UHT-heated non-inoculated LBMA.

For optimization of EPS extraction, EPS of selected samples were extracted with another solvent, namely 2-propanol. Besides, another EPS isolation technique was evaluated (cf. Figure 6) according to Folkenberg et al. (2006a) with slight modifications. Therefore, samples were diluted with distilled water as described above and were neutralized. Then, the samples were incubated with Pronase E (500 mg/l LBYA), a mixture of endo- and exoprotease activities from *Streptomyces griseus* or Alcalase ® 2.4 L FG (500 mg/l LBYA), an endoprotease from *Bacillus licheniformis*, at 30 °C for 24 h. Additional, sodium azide (1000 mg/l LBYA) was added to restrict bacterial growth. The enzymatic reaction was stopped by incubation at 100 °C for 30 min and hereafter the samples were centrifuged as described above to remove cells and proteins. EPS was precipitated, dialyzed and lyophilized as already described.

3.7.4 Chemical characterization of extracted exopolysaccharides

Dry matter was determined according to AOAC methods (AOAC, 2005a). The protein concentration of the samples was analyzed by the calculation of the nitrogen content (N x 6.25) based on the Dumas method (AOAC, 2005b). Detection of monosaccharides was kindly carried out by Dr. Broder Rühmann from Chair of Chemistry of Biogenic Resources, Technical University Munich using High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS). Procedure of monosaccharide detection was performed according to Rühmann et al. (2014).

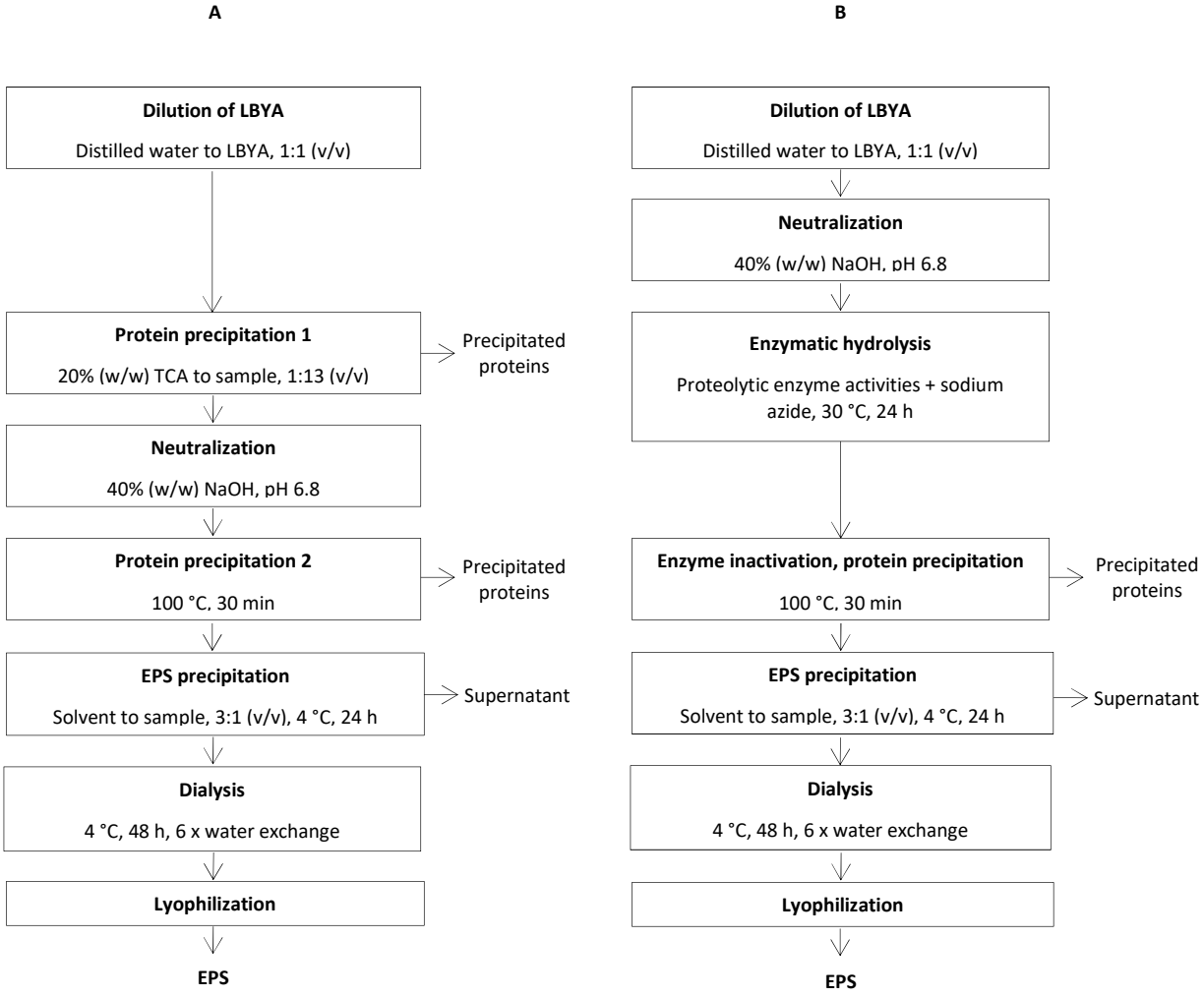


Figure 6: Isolation of EPS out of LBYA with different isolation techniques: Separation of proteins by TCA and heat (A) or by enzymatic hydrolysis (B) according to Amatayakul et al. (2006b); Folkenberg et al. (2006a).

3.8 Analytical characterization of heat-treated lupin-based milk alternatives

To monitor changes throughout the production process of LBMA (cf. Figure 4), five different samples were drawn upon manufacture: LPI solution (2% (w/v)); LBMA prior to homogenization; LBMA after homogenization: unheated, pasteurized and UHT-heated. These aliquots were analyzed with differential scanning calorimetry (DSC), content of free SH-groups, total SH-groups and SS-bondings was determined, as well as particle size distribution and sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE). Two independent measurements were performed with triplicate repetition.

3.8.1 Differential scanning calorimetry measurements

The thermal denaturation of lupin proteins in LPI (2% (w/v)) and LBMA was examined using DSC Q 2000 system (TA Instruments, New Castle, USA). An aliquot of 100 ml of the samples were gently evaporated under vacuum to 20 ml by means of a rotary evaporator (Rotavapor R-215, BÜCHI Labortechnik GmbH, Essen, Germany) to obtain a higher protein concentration. About 15 mg of concentrated samples were weighed into aluminum pans and were sealed hermetically. An empty DSC pan was taken as reference. Samples were heated with a heating rate of 2 °C/min from 30 °C to 130 °C. Peak denaturation temperature (T_d), onset temperature (T_{onset}) and enthalpy of denaturation (ΔH) for respective endothermic peaks were calculated by TA Universal Analysis software (TA Instruments).

3.8.2 Sulfhydryl-disulfide transitions

The content of free SH-groups (Koka et al., 1968), of total SH-groups (free and masked groups) (Beveridge et al., 1974) and total SS-bondings (Beveridge et al., 1974; Huang et al., 2006) was determined with Ellmans reagent (5,5′-dithiobis(2-nitrobenzoic acid)), DTNB) based on the general procedure of Ellman (1959).

For free SH-group determination, 5 ml of each sample was purged with nitrogen for 10 s in the test tubes. Then, 0.1 ml DTNB solution (39.37 mg DTNB in 10 ml 0.1 M phosphate buffer pH 7.0) was added. After the yellow color was developed at room temperature for 3 min, 1 ml 0.1 M phosphate buffer (pH 8.0) and 4 ml distilled water was added and mixed carefully. For clarification, the mixture was treated with 2 g ammonium sulfate and was centrifuged at 4440 *g* at 20 °C for 10 min (Mini Spin, Eppendorf AG, Hamburg, Germany). The supernatant was sterile filtered with a 0.45 µm filter (Chromafil® O-45/15 MS Macherey-Nagel GmbH & Co. KG, Düren, Germany) and the absorbance of the filtrate was read at a wavelength of 412 nm with a spectrophotometer (Specord® 210 Plus, Analytik Jena AG, Jena, Germany) versus reagent blanks, treated the same way. The concentration of free SH-groups was calculated based on the following equation 1 with a dilution factor of 2.02:

$$\mathrm{c\ (SH)}\ \left[\frac{\mu\mathrm{mol}}{\mathrm{g}}\right] = \frac{\Delta A_{412}}{\varepsilon * z} \times \frac{D}{C} \times 1000 \qquad (1)$$

with ΔA_{412} = absorbance at 412 nm; ε = extinction coefficient of 9500 l mol^{-1} cm^{-1} (Koka et al., 1968); z = path length of 1 cm; D = the dilution factor; C = protein concentration in g ml^{-1}.

For total SH-group determination, 1 ml of each sample was mixed with 5 ml 8 M urea in Tris-glycine buffer (10.4 g Tris, 6.9 g glycine, 1.2 g EDTA per l) and 0.02 ml DTNB-solution (2 ml DTNB in Tris-glycine buffer). After 5 min of color development, samples were centrifuged at 4440 *g* for 10 min (Mini Spin). Again, the supernatant was sterile filtrated with a 0.45 µm filter and the absorbance of the filtrate was read at a wavelength of 412 nm versus reagent blank. The concentration of total SH-groups was calculated based on equation (1) with ε = 13600 l mol^{-1} cm^{-1} (Ellman, 1959) and D = 6.04.

For the determination of SS-content, 1 ml of each sample was mixed with 0.05 ml of 2-mercaptoethanol and 4 ml urea-guanidine hydrochloride (8 M urea and 5 M guanidine hydrochloride) in 0.1 M phosphate buffer pH 8.0, 1 mM EDTA and 1% (w/v) SDS and were incubated for 1 h at 25 °C. Then, 10 ml of 12% (w/v) TCA were added and were incubated for an additional hour at 25 °C. After incubation, samples were centrifuged at 4440 *g* for 10 min (Mini Spin). The precipitate was resuspended twice in 5 ml of 12% (w/v) TCA and was centrifuged to remove 2-mercaptoethanol. The washed precipitate was dissolved thoroughly in 10 ml of 0.1 M phosphate buffer (pH 8.0), 1 mM EDTA and 1% (w/v) SDS. For color development 0.08 ml DTNB solution was added after 5 min and absorbance was measured at 412 nm versus reagent blank. Prior to measurement, the samples were sterile filtered as described above. The concentration of total SH and SS-groups was calculated based on equation (1) with ε = 13600 l mol^{-1} cm^{-1} (Ellman, 1959) and D = 10.08. Finally, the SS-content was calculated with equation 2:

$$\mathrm{c\ (SS)}\ \left[\frac{\mu\mathrm{mol}}{\mathrm{g}}\right] = \mathrm{c\ (total\ SH + SS)} - \mathrm{c\ (total\ SH)} \qquad (2)$$

The content of free and total SH-groups as well of the SS-content was expressed as a percentage ascribing the SS-content of UHT-heated LBMA to 100%.

3.8.3 Molecular weight distribution

The molecular weight distribution was determined based on the general procedure of Laemmli (1970) modified by Meinlschmidt et al. (2015) using SDS-PAGE under reducing and non-reducing conditions. For reducing conditions, the samples were treated with Tris-HCl treatment buffer (0.125 mol l^{-1} Tris-HCl, 4% SDS, 20% (v/v) glycerol, 0.2 mol l^{-1} DTT, 0.02% bromophenol blue, pH 6.8), boiled for 3 min to cleave covalent bonds and centrifuged at 9840 *g* for 4 min (Mini Spin, Eppendorf AG, Hamburg, Germany). For non-reducing

conditions, samples were treated the same way, but were suspended in 1x Tris-HCl treatment buffer without DTT and were not boiled. The electrophoresis was performed in a Midi Criterion™ Cell on 4 – 20% midi Criterion™ TGX Stain-Free™ precast gels (Bio-Rad Laboratories Ltd., Hempstead, UK). A molecular weight marker (10 – 250 kDa, Precision Plus Protein™ Unstained Protein Standard) was additionally loaded onto the gel. Electrophoresis conditions were 200 V, 60 mA, 100 W at room temperature and protein visualization was performed by Criterion Stain- Free Gel Doc™ EZ Imager (Bio-Rad).

3.8.4 Particle size distribution

The particle size distribution of LPI and different LBMA samples was determined using Mastersizer S Long bench model MSS software version 2.19, equipped with the small sample dispersion unit MS 1 (volume max. 150 ml), the 300 mm RF lens and with a He-Ne laser (λ = 632.8 nm) (Malvern Instruments Ltd., Worcestershire, UK). During the measurement the samples were diluted with HPLC water and were circulated through the detector. To arrange the sample concentration, the obscuration rate, which denotes the amount of laser light that has been lost by passing through the sample, was adjusted to 14 ± 1%. For the calculation of the droplet size, a polydisperse distribution was chosen as analysis model and Mie Theory with the optical density for the wet phase, 1.33, and for the disperse part, 1.46, were set (Software Model: 3NHD). The measurement was started after 2–3 min of dispersion time. The mean values of the droplet sizes were calculated as mean of three samples which were measured twice. The measurement was performed the day after production of the LBMA.

3.9 Physical characterization of lupin-based yogurt alternatives

For physical characterization of products, set-type LBYA was prepared with the general procedure unless stated otherwise (cf. Section 3.6). Inoculated LBMA was filled in different containers in relation to the different measurements. For rheological measurements, Texture Profile Analysis and syneresis performed with the siphon method, cylindrical glass containers (J. Weck) were filled with 30 ml inoculated LBMA and were fermented as described above (fivefold replication). For syneresis measured with centrifugation method, 30 ml of inoculated LBMA was filled in centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany), as well with five technical replicates.

3.9.1 Rheological measurements

LBYA exhibiting 4 °C were gently stirred for 10 s prior to measurement with a Physica MCR 301 rheometer equipped with cone-plate geometry was conducted (CP40; diameter: 40 mm, cone angle: 0.3°, gap size 0.4 mm, Anton Paar, Ostfildern-Scharnhausen, Germany). For all rheological analysis, temperature was maintained at 4 °C and a pre-shearing was implemented with shear stress of τ = 0.1 Pa for 60 s. Flow curves were obtained by increasing the shear rate at a linear ramp from 0 to 500 s^{-1} within 3 min (upward flow curve), followed by a 5 min holding time. Subsequently, a linear decrease of the shear rate from 500 to 0 s^{-1} was performed within another 3 min (downward flow curve). The apparent viscosity η_a with a shear rate of 50 s^{-1} was calculated with RheoCompass™ Software version 3.0× (Anton Paar). Additionally, the area between the upward and downward flow curve (A_H) referred to the hysteresis loop was determined to by RheoCompass™ Software measure the degree of structure degradation (Mezger, 2006). For oscillatory measurements a shear strain amplitude sweep was performed to determine the linear viscoelastic region. Therefore, shear strain was varied in a logarithmic ramp from 0.01 to 100% at a constant angular frequency of 10 rad/s. A structural change of the samples was visible when the slopes of curves changed and is expressed as yield point γ_y (tolerance: ± 5 %). Further, the flow point γ_f was calculated, which refers to the intercept point of G' and G″ (Mezger, 2006). Subsequently, a small strain frequency sweep was followed at a constant shear strain of 0.05% within the linear viscoelastic region. Angular frequency was recorded from 100 rad/s to 0.1 rad/s. The elastic modulus G', the viscous modulus G″, the complex modulus G* and the phase angle δ at an angular frequency ω of 10 rad/s was calculated using RheoCompass™ Software in accordance equation 3 and 4:

$$|G^*| = \sqrt{(G')^2 + (G'')^2} \quad (3)$$

$$tan\ \delta = \frac{G''}{G'} \quad (4)$$

3.9.2 Texture Profile Analysis

Texture evaluation was performed with samples in cylindrical glass containers using a TA.XT plus Texture analyzer (Stable Micro Systems, Surrey, United Kingdom) equipped with a 5 kg load cell and a 25 mm cylindrical probe. The depth of immersion was 5 mm at a constant speed of 0.5 mm/s. The compression was carried out one time using a trigger force of 0.03 N. Force–time curves were recorded and textural parameters were calculated with

Exponent software 6: firmness (maximum compression force during compression i.e. rupture point of the gel [N]); consistency (positive peak area within curve during extrusion thrust [Ns]); cohesiveness (maximum compression force during probe return [N]); index of viscosity (area within negative region of curve during probe return [Ns]) (Angioloni and Collar, 2009).

3.9.3 Syneresis measurement

The susceptibility of LBYA to spontaneous syneresis was determined using the siphon method developed by Lucey et al. (1998) with slight modifications adapted from Amatayakul et al. (2006b). Empty yogurt cups were weighed and filled with 30 ml of inoculated LBMA and fermented as described in Section 3.6. Full glasses were weighed again after incubation to determine the net weight. After 24 h storage at 1 °C, glasses were held horizontally for 10 s and the exceeding water was collected and weighed. Syneresis was expressed as the percentage of exceeded water over the initial weight of the LBYA sample.

Syneresis was also measured by the centrifugation method also referred as water-holding capacity. Inoculated LBMA was filled in centrifuge tubes (Greiner Bio-One), which were weighed prior to filling and incubated as described in Section 3.6. Full tubes were centrifuged at 877 *g* at 20 °C for 10 min. The separated water was collected and weighed. Syneresis was calculated as the percentage of exceeded water in relation to the yogurt sample (Amatayakul et al., 2006b).

3.9.4 Color measurement

For color measurements, LBYA and different commercial samples were subjected to the non-contact color imaging system DigiEye V2.62 (VeriVide, Leicester, UK) to measure the color attributes. Samples were placed into a petri dish at room temperature in the enclosed 'DigiEye Cube' eliminating all ambient light. A digital camera (Nikon D90, Düsseldorf, Germany) was centrally positioned on top of the cube and captured the images from above. Calibration was performed with a white uniformity board and a DigiTizer Chart. L*: lightness-to-darkness (100-0), a*: red-green (+a, -a) and b*: yellow-blue (+b, -b) were calculated by the DigiEye measurement system. Color difference ΔE* between cow milk yogurt as reference (mean value of yogurt from *Danone*, *Ehrmann* and *Weihenstephan*) and the soy-based yogurt alternative and LBYA was calculated using equation 5:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (5)$$

3.10 Microstructural observations

3.10.1 Confocal laser scanning microscopy

For confocal laser scanning microscopy (CLSM) measurements, 400 µl of inoculated LBMA (as described in 3.6) was stained with 5 µl rhodamine B dye (stock solution: 1 mg rhodamine B per 1 ml distilled water, working solution: 1:1000 (v/v)) for protein visualization according to Heilig et al. (2009). The sample was then filled in chambered coverslips (8 well uncoated µ-slide; Ibidi, Martinsried, Germany) and was fermented as described in Section 3.6. After 24 h storage, CLSM measurements were carried out. Therefore, a Ti-U inverted microscope with an e-C1 plus confocal system (Nikon, Düsseldorf, Germany) and a 60x oil objective was used to analyze the protein microstructure. Proteins, visualized in green, were monitored in fluorescence micrographs ($\lambda_{excitation}$ = 543 nm, $\lambda_{emission}$ = 590 nm) with resolution of 1024 x 1024 pixels (212 x 212 µm) in a constant z-position. For fat coloration, LBYA was labeled with 2.5 - 10 µl nile red (stock solution: 1 mg nile red per 1 ml acetone, working solution: 1:10 (v/v)) and EPS with 2.5 - 10 µl alexa flour® 488 (stock solution: 1 mg alexa flour 488 per 1 ml distilled water; working solution: 1:20 (v/v)) according to Cruz et al. (2009) and to Zhang et al. (2016), respectively. At least five different positions on the x-y-axis were captured for each LBYA sample with two independent replications.

3.10.2 Cryo-scanning electron microscopy

Cryo-scanning electron microscopy (Cryo-SEM) was performed with LBYA out of unheated and UHT-heated LBMA fermented with *L. plantarum* TMW 1.460 with a procedure described previously (Muranyi et al., 2013). About 5 mg of the sample was mounted onto a sample carrier and was plunged into slush nitrogen at atmospheric pressure (−210 °C). The frozen specimen was then transferred to a Polaron SC 7600 preparation stage (Quorum Technologies Ltd, U.K.) under high vacuum and was freeze-fractured at −150 °C. The surface water was slightly sublimated at −80 °C for 20 min and the fractured sample was then sputter coated at −150 °C with 5 nm of platinum. The platinum-coated specimen was transferred into a scanning electron microscope (SUPRA 40VP, EMITECH K1250X, Zeiss, Oberkochen, Germany) using a transportable cryo-stage. On that cryo-stage, the specimen was maintained below −130 °C and was observed at an accelerating voltage of 5 kV. Two independent measurements were performed and at least five different positions were captured of each sample.

3.11 Statistical analysis

According to Granato et al. (2014), statistical analysis was performed with SPSS Statistics for Windows, Version 20.0 (IBM Corp. Armonk, New York, USA). Mean values and standard deviations were calculated. Statistically significant differences of presented data were identified by one-way Analysis of Variance (ANOVA) at a significance level of $P < 0.05$. Prior to ANOVA, Levene's test for homogeneity of variances and normality test by Shapiro-Wilk were conducted. Post-hoc tests were performed using Tukey in case of variance homogeneity and Games-Howell for variance heterogeneity. Study designs regarding pairwise comparison of means were analyzed with Student's t-test. Thereby, variance equality was tested with Levene's test. In case of variance heterogeneity Welch's test was included. Additionally, bivariate correlation analysis of the different rheological, textural and syneresis measurements was performed using Pearson's linear correlation coefficient.

4 Results

4.1 Selection and characterization of suitable strains for lupin protein fermentation

4.1.1 Acidification, yogurt-like texture and odor

In total, 35 strains were screened for their acidification performance and ability to produce yogurt-like texture and odor on LPI (Table 11). Strains that are typically associated with yogurt as primary (e.g. *L. delbrueckii* ssp. *bulgaricus*, *S. salivarius* ssp. *thermophilus*) or secondary (e.g. *L. acidophilus*, *L. casei*) microorganisms were evaluated, as well as strains that are used in other cultured dairy products e.g. *Lc. lactis* ssp. *lactis*, *L. perolens* (Chandan and Kilara, 2013; Leroy and De Vuyst, 2004) and dairy-free yogurt alternatives e.g. *L. plantarum* (Li et al., 2014). Probiotic Bifidobacteria were examined (Chandan and Kilara, 2013), strains that are common in sourdoughs such as *L. fermentum* (Vogel et al., 1999) and strains referred as EPS-producers e.g. *L. casei* (De Vuyst and Degeest, 1999). In addition, different slime-producing beer spoilage bacteria e.g. *L. brevis*, were selected, isolated from spoiled beverages, since they may be as well EPS-producers and may have a positive impact on yogurt texture. Furthermore, strains that can degrade antinutritive substances like phytic acid or oligosaccharides were considered in the selection e.g. *L. buchneri* (Fritsch et al., 2015) as these secondary plant metabolites were found in lupin in distinct amounts (Macrae and Zand-Moghaddam, 1978). Finally, strains that generate key aroma compounds in cereal flours were evaluated (e.g. *Kluyveromyces fragilis*) (Opperer, 2014).

Fermentation of LPI suspensions with glucose showed that all strains were able to grow as the pH of 7.0 ± 0.1 was decreased after 48 h with declines between 1.0 and 2.9 (Table 11). A pH difference of more than 2.0 was favored due to product safety, which was achieved by 19 strains. Besides, fermented products were evaluated regarding their textures. Thereby, three main types of textures were observed (Figure 7). Type A was characterized by a typical yogurt-like texture with different pronounced gel firmness and syneresis. Type B gels exhibited a rather weak network, with a porous structure and fissures embedding water or bubbles, as well with different pronounced gel firmness and syneresis. Highest diversity was seen upon type C gels: precipitated, cottage cheese-like formations were built, often with a high amount of syneresis. Upon the screening, 17 of the strains were able to produce the favored type A gels. Another criterion concerned yogurt-like odor generated during fermentation. Odor impressions like ‘milky’, ‘buttery’, ‘sour’, ‘sweet’ or ‘fruity’ were referred as yogurt-like, which was achieved by 13 strains. The rest of the strains were excluded due to negative or not yogurt-like odor attributes such as ‘sulfurous’, ‘metallic’, ‘faecal’ or ‘cheesy’.

Table 11: Fermentation of different LAB on LPI suspensions supplemented with glucose – Criteria for selection and evaluation: acidification, yogurt-like texture and odor.

Strain	Origin	Criteria for selection	Acidification ΔpH (48 h)	Yogurt-like texture	Yogurt-like odor
L. delbrueckii ssp. *bulgaricus*	DSM 20080	1, 3, 5	1.9	B	+
S. salivarius ssp. *thermophilus*	DSM 20259	1, 3	2.3	A	–
L. acidophilus	DSM 20079	1, 4, 5	2.5	A	+
L. casei	DSM 20011	1, 3, 4	1.8	C	+
L. helveticus	DSM 20057	1, 4	2.5	A	+
L. perolens	DSM 12744	6	2.5	A	+
Lc. lactis ssp. *lactis*	DSM 20384	1, 7	2.0	A	+
Lc. lactis ssp. *cremoris*	DSM 20069	1, 3	2.2	A	–
Leuconostoc mesenteroides ssp. *cremoris*	DSM 20200	1	2.2	A	–
Brevibacterium linens	DSM 1233	1	1.8	A	+
Propionibacterium freundenreichii ssp. *shermanii*	DSM 4902	1	1.5	C	–
Geotrichum candidum	DSM 10452	1	2.5	A	–
Bifidobacterium bifidum	DSM 20239	1, 4	1.5	B	++
Bifidobacterium animalis ssp. *lactis*	DSM 10140	4, 7	1.9	B	–
L. plantarum	TWM 1.460	2, 3, 4, 5, 7	2.8	A	++
L. plantarum	TMW 1.1468	2, 3, 4, 5	2.6	A	+
L. fermentum	DSM 20391	4, 5	1.9	C	–
L. pontis	TMW 1.1086	5	2.0	C	–
L. sanfranciscensis	DSM 20451	5, 8	2.1	A	–
Weissella cibaria	TMW 2.1333	5	2.4	B	–
L. brevis	TMW 1.1326	4, 5, 6	1.7	C	–
L. brevis	BGT L150	4, 5, 6	2.0	A	+
L. amylolyticus	BGT TL3	6	1.3	C	–
L. amylolyticus	BGT TL5	6	2.9	A	–
L. species	BGT TL11	6	1.0	C	–
L. species	BGT TL13	6	1.1	C	–
L. rossiae	BGT L1202	6	2.1	C	–
P. pentosaceus	BGT B34	6	2.7	A	+
P. pentosaceus	DSM 20336	7	2.4	A	+
L. curvatus	TMW 1.624	3	1.9	A	–
L. reuteri	DSM 20016	4, 5, 7	2.0	B	–
L. buchneri	DSM 20057	7	1.8	C	–
L. gasseri	DSM 20243	7	1.9	B	–
Saccharomyces cerevisiae	TMW 3.210	8	1.4	B	–
Kluyveromyces fragilis	TMW 3.188	8	1.8	A	–
Blank			1.6	C	–

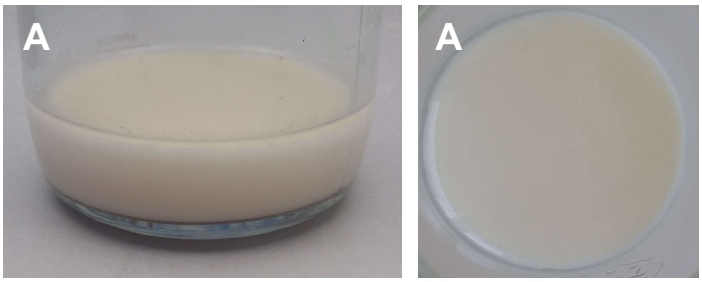
1: Strains of this species are often used for cultured dairy products (Chandan and Kilara, 2013; Leroy and De Vuyst, 2004)
2: Strains of this species are used in soy yogurt (Li et al., 2014)
3: Strains of this species are partially EPS-producers (De Vuyst and Degeest, 1999; Palomba et al., 2012)
4: Strains of this species are partially probiotic (Chandan and Kilara, 2013)
5: Strains of this species are often used in sourdoughs (Leroy and De Vuyst, 2004; Vogel et al., 1999)
6: Isolates from spoiled beer or lemonade, some are slime producers
7: Strains degrade antinutritive substances e.g. phytic acid, oligosaccharides (Fritsch et al., 2015)
8: Strains generate key aroma compounds in cereal flours (Opperer, 2014)

A yogurt-like texture
B porous structures with fissures
C aggregated formations

– no yogurt-like odor
+ adequate yogurt-like odor
++ strong yogurt-like odor

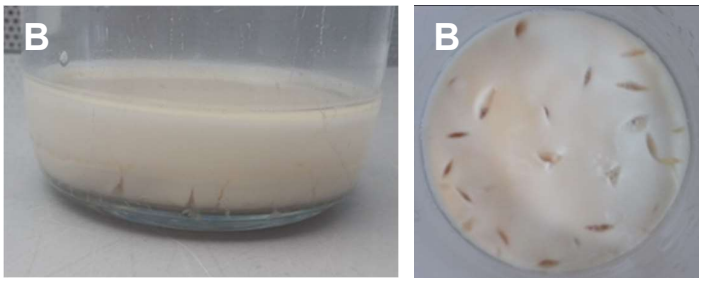
Type A:

Yogurt-like gel with different pronounced gel firmness and syneresis



Type B:

Gels with porous structures and fissures embedding water or bubbles, with different pronounced gel firmness and syneresis



Type C:

Precipitated, cottage cheese-like formations, with a high amount of syneresis

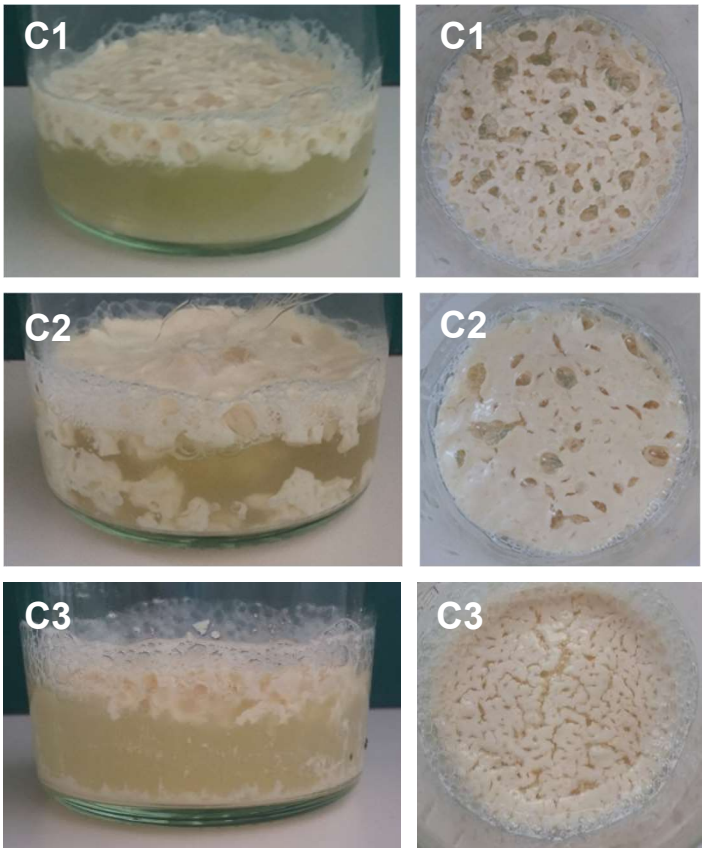


Figure 7: Three types of texturized LPI suspensions: A) *L. plantarum* TMW 1.460, B) *Weisella cibaria* TMW 2.1333, C1) *L. fermentum* DSM 20391, C2) *L. casei* DSM 20011, C3) *L. rossiae* BGT L1202.

Several strains were evaluated additionally on sucrose beside glucose in lupin protein suspensions. Most of the strains showed a similar appearance when grown on glucose or sucrose, while fermented lupin protein suspensions with *L. curvatus* TMW 1.624 showed a fundamental different visual appearance (Figure 8). Grown on LPI suspensions supplemented with glucose type A gels were obtained, whereas type C gels were received with sucrose. This was also seen for *Brevibacterium linens* DSM 1233 fermentates.

Figure 8: Influence of carbon source on the formation of LPI gels. LPI suspensions were fermented with *L. curvatus* TMW 1.624 and glucose (A and B, left) and sucrose (A and B, right).

4.1.2 Exopolysaccharide-production in media

As LPI are associated with weak gelling properties (Batista et al., 2005; Berghout et al., 2015) the screening was also focused on EPS-producers as they can improve textural properties of yogurts (De Vuyst and Degeest, 1999; Folkenberg et al., 2006b). Therefore, strains were spread on MRS agar plates with increased amount of sugar and were incubated strain-specifically. Strains were classified as EPS-producers with ropy phenotype, when they built strands when touched with a toothpick (Figure 9B) or as slimy/mucoid, when they showed slimy appearance (Figure 9C). Those which neither built strands nor showed slimy appearance were referred as EPS non-producers (Figure 9A).

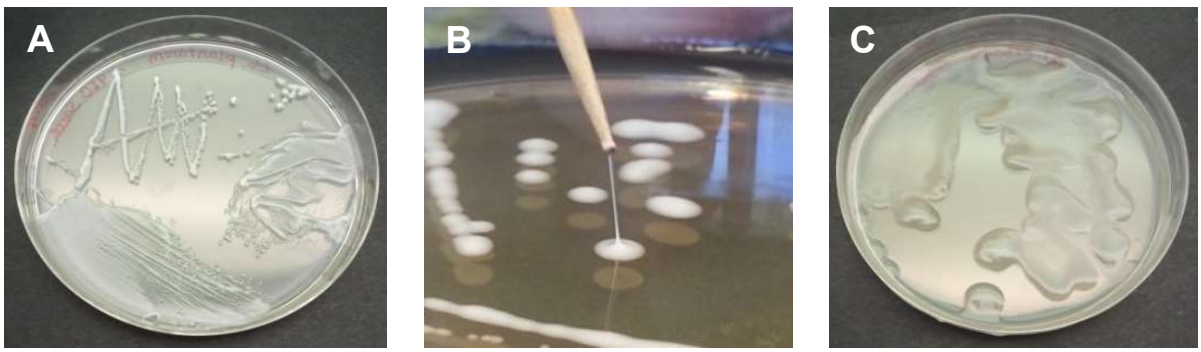


Figure 9: EPS phenotype on MRS agar plates: no EPS (A), ropy EPS (B) and slimy/mucoid EPS (C).

All 35 strains were tested regarding their EPS-expression on their recommended media fortified with glucose, galactose, maltose or sucrose (Table 12). Out of the 35 tested strains seven were able to build EPS, namely *L. plantarum* TMW 1.460 and TMW 1.1468, *P. pentosaceus* BGT B34, *L. brevis* BGT L150 and *L. rossiae* BGT L1202 on glucose, galactose and maltose as well as *L. curvatus* TMW 1.624 and *Weissella cibaria* TMW 2.1333 on sucrose. When strains produce slimy EPS on sucrose, they are referred as HoPS, when grown on other sugar sources HePS are built. Both *L. plantarum* species, as well as *P. pentosaceus*, *L. brevis* and *L. rossiae* built ropy strands and can be classified as HePS, while *L. curvatus* and *Weissella cibaria* were slimy on sucrose and can therefore build HoPS.

Table 12: EPS expression on agar plates supplemented with increased amount of glucose, galactose, maltose and sucrose (phenotype).

Strain	Origin	Carbohydrate			
		glucose	galactose	maltose	sucrose
L. plantarum	TMW 1.460	++	+	+	++
L. plantarum	TMW 1.1468	+	–	–	–
P. pentosaceus	BGT B34	++	++	++	–
L. brevis	BGT L150	++	++	++	–
L. rossiae	BGT L1202	++	++	+	–
L. curvatus	TMW 1.624	–	–	–	++
Weissella cibaria	TMW 2.1333	–	–	–	++

When *L. plantarum* TMW 1.460 was grown in MRS broth and was centrifuged, the pellet showed a slimy appearance. This is an indication that the strain produces *capsular* EPS. To verify this, *L. plantarum* TMW 1.460 and TMW 1.1468 (as control) were evaluated for their production of *free* and *capsular* EPS. Prior to EPS production, a suitable growth medium had to be found. Therefore, LAB were propagated in mMRS, a MRS medium without meat and yeast extract, as those ingredients contain polysaccharides (Kimmel and Roberts, 1998). Figure 10 depicts that growth of *L. plantarum* TMW 1.460 was significantly reduced in mMRS (cf. Figure 10B) compared to common MRS medium (cf. Figure 10A). Only mMRS media inoculated with 8.0 $\log_{10}$ CFU/ml showed sufficient growth. Afterwards, several growth stimulating media supplements were added such as vitamins, nucleobases, salts and cysteine (Mende et al., 2012b). The addition of vitamins to mMRS enhanced growth performance of *L. plantarum* TMW 1.460 for all inoculum sizes (C), whereas the addition of nucleobases and salts did only slightly stimulate growth further (D and E). mMRS broth

supplemented with additional cysteine beside the vitamins, nucleobases and salts, enhanced growth significantly (F). Samples inoculated with 5.0 $\log_{10}$ CFU/ml had a lag phase of 20 h previously, which was more than halved by the addition of cysteine. These measurements were also performed with *L. plantarum* TMW 1.1468 and showed comparable results (data not shown). Therefore, mMRS with all four supplements was chosen for further propagations. The medium was inoculated with 7.0 $\log_{10}$ CFU/ml of the respective strains, as the lag phase was acceptable. Then, fermentations were stopped after 15 h at a pH of ~4.3 (cf. Figure 10F) as growth kinetics showed that stationary phase begins immediately. The isolation of *free* and *capsular* EPS showed that *L. plantarum* TMW 1.460 was able to produce both types of EPS with 269 mg/l and 69 mg/l, respectively (Table 13). However, *L. plantarum* TMW 1.1468 – without slimy pellet – only produced *free* EPS with 226 mg/l and negligible amounts of *capsular* EPS. Therefore, the previous consumption that the slimy pellet can be ascribed to *capsular* EPS was corroborated.

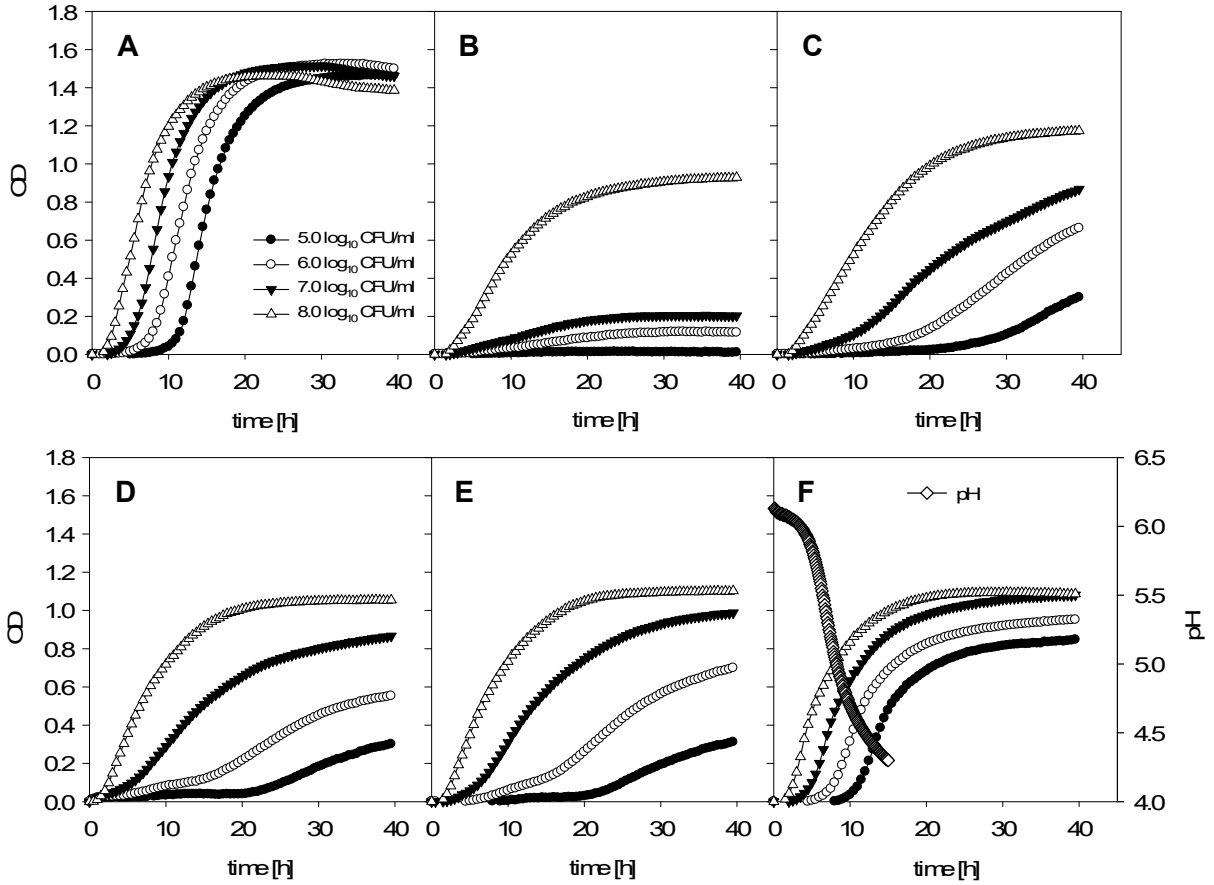


Figure 10: Growth kinetics of *L. plantarum* TMW 1.460 propagated in different media: MRS (A), mMRS (B), mMRS with vitamins (C), mMRS with vitamins and nucleobases (D), mMRS with vitamins, nucleobases and salts (E), mMRS vitamins, nucleobases, salts and cysteine (F) (cf. composition of different media Table 10). OD: optical density.

Table 13: Content of *free* and *capsular* EPS produced by *L. plantarum* TMW 1.460 and 1.1468 isolated from mMRS supplemented with vitamins, nucleobases, salts and cysteine (cf. content of supplements Table 10).

Strain	*Free* EPS [mg/l]	*Capsular* EPS [mg/l]
L. plantarum TMW 1.460	269 ± 2	69 ± 3
L. plantarum TMW 1.1468	226 ± 14	9 ± 5

Finally, 19 of the tested strains showed reasonable acidification rates, among them, 15 strains built the favored type A gel and in turn, nine showed additionally yogurt-like aroma. As LPI comprise weak gelling properties (Batista et al., 2005; Berghout et al., 2015), EPS-producing strains were preferred. Consequently, the four EPS-producers out of these nine strains were chosen and were used throughout this study. These were: *L. plantarum* TMW 1.460 and 1.1468, *P. pentosaceus* BGT B34, *L. brevis* BGT L150.

4.2 Growth pattern of lactic acid bacteria in lupin-based milk alternatives

4.2.1 Acidification

The four chosen EPS-producing LAB were subjected to heated LBMA to test their growth performance. As displayed in Figure 11A and B, the selected strains were able to grow in LBMA and produced organic acids, as the pH decreased continuously. Differences between strains and heat treatment were obvious: total fermentation time differed about 11 h between the UHT and pasteurized LBMA except for *L. plantarum* TMW 1.1468 (7 h). Between 25 h and 35 h of fermentation time was needed to reach a pH-value of 4.5 using the four different strains in the UHT-heated LBMA compared to 14 and 24 h for the pasteurized LBMA. Furthermore, the prolonged lag phase of around 10 h for *L. brevis* BGT L150 for both heat treated milks was notable.

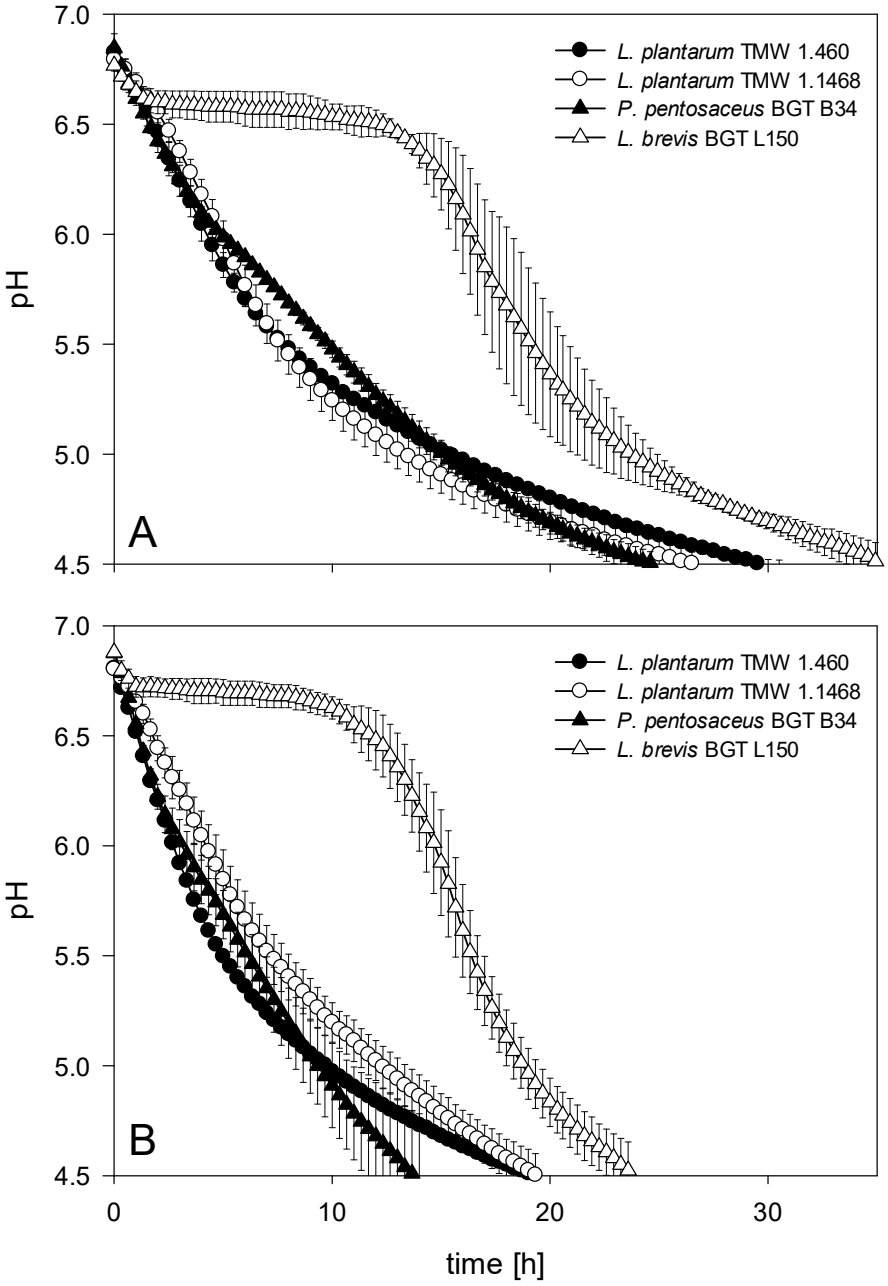


Figure 11: Acidification rate of different test strains in UHT-heated (A) and pasteurized (B) LBMA.

4.2.2 Back-slopping

To investigate the reason for the prolonged lag phase in the growth pattern of *L. brevis* BGT L150, which might be due to a slow metabolism of the strain in the thermally treated LBMA or a lack of adaption to the media, back-slopping experiments were performed. Fresh pasteurized LBMA was inoculated with fermented LBMA by back-slopping. Figure 12 shows that fermentation time decreased significantly by back-slopping with a difference of 8.5 h

between standard and back-slopped procedure. Furthermore, lag phase almost disappeared. Second back-slopping resulted in a slightly faster acidification and the lag phase completely disappeared. Back-slopped acidification curves of *L. brevis* BGT L150 are more similar to those from *L. plantarum* TMW 1.460 and 1.1468 and *P. pentosaceus* BGT B34.

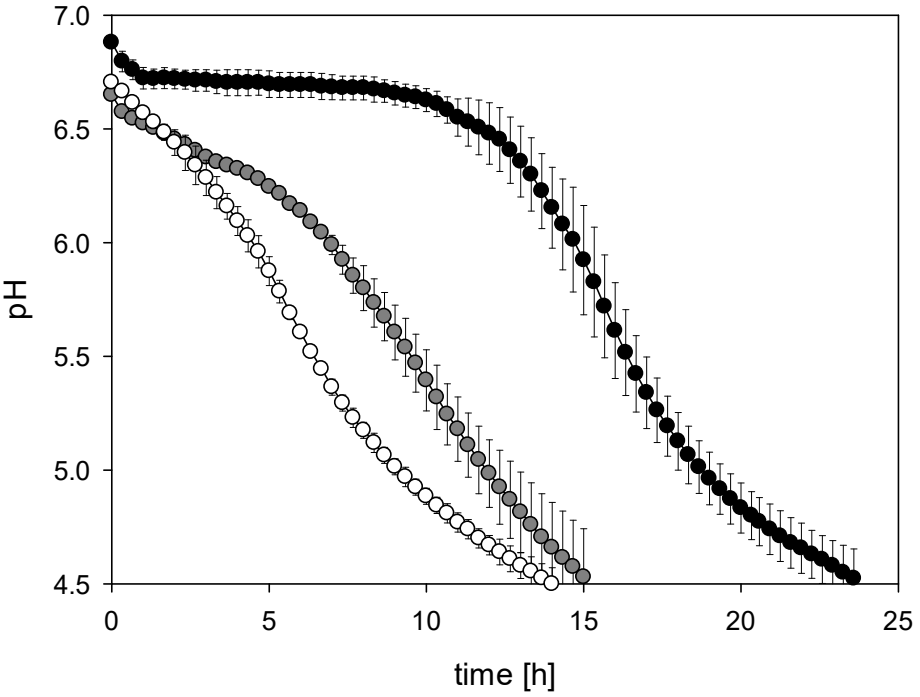


Figure 12: Acidification rate of *L. brevis* BGT L150 in pasteurized LBMA (black circles), after first back-slopping (grey circles) and after second back-slopping (white circles).

4.2.3 Cell counts and exopolysaccharide-production

As given in Table 14, heat treatment of milk alternatives influenced initial cell counts. Cell counts of UHT-heated LBMA prior to inoculation were below the detection limit whereas pasteurized LBMA showed cell counts of 2.2 $\log_{10}$ CFU/ ml. Unheated LBMA had a cell count of 5.4 $\log_{10}$ CFU/ml. UHT-heated and pasteurized LBMA were inoculated with the four test strains with 8.0 $\log_{10}$ CFU/ ml. After 10 h, the cell counts of the different strains were higher than at t = 0 h, irrespective whether a pasteurized or UHT-heated LBMA was used, except for *L. brevis* BGT L150, in those samples cell counts remained in the same range. In total, bacterial counts of all strains increased about one log cycle until a pH of 4.5 was reached, where fermentation was stopped. Finally, MALDI-TOF MS analyzes were performed, which proved that the added strains were competitive towards the endogenous biota of LPI, as only the inoculated strains were determined.

Interestingly, higher amounts of extracted EPS were recorded for LBYA prepared out of UHT-heated LBMA than for pasteurized LBMA (Table 14). Both *L. plantarum* strains and *P. pentosaceus* BGT B34 produced around 0.85 g/l EPS. In contrast, about 0.2–0.3 g/l less EPS was detected in LBYA obtained from pasteurized LBMA. Amounts of EPS in LBYA inoculated with *L. brevis* BGT L150 differed in both LBMA varieties but deviation was lower than for the other strains. Additionally, an unheated LBMA was inoculated with *L. plantarum* TMW 1.460 and EPS was extracted after fermentation with a content of 0.52 g/l, which was slightly reduced compared to the EPS content of the LBYA obtained from the thermally treated LBMA. Extraction of EPS in control LBMA (without inoculation and propagation) showed ~0.3 g/l EPS, which is quite high. Therefore, other EPS isolation techniques were performed, which are frequently found in literature for cow milk yogurt. Neither the use of another solvent – 2-propanol instead of ethanol absolute – was expedient, nor was the use of enzymes for the cleavage of the peptide bonds pf of the proteins beneficial (Table 15). Although the yield of the extracted EPS was rising slightly using 2-propanol and strongly increased applying the proteolytic enzyme preparations Pronase E and Alcalase. However, the protein content of the extracted EPS also increased assuming that the extracted EPS was more contaminated with lupin protein.

Table 14: Microbial growth of different test strains in UHT-heated and pasteurized LBMA and EPS production in LBYA.

Strain	LBMA heating	Viable counts of LBMA and LBYA [log_{10} CFU/ml]			Total EPS [g/l]
		t = 0 h	t = 10 h	t (pH = 4.5) = end	
–	UHT	< 1	–	–	0.35 ± 0.03 ^{A}
–	Pasteur.	2.2 ± 0.2	–	–	0.29 ± 0.07^{A}
–	Unheated	5.4 ± 0.5	–	–	–
L. plantarum TMW 1.460	UHT	7.9 ± 0.1^{A}	8.5 ± 0.1^{A}	8.8 ± 0.1^{A}	0.86 ± 0.01^{A}
	Pasteur.	7.9 ± 0.1^{A}	8.6 ± 0.0^{A}	8.8 ± 0.2^{A}	0.56 ± 0.04^{B}
	Unheated	8.2 ± 0.1^{B}	8.6 ± 0.1^{A}	8.8 ± 0.2^{A}	0.52 ± 0.02^{B}
L. plantarum TMW 1.1468	UHT	7.9 ± 0.0^{A}	8.3 ± 0.1^{A}	8.6 ± 0.3^{A}	0.83 ± 0.07^{A}
	Pasteur.	8.0 ± 0.1^{A}	8.5 ± 0.1^{A}	8.7 ± 0.0^{A}	0.59 ± 0.06^{B}
P. pentosaceus BGT B34	UHT	7.9 ± 0.0^{A}	9.0 ± 0.1^{A}	9.0 ± 0.0^{A}	0.87 ± 0.04^{A}
	Pasteur.	8.0 ± 0.0^{A}	8.9 ± 0.0^{A}	8.9 ± 0.0^{B}	0.69 ± 0.07^{B}
L. brevis BGT L150	UHT	8.1 ± 0.1^{A}	8.1 ± 0.1^{A}	9.3 ± 0.6^{A}	0.48 ± 0.01^{A}
	Pasteur.	8.1 ± 0.1^{A}	8.2 ± 0.2^{A}	9.2 ± 0.2^{A}	0.39 ± 0.04^{B}

$^{A, B}$ Pairwise comparison of two (or three for *L. plantarum* TMW 1.460) differently heated LBMA: different capital letters indicate significant differences for one strain grown in two (or three) different media with $P < 0.05$.

Table 15: Comparison of different EPS extraction procedures for LBYA produced out of UHT-heated LBMA with *L. plantarum* TMW 1.460.

Applied extraction technique	Total EPS [g/l]	Protein content [%] of dry matter
Ethanol absolute	0.86 ± 0.01	30.73 ± 1.03
2-Propanol	0.93 ± 0.06	33.16 ± 2.09
Pronase E	2.77 ± 0.12	79.13 ± 8.09
Alcalase	4.35 ± 0.83	91.97 ± 9.27

Extracted EPS were also evaluated according to their monosaccharide composition with HPLC-MS (Table 16). It was remarkable that all extracted EPS were composed of a similar monosaccharide pattern; mainly galactose, arabinose/ xylose and glucose were found. Further, the method of Rühmann et al. (2014) has one drawback: during sample preparation parts of the sample remained insoluble and can be consequently not analyzed. This was also true for our samples.

Table 16: Monosaccharide composition of EPS extracted out of LBYA.

Strain	LBMA heating	Amount [mg/l]						
		galactose	arabinose/ xylose	glucose	mannose	rhamnose	galactos-amine	ribose
L. plantarum TMW 1.460	UHT	223 ± 3	34 ± 2	8 ± 0	8 ± 0	7 ± 0	6 ± 0	8 ± 1
	Pasteur.	263 ± 13	38 ± 2	14 ± 1	7 ± 0	6 ± 0	6 ± 0	8 ± 1
L. plantarum TMW 1.1468	UHT	256 ± 24	37 ± 4	10 ± 3	7 ± 0	5 ± 0	6 ± 0	–
	Pasteur.	230 ± 9	35 ± 1	10 ± 2	8 ± 0	5 ± 0	6 ± 0	–
P. pentosaceus BGT B34	UHT	135 ± 7	27 ± 2	19 ± 12	10 ± 1	5 ± 0	5 ± 0	–
	Pasteur.	195 ± 2	31 ± 1	10 ± 2	10 ± 0	5 ± 0	5 ± 0	–
L. brevis BGT L150	UHT	171 ± 9	26 ± 0	13 ± 1	11 ± 0	7 ± 1	5 ± 0	8 ± 0
	Pasteur.	200 ± 13	31 ± 2	34 ± 1	13 ± 1	6 ± 1	5 ± 0	7 ± 0

4.3 Physical characterization of lupin-based milk alternatives

4.3.1 Differential scanning calorimetry

To assess the effect of homogenization and heating upon LBMA production on protein denaturation, DSC analysis was performed. LPI (2% (w/v)) exhibited two endothermic thermal transitions. The major peak denaturation temperatures (T_d) were at approximately 82.6 °C (T_{onset} = 81.0 °C) and 92.6 °C (T_{onset} = 88.7 °C) along with denaturation enthalpies of 0.018 J/g and 0.052 J/g, respectively (Table 17). LBMA prior to homogenization showed nearly the same endothermic transitions like LPI (2% (w/v)), while after homogenization of LBMA an additional peak emerged at T_d = 71.7 °C (T_{onset} = 67.7 °C; ΔH = 0.015 J/g). After pasteurization of the homogenized LBMA the additional peak disappeared in the thermograms and solely the two peaks with T_d = 84.3 °C and T_d = 96.8 °C appeared. Harsher thermal conditions like UHT-heating of LBMA caused a complete denaturation of the peak around 84 °C and solely the peak with the higher denaturation temperature (T_d = 96.8 °C) occurred in the thermograms.

4.3.2 Sulfhydryl-disulfide transitions

Along with stepwise denaturation and the change of the native conformation of proteins, SH-SS transitions were apparent (Figure 13). The contents of free SH-groups and total SH-groups (free and masked SH-groups) were initially constant upon the LBMA production in LPI (2% (w/v)) and LBMA prior and after homogenization with ~12% and ~20-22%, respectively. Pasteurization of LBMA led to a slight increase of free SH-groups, while the content of total SH-groups decreased slightly. The more intense UHT-heating of the LBMA resulted in a decrease of both free and total SH-groups. The amount of SS-bondings in LPI (2% (w/v)) and LBMA before homogenization was the same with ~71%. After homogenization the content of SS-bondings was slightly increased. Heating of LBMA, namely pasteurization and UHT-heating resulted in further increases of the content of SS-bondings with ~97% and ~100%, respectively.

Table 17: DSC analyzes upon LBMA production and different heat treatments. T_d: Peak denaturation temperature, T_{onset}: onset temperature and ΔH: enthalpy of denaturation.

	Transition 1			Transition 2			Transition 3		
	T_{onset} [°C]	ΔH [J/g]	T_d [°C]	T_{onset} [°C]	ΔH [J/g]	T_d [°C]	T_{onset} [°C]	ΔH [J/g]	T_d [°C]
LPI 2% (w/v)				81.0 ± 2.1^{A}	0.018 ± 0.010^{A}	82.6 ± 2.0^{A}	88.7 ± 0.8^{A}	0.052 ± 0.024^{A}	92.6 ± 2.2^{A,B}
LBMA				80.6 ± 2.1^{A}	0.026 ± 0.014^{A}	82.9 ± 1.4^{A}	89.3 ± 2.2^{A,B}	0.060 ± 0.036^{A}	93.7 ± 0.8^{A}
LBMA, homog.	67.7 ± 0.7	0.015 ± 0.006	71.7 ± 0.2	79.6 ± 0.6^{A}	0.087 ± 0.007^{B}	85.4 ± 0.2^{A}	92.1 ± 0.4^{B}	0.027 ± 0.005^{A}	96.3 ± 0.5^{A,B}
LBMA, homog., pasteur.				80.1 ± 0.9^{A}	0.030 ± 0.001^{A}	84.3 ± 0.4^{A}	91.8 ± 0.3^{A,B}	0.027 ± 0.007^{A}	96.8 ± 0.1^{A,B}
LBMA, homog., UHT-heated							91.4 ± 1.2^{A,B}	0.034 ± 0.004^{A}	96.8 ± 0.7^{B}

$^{A, B}$ Different capital letters within same columns indicate significant difference with $P < 0.05$.
LPI 2% (w/v): 2% (w/v) LPI solution
LBMA: lupin-based milk alternative
LBMA, homog.: lupin-based milk alternative, homogenized
LBMA, homog., pasteur.: lupin-based milk alternative, homogenized, pasteurized
LBMA, homog., UHT-heated: lupin-based milk alternative, homogenized, ultra-high temperature heated

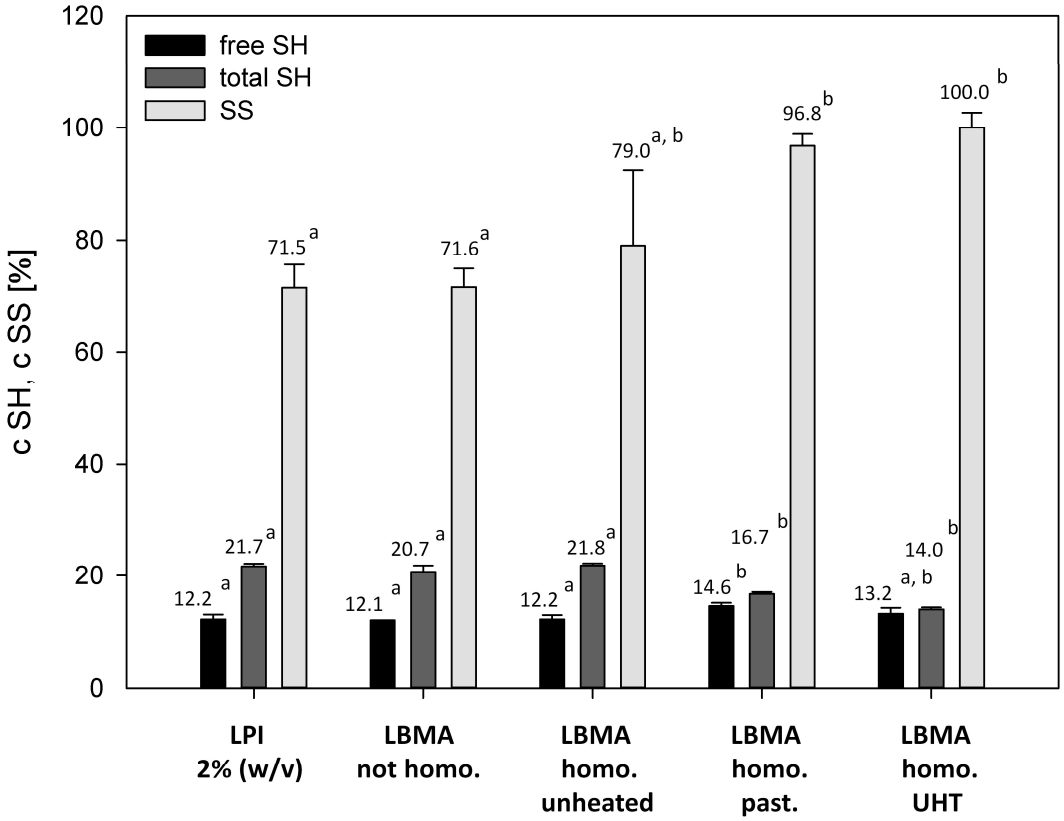


Figure 13: Free SH, total SH and SS-content upon LBMA production and different heat treatments. [a, b] Different lower case letters indicate significant difference with P < 0.05. Data for free SH, total SH and SS were analyzed separately. homog.: homogenized; pasteur.: pasteurized; UHT: ultra-high temperature heated.

4.3.3 Molecular weight distribution

Characteristic electrophoretic profiles of lupin protein samples under reducing and non-reducing conditions are shown in Figure 14. Under reducing conditions, bands did not differ considerably among LPI 2% (w/v) and LBMA (unheated, pasteurized and UHT-heated). Under non-reducing conditions differences were apparent upon heat treatment: A band at 30 kDa and at 40 kDa from LPI (2% (w/v) and unheated LBMA gradually disappeared upon thermal processing. Additionally, new bands between 30 kDa and 40 kDa emerged in the pasteurized LBMA sample and broadened upon LBMA heat treatment, especially in UHT-heated LBMA. Moreover, the pockets of the gradient gel showed different intensities under reducing and non-reducing conditions, with the highest intensities for the UHT-heated samples.

To assign the single bands of the SDS-PAGE pattern of Figure 14 to conglutin α and β, the pattern was compared to SDS-PAGE patterns of pure conglutin α and β samples (data not shown and not yet published). Thereby, it was possible to assign different bands to conglutin α and to subdivide the bands of conglutin β to three different regions by their molecular weight in accordance to Duranti et al. (2008), who found a HMW region with 53 kDa to 64 kDa, an IMW region with 25 kDa to 46 kDa and a LMW region with 17 kDa to 20 kDa. In the present pattern the band at 59 kDa can be assigned to HMW, two bands at 30 kDa and 40 kDa can be related to the IMW region and two other bands at 17 kDa and 21 kDa can be referred to the LMW region. These bands appear under reducing and non-reducing conditions, as conglutin β has no SS-bondings (Duranti et al., 2008). Thus, the bands, which are of special interest and which disappeared at 30 kDa and 40 kDa upon heat treatment, can be referred to conglutin β of the IMW region. According to Duranti et al. (2008) each conglutin α monomer is composed of two subunits, an acidic and a basic chain, with 42 kDa to 52 kDa and 20 kDa to 22 kDa, respectively. However, to assign the bands of conglutin α to the acidic and basic chain was not possible, as additional bands beyond these regions were found.

4.3.4 Particle size distribution

Particle size distribution curves are depicted in Figure 15. The particle size distribution of the LPI (2% (w/v)) and non-homogenized LBMA was unimodal with particle diameters from about 0.1 to 70 µm, with slightly smaller particle sizes for the latter. Homogenization of LBMA (unheated, pasteurized and UHT-heated) shifted the particle diameters towards smaller particles, however, a bimodal distribution with a large volume % of particle diameters from 0.05 to 5 µm and a small volume % with particle diameters from 5 to 100 µm was seen for the unheated LBMA. Heat treatment promoted the formation of smaller particles from 0.1 to 5 µm with almost unimodal distribution, independent of the time-temperature regimes applied as the pasteurized LBMA and UHT-treated LBMA samples did not differ in their particle size.

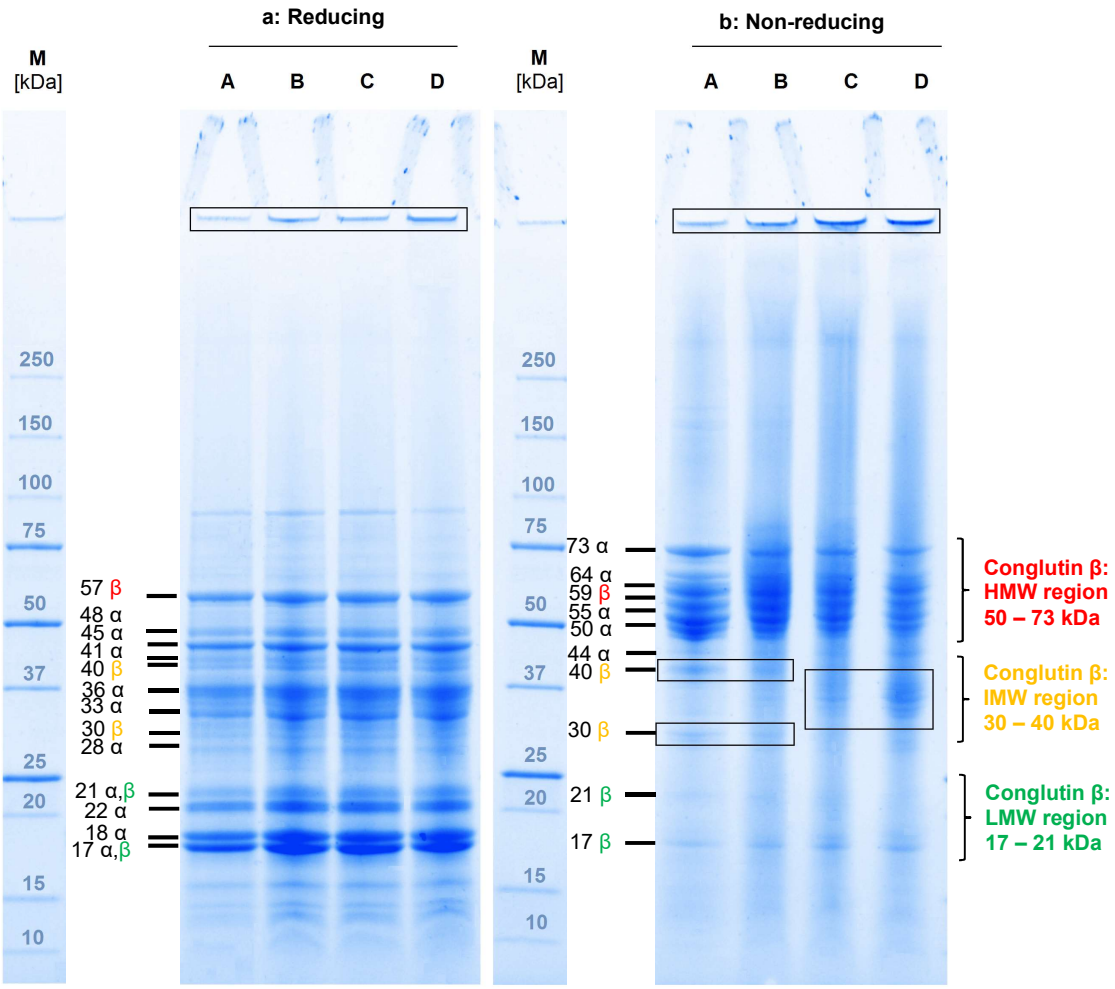


Figure 14: SDS-PAGE pattern upon LBMA production and different heat treatments under reducing (a) and non-reducing (b) conditions, as well as the assignment of single bands to conglutin α (black) and conglutin β (red, yellow and green: HMW: high molecular weight; IMW: intermediate molecular weight; LMW: low molecular weight). M: molecular weight standard in kilo Dalton (kDa); A: LPI 2% (w/v); B: LBMA, homog.; C: LBMA, homog., pasteur.; D: LBMA, homog., UHT.

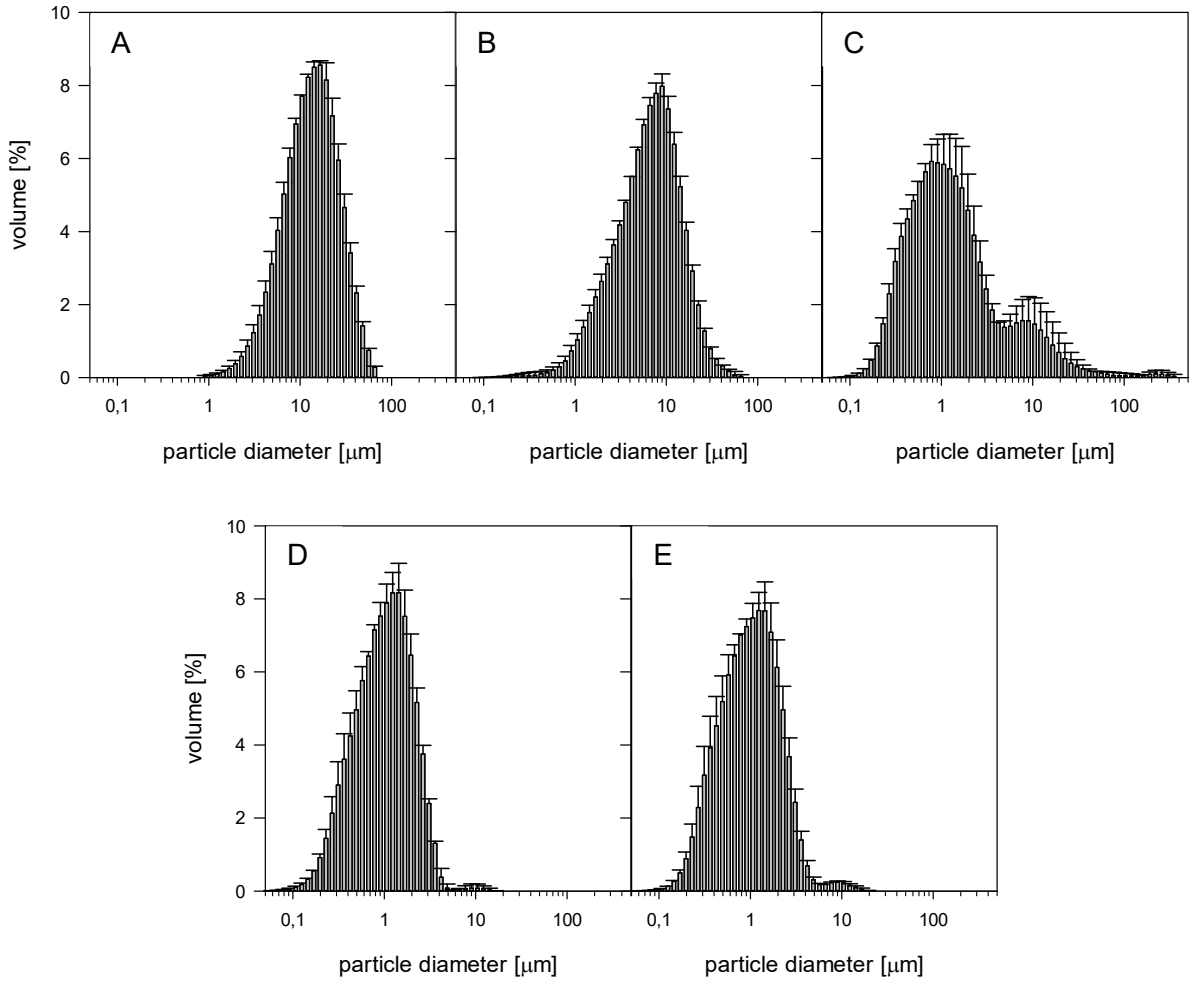


Figure 15: Particle size distribution upon LBMA production as determined by laser light-scattering. A: LPI 2% (w/v); B: LBMA; C: LBMA, homog.; D: LBMA, homog., pasteur.; E: LBMA, homog., UHT.

4.4 Physical characterization of lupin-based yogurt alternatives

4.4.1 Influence of inoculum sizes on the texture of lupin-based yogurt alternatives

The influence of different initial inoculum sizes such as 7.0 and 8.0 $\log_{10}$ CFU/ml on the texture of the respective LBYA was evaluated in pasteurized and UHT-heated LBMA (Figure 16). Initial inoculum sizes lower than 7.0 $\log_{10}$ CFU/ml were not considered in this study design, as the acidification rates were quite low with lower inoculation sizes and consequently the risk of malfermentation quite high. Firmness, the maximum force that occurs when the gel initially breaks (Angioloni and Collar, 2009), was significantly higher in both types of LBYA that were prepared out of UHT-heated LBMA (~ 1.0 N for

7.0 $\log_{10}$ CFU/ml and 1.1 N for 8.0 $\log_{10}$ CFU/ml) than for pasteurized LBMA (~ 0.5 N for 7.0 $\log_{10}$ CFU/ml and 0.7 N for 8.0 $\log_{10}$ CFU/ml). Besides, the inoculum size influenced firmness significantly: the firmness was higher when LBMA of both heat treatments was inoculated with 8.0 $\log_{10}$ CFU/ml instead of 7.0 $\log_{10}$ CFU/ml. Similar observations were made regarding the thickness of LBYA expressed as consistency. A higher influence on the consistency of LBYA can be ascribed to the heat treatment, favoring harsher treatments, but again the initial inoculum size was also decisive: higher inoculum sizes caused higher levels of consistency. Concerning cohesiveness (the resistance of the sample to probe return) and index of viscosity (the resistance to flow), the initial inoculum size seemed to have a smaller effect: no statistically verifiable difference was recorded regarding different inoculum sizes, while heat treatment influenced cohesiveness significantly.

Due to these results, the following experiments were continued with inoculum sizes of 8.0 $\log_{10}$ CFU/ml. The influence of heat treatment on textural properties was evaluated more detailed in Section 4.4.2, 4.4.3 and 4.4.4.

4.4.2 Rheological properties of lupin-based yogurt alternatives

To determine the rheological properties of set-type LBYA, three different rheological tests were performed as displayed in Table 18. Rotation measurements of all prepared LBYA showed time-dependent shear-thinning behavior of a non-Newtonian fluid as shear rate increased from 0 – 500 s^{-1} with a decline for apparent viscosity η_a (data not shown). At η_a(50 s^{-1}), which represents the approximate viscosity felt in the mouth (Bourne, 2002), values for η_a ranged from 353 to 1176 mPas and seemed to depend to a large extent on the previously applied heat-treatment of LBMA. Apparent viscosity was significantly higher for LBYA made out of UHT-heated LBMA then for pasteurized LBMA.

The hysteresis loop area A_H, also referred as thixotropic behavior, was determined of the prepared LBYA. A_H, which is a measure for regeneration after shear-induced structure breakdown was significantly higher for cultures when grown in the UHT-heated LBMA (Table 18). This difference between A_H suggests a more stable gel structure against shear stress when the LBYA were prepared with UHT-heated LBMA. *L. plantarum* TMW 1.1468 was an exception with nearly the same A_H for differently heat treated LBMA. Interestingly, LBYA from *L. brevis* BGT L150 differed to a large extent compared to the other LBYA, with two- to threefold higher A_H and higher η_a.

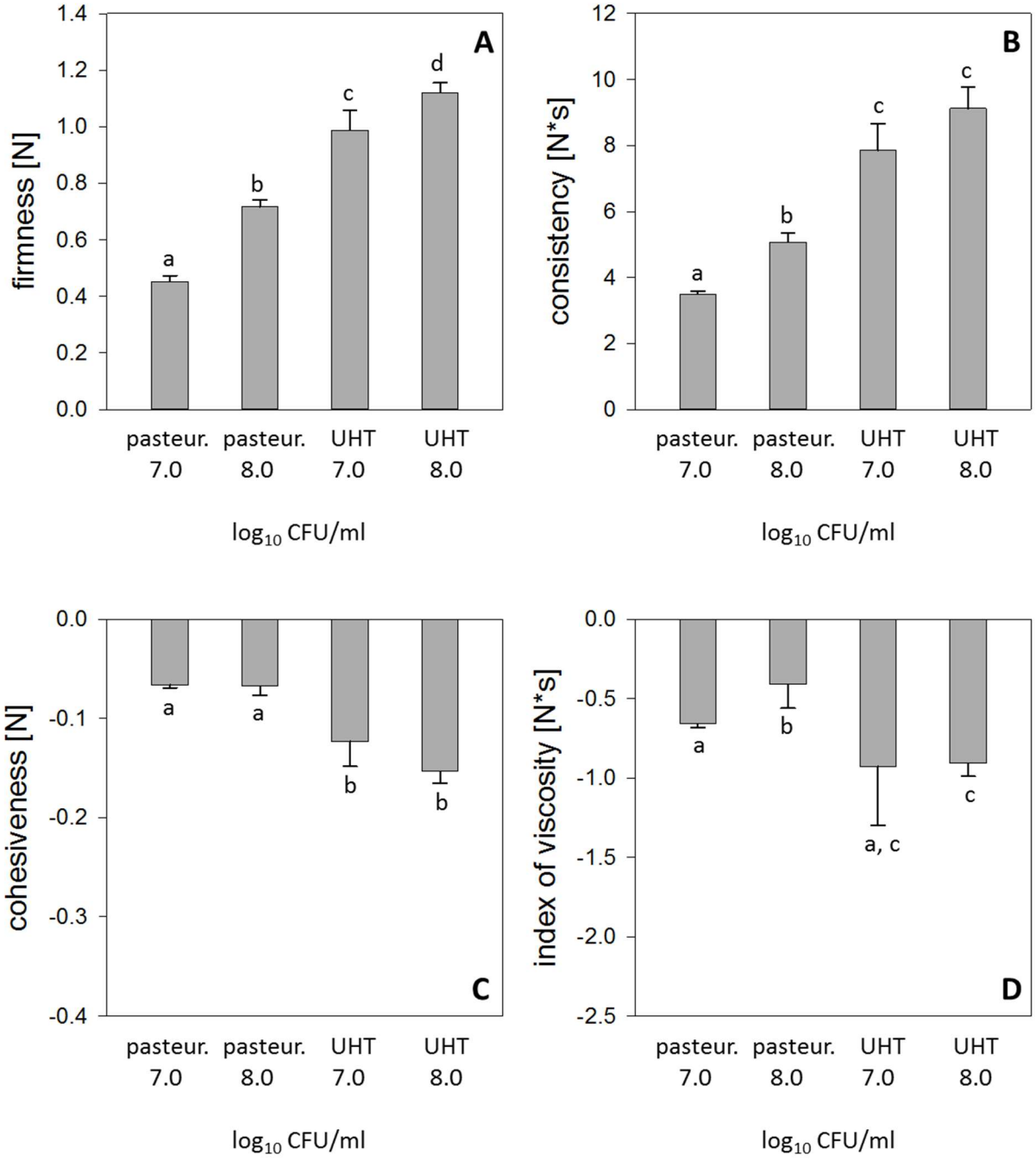


Figure 16: Texture Profile Analysis of different LBYA obtained from UHT-heated and pasteurized LBMA with different initial inoculum sizes (7.0 and 8.0 log_{10} CFU/ml); A) firmness, B) consistency, C) cohesiveness, D) index of viscosity. [a, b, c, d] Different lower case letters indicate significant difference with $P < 0.05$.

Amplitude sweeps were performed to characterize the samples according to their yield and flow point (cf. Figure 17). In early stages of this test, G' and G'' were constant with increasing strain. It is striking that G' and G'' were higher in LBYA made from UHT-heated than from

pasteurized LBMA, indicating that the viscoelastic properties are more pronounced in these samples. In this region, the sample can regain its original structure when force is stopped, which is called the linear viscoelastic region. The end of this region is marked with the yield point γ_y. After that, the curves progressively decrease until G' and G'' cross, which is referred as flow point γ_f. Exceeding this point, the sample is flowing and the gel character is no longer maintained with G'' higher than G' (Mezger, 2006). As displayed in Figure 17 and expressed numerical in Table 18 the yield points for LBYA made out of UHT-heated LBMA are significantly higher for all tested LAB than for pasteurized LBMA. The longer the sample can maintain the structure, the more rigidity and elasticity it has. Highest yield points were observed for *L. plantarum* TMW 1.460, followed by *L. plantarum* TMW 1.1468 and *P. pentosaceus* BGT B34 and *L. brevis* BGT L150 with the lowest values.

The flow point γ_f is referred as the point where the gel character is lost and the breakdown of the gel (Mezger, 2006). LBYA prepared out of UHT-heated LBMA and *L. plantarum* TMW 1.460 reached the highest flow point of all samples (49.4 ± 8.0 %), maintaining the gel character at the highest strain. In contrast LBYA made with the same culture but with pasteurized LBMA resulted in a flow point reduced by half (25.0 ± 4.8 %). However, flow points for all LBYA made of pasteurized LBMA were significantly lower than for the UHT-heated LBMA ($P > 0.05$). *L. brevis* BGT L150 showed the lowest resistance to increasing strain with 20.4 ±7.4 % for the UHT-heated LBMA and 5.5 ±1.4 % for the pasteurized LBMA.

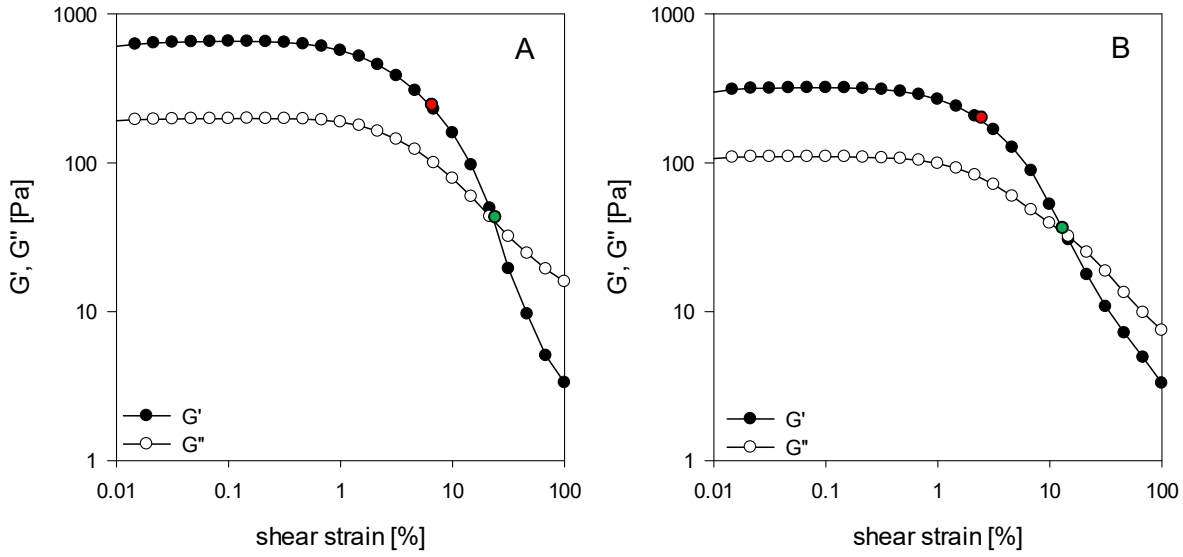


Figure 17: Amplitude sweeps of LBYA produced out of UHT-heated (A) and pasteurized (B) LBYA prepared with *L. plantarum* TMW 1.460. The red dots are referred to the yield points, the green dots to the flow points.

Frequency sweeps determine the elastic and viscous properties of the gels, represented as two critical factors, namely storage or elastic (G') and loss or viscous (G'') modulus, respectively. G' is a measure of energy stored per oscillation cycle, while G'' is a measure of the energy dissipated as heat per cycle (Lucey et al., 1998). In Figure 18, log (G') and log (G'') values were plotted against log (angular frequency), resulting in linear curves for LBYA composed of both types of LBMA. All fermented LBMA showed characteristics typical for a weak viscoelastic gel, with G' higher than G'' across the entire measuring range. Both, the storage and loss modulus exhibited frequency dependency in the linear viscoelastic region, with G' and G'' values rising gradually with increasing frequencies. Only LBYA processed with *L. brevis* BGT L150 showed irregularities in its strait lines. The heat treatment of LBMA prior to fermentation also affected frequency induced behavior. LBYA made with UHT-heated LBMA had remarkably higher values for G' and G'' than the corresponding yogurts out of pasteurized LBMA for all selected cultures (Table 18, Figure 18). The stiffest gels were observed at approximately G' (ω = 10 rad/s) = 640 Pa (*L. plantarum* TMW 1.460, UHT-heated LBMA) and the weakest at approximately G' (ω = 10 rad/s) = 140 Pa for *L. brevis* BGT L150 in pasteurized LBMA (Table 18).

Complex shear modulus G* is a measure of rigidity, which represents total resistance of a material against applied strain (Mezger, 2006). LBYA out of UHT-heated LBMA exhibited higher G* (ω = 10 rad/s) values (approximately 670 – 422 Pa) while those obtained from pasteurized LBMA were significantly lower (approximately 319 – 164 Pa) (Table 18). Moreover, the viscoelastic behavior of the samples was calculated and expressed as the phase angle δ. Exceeding δ = 45 °, the sol/gel transition point, the samples are denoted by their increasing viscous, liquid-like character. But, with δ moving towards zero, the extent of elastic, solid-like behavior is rising. Phase angle values showed no differences within one type of LBMA fermented with different cultures. Only *L. plantarum* TMW 1.460 in pasteurized LBMA differed slightly from the other microorganisms in pasteurized LBMA. LBYA manufactured out of UHT-heated LBMA showed a slightly higher solid-like behavior with data of approximately 17 °. The corresponding pasteurized LBYA had higher phase angles ranging from 19.5 ° to 17 °.

L. plantarum TMW 1460 was additionally propagated in unheated LBMA beside pasteurized and UHT-heated LBMA (cf. Table 18). Rotational measurements showed, that LBYA out of unheated LBMA had significantly lower values for A_H and η_a compared to LBYA out of pasteurized or UHT-heated LBMA. Differences for oscillation measurements were also seen, but were not as pronounced as for rotational measurement: Values for flow and yield point, as well as the elastic, viscous and complex modulus were slightly lower for LBYA out of unheated LBMA, but not statistically different to values compared to LBYA out of pasteurized

LBMA. Solely the phase angle of LBYA out of unheated LBMA was more similar to LBYA out of UHT-heated LBMA as for LBYA out of pasteurized LBMA.

Table 18: Rheological properties of different LBYA prepared with differently heated LBMA and different test strains.

Parameter	LBMA heat treatment	Strains			
		L. plantarum TMW 1.460	*L. plantarum* TMW 1.1468	*P. pentosaceus* BGT B34	*L. brevis* BGT L150
Rotation					
Apparent viscosity[+] η_a [mPas]	UHT	507 ± 45$^{a, b, A}$	409 ± 25$^{a, A}$	594 ± 28$^{b, A}$	1176 ± 104$^{c, A}$
	Pasteur.	353 ± 38$^{a, B}$	375 ± 21$^{a, b, B}$	463 ± 35$^{b, B}$	623 ± 35$^{c, B}$
	Unheated	239 ± 37^{C}			
Hysteresis loop area[++] A_H [Pas^{-1}]	UHT	4557 ± 377$^{a, A}$	4048 ± 198$^{a, A}$	5496 ± 257$^{b, A}$	12552 ± 559^{c,}
	Pasteur.	3604 ± 311$^{a, B}$	4056 ± 146$^{a, b, A}$	4365 ± 255$^{b, B}$	7894 ± 206$^{c, B}$
	Unheated	2001 ± 309^{C}			
Amplitude Sweep					
Yield point τ_y [%]	UHT	6.8 ± 0.6$^{a, A}$	4.0 ± 0.3$^{b, A}$	4.9 ± 1.9$^{a, b, A}$	3.8 ± 1.6$^{b, A}$
	Pasteur.	2.7 ± 0.7$^{a, b, B}$	2.9 ± 0.4$^{a, B}$	2.0 ± 0.9$^{a, b, B}$	1.0 ± 0.1$^{b, B}$
	Unheated	2.6 ± 0.3^{B}			
Flow point τ_f [%]	UHT	49.4 ± 8.0$^{a, A}$	26.0 ± 2.0$^{b, c, A}$	32.7 ± 6.9$^{b, A}$	20.4 ± 7.4$^{c, A}$
	Pasteur.	25.0 ± 4.8$^{a, B}$	20.5 ± 3.7$^{a, B}$	17.1 ± 1.2$^{a, B}$	5.5 ± 1.4$^{b, B}$
	Unheated	23.2 ± 1.2^{B}			
Frequency Sweep					
Elastic modulus[+++] G' [Pa]	UHT	638 ± 68$^{a, A}$	404 ± 78$^{b, A}$	435 ± 42$^{b, A}$	415 ± 66$^{b, A}$
	Pasteur.	301 ± 44$^{a, B}$	254 ± 31$^{a, b, B}$	247 ± 31$^{a, b, B}$	139 ± 34$^{b, B}$
	Unheated	297 ± 5^{B}			
Viscous modulus[+++] G'' [Pa]	UHT	196 ± 20$^{a, A}$	122 ± 20$^{b, A}$	135 ± 13$^{b, A}$	128 ± 20$^{b, A}$
	Pasteur.	107 ± 15$^{a, B}$	85 ± 10$^{a, B}$	82 ± 8$^{a, B}$	43 ± 10$^{b, B}$
	Unheated	95 ± 1^{C}			
Complex modulus[+++] G* [Pa]	UHT	667 ± 79$^{a, A}$	422 ± 90$^{b, A}$	456 ± 49$^{b, A}$	434 ± 77$^{b, A}$
	Pasteur.	319 ± 52$^{a, B}$	268 ± 36$^{a, B}$	260 ± 36$^{a, B}$	164 ± 40$^{b, B}$
	Unheated	314 ± 5^{B}			
Phase angle[+++] δ [°]	UHT	17.1 ± 0.1$^{a, A}$	16.9 ± 0.6$^{a, A}$	17.3 ± 0.2$^{a, A}$	17.1 ± 0.4$^{a, A}$
	Pasteur.	19.5 ± 0.2$^{a, B}$	18.6 ± 0.1$^{b, B}$	18.5 ± 0.5$^{b, B}$	17.0 ± 0.7$^{b, A}$
	Unheated	17.5 ± 0.4^{A}			

$^{a, b, c}$ Different lower case letters within same row indicate significant difference with $P < 0.05$.
$^{A, B, C}$ Different capital letters within same column indicate significant difference with $P < 0.05$. Data for each parameter were analyzed separately.
[+] Apparent viscosity at shear rate 50 s^{-1} (upward curve)
[++] Area between the upward and downward curve when plotting shear stress vs. shear rate
[+++] Dynamic moduli and phase angle at angular frequency ω of 10 $rads^{-1}$

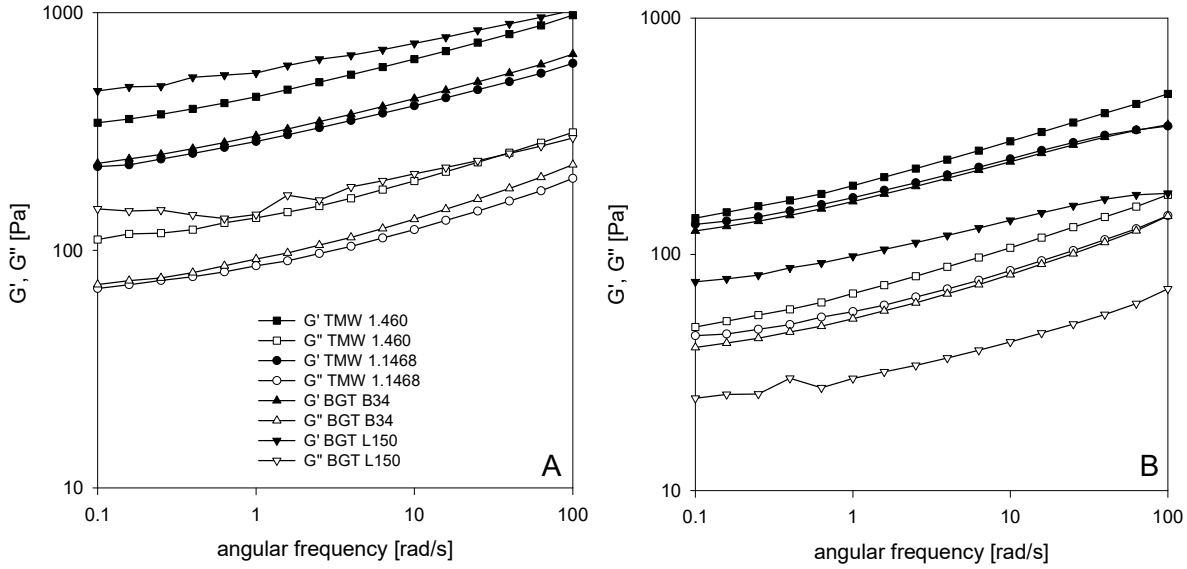


Figure 18: Frequency sweeps of LBYA made out of UHT-heated (A) and pasteurized LBMA (B) with G' (storage modulus) and G'' (loss modulus).

4.4.3 Textural properties of lupin-based yogurt alternatives

While oscillation measurement characterizes samples under small deformation within linear viscoelasticity, large deformation tests are needed to simulate breakdown of food as it occurs in the mouth or during processing. Results in Figure 19 demonstrate that a more severe heat treatment of LBMA (UHT) improved the textural properties for most of the samples. Firmness was significantly higher for LBYA prepared from the UHT-heated LBMA than for the pasteurized LBMA for *L. plantarum* TMW 1.460, *P. pentosaceus* BGT B34 and *L. brevis* L150. In addition, all LBYA samples out of the UHT-heated LBMA exhibited a significantly higher consistency. The same was true for cohesiveness except for *P. pentosaceus* BGT B34. Finally, the index of viscosity for *L. plantarum* TMW 1.460 and *L. brevis* BGT L150 was significantly higher for LBYA from the UHT-heated LBMA and statistically equal for the other two microorganisms.

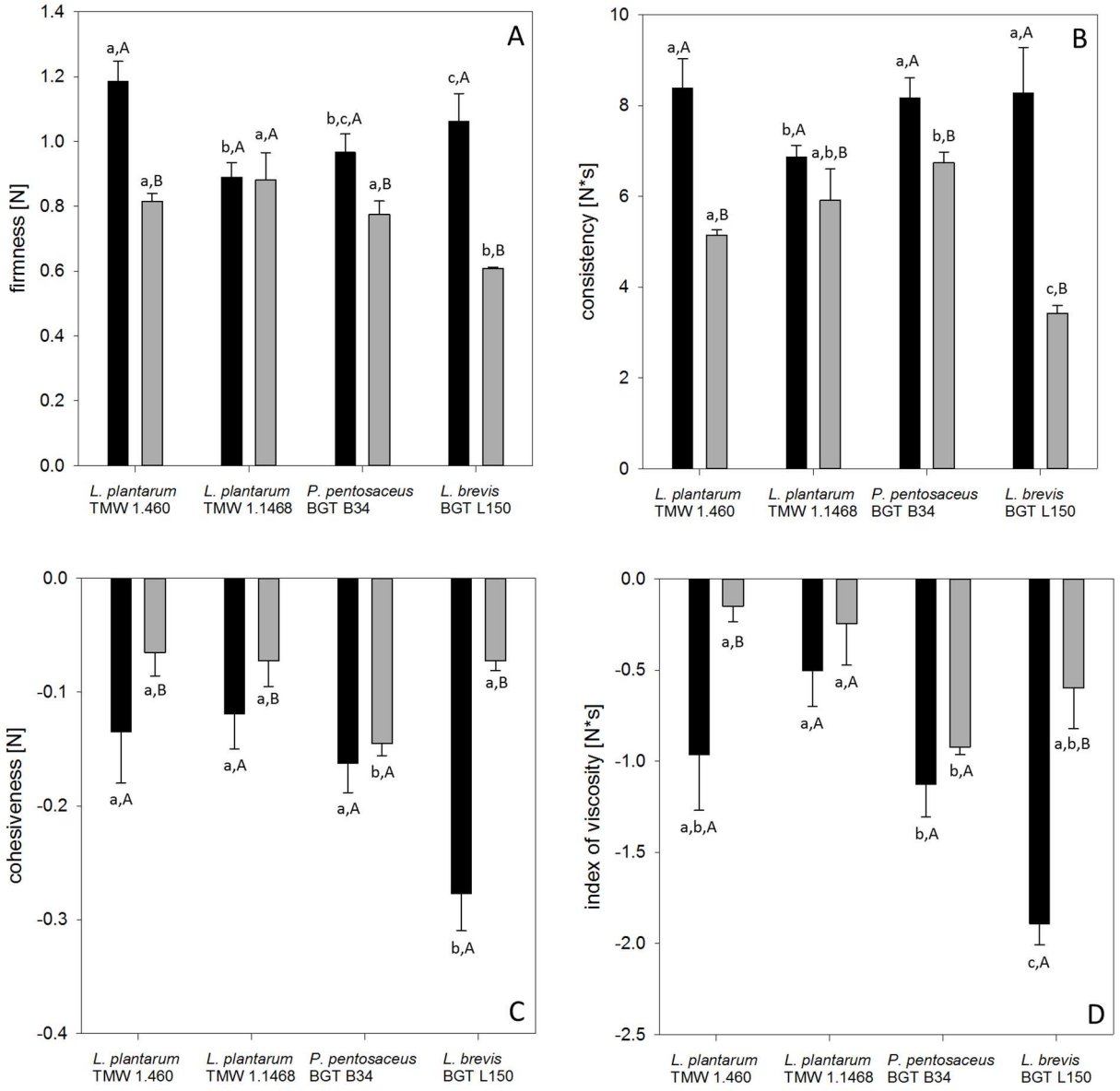


Figure 19: Texture Profile Analysis of different LBYA made out of UHT-heated (black bars) and pasteurized LBMA (grey bars); A) firmness, B) consistency, C) cohesiveness, D) index of viscosity. [a, b, c] Different lower case letters within one applied heat treatment indicate significant difference with P < 0.05. [A, B] Different capital letters within one strain indicate significant difference with P < 0.05.

4.4.4 Susceptibility to syneresis of lupin-based yogurt alternatives

Results of susceptibility to syneresis are summarized in Table 19. Different heat treatments of LBMA and different microorganisms strongly influenced the degree of syneresis. Significantly more expelled water was measured for LBYA made of pasteurized LBMA for both applied methods than for LBYA out of UHT-heated LBMA. Except for LBYA fermented

with *P. pentosaceus* BGT B34 (siphon method) and *L. plantarum* TMW 1.1468 (centrifugation method) as levels of syneresis were nearly the same for both LBYA. For *L. plantarum* TMW 1.460 and TMW 1.1468 and *L. brevis* BGT L150 the majority of the expelled water was on top of the LBYA. In contrast, *P. pentosaceus* BGT B34 showed comparatively low values of expelled water with the siphon method and higher values for the centrifugation method. The highest discrepancy in syneresis for different heated LBMA was measured for *L. brevis* BGT L150 with 0.62 ± 1.24% expelled water in LBYA out of UHT-heated LBMA and 20.81 ± 1.04% in pasteurized LBMA. Additionally, for *L. plantarum* TMW 1.460, unheated LBMA was used for yogurt manufacture. Respective yogurts showed, that degree of syneresis was significantly more pronounced as for heated LBMA for both applied methods.

Table 19: The level of syneresis [%] determined by the siphon and centrifugation method in LBYA out of pasteurized and UHT-heated LBMA and different strains.

Applied method	Milk heat treatment	Water separation of different strains [%]			
		L. plantarum TMW 1.460	*L. plantarum* TMW 1.1468	*P. pentosaceus* BGT B34	*L. brevis* BGT L150
Siphon	UHT	$10.9 \pm 3.9^{a, A}$	$5.6 \pm 1.1^{a, A}$	$0.9 \pm 1.8^{b, A}$	$0.6 \pm 1.2^{b, A}$
	Pasteur.	$19.7 \pm 2.4^{a, B}$	$8.5 \pm 0.7^{b, B}$	$1.8 \pm 2.2^{c, A}$	$20.8 \pm 1.0^{a, B}$
	Unheated	31.5 ± 0.4^{C}	–	–	–
Centrifugation	UHT	$11.2 \pm 0.5^{a, A}$	$9.6 \pm 0.8^{a, b, A}$	$8.9 \pm 0.4^{b, A}$	$2.2 \pm 0.2^{c, A}$
	Pasteur.	$19.6 \pm 1.3^{a, B}$	$9.6 \pm 0.5^{b, A}$	$10.4 \pm 0.7^{b, B}$	$17.6 \pm 0.6^{c, B}$
	Unheated	30.3 ± 0.6^{C}	–	–	–

$^{a, b, c}$ Different lower case letters within same row indicate significant difference with $P < 0.05$.
$^{A, B, C}$ Different capital letters within same column indicate significant difference with $P < 0.05$. Data for siphon and centrifugation method were analyzed statistically separately.

4.4.5 Correlation of textural properties and syneresis parameters of lupin-based yogurt alternatives

The bivariate correlation analysis after Pearson showed significant correlations between some of the rheological, textural and syneresis parameter (Table 20). Particularly, parameters within one type of measurement correlated highly, e.g. syneresis measurement applied with two different methods ($P < 0.01$, $R = 0.903$) or rotation measurements, including the parameters η_a and A_H ($P < 0.01$, $R = 0.976$). Moreover, within oscillation measurements correlations were obvious, amplitude sweep parameters τ_y and τ_f were positively correlated with the parameters of the frequency sweep G', G'' and G* ($P < 0.01$). Firmness and cohesiveness, the positive and negative maximum compression force obtained from Texture Profile Analysis correlated positively ($P < 0.01$ for firmness and $P < 0.05$ for cohesiveness) with all oscillation derived parameters, namely γ_y, γ_f, G', G'' and G*. In contrast consistency and index of viscosity, the positive and negative peak area during extrusion thrust and probe return correlated with data from the rotational measurements, namely η_a and A_H. The extent of syneresis measured by the siphon method was positively correlated with consistency and negatively with cohesiveness. Expelled water measured by centrifugation method showed significant correlations to consistency (positively), cohesiveness (negatively) and index of viscosity (positively).

Table 20: Correlation coefficients between dependent variables.

	Rotation		Amplitude Sweep		Frequency Sweep			Texture Profile Analysis				Syneresis	
	η_a^x	A_H	τ_y	τ_f	G' ^{xx}	G'' ^{xx}	G* ^{xx}	firmness	consis-tency	cohesive-ness	index of viscosity	siphon	centrifu-gation
η_a^x	1	0,976	0.086	-0.154	0.155	0.106	0.150	0.320	-0.858	0.385	-0.901	-0.398	-0.633
A_H	0.976	1	-0.083	-0.322	-0.018	-0.071	-0.023	0.147	-0.732	0.181	-0.800	-0.238	-0.513
τ_y	0.086	-0,083	1	0.953	0.981	0.975	0.981	0.931	-0.368	0.817	-0.331	-0.384	-0.391
τ_f	-0.154	-0,322	0.953	1	0.937	0.958	0.940	0.848	-0.162	0.707	-0.118	-0.231	-0.164
G' ^{xx}	0.155	-0,018	0.981	0.937	1	0.994	1.000	0.938	-0.443	0.832	-0.397	-0.381	-0.394
G'' ^{xx}	0.106	-0,071	0.975	0.958	0.994	1	0.995	0.938	-0.407	0.821	-0.348	-0.353	-0.350
G* ^{xx}	0.150	-0,023	0.981	0.940	1.000	0.995	1	0.939	-0.440	0.831	-0.393	-0.379	-0.390
Firmness	0,320	0,147	0,931	0.848	0.938	0,938	0.939	1	-0.577	0.890	-0.510	-0.499	-0.587
Consistency	-0.858	-0,732	-0.368	-0.162	-0.443	-0.,407	-0.440	-0.577	1	-0.759	0.958	0.758	0.840
Cohesiveness	0.385	0,181	0,817	0.707	0.832	0.821	0.831	0.890	-0.759	1	-0.669	-0.813	-0.772
index of viscosity	-0.901	-0,800	-0.331	-0.118	-0.397	-0.348	-0.393	-0.510	0.958	-0.669	1	0.654	0.752
Siphon	-0.398	-0,238	-0.384	-0.231	-0.381	-0.353	-0.379	-0.499	0.758	-0.813	0.654	1	0.903
Centrifugation	-0.633	-0,513	-0.391	-0.164	-0.394	-0.350	-0.390	-0.587	0.840	-0.772	0.752	0.903	1

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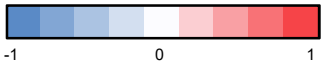

[x] Apparent viscosity at shear rate 50 s^{-1}

[xx] Dynamic moduli at angular frequency ω of 10 rad/s

4.4.6 Comparison of lupin-based yogurt alternatives with increased dry matter to commercially available products

With regard to the composition of cow milk yogurts, as well as soy-based yogurt alternatives from the German supermarket (cf. Table 21), the dry matter of LBYA was adapted by increasing the protein and sugar content. The dry matter of commercial cow milk yogurt ranged from 13.5 to 14.4% and therefore the dry matter of LBYA was increased from 10.0% to 14.3%. As commercial samples are stirred yogurts, stirred versions of LBYA were prepared as described in Section 3.6.

Table 21: Composition of commercially available yogurts from the German supermarket compared to LBYA with standard and increased dry matter.

Supplier	Source	Style of manufacture	Content [g/ 100g]				Total [g/ 100 g]
			Fat	Protein	Sugar	Others	
*Alpro Soja**	soy	stirred	2.3	4.0	2.1	1.3	9.7
*Berchtesgardener**	cow milk	stirred	4.0	4.9	4.6	0.3	13.8
*Danone**	cow milk	stirred	3.5	4.8	5.8	0.3	14.4
*Ehrmann**	cow milk	stirred	3.8	4.3	5.0	0.1	13.2
*Weihenstephan**	cow milk	stirred	3.5	4.4	5.3	0.3	13.5
Adaption to LBYA							
LBYA with standard dry matter	lupin	set, stirred	4.0	2.0	4.0**	–	10.0
LBYA with increased dry matter	lupin	set, stirred	4.0	4.8	5.5**	–	14.3

* Data of composition as by manufacturer declaration
** Prior to fermentation

Flow curves of LBYA with standard and increased dry matter compared to commercial products are shown in Figure 20. Flow curves differed in their shape, maximal reached shear stress and the area of the hysteresis loop. The flow curve of cow milk yogurt (Berchtesgadener Land) visualizes the characteristic rheological behavior of a weak, viscoelastic gel. In the first stage of the measurement, the shear stress increased with rising shear rate, reached a maximum and subsequently flattened. Soy-based yogurt alternative *Alpro Soja* reached clearly higher maximum shear stress values that are constantly increasing with increasing shear rate, suggesting less damage of the gel structure. However, yogurt alternatives of *Alpro Soja* are fortified with 1% pectin. Therefore, this yogurt is not only recognized as a pure protein gel. LBYA with increased dry matter showed a more comparable curve shape to cow milk yogurt *Berchtesgardener* than to *Alpro Soja*, however,

with a lower maximum shear stress and area of hysteresis loop and the upward curve reached a maximum and constantly decreased afterwards. The increase of dry matter in LBMA resulted in higher maximum shear stress and area of hysteresis loop compared to the standard dry matter LBYA, but as already mentioned, not with the same magnitude as for cow milk yogurt.

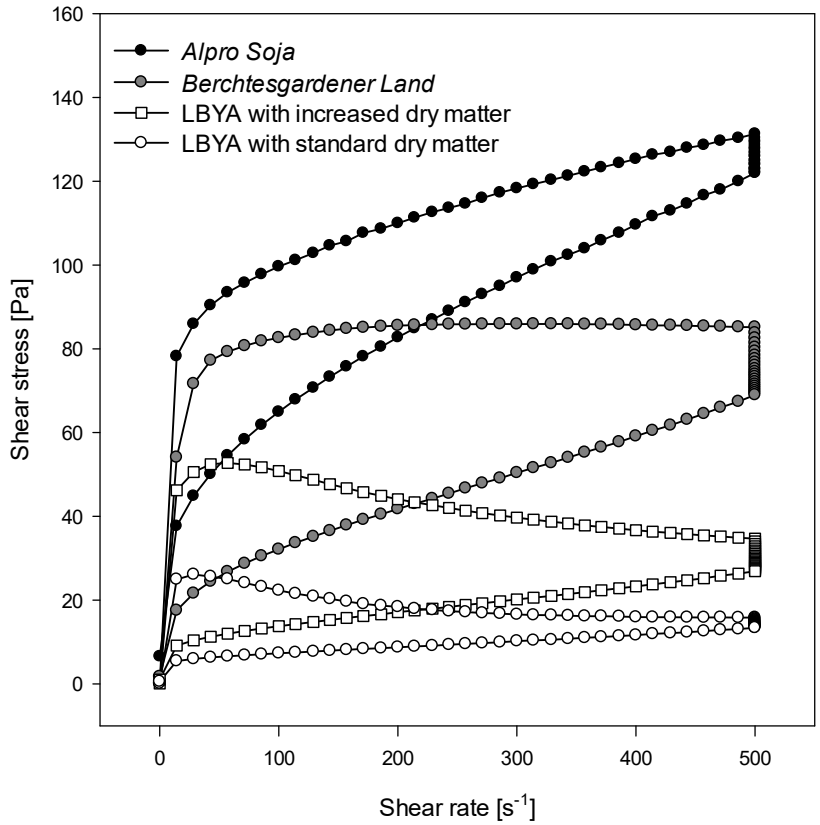

Figure 20: Flow curve with hysteresis loop area of LBYA with increased dry matter compared to commercial products.

The firmness and consistency of LBYA with increased dry matter was twice as high compared to standard dry matter (Figure 21A and B, left), although the dry matter was only increased from 10 to 14.5%. Cohesiveness and index of viscosity also showed significant differences between standard and increased dry matter preferring the higher dry matter account.

LBYA with different dry matters revealed high differences regarding susceptibility to syneresis (Figure 22). While levels of syneresis for both applied syneresis measurements ranged about 11% with standard dry matter, levels of syneresis were significantly decreased

to 0.2% (siphon method) and 5.4% (centrifugation method) when dry matter was increased to 14.5%.

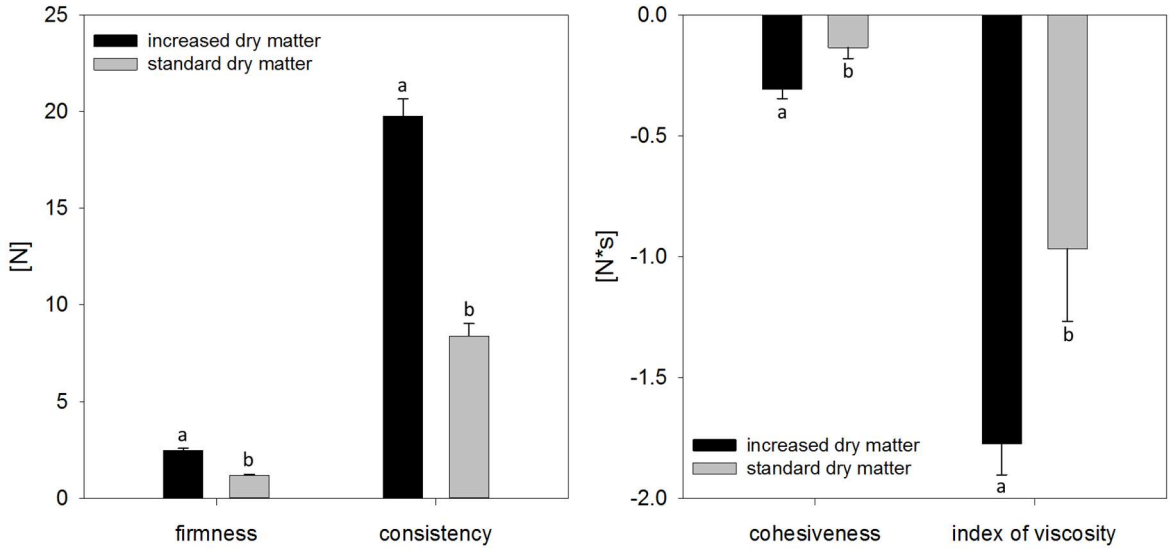

Figure 21: Texture Profile Analysis of different set-type LBYA made out of UHT-heated LBMA and *L. plantarum* TMW 1.460 with standard and increased dry matter. A) Firmness and cohesiveness, B) consistency and index of viscosity. [a, b] Different lower case letters indicate significant difference with P < 0.05.

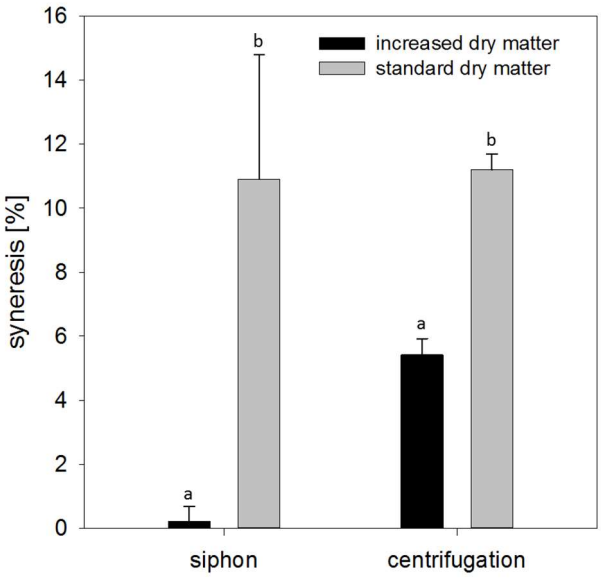

Figure 22: The level of syneresis [%] determined by the siphon and centrifugation method in set-type LBYA out of UHT-heated LBMA and *L. plantarum* TMW 1.460 with standard and increased dry matter. [a, b] Different lower case letters indicate significant difference with P < 0.05.

The firmness of the different commercial stirred yogurts and yogurt alternatives was measured with Texture Profile Analysis (Table 22). Data of commercial samples for firmness ranged from around 0.12 to 0.15 N, whereas firmness of a stirred standard LBYA was significantly lower with 0.06 N. However, stirred LBYA with increased dry matter was in the same range of commercial samples with around 0.12 N.

Beside texture, another important attribute for the acceptance of a food is its color. The color value L*, which is a measure of whiteness of a sample, shows that LBYA was slightly brighter as the soy-based yogurt alternative, but not as bright as cow milk yogurt (Table 22). Moreover, the ratio of red to green (a*) and yellow to blue (b*) of LBYA was closer to cow milk yogurt, than the soy-based sample to cow milk yogurt. This emphasizes also the ΔE*: the color difference of LBYA to cow milk yogurt is smaller as of the soy-based sample to cow milk yogurt.

Table 22: Firmness and L*, a* and b* values of different commercial purchased cow milk-based yogurts and soy-based yogurt alternative, as well as stirred LBYA with standard and increased dry matter.

Sample	Source	Firmness [N]	L*	a*	b*	ΔE*
Alpro Soja	soy	0.12 ± 0.00^{A}	89.8 ± 0.1^{A}	0.68 ± 0.02^{A}	10.90 ± 0.13^{A}	13.48
Berchtesgardener	cow milk	0.14 ± 0.00^{B}	96.1 ± 0.1^{B}	-2.13 ± 0.03^{B}	10.95 ± 0.146^{A}	reference
Danone	cow milk	0.15 ± 0.00^{B}	96.7 ± 0.1^{B}	-2.30 ± 0.04^{B}	10.46 ± 0.16^{B}	reference
Ehrmann	cow milk	0.12 ± 0.00^{A}	95.6 ± 0.2^{B}	-1.67 ± 0.04^{B}	11.22 ± 0.10^{A}	reference
Weihenstephan	cow milk	0.15 ± 0.01^{B}	96.0 ± 0.0^{B}	-2.03 ± 0.04^{B}	10.93 ± 0.16^{A}	reference
LBYA with standard dry matter	lupin	0.06 ± 0.01^{C}	–	–	–	–
LBYA with increased dry matter	lupin	0.12 ± 0.01^{A}	93.5 ± 0.4^{C}	-0.14 ± 0.04^{C}	9.43 ± 0.12^{C}	8.13

$^{A, B, C}$ Different lower case letters within same column indicate significant difference with $P < 0.05$.
The color difference ΔE* of "Alpro Soja" and "LBYA with increased dry matter" were related to the mean values of L*, a* and b* of the cow milk-based yogurts *Berchtesgardener, Danone*, *Ehrmann* and *Weihenstephan*.
L*: lightness (100) to darkness (0), a*: red (+) to green (-) and b*: yellow (+) to blue (-).

4.5 Microstructural observations

4.5.1 Confocal laser scanning microscopy

Figure 23 shows characteristic CLSM micrographs of LBYA obtained from unheated, pasteurized and UHT-heated LBMA, fermented with *L. plantarum* TMW 1.460 and TMW 1.1468, as well as *P. pentosaceus* B34 and *L. brevis* L150. It was obvious that both, choice of microorganism and intensity of heat treatment, played decisive roles in microstructural appearance. However, the choice of microorganism had a higher impact on the microstructure of the LBYA than the heat treatment. Both *L. plantarum* species showed a relatively close-meshed lupin protein network with interspaces of small sizes appearing as black, unstained regions (Figure 23: A1-3; B1-3). Network of *P. pentosaceus* revealed a more porous structure with larger pores, but of similar size within one sample (Figure 23: C1-C3). In contrast, LBYA from *L. brevis* had a completely different microstructural appearance compared to the other strains (Figure 23: D1-3). Yogurt alternatives obtained from unheated LBMA and propagated with *L. brevis* showed a loose formation of aggregated proteins without the formation of a network. For pasteurized samples still an interrupted structure with aggregated spots was observed, but with some branchings forming a loose network. In LBYA obtained from UHT-heated LBMA a denser network was detected with voids of different sizes, which were unevenly distributed. Compared to the networks of the other strains, a heterogeneous pattern still appeared in this sample. For the three other species smaller differences were apparent: LBYA obtained from a more intense heated LBMA revealed a denser network with smaller interspaces. This was especially obvious for *P. pentosaceus* yogurts out of unheated LBMA: Network was spiked with larger pores, while pores from LBYA from heated LBMA were visibly smaller.

To get more insight to the network, staining of coconut fat with nile red and staining of EPS with concanavalin A was performed. Unfortunately, stained spots were too small to display them adequately in the micrographs. Therefore, cryo-SEM was performed with representative yogurt alternatives (cf. Section 4.5.2).

4.5.2 Cryo-scanning electron microscopy

Due to its promising microstructural properties obtained by CLSM measurements, LBYA produced out of UHT-heated LBMA and fermented with *L. plantarum* TMW 1.460 was exemplarily analyzed using cryo-SEM (Figure 24) (cf. Section 4.5.1). Figure 24A and B depicts an overview of LBYA microstructure: a fine stranded, ordered, dense and close-meshed network was observed including interspaces with different sizes and shapes. The

variety of the interspaces was more clarified in further images: oblonged, lamellar-like shapes were seen (Figure 24E), also edgy- (Figure 24E) and spherical-shaped pores (Figure 24F) in both, smaller and larger sizes. The network was further stabilized by fine strands (e.g. Figure 24D, E and F). The areas of dark coloration formed by the network are the pores (Figure 24E, F). Fat globules were also attached onto the network (Figure 24C, G), which were about 1 µm sized.

Additionally, cryo-SEM pictures were captured from LBYA prepared from unheated LBMA and *L. plantarum* TMW 1.460 (Figure 25). Just like the pictures of LBYA obtained from UHT-heated LBMA, a high diversity of pore sizes was recognized, but not at adjacent pores: while pores in Figure 25A were mainly spherical and edgy and had a honey-comb like appearance, pores in Figure 25B were exclusively oblonged, lamellar-like. In Figure 25C, D and F fine strands are visible. Moreover, it seemed to be decisive how the sample was mounted onto the stage and how the frozen specimen was broken, as it is seen in Figure 25E and F: the specimen was broken straight while for the other picture it was broken more or less oblique. Furthermore, time of drying has a high impact: the unheated samples seem harsher heated than the UHT-heated samples.

Altogether, the gel network of the LBYA prepared from UHT-heated LMA seemed to be more ordered than from the unheated LBMA, but as mentioned before, it is decisive how the specimen was mounted and how it was broken, as well as the drying time.

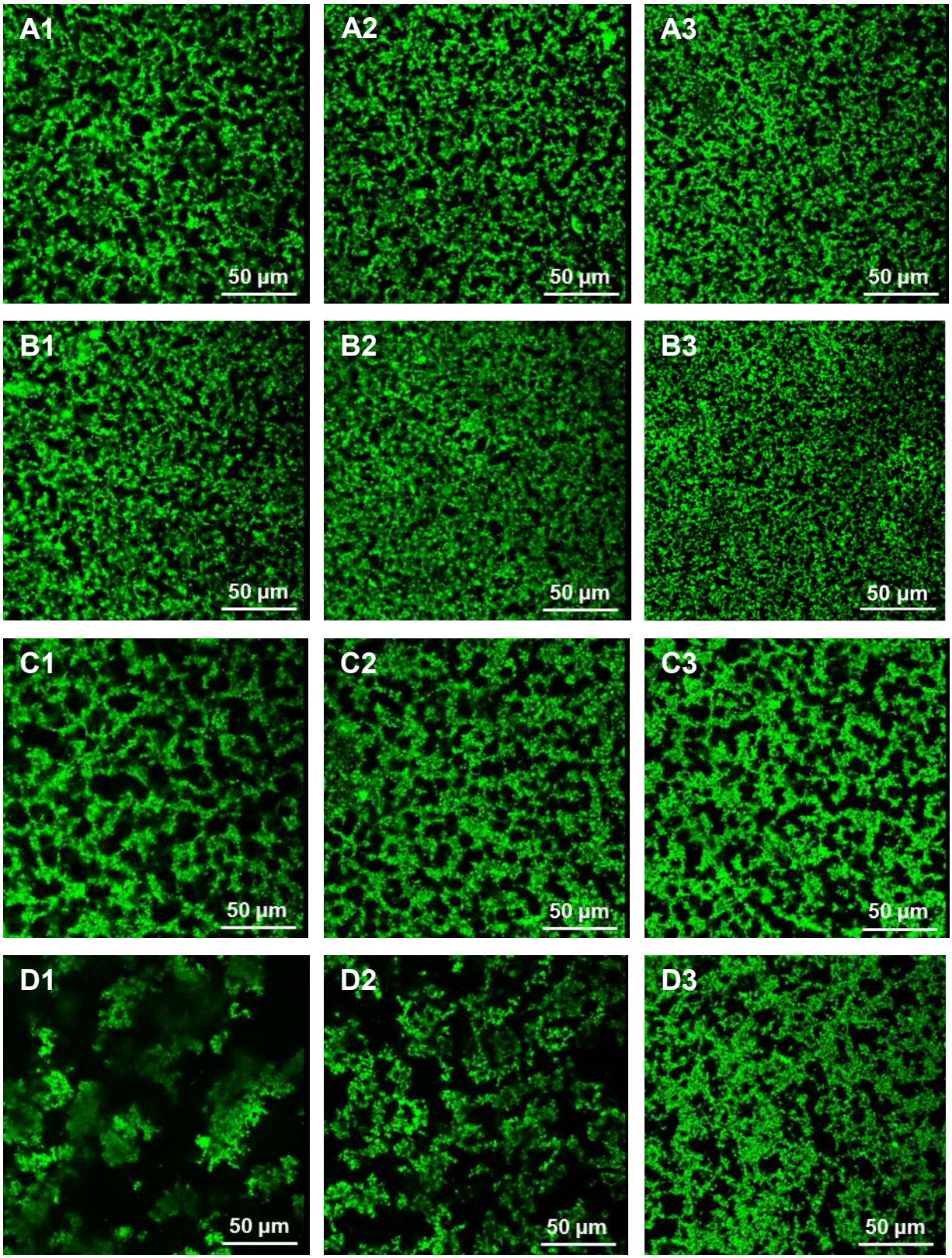

Figure 23: Microstructure of LBYA obtained by CLSM from unheated (1), pasteurized (2) and UHT-heated (3) LBMA, fermented with *L. plantarum* TMW 1.460 (A), TMW 1.1468 (B), *P. pentosaceus* BGT B34 (C) and *L. brevis* BGT L150 (D). The rhodamine B stained lupin protein appears green. The scale bars are 50 µm in length.

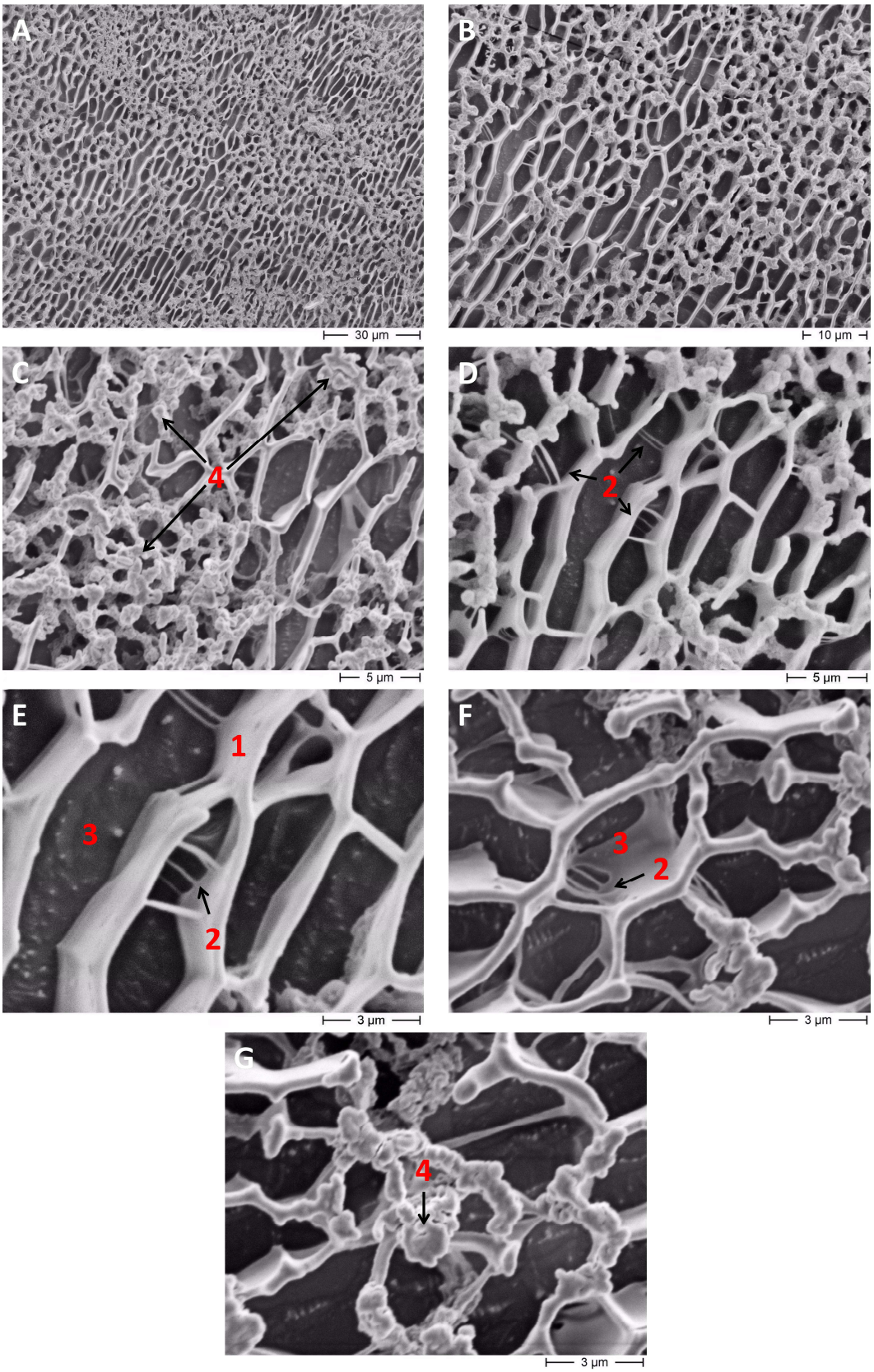

Figure 24: Microstructure of LBYA obtained by cryo-SEM from UHT-heated LBMA fermented with *L. plantarum* TMW 1.460 at different magnification (A-G); Scale bars are 3-30 µm in length; 1: lupin protein network; 2: fine strands; 3: pores; 4: fat globules.

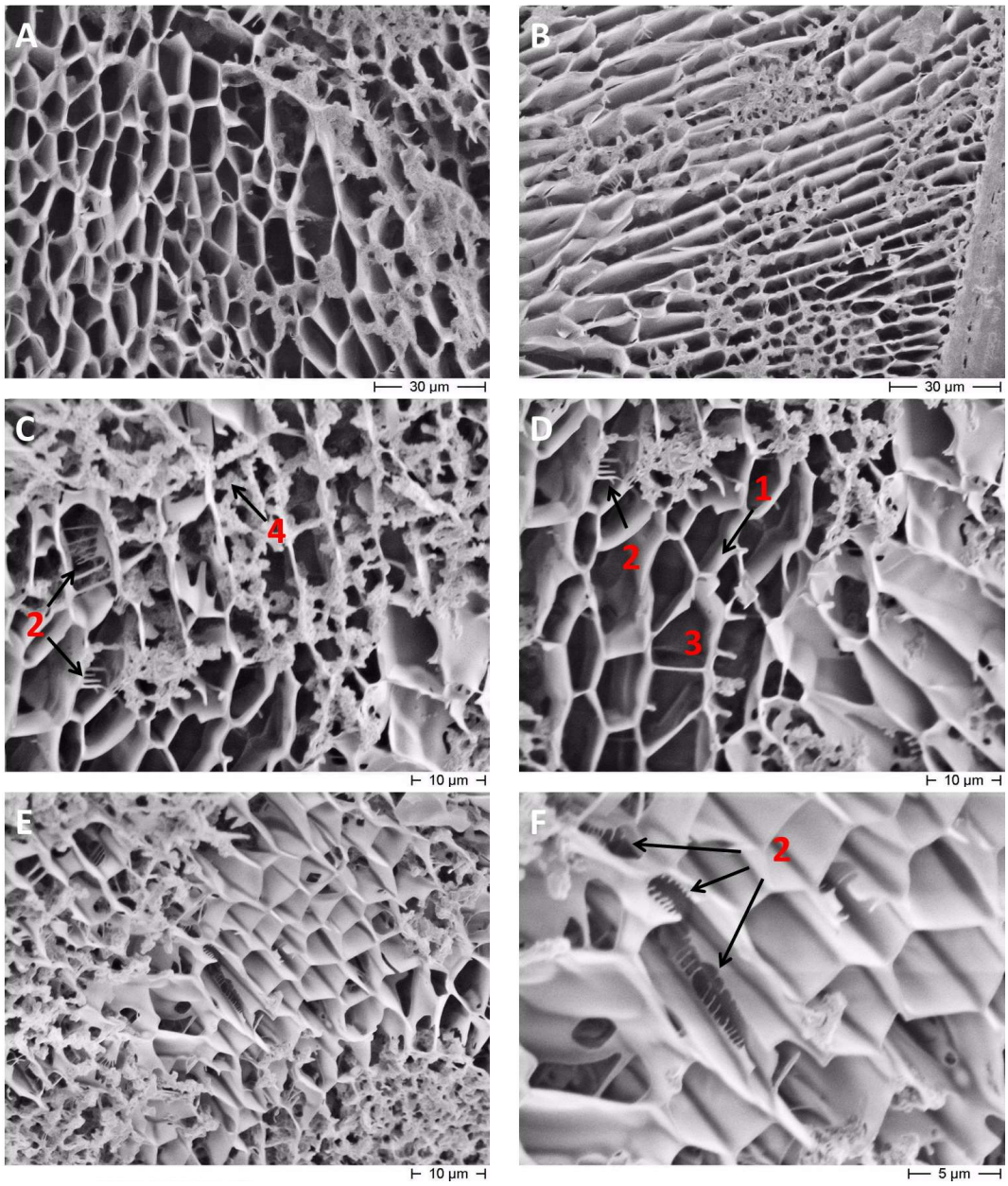

Figure 25: Microstructure of LBYA obtained by cryo-SEM from unheated LBMA fermented with *L. plantarum* TMW 1.460 at different magnification (A-F); Scale bars are 5-30 µm in length; 1: lupin protein network; 2: fine strands; 3: pores; 4: fat globules.

5 Discussion

The present study successfully demonstrated the applicability of lupin protein for the production of plant-based dairy alternatives taking yogurt alternatives as an example. For its production, the process of cow milk yogurt was adapted; thereby several key factors, which have a considerable impact on the textural properties of LBYA, were identified such as choice of microorganisms, LBMA heat treatment, inoculation size and the dry matter content. In particular, individual strains were investigated regarding their growth performance as well as their aroma and texture formation during LBYA production. These strains were further evaluated for their *in situ* EPS-expression and the influence of their produced EPS on the texture of respective LBYA. Moreover, different heat regimes were suspected to LBMA, which altered the characteristics of lupin proteins in terms of their degree of denaturation, content of SH-groups and SS-bondings, as well as their molecular weight and particle size distribution. In addition, LBMA heat treatment had a huge impact on the texture and microstructure of respective LBYA, as well as for the growth performance of different strains. These results are discussed more detailed in the following Sections to broaden the knowledge, how the texture of a plant-based yogurt alternative can be modified and optimized and how the quality of these and further upcoming plant-based products can be enhanced.

5.1 Selection of strains for lupin fermentation

The typical strains for yogurt production, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, are evolutionary adapted to grow in milk, with caseins and lactoglobulins as nitrogen source and lactose as carbon source (Zourari et al., 1992). Therefore, the initial screening for suitable strains, which were mainly representatives of LAB (cf. Table 4), was quite surprising: Although the LPI suspensions (5% (w/v)) fortified with 2% (w/v) glucose did not contain cow milk-based proteins or lactose, all tested strains were able to grow in this media: 19 out of the 35 strains even showed acidification of ΔpH ≥ 2.0 starting at a pH of 7.0, which can be recognized as a high acidification rate. A high acidification rate is quite favorable regarding product safety. However, the blank samples, which were not inoculated with strains, also showed sufficient acidification rates of 1.6. Consequently, the endogenous biota might not be eliminated completely by pasteurization (65 °C, 30 min) and might be responsible for the drop of pH. This might have also occurred in samples, e.g. with yeasts such as *Saccharomyces cerevisiae*. In these samples, the pH was as well reduced by ΔpH = 1.4,

although yeasts do not produce acids upon sugar metabolism (Rodrigues et al., 2006). Consequently the drop of pH might be attributed as well to the endogenous biota.

Fermentation of LPI solutions resulted in three main types of textured gels (cf. Figure 7): typical yogurt gels were categorized as type A gels, gels with porous structures and fissures embedding water or bubbles were designated as type B gels and finally type C emerged, which contained precipitated, cottage-cheese like formations. In general, gel formation involves three main steps: molecular unfolding (denaturation) and thereby exposing buried residues, dissociation–association of the exposed residues to form aggregates and arrangement of the aggregates into a continuous network (Batista et al., 2005; Hermansson, 1986). This arrangement of aggregated proteins can either lead to gel formation or protein precipitation (Bremer et al., 1995). In this study, it is stated, that type A gels are attributed to gel formation and type C structures lead to lupin protein precipitation. However, aggregation is dependent on the protein content of the milk alternative: when the protein concentration is high enough, aggregation leads to the formation of a gel, at lower concentrations proteins are more prone to precipitation (Renkema, 2001). In this study, all LPI solutions contained the same protein concentration; therefore the formation of different gel types cannot be attributed to protein concentration. Another reason mentioned are extensive rearrangements that may occur during or after aggregation, which may result in protein precipitation instead of gel formation. Proteins may change their position e.g. by rolling around until the protein particles acquired bonds, which leads to gel formation or precipitation (Bremer et al., 1995; Renkema, 2001). However, why strains create environmental conditions for gel formation or protein precipitation cannot be answered at this point and more studies are needed. Type B gels may also be attributed to gel formation instead of protein precipitation. However, the water inclusions of type B gels indicate, that the gel structures might not be as firm as for type A gels. Moreover, type B gels contained bubbles, indicating that gas-producing heterofermentative strains may be involved, as they metabolize glucose to lactic acid, ethanol and CO_2. However both, heterofermentatives e.g. *Bifidobacterium animalis* ssp. *lactis* DSM 10140 (Fritsch et al. (2015)) and homofermentatives e.g. *L. delbrueckii* ssp. *bulgaricus* DSM 20080 (Tamime and Robinson (2007)), belonged to type B, suggesting that heterofermentative, endogenous biota could be involved as well. Furthermore, it could be observed that the type of carbohydrate source was decisively for gel formation or protein precipitation: *L. curvatus* TMW 1.624 as well as *Brevibacterium linens* DSM 1233 built type A gels on glucose and type C gels on sucrose.

In conclusion, it was not feasible to predict the aggregation behavior of lupin proteins induced by different strains, meaning the formation of type A, B or C gels, as the chosen strains assigned to the different types of gels were quite heterogeneous. Neither the origin, nor the

metabolism of the strains gave clear information about the aggregation behavior of the lupin proteins. Nevertheless, four suitable strains were found meeting aroma perception suitable for yogurt-like products and building type A gels, although they are not commonly used as primary or secondary starter cultures for cow milk yogurt production: *L. plantarum* TMW 1.460, *L. plantarum* TMW 1.1468, *P. pediococcus* BGT B34 and *L. brevis* BGT L150.

The four selected strains were further characterized as EPS-producers (Table 12). Growth and EPS expression of *L. plantarum* TMW 1.460 and TMW 1.1468 was then further specified. Therefore, both *L. plantarum* species were cultivated in mMRS media lacking of meat and yeast extract, as those contain as well polysaccharides. The strains showed poor growth in this media (Figure 10). To stimulate cell growth and to investigate nutritional requirements, it was necessary to spike mMRS with different supplements including nucleobases, vitamins, mineral salts and cysteine. The supplemented nucleobases and salts had hardly any effect on growth, whereas vitamins (folic acid, riboflavin, cobalamin) and cysteine were identified as most sufficient growth stimuli, which could be partly confirmed by many other studies. Folic acid and cobalamin were not characterized as essential substances for the growth of *L. plantarum* (Wegkamp et al., 2010). However, riboflavin was found to be essential for many LAB (Hayek and Ibrahim, 2013): when riboflavin was omitted from a minimal medium of a *L. plantarum* strain, a severe inhibition of growth was observed (Hayek and Ibrahim, 2013; Wegkamp et al., 2010). Cysteine was shown to be stimulatory for growth of different LAB (Greene and Jezbski, 1957). Furthermore, cysteine can activate key enzymes in the EPS pathway such as phosphoglucomutase (Milstein, 1961) and UDP-glucose pyrophosphorylase (Martínez et al., 2011). As the sulfur-containing amino acid cysteine is rather low concentrated in lupin (Lqari et al., 2002; Sujak et al., 2006), the addition of cysteine-containing raw materials in applications would be useful to promote EPS production of respective strains.

EPS production in thermophilic LAB largely appears to be growth-associated (De Vuyst et al., 1998; Mende et al., 2012b; Tallon et al., 2003) and the highest EPS production was observed during the exponential growth phase (Degeest and De Vuyst, 1999; Mende et al., 2012b). Therefore, *free* and *capsular* EPS of the thermophilic *L. plantarum* TMW 1.460 was isolated shortly before the end of the exponential growth phase was reached. *L. plantarum* TMW 1.460 produced *free* and *capsular* EPS upon fermentation, which is in accordance with literature (Tallon et al., 2003), whereas *L. plantarum* TMW 1.1468 only produced *free* EPS. The function of these extracted *free* and *capsular* EPS in yogurt should be characterized, as *free* EPS are addressed to ropiness in yogurt and *capsular* EPS are known to support viscosity, creaminess and mouth thickness in yogurt (Folkenberg et al., 2006a).

5.2 Role of exopolysaccharide-producing lactic acid bacteria in lupin-based milk alternatives

The fermentation performance of four different EPS-producing LAB was monitored on differently heated LBMA (cf. 4.2). The selected strains were able to grow in LBMA while CFU/ml increased and pH dropped (Figure 11, Table 14). Growth ability of *L. plantarum* species TMW 1.460 and 1.1468 on LPI was described previously (Fritsch et al., 2015). The acidification rates were different for the selected strains. While both *L. plantarum* species and *P. pentosaceus* BGT B34 reduced the pH immediately after incubation, *L. brevis* BGT L150 had a prolonged lag phase, with stagnating cell counts in the first 10 h. This delay might be attributed to both, slow metabolism of the strain and a lack of adaptation. Slow growth is typical for beer spoilage bacterium *L. brevis* BGT L150. Indeed in beer several months can pass by until subsequent cell count can be detected (Suzuki et al., 2002). A prolonged lag phase might pose safety risks, especially in pasteurized LBMA with initial cell counts of 2.2 $\log_{10}$ CFU/ml after heat treatment. The surviving endogenous microorganisms, which are already adapted to the lupin medium, could grow rapidly during the lag phase of *L. brevis* BGT L150 and dominate the fermentation process. Beside malfermentation, also pathogenic bacteria could pose a considerable risk. In fermented LPI food pathogen *Bacillus cereus* was detected previously (Fritsch et al., 2015). But MALDI-TOF MS analyzes verified that all test strains – also the *L. brevis* strain with the prolonged lag-phase – were competitive towards the endogenous biota. This might be attributed to the high inoculation rate of 8.0 ± 0.1 $\log_{10}$ CFU/ml and the assertiveness of the strains. Finally, back-slopping experiments confirmed that slow-metabolizing *L. brevis* BGT L150 can be adapted to lupin protein medium (Figure 12), as acidification rates were increased upon the first back-slopping cycle and *L. brevis* BGT L150 was fully adapted with a completely disappeared lag phase upon the second back-slopping cycle. However, the impact of back-slopping on the texture of LBYA was not tested yet and would be an interesting approach for future studies. An additional way to accelerate the growth of this strain might be the addition of cysteine, as in mMRS media it was shown that growth can be promoted.

The intensity of the heat treatment of LBMA prior to fermentation was crucial for fermentation duration. UHT-heating provoked longer fermentation times until a pH-value below 4.5 was reached than pasteurization for all tested strains (Figure 11). Contradictory findings are stated in literature for growth behavior of LAB in cow milk: De Brabandere and De Baerdemaeker (1999) found a shorter lag phase for LAB in UHT-heated milk than in pasteurized milk, but pH decrease was nevertheless slower in UHT-heated milk. Stulova et al. (2011) detected that growth in UHT-heated cow milk was decreased or completely inhibited compared to pasteurized milk. Others did not find significant differences in

acidification (Shaker et al., 2000) or cell counts (Krasaekoopt et al., 2004) for differently heated milks. Regarding UHT-heated LBMA, it can only be assumed that growth retardation might be induced by Maillard products, arising during UHT-heating and the stronger degradation of nutrients which was already described for UHT-heated cow milk (Van Boekel, 1998). Especially the advanced Maillard products have shown high antimicrobial activity on LAB (Lanciotti et al., 1999; Stecchini et al., 1991). Beside Maillard products, other stimulatory and inhibitory components can arise upon heating such as cysteine, glutathione or thioglycolate and the expulsion of oxygen. The effect of cysteine is concentration-dependent: small amounts are stimulatory, while higher amounts are inhibitory (Greene and Jezbski, 1957). Measuring the cysteine content in differently heated LBMA would be interesting for prospective studies, as a growth stimulating effect was seen in mMRS supplemented with cysteine for *L. plantarum* TMW 1.460 (Figure 10).

Another difference from cow milk to LBMA as fermentation medium for LAB is the carbon source. In cow milk lactose is present, in LBMA glucose. However, it seems obvious that LAB are more prone to metabolize lactose than glucose, as LAB are evolutionary adapted to lactose (Zourari et al., 1992), wherefore the slow acidification might be explained as well. This was also found by Jiménez-Martínez et al. (2003), who showed that LAB metabolize lactose faster than sucrose. In addition, 14 – 35 h fermentation time is economically not efficient for LBYA production, particularly as the production of cow milk yogurt only requires 4 - 8 h fermentation time (Soukoulis et al., 2007).

EPS yields found in LBYA were comparable to EPS yields reported for cow milk yogurt (Cerning, 1995). EPS expression is growth-associated for many LAB (De Vuyst et al., 1998; Mende et al., 2012b; Tallon et al., 2003). Strains in this study showed similar cell counts when grown in UHT-heated or pasteurized LBMA, but had differently pronounced EPS yields (Table 14). Environmental factors are regulating EPS expression. Conditions or substances that boost or weaken EPS expression are strain-dependent (De Vuyst and Degeest, 1999). It is suggested that the composition of LBMA might have changed due to the heat treatment (e.g. Maillard products, degraded nutrients, stimulatory or inhibitory components) and could be the reason for different EPS expressions of LAB in UHT-heated and pasteurized LBMA. Further, the prolonged fermentation duration in UHT-heated LBMA might be related to the higher EPS-contents. So far, no profound studies dealing with the influence of heat-induced changes of media composition and prolonged fermentation durations on the expression of EPS were found. Therefore, analyzes regarding changed composition of LBMA due to thermal processing are needed and the behavior of LAB on this media has to be studied in more detail.

Isolation of EPS out of complex media like fermented milk includes limitations due to the purity of the EPS extracts (Cerning, 1995; Ruas-Madiedo and de los Reyes-Gavilán, 2005). This was also seen in our experiments irrespective whether the EPS was isolated with ethanol, 2-propanol or enzymes such as Pronase E or Alcalase (cf. Table 15). EPS is very likely embedded in a network out of proteins and other constituents of the fermentation substrate which can hardly be separated (Cerning, 1995). In this study, EPS was also extracted out of unfermented LBMA (Table 14) resulting in amounts of about 0.3 g/L EPS. Although two precipitation steps were performed to separate proteins, the result implies that the applied EPS extraction is not only addressed to microbial polysaccharides. Impurities might include residual lupin protein or plant polysaccharides like e.g. galactans and arabinogalactans known to occur in lupin (al-Kaisey and Wilkie, 1992; Duranti et al., 1981). Determination of the monosaccharide composition confirmed this observation (Table 16). All EPS samples extracted out of LBYA revealed mean values of monosaccharides at a ratio of:

galactose : arabinose / xylose : rhamnose : galactosamine = 77 : 12 : 2 : 2.

Indeed, commercial polysaccharides of *Lupinus angustifolius* L. have a similar ratio of the same monosaccharides of 77 : 14 : 3 : 5.4 (pectin galactan from Megazyme Inc., Chicago, USA). Obviously a high amount of lupin polysaccharides might be extracted. The other monosaccharides evaluated in this determination – glucose : mannose : ribose = 15 : 9 : 8 – might belong to the microbial EPS, as well as the unsolube non-detectable part of the EPS sample and possibly also galactose, rhamnose and galactoseamine units.

5.3 Thermal processing of lupin-based milk alternatives

5.3.1 Influence of thermal processing of lupin-based milk alternatives on the properties of lupin proteins

Influence of thermal processing on the thermodynamic properties of lupin proteins in lupin-based milk alternatives

The influence of homogenization and heat treatment upon LBMA production on the alteration of lupin proteins was monitored with DSC analysis (cf. 4.3.1). Prior to homogenization, a LPI suspension with 2% (w/v) of proteins and LBMA exhibited similar endothermic thermal transitions with T_d around 83 °C and 93 °C (cf. Table 17). These results are consistent with Bader et al. (2011a), who found denaturation temperatures of around 81 °C and 96 °C for LPI attributed to conglutin β and α, respectively. Conglutin β and α are the major protein

fractions of lupin proteins with about 42% and 51% followed by conglutin γ and δ, with 5% and 5 to 11% of the total lupin protein, respectively (Gulewicz et al., 2008; Plant and Moore, 1983).

Furthermore, it seemed that the individual components of the LBMA, namely fat and glucose, as well as the Ultraturrax treatment did not influence the DSC measurements as the LBMA sample prior to homogenization showed the same endothermic transitions compared to the untreated LPI. Similar results regarding mechanical stress have been found by Sirtori et al. (2010) investigating the impact of different Ultraturrax treatments on the endothermic transition of LPI solutions. Further, it was surprisingly, that the homogenization of the LBMA led to an additional peak at T_d = 71.7 °C (T_{onset} = 67.7 °C; ΔH = 0.015 J/g). Bader et al. (2011a) also described a transition peak at T_d = 70 °C as conglutin γ for defatted lupin flakes. However, conglutin γ is not contained within the LPI, as conglutin γ is removed by a previous acid-aqueous extraction step applied during LPI production. Therefore, the unknown peak was tentatively assigned to conglutin δ, as Sousa et al. (1995), studying another species of lupin (*Lupinus luteus*), found three thermal transitions, assigning the peak with the lowest T_d to conglutin δ. The occurrence of this peak after homogenization leads to the assumption that conglutin δ was released after high pressure homogenization. However, it cannot be excluded that this peak could also be an artefact, which should be investigated more carefully in further studies.

The DSC thermograms of the pasteurized samples showed that the two transition peaks for conglutin α and β remain unchanged. In contrast, the small peak at T_d= 71.7 °C that emerged after homogenization, disappeared, which indicates that this protein fraction was completely denatured upon pasteurization. UHT-heating of homogenized LBMA led to a complete denaturation of the β-fraction, since the transition peak at T_d = 83 °C completely disappeared, but conglutin α still occurred in the thermograms. Denaturation of proteins is accompanied by the cleavage of inter- and intramolecular bonds and causes thereby changes in the secondary, tertiary and quaternary protein structure (Tanford, 1968). Subunits of conglutin α are mainly linked by covalent bonds such as SS-bondings. These bonds are more heat stable than non-covalent bonds, e.g. hydrogen bonds, hydrophobic interactions and electrostatic forces, which form the protein structure of conglutin β and show lower stability at thermal processing (Duranti et al., 2008; Sirtori et al., 2010). A complete denaturation of protein fractions would be interesting in terms of textural properties of resulting products. However, Sirtori et al. (2010) showed that thermal treatments of LPI solutions with 200 °C for 15 min still showed endothermic transitions for conglutin α.

Influence of thermal processing on the sulfhydryl-disulfide transitions in lupin-based milk alternatives

The stepwise denaturation of lupin proteins was accompanied by SH-SS-transitions (Figure 13). Initially, the contents of free and total SH-groups were constant in LPI (2% (w/v)) and LBMA prior and after homogenization. This result points out that the LBMA constituents, coconut fat and glucose, as well as the Ultraturrax treatment, again did not interfere with the measurement. Pasteurization of LBMA led to a slight increase of free SH-groups, indicating that formerly masked SH-groups were partially exposed and detectable (= SH-group activation) due to partial unfolding of conglutin β (see Section 4.3.1, Table 17). The content of total SH-groups decreased slightly in pasteurized LBMA, which may be attributed to further transformations of reactive SH-groups. UHT-heating of the LBMA led to a decrease of both free and total SH-groups, reflecting that the advanced unfolding caused more exposed SH-groups which might have reacted inter- or intramolecular. The amount of SS-bondings in LPI (2% (w/v)) and LBMA before homogenization was constant with about 71% to 72%. After homogenization the content of SS-bondings was slightly increased but without statistical significance, whereas the amount of total and free SH-groups remained unchanged. Pasteurization and UHT-heating of LMBA resulted in further increases of SS-bondings contents of about 97% and 100%, respectively, compared to the unheated LBMA. Concomitantly, a drop in total SH-groups from about 21% to 17% and 14% occurred after pasteurization and UHT-treatment, respectively. This might indicate that during thermal treatment functional groups get exposed, which might be prone to covalent cross-linking.

Similar tendencies were seen upon thermal processing on cow milk: mild heating resulted in an activation of masked SH-groups (increased content of free SH-groups) while harsher heat treatment was followed by the oxidation to SS-bondings (decreased content of free SH-groups). The content of total SH-groups slightly dropped with mild heat treatment, upon severe heat treatment a more pronounced decrease was observed (Lyster, 1964; Owusu-Apenten, 2005). Besides, a positive correlation between SH-group losses and whey protein denaturation during thermal treatment in milk was found (Parnell-Clunies et al., 1988). This tendency could be also observed in our study: the more the proteins were unfolded, the more cross-linking occurred.

However, the transitions of SH- and SS-groups during thermal treatment are in contrast to the data from Berghout et al. (2015): they ascribed LPI solutions low cross-linking abilities and thus the formation of weak and deformable gels, as the content of free SH-groups increased during thermal treatment with heating performances of 75 °C, 85 °C and 95 °C for 30 min or of 80 °C for 8 h compared to a unheated sample. Unfortunately, the authors did not

give an explanation for the strong increase of the free SH-bonds. In contrast, the same authors observed a decrease of free SH-groups in soy protein isolate solutions upon the heating (Berghout et al., 2015). The discrepancy between the results from Berghout et al. (2015) and this study might be attributed to the different selected temperature-time regimes: Berghout et al. (2015) chose moderate temperatures but high heating times of 30 min or 8 h, while in this thesis pasteurization was performed at 80 °C for 60 s and UHT-heating at 140 °C for 10 s. It would be helpful to gather more data about SH-SS-transitions during heat treatment of lupin proteins taking different LPI concentrations and temperature-time heat regimes into consideration. In addition, influence of cooling of the LPI solutions should also be taken into account.

Influence of thermal processing on the molecular weight distribution of lupin proteins in lupin-based milk alternatives

Alterations of the molecular weight pattern of the lupin protein molecules were displayed in Figure 14 a und b. Under reducing conditions (Figure 14a), the band intensities of the LBMA samples (line B-D) were more intensive than the band intensities of the LPI (2%, w/w) solutions (line A). Furthermore, the bands of the LBMA samples showed a slight smearing in comparison to the bands of the LPI solution. However, the protein pattern did not differ considerably among the samples. These findings implicate that the molecular weight distribution of the protein fractions were not affected during thermal treatment. In contrast, some proteins accumulate in the pocket of the gradient gel (Figure 14a, lines B-D), which is in accordance to Meinlschmidt et al. (2016b). It seems that the amount of accumulated proteins was higher in the UHT treated sample (line D) than in the non-treated and pasteurized ones. Probably, UHT-treatment caused an aggregation of protein molecules resulting in large molecular weight proteins unable to shift into the gels. These observations were even more pronounced for the experiments under non-reducing conditions (b). The amount of proteins accumulating in the pockets was dependent on the respective processing. Thermal treatment enhanced the accumulation and consequently the denaturation, which corroborate the data of the DSC measurements (cf. Section 4.3.1). Under non-reducing conditions differences were apparent: Two bands at a molecular weight of approximately 30 kDa and 40 kDa gradually disappeared upon thermal treatment and new bands between 30 kDa and 40 kDa emerged in the pasteurized LBMA sample and broadened in UHT-heated LBMA. These disappearing bands in the IMW range can be assigned to conglutin β (cf. Figure 14). Conglutin β is thermally less stable as conglutin α, as it is not stabilized via covalent SS-bondings (Duranti et al., 2008), therefore it is more prone

to alterations. However, mass spectrometric determination for the identification of the individual bands should be performed in future studies. The alteration of the IMW range of conglutin β may have a positive impact on gel firmness, as rheological and textural properties were enhanced upon successive protein denaturation (cf. Table 18).

Obviously more changes were seen for the lupin protein fraction conglutin β. Therefore, it would be interesting to investigate other lupin species with a higher pronounced content of conglutin β. *Lupinus albus* L. could be taken into account as the ratio of conglutin α to β amounts to 0.9 : 1, whereas the ratio of conglutin α to β in *Lupinus angustifolius* L. is 2 : 1 (Sirtori et al., 2008). However, it was just assumed that conglutin β has a higher contribution to gel strength than conglutin α, as conglutin α was not denatured completely in this study. Therefore, the effect of denatured conglutin α on its gelling ability has to be investigated furthermore. In general, the content of the different protein fractions, their degree of denaturation and their ability for cross-linking such as SS-bondings, as well as hydrophobic interactions determine their contribution to gel firmness. To prove the contribution of the individual fractions to gel firmness, pure protein fractions of α and β must be gained and characterized.

Influence of thermal processing on the particle size distribution of lupin proteins in lupin-based milk alternatives

Particle size distribution was unimodal for LPI (2% (w/v)) and non-homogenized LBMA with particle diameters ranging from 0.1 to 70 µm, with slightly smaller particle sizes for the latter (Figure 15). Upon homogenization of the LBMA, particle sizes were shifted towards smaller particles resulting in a bimodal distribution. Furthermore, heat treatment of LBMA promoted the formation of even smaller particles with an almost unimodal distribution with particle diameters from 0.1 – 5 µm. Generally, heat denaturation results in aggregation of proteins and therefore the development of larger particle sizes (Cheftel et al., 1992), which is in accordance to the SDS-PAGE pattern, as the coloration of the pockets – caused by the aggregated proteins – increased with increasing heat treatment (Figure 14). However, in this study, particle size distribution of all samples was measured one day after LBMA production. The bigger particles of unheated LBMA might be attributed to the overnight storage accompanied by coalescence of particles, which obviously not occurred in heated samples. In general, particle sizes are highly correlated to the stability of milk products, with the most stable milk having the smallest particles taking both cow milk and plant-based milk alternatives into consideration. Particle size distribution of the UHT-heated LBMA was similar to UHT-heated cow milk and soy-based milk alternative, as particle diameters ranged

unimodal distributed from 0.1 – 3 µm. However, when separation of the UHT-heated cow milk and soy-based milk alternative was induced by a light centrifugation, a bimodal particle size distribution occurred, with particle sizes comparable to the unheated LBMA in this study (Durand et al., 2003). This supports the assumption, that the bigger particles in the unheated milk might be attributed to the coalescence of particles.

5.3.2 Influence of thermal processing of lupin-based milk alternatives on the texture of respective lupin-based yogurt alternatives

The texture of LBYA was significantly influenced by milk heating. UHT-heating of LBMA led to better rheological (Table 18, Figure 17 and Figure 18) and textural (Figure 16) properties in comparison to pasteurization, as well as lower susceptibility to syneresis (Table 19) of the respective LBYA compared to pasteurization of LBMA. In detail, LBYA prepared out of UHT-heated LBMA exhibited increased levels of apparent viscosity. These findings are in accordance with results reported for cow milk. A more intense heat treatment led to a higher level of apparent viscosity in cow milk yogurt. In comparison, apparent viscosity (at 50 s^{-1}) of cow milk yogurt was on an average level significantly higher (~2000-2400 mPas) than for LBYA (~350-1180 mPas) (Lee and Lucey, 2006), indicating that LBYA gels are not characterized by a strong gel network. Besides, LBYA prepared out of the more intense heated UHT-heated LBMA showed a higher stability against shear expressed as hysteresis loop area. Hysteresis loop area is a measure for structure regeneration after shear-induced structure breakdown. In general, yogurt is categorized as ‘incomplete’ or ‘false’ thixotropic material since structural breakdown during the shearing cycle is not completely reversible once the shear stops and during a period of rest (Lee and Lucey, 2010). This is also true for LBYA: the samples cannot regain its initial structure completely, which was more obvious when samples were only pasteurized. Yield points, as well as flow points occurred at higher amplitudes in UHT-heated LBYA than in the corresponding pasteurized LBYA, marking a higher level of rigidity and elasticity in these samples. Higher dynamic moduli, namely G’, G’’ and G* were observed for LBYA manufactured of UHT-heated LBMA. In accordance, the correlation of milk heat treatment and rheological parameters has frequently been found for cow milk. Lucey (2004) reported that a more intense heat treatment resulted in higher values for G’ of the respective yogurts. These results were emphasized by the propagation of *L. plantarum* TMW 1.460 in unheated LBMA: Resulting LBYA showed lower measured values for all rheological parameters.

Syneresis was determined with two different methods. The siphon method regards only the expelled water on top of the yogurt, which is built without any external force. Hence, it

reflects the spontaneous syneresis. The centrifugation method represents the water-holding capacity, measuring the water on top of the product and the water entrapped in the network with a strong susceptibility to flow out during storage (Amatayakul et al., 2006b). LBYA prepared out of UHT-heated LBMA had a lower tendency to syneresis independently which strain and measuring method was used. For most strains, similar volumes were measured with both methods with the exception of *P. pentosaceus* BGT B34. After fermentation and cooling only little syneresis (< 2%, siphon method) for both LBYA varieties was observed, while the centrifugation method showed five times higher volumes, indicating that these samples are more likely to syneresis during storage. Another factor that may influence the extent of syneresis and the texture of yogurt is the fermentation duration (Beal et al., 1999). Some researchers stated that longer fermentation times might lead to structural rearrangements and therefore, lower gel stability and higher levels of syneresis, as yogurt networks are relatively dynamic systems that are prone to structural rearrangements (Lee and Lucey, 2004; Purohit et al., 2009). Others found that the most viscous yogurt was obtained with the longest fermentation times (Beal et al., 1999), which is in agreement to the results found in this study. The effect of EPS on the texture of yogurt and syneresis was frequently discussed in literature. EPS have the ability to bind water and enhance viscosity and firmness of gels (De Vuyst and Degeest, 1999; Folkenberg et al., 2006b; Hassan et al., 2001). The functionality of EPS depends on their structural characteristics (e.g. molar mass, degree of branching, charge) (De Vuyst and Degeest, 1999) and also the concentration of EPS (Mende et al., 2013b). Interestingly, in our study higher EPS concentrations were found in LBYA produced out of UHT-heated LBMA (Table 14), which showed also better textural properties and a lower degree of syneresis. However, similar amounts of EPS of both *L. plantarum* species and *P. pentosaceus* BGT B34 were extracted from UHT-heated LBYA with different textural behavior and degree of syneresis. Therefore, structural characterizations of the extracted EPS are needed to determine structure-function relationship.

The effects of the different applied LAB on the texture of LBYA showed irregularities among the different texture measurements. While rotational measurements favored *L. brevis* BGT L150 with the highest hysteresis loop area and apparent viscosity by far, oscillation measurements of this culture showed the lowest values, e.g. lowest flow point or dynamic moduli. In contrast, *L. plantarum* TMW 1.460 had remarkably the highest values in oscillatory measurements compared to the other cultures. Within this study it was obvious that LBYA prepared out of *L. brevis* BGT L150 clearly differed in its visual appearance to the others. While LBYA out of the other cultures had a yogurt-like texture, *L. brevis* LBYA was visibly slimy, which can be ascribed to the *in situ* produced EPS. The sliminess can be related to the

high values in rotational measurements. The differences in the parameters can also be attributed to the different magnitude of applied force. Oscillation measurements were performed within the linear viscoelastic region, where the structure of the network is not broken. Its sensitivity to detect the different interactions is higher than in rotational tests or with the texture analyzer, in which the macroscopic failure of samples is detected (Ferragut et al., 2009). Nevertheless, measurements under small and large deformations measurements must be performed to closely imitate stress-strain-conditions which are relevant for food production and consumption (Angioloni and Collar, 2009).

Significant correlations were explored between many of the rheological and textural properties, as well as syneresis parameters. As described before, parameter within one type of measurement correlated highly e.g. η_a and A_H. A correlation between η_a and A_H was also reported previously in cow milk yogurt (Mende et al., 2012a). Moreover, the positive and negative maximum compression forces firmness and cohesiveness were related to all oscillation parameters. However, consistency and index of viscosity the positive and negative peak areas are correlated to the rotational measurements. This is quite surprising as already stated before oscillation measurements are performed under small deformation, without breaking the network, whereas rotational and texture analyzer measurements are performed under higher deformation. Interestingly, the extent of syneresis of both measurements was positively correlated with consistency and negatively with cohesiveness. It is proposed that the parameters consistency and cohesiveness can be identified as key textural parameters that act as indicators to predict syneresis. Susceptibility to syneresis is an important quality defect, which is induced without any external force (Amatayakul et al., 2006b). Manufacturers may add stabilizer to prevent syneresis. However, there is a growing demand of consumers for natural products, so called “clean label” products. Therefore, a better understanding of rheological and textural properties may be helpful to improve yogurt quality.

Different inoculum sizes and increased dry matter were further evaluated as another approach to enhance textural properties. Magnitude of inoculum size can be included to the texture-enhancing parameter for LBYA, as especially levels of firmness and consistency were increased when LBMA were inoculated with 8.0 instead of 7.0 $\log_{10}$ CFU/ml (Figure 16). Such an impact of inoculum sizes was also seen for cow milk yogurt by other authors (Kristo et al., 2003; Lee and Lucey, 2004). Nevertheless, an initial bacterial load of 8.0 $\log_{10}$ CFU/ml is quite high and from an economic perspective cost-ineffective and difficult in terms of realization. Moreover, for cow milk yogurt production, inoculum sizes of only 5.0 – 6.0 $\log_{10}$ CFU/ml are utilized as LAB are adapted to lactose and cow milk proteins (Zourari et al., 1992). However, Kristo et al. (2003) as well as Lee and Lucey (2004) found not only a relation between inoculum sizes and the texture of respective yogurts for cow milk-based

products, favoring higher inoculum sizes. Furthermore, they investigated that this effect was enhanced, when the incubation temperature was decreased. To include different incubation temperatures into the setup described in this thesis could be another approach for future studies enhancing textural properties of LBYA.

Dry matter fortification of cow milk is a common method to enhance textural properties of cow milk yogurt, e.g. by adding skimmed milk powder, caseins, coagulants or stabilizers (Tamime and Robinson, 2007). In contrast to cow milk, which has a naturally defined composition, for LBYA the formula of the LBMA can be altered quite easy e.g. by raising the protein content instead of adding other substances. In this study, the lupin protein content was raised from 2.0% up to 4.8% and the sugar content from 4.0% to 5.5%. This increase had a massive effect on the rheological and textural properties, as well as for susceptibility to syneresis of LBYA. Higher maximum shear rates and area of hysteresis was observed for LBYA with increased dry matter compared to standard LBYA (Figure 20). The firmness and consistency was twice as high compared to the standard dry matter content (Figure 21). It is suggested that the increased firmness is related to a higher amount of cross-linkings, as the reactive residuals are closer together (Cheftel et al., 1992). The level of syneresis was reduced from 11% to 0.2% for siphon method and from 11% to 5.4% for the centrifugation method (Figure 22). However, rotational measurement displayed distinct differences between the LBYA and commercial samples, such as different cow milk yogurts and a soy-based yogurt: Regarding textural properties such as firmness, LBYA with increased dry matter contents were comparable to commercial products without adding additives such as stabilizers. However, rotational measurement showed striking differences to a commercial cow milk yogurt and soy milk-based yogurts. The flow curve of the cow milk-based yogurt showed the typical rheological behavior of yogurt, at first the shear stress increased and was then constant. However, the soy-based sample showed an increasing shear stress over the entire upward flow curve, which can be explained as this sample can be recognized as a mixed gelling system containing protein and pectin as gelling agent. The flow curve of the LBYA, which is a protein gel solely, with standard as well as increased dry matter also showed an increasing shear stress at first, which then flattened slightly instead of being constant. Also lower maximum shear stress were obvious for LBYA compared to cow milk-based yogurt, which suggests that LBYA are less stable and can therefore resist less to the applied shear rate. In accordance to these results further dry matter fortifications might be useful and other texture-promoting actions should be taken into account.

5.3.3 Influence of thermal processing of lupin-based milk alternatives on the microstructure of respective lupin-based yogurt alternatives

CLSM micrographs clearly demonstrated that the microstructure of LBYA is primary influenced by the choice of microorganisms and secondary by the intensity of heat treatment of LBMA (4.5.1, Figure 23). LBYA out of *L. plantarum* species revealed a relatively close-meshed lupin protein network, while networks of LBYA out of *P. pentosaceus* were significantly more porous. In contrast, LBYA from *L. brevis* had a completely different microstructural appearance compared to the other strains: Depending on the applied heat treatment a relatively loose network was observed. It was shown before that all applied strains produce EPS in LBMA (cf. 4.2.3). Thereby, the EPS produced by *L. brevis* BGT L150 was visibly slimy in LBYA compared to the LBYA of the other strains. It can be assumed that these high viscous EPS of *L. brevis* may act as inert fillers and may prevent protein-protein interaction such as SS-bondings, which may explain the loose network especially in unheated LBMA for *L. brevis* BGT L150. Interference or prevention of protein-protein interactions was described before for EPS in cow milk yogurt (Zhang et al., 2016) and in protein-polysaccharide solutions (De Kruif and Tuinier, 2001). In contrast, EPS of both *L. plantarum* strains and of *P. pentosaceus* might not disturb the formation of a protein network.

Moreover, heat treatment of LBMA conspicuously influenced microstructure of LBYA: The more intense the LBMA was heated (UHT-heating), the more close-meshed microstructures were observed. This was discovered for all tested strains, but especially for *L. brevis* BGT L150. Network density might be attributed to the number of physiochemical bonds due to the denatured character of the proteins e.g. the content of SS-bondings (cf. Section 4.3.1 and 4.3.2), which is more thoroughly described in Section 5.4. Influence of heat treatment prior to fermentation on the pore pattern was also seen for cow milk yogurt: smaller pores appeared in the network of respective yogurts when milk was stronger heated; moreover, network was thicker, less tortuous clustered and more branched applying higher temperatures for longer times (Lee and Lucey, 2006; Lucey et al., 1999).

A deeper insight allowed micrographs obtained from cryo-SEM (cf. Section 4.5.2 and Figure 24 and Figure 25). The relatively closed-meshed LBYA prepared with *L. plantarum* TMW 1.460 was characterized in more detail: A fine stranded, ordered and dense network was seen including interspaces of different sizes and shapes: oblonged, lamellar-like, edgy and spherical shaped pores. These fine stranded pores might be caused by a high number of physiochemical bonds due to the denatured character of the proteins, as a high content of SS-bondings in UHT-heated LBMA was seen previously (cf. Section 4.3.1 and 4.3.2). Moreover, these pores might act as pockets where it is assumed that LAB, EPS, fat particles

and water (that has sublimed during sample preparation) are embedded or affiliated. Fat particles appeared quite small as 1 µm sized spheres. It seems that this is why it was not possible to detect them with CLSM.

5.4 Impact of thermal-induced protein alteration on the texture and microstructure of lupin-based yogurt alternatives

LBYA manufactured out of unheated, pasteurized or UHT-heated LBMA revealed fundamental different physical properties: yogurt alternatives from UHT-heated LBMA exhibited better textural and rheological properties and had lower tendencies to syneresis than LBYA out of pasteurized or unheated LBMA (Section 4.4). Furthermore, LBYA made from UHT-heated LBMA exhibited denser and more close-meshed networks for all tested LAB (Section 4.5). The UHT-heating of the LBMA resulted in higher amounts of unfolded lupin proteins compared to the samples obtained from the pasteurized and the non-heated LBMA, respectively. This subsequent unfolding was accompanied by the decrease of free and total SH-content and an increase of SS-bondings with harsher heating (Section 4.3).

This work suggests that untreated more native lupin proteins have only little impact on the texture of LBYA and its ability to embed water properly in the pores. However, when lupin protein fractions were denatured partially or completely upon heating, reactive side chains get exposed due to unfolding of the proteins. These reactive side chains favor intra- und intermolecular cross-linkings such as SS-bondings. After heat treatment, LBMA were propagated with LAB converting glucose to lactic acid and thereby lowering the pH-value. It seems reasonable that upon acidification the LBMA with the higher ratio of unfolded lupin proteins were more prone to build further linkages upon fermentation as higher viscous and elastic moduli were observed for LBYA manufactured out of the harsher heated LBMA (Table 18). The viscous and elastic moduli are proportional to the number of bonds in a gel network and thus to the gel strength (Lucey et al., 1997; Yang et al., 2012). Further, less syneresis occurred in LBYA out of the harsher heated LBMA with the higher ratio of denatured lupin proteins, which also indicates that more linkages were built embedding serum water properly. Moreover, CLSM micrographs pictured that LBMA out of more denatured LBMA had a more close-meshed network, which also indicates that more linkages were built upon acidification with UHT-heated LBMA than with pasteurized or unheated LBMA. Finally, denatured lupin proteins aggregate when the pH approaches the IP of the lupin proteins (between 5.0 – 6.0 for conglutin α and β (Duranti et al., 2008)).

These relations were also found for cow milk yogurt: stepwise unfolding of whey proteins occurred due to thermal treatment, consequently sidechains like SH-groups were disclosed, which were more prone to covalent cross-linking to SS-bondings with the casein micelles (Lee and Lucey, 2003; Lyster, 1964). The extent of unfolding is therefore crucial: if denaturation of proteins was increased by more intense heat treatments and thus the extent of linkages, firmness and viscosity of the respective yogurts were affected proportionally (Dannenberg and Kessler, 1988; Lucey et al., 1997).

In addition, it seems that the fermentation duration is also related to textural properties of LBYA: During acidification LAB needed significantly longer to reduce the pH to 4.5 in UHT-heated LBMA than in pasteurized LBMA (Figure 11). It is suggested that longer fermentation times lead to denser networks and thus better textural properties, as more extensive structure reorganization and rearrangements during gel formation can occur. Moreover the susceptibility to syneresis, which is a consequence of network density, can be influenced by heat treatment. It was shown that weak cow milk yogurt gels with a low extent of linkages were not likely to entrap water within their network (Remeuf et al., 2003). This was also seen for LBYA: best enclosing of water was investigated for LBYA out of UHT-heated LBMA, with the highest network density and highest content of SS-bondings (Table 19, Figure 13).

The network of cow milk yogurt is described as an ordered, honeycomb-like net with interspaces of different sizes which is associated with its soft texture (Tamime et al., 1984; Yang et al., 2012), while microstructures of conventionally manufactured soy-based yogurt alternatives are related to compact and rigid networks accounting to firm and brittle textures. Thus, approaches for soy-based yogurt alternatives are directed towards lowering gel strength and smoothening the texture and therefore stretching the microstructure through a lower number of physical bonds (Yang et al., 2012). However, microstructure of LBYA showed a high diversity of network formations and differently shaped pores depending on the used strain and LBMA (Section 4.5); it is assumed that the oblonged-shaped pores, lacking of stabilizing strands, might weaken the network and should be strengthened by more linkages (Figure 24E). Therefore, optimizations should be addressed with regard to higher branched, more evenly arranged networks, especially in terms of storage and to receive a network more adapted to cow milk yogurt with lower susceptibility to syneresis. As mentioned before a lupin variety with a higher ratio of conglutin β to α could be a way to achieve more branchings, as conglutin β is more heat labile and therefore more prone to unfolding, thereby exposing reactive groups and potentially building more linkages. Another approach could involve an intensified time-temperature heat regime for LBMA before fermentation: as it was shown for UHT-heated LBMA conglutin α was not completely denatured and thus potentially some reactive groups are still embedded in the core.

6 Summary

Interest of consumers in vegetarian or vegan nutrition has been rising steadily in recent years. Health related reasons, ethical or sustainability issues lead to diets free from animal-based products. An increase of the consumption of plant-derived products like dairy alternatives can only be achieved on a long-term basis, if these products meet consumer's acceptance regarding taste and texture. So far, main research of dairy alternatives has been focused on soy-based products like milk and yogurt alternatives. However, the demand for other plant-based protein sources increases, due to consumers rising awareness about genetically modified crops and their demand for sustainable and regional cultivated crops. Therefore, lupin proteins are a promising source for the production of dairy alternatives, as lupins are cultivated regionally and genetically-free. Besides, the protein content in the seeds is comparable to soy and of high nutritional value.

This study was dedicated to the development of a plant-based yogurt alternative taking lupin protein as raw material. For the fermentation of lupin proteins, a screening of different microorganisms was performed, to find appropriate strains for the production of lupin-based yogurt alternatives. Concomitantly, a new process for the production of this yogurt alternative was established, adapting the partial steps of traditional cow milk yogurt production. Thereby, key process parameters influencing yogurt texture were identified. Furthermore, the impact of heat treatment of lupin-based milk alternatives on the growth performance of selected strains was evaluated. The *in situ* exopolysaccharide production of the selected strains and the influence of exopolysaccharides on the texture of respective lupin-based yogurt alternatives were also investigated. Finally, the impact of heat treatment of lupin-based milk alternatives on gel properties in the respective lupin-based yogurt alternatives was examined. These findings are important for gel-network formation and concomitantly for texture perception and water-holding capacity of the respective products.

First of all, a screening of 35 different microorganisms on lupin protein isolate suspensions was performed focusing on their ability to acidify the medium sufficiently and to impart the products yogurt-like aroma and texture. *Lactobacillus plantarum* TMW 1.460 and TMW 1.1468, *Pediococcus pentosaceus* BGT B34 and *Lactobacillus brevis* BGT L150 turned out to produce the most acceptable aroma notes and a creamy yogurt-like texture, while the growth of the other microorganisms were characterized by a unpleasant smell or atypical yogurt texture. All four strains are not known as common primary or secondary starters for yogurt, but were more promising on the plant material as the well-known yogurt starters.

For the production of a lupin-based yogurt alternative the adaption of the traditional process for cow milk yogurt was necessary. Prior to the fermentation step, a stable emulsion containing certain amounts of lupin protein, fat and sugar, respectively, was developed. The milk proteins were substituted by lupin proteins, which behave fundamentally different upon heating and fermentation. Moreover, lactose was replaced by glucose and coconut fat was used instead of milk fat. Beside the types of milk alternative compounds, the amounts of lupin proteins and the sugar were also varied to meet dry matter contents in the range of cow milk yogurt. After fermentation, the lupin-based yogurt alternatives showed the behavior of weak, viscoelastic gels as typical for cow milk yogurt and can be recognized as a shear-thinning fluid. However, even with increased dry matter, maximum shear stress and the area of hysteresis loop were significantly lower than for cow milk yogurt, which can be attributed to the poor gelling abilities of lupin proteins. Further differences to the production of cow milk yogurt turned out: Inoculation sizes of 8.0 $\log_{10}$ colony-forming units per millimeter were necessary to obtain a yogurt-like texture of the products. Although the inoculum sizes were very high, the fermentation time took about 14 h up to 35 h to reduce the pH from 7.0 to 4.5 depending on the strain and the applied heat treatment for the lupin-based milk alternative. These quite long fermentation durations might be attributed to the fact that strains are not adapted to the lupin protein and glucose containing substrate. This hypothesis was confirmed with back-slopping experiments performed with *Lactobacillus brevis* BGT L150 in the lupin-based milk alternative. It was shown, that the fermentation time was reduced from 24 h to 14 h, as the lag phase of 10 h completely disappeared by a triple repeated reinoculation circle of *Lactobacillus brevis* BGT L150 in fresh lupin-based milk alternative.

To modify the functional properties of lupin protein, the influence of the thermal treatment on the lupin protein properties within the emulsion was evaluated. Therefore, the emulsion was pasteurized applying 80 °C for 60 s and ultra-high temperature at 140 °C for 10 s. A sample, which was not thermally treated, was used as reference. The thermal treatment led to a stepwise denaturation of the lupin protein fractions, conglutin α and conglutin β, which was accompanied by the exposure of masked sulfhydryl groups, the decrease of free sulfhydryl groups and reoxidation to disulfide bondings. These alterations were dependent on the strength of the chosen heat treatment and were more pronounced with a more intense heat treatment. Moreover, the molecular weight distribution of conglutin β in the intermediate molecular weight region between 30 kDa und 40 kDa was influenced upon heat treatment. Concise bands in this region disappeared, while new bands emerged. Additionally, particle size distribution showed that homogenization and heat treatment promoted the formation of smaller particles.

The effect of thermal treatment on the growth behavior and exopolysaccharide formation of lactic acid bacteria, on the texture formation of lupin-based yogurt alternatives, on the susceptibility to syneresis and on the network of lupin-based yogurt alternatives was investigated. Therefore, the differently heated lupin-based milk alternatives were inoculated with the four chosen lactic acid bacteria. It turned out, that the fermentation duration was strongly affected by the intensity of heat treatment of the lupin-based milk alternatives: the four different strains needed significantly longer to reach a pH of 4.5, when grown in ultra-high temperature heated lupin-based milk alternative (between 25 to 35 h), compared to their growth in pasteurized lupin-based milk alternative (14 to 24 h). Furthermore, slightly higher amounts of exopolysaccharides were metabolized in ultra-high temperature heated lupin-based milk alternatives than in pasteurized lupin-based milk alternatives, which might be attributed to longer fermentation durations. Moreover, heat treatment of lupin-based milk alternatives significantly influenced the physical properties of respective lupin-based yogurt alternatives: The more intensive heat treatment of lupin-based milk alternatives (ultra-high temperature heating) resulted in higher levels of apparent viscosity and hysteresis loop area, as well as storage and loss moduli of the respective lupin-based yogurt alternatives than pasteurization. Furthermore, lupin-based yogurt alternatives produced out of ultra-high temperature heated lupin-based milk alternatives revealed a lower tendency to syneresis.

The protein networks were visualized with confocal laser scanning microscopy and cryo-scanning electron microscopy. It was shown that the ultra-high temperature heating of lupin-based milk alternatives led to more close-meshed microstructures of respective lupin-based yogurt alternatives prepared with different strains than pasteurized or unheated lupin-based milk alternatives. Beside the thermal treatment, the choice of microorganisms seemed to play also a decisive role in microstructural appearance of respective lupin-based yogurt alternatives as the composition of the networks were fundamental different. *Lactobacillus plantarum* TMW 1.460 and TMW 1.1468 resulted in quite close-meshed networks with interspaces of small sizes, *Pediococcus pentosaceus* BGT B34 revealed a more porous structure with larger pores, whereas for *Lactobacillus brevis* BGT L150 the microstructural appearance differed considerably in dependence on the applied heat treatment: in yogurt out of unheated lupin-based milk alternatives only a loose formation of aggregated proteins was formed, which can hardly be considered as network. Using the pasteurized lupin-based milk alternative, a loose network with some branchings was formed, whereas a denser network with unevenly distributed voids of different sizes was obained in yogurts produced out of ultra-high temperature heated lupin-based milk alternative.

These studies in lupin-based milk and yogurt alternatives suggest that non-treated lupin proteins have less impact on the texture of lupin-based yogurt alternatives. Moreover the

ability of non-treated lupin proteins to embed water properly in the pores of the network was poor. However, when lupin protein fractions were denatured partially or completely upon heating, reactive side chains get exposed due to unfolding of the proteins. These reactive side chains facilitate cross-linkings such as disulfide bondings. Upon the followed fermentation, it seems reasonable that the lupin-based milk alternatives with the higher ratio of unfolded lupin proteins were more prone to build further linkages as higher viscous and elastic moduli were observed for lupin-based yogurt alternatives manufactured out of the harsher heated lupin-based milk alternatives. Therefore, heat treatment of lupin-based milk alternatives is crucial to reveal highly stranded protein networks of respective lupin-based yogurt alternatives with textures of high firmness and low susceptibility to syneresis.

This study shows, that lupin protein isolate from *Lupinus angustifolius* cv. Boregine is a suitable substrate for the production of lupin-based yogurt alternatives, although lupin protein is considered as poor gelling agent in comparison to soy. This work contributes to the elucidation of the mechanisms for the modification of the texture of yogurt alternatives and creates the basis for upcoming studies with other plant-based raw materials. The findings of this work are already in application: The Prolupin Ltd sells the lupin-based yogurt alternative with the brand “Made with LUVE” since 2015 in Germany, which is based on the recipes and the production process of this study. Since November 2017 starter cultures containing a combination of *Lactobacillus plantarum* and *Pediococcus pentosaceus* are commercially available, which are in particular intended for plant-based dairy alternatives. Those strains were designated as appropriate starter cultures for dairy alternatives for the first time in the publication Hickisch et al. 2016 included in this work.

7 Zusammenfassung

Das Interesse der Verbraucher an vegetarischer oder veganer Ernährung ist in den letzten Jahren stetig angestiegen. Gesundheitsbedingte Aspekte, ethische Vorbehalte oder der Wunsch nach nachhaltigeren Produkten führen zu Ernährungsstilen frei von tierischen Produkten. Ein steigender Verzehr pflanzlicher Erzeugnisse wie von Milchersatzprodukten kann langfristig nur erreicht werden, wenn die Produkte den Erwartungen der Konsumenten bezüglich Geschmack und Textur gerecht werden. Bislang lag der Schwerpunkt der Forschung zu Milchersatzprodukten hauptsächlich auf soja-basierten Produkten wie Milch- und Joghurtalternativen. Jedoch steigt die Nachfrage nach anderen pflanzlichen Proteinquellen, aufgrund der wachsenden Vorbehalte der Konsumenten gegenüber gentechnisch veränderten Pflanzen und ihrer Forderung nach nachhaltigen und regional angebauten Pflanzen. Lupinenproteine stellen eine vielversprechende Quelle für Milchersatzprodukte dar. Nicht nur aufgrund ihrer regionalen, gentechnikfreien Kultivierung, sondern weil der Proteingehalt in den Samen vergleichbar zu dem in Soja ist und einen hohen Nährwert aufweist.

Ziel dieser Dissertation war die Entwicklung einer pflanzlichen Joghurtalternative am Beispiel von Lupinenproteinen als Rohmaterial. Für die Fermentation von Lupinenproteinen wurde ein Screening mit verschiedenen Mikroorganismen durchgeführt, um geeignete Stämme für die Herstellung der lupinen-basierten Joghurtalternative zu finden. Gleichzeitig wurde ein neuer Prozess für die Herstellung dieser Joghurtalternative entwickelt, dessen Teilschritte sich am Prozess der traditionellen Kuhmilchjoghurt Herstellung orientieren. Dabei wurden Schlüsselparameter identifiziert, die die Joghurttextur beeinflussen. Zusätzlich wurde der Einfluss der Milcherhitzung der lupinen-basierten Milchalternative auf die Wachstumsleistung der ausgewählten Stämme evaluiert, die *in situ* Exopolysaccharid-Produktion der ausgewählten Stämme betrachtet und der Einfluss der gebildeten Exopolysaccharide auf die Textur der resultierenden lupinen-basierten Joghurtalternativen untersucht. Zuletzt wurde der Einfluss der thermischen Behandlung der lupinen-basierten Milchalternative auf die Geleigenschaften in den resultierenden Joghurtalternativen betrachtet. Diese Erkenntnisse sind wichtig für die Bildung eines stabilen Gelnetzwerks und gleichzeitig für die Texturausbildung und die Wasserhaltekapazität der daraus resultierenden Produkte.

Zunächst wurde ein Screening mit 35 Mikroorganismen in Lupinenproteinisolat-Suspensionen durchgeführt, auf die Fähigkeit der Mikroorganismen ausgerichtet war, das Medium abzusäuern und den Produkten joghurt-typische Aroma- und Textureindrücke zu verleihen. Mit *Lactobacillus plantarum* TMW 1.460 und TMW 1.1468, *Pediococcus pentosaceus* BGT B34 und *Lactobacillus brevis* BGT L150 konnten die angenehmsten

Aromanoten und cremige, joghurt-ähnliche Texturen erreicht werden, während das Wachstum der anderen Mikroorganismen mit einem unangenehmen Geruch oder einer für Joghurt untypischen Textur verbunden war. Alle vier Stämme sind nicht als primäre oder sekundäre Starterkulturen für Joghurt bekannt, aber konnten sich an den pflanzlichen Rohstoff besser anpassen, als die für Kuhmilchjoghurt bekannten Starterkulturen.

Für die Herstellung der lupinen-basierten Joghurtalternative war es notwendig den traditionellen Prozess der Kuhmilchjoghurt-Herstellung anzupassen. Zunächst musste eine stabile Emulsion entwickelt werden, die Wasser, Lupinenprotein, Fett und Zucker enthielt. Die Milchproteine wurden durch Lupinenproteine ausgetauscht, welche sich während der Erhitzung und der Fermentation grundverschieden verhalten. Weiterhin wurde Laktose durch Glukose ersetzt und Milchfett durch Kokosnussfett. Neben den unterschiedlichen Zutaten der Milchalternativen wurden auch die Gehalte an Lupinenprotein und Zucker variiert, um eine Trockenmasse im Bereich von Kuhmilchjoghurt zu erreichen. Nach der Fermentation zeigten die lupinen-basierten Joghurtalternativen genauso wie Kuhmilchjoghurt das Verhalten eines schwachen, viskoelastischen Gels, dass als scherverdünnendes Fluid charakterisiert werden kann. Jedoch zeigte sich, dass sogar mit erhöhter Trockenmasse die maximale Schubspannung und die Fläche der Hystereseschleife signifikant geringer waren als für Kuhmilchjoghurt, was den schwachen Gelbildungseigenschaften der Lupinenproteine zugeschrieben werden kann. Weitere Unterschiede zu der Herstellung von Kuhmilchjoghurt zeigten sich: Animpfmengen von 8,0 $\log_{10}$ Kolonie bildende Einheiten pro Milliliter waren notwendig, um eine joghurt-ähnliche Textur zu erhalten. Trotz der hohen Animpfmengen, lagen die Fermentationszeiten bei ungefähr 14 bis zu 35 Stunden, um den pH-Wert von 7,0 auf 4,5 zu reduzieren, abhängig davon, welcher Stamm und welche Art der thermischen Behandlung der lupinen-basierten Milchalternative angewendet wurden. Diese recht langen Fermentationszeiten könnten der fehlenden Adaption der Stämme an das Lupinenprotein und Glukose enthaltendem Substrat geschuldet sein. Diese Hypothese wurde durch Überimpfungsexperimente mit *Lactobacillus brevis* BGT L150 in der lupinen-basierten Milchalternative bestätigt. Es wurde gezeigt, dass die Fermentationszeit von 24 auf 14 Stunden reduziert werden kann, da die lag-Phase von 10 Stunden durch ein dreimalig wiederholtes Überimpfen von *Lactobacillus brevis* BGT L150 in frischer lupinen-basierter Milchalternative vollständig verschwand.

Um die funktionellen Eigenschaften der Lupinenproteine zu modifizieren, wurde der Einfluss der Erhitzung auf die Lupinenproteine untersucht. Dafür wurde die Emulsion bei 80 °C für 60 s pasteurisiert oder alternativ bei 140 °C für 10 s ultrahocherhitzt und mit einer unerhitzten Probe als Referenz verglichen. Die thermische Behandlung führte zu einer stufenweisen Denaturierung der Lupinenproteinfraktionen, Conglutin β und Conglutin α, was von der

Exposition maskierter Sulfhydryl-Gruppen, der Abnahme freier Sulfhydryl-Gruppen und der Reoxidation zu Disulfid-Bindungen begleitet wurde. Diese Veränderungen waren abhängig von der Intensität der gewählten Hitzebehandlung und waren ausgeprägter bei einer intensiveren Hitzebehandlung. Außerdem wurde die Molekulargewichtsverteilung von Conglutin β bei mittleren Molekulargewichten zwischen 30 kDa und 40 kDa im Verlauf der Erhitzung beeinflusst. Prägnante Banden in diesem Bereich verschwanden, während neue Banden in dieser Region auftauchten. Zusätzlich zeigte die Partikelgrößenverteilung, dass die Homogenisierung und Erhitzung die Bildung kleinerer Partikel förderte.

Der Einfluss der thermischen Behandlung auf das Wachstumsverhalten der Milchsäurebakterien, deren Exopolysaccharid-Bildung, auf die Texturbildung und das Proteinnetzwerk der lupinen-basierten Joghurtalternativen, sowie ihre Anfälligkeit für Synärese wurde untersucht. Dafür wurden die unterschiedlich erhitzten lupinen-basierten Milchalternativen mit den vier verschiedenen Milchsäurebakterien angeimpft. Es stellte sich heraus, dass die Fermentationsdauer durch die Intensität der Erhitzung der lupinen-basierten Milchalternativen deutlich beeinflusst wird: Die vier verschiedenen Stämme brauchten signifikant länger, um nach der Ultrahocherhitzung auf einen pH-Wert von 4,5 abzusäuern (zwischen 25 und 35 Stunden), verglichen mit ihrem Wachstum nach der Pasteurisation (zwischen 14 und 24 Stunden). Allerdings wurden etwas höhere Gehalte an Exopolysacchariden in der ultrahocherhitzten lupinen-basierten Milchalternative gebildet, als in der pasteurisierten, was sehr wahrscheinlich auf die längeren Fermentationszeiten zurück zu führen ist. Außerdem beeinflusste die Erhitzung die physikalischen Eigenschaften der jeweiligen Joghurtalternativen signifikant: Die intensivere Erhitzung der lupinen-basierten Milchalternative (Ultrahocherhitzung) resultierte in höhere Werte der scheinbaren Viskosität und der Hysteresefläche, als auch der Speicher und Verlust Moduln der resultierenden lupinen-basierten Joghurtalternativen im Vergleich zu den pasteurisierten Milchalternativen und deren resultierender Produkte. Weiterhin hatten lupinen-basierte Joghurtalternativen aus ultrahocherhitzter lupinen-basierten Milchalternativen eine geringere Tendenz zur Synärese.

Die Proteinnetzwerke wurden mittels konfokaler Laserscanningmikroskopie und Cryo-Scanningelektronenmikroskopie visualisiert. Es wurde gezeigt, dass die Ultrahocherhitzung der lupinen-basierten Milchalternative mit anschließender Fermentation mit verschiedenen Stämmen generell zu engmaschigeren Mikrostrukturen der jeweiligen lupinen-basierten Joghurtalternativen führte, als die Verwendung von pasteurisierter oder unerhitzter lupinen-basierten Milchalternativen. Neben der thermischen Behandlung scheint zudem auch die Auswahl an Mikroorganismen eine wichtige Rolle in der mikrostrukturellen Erscheinung zu spielen, da die Zusammensetzung der Netzwerke grundlegend verschieden waren. Der Einsatz von *Lactobacillus plantarum* TMW 1.460 und TMW 1.1468 führte zu recht

engmaschigen Netzwerken mit kleinen Zwischenräumen, mit *Pediococcus pentosaceus* BGT B34 wurde eine porösere Struktur mit größeren Poren erhalten, während sich mit *Lactobacillus brevis* BGT L150 die mikrostrukturelle Erscheinung in Abhängigkeit von der Erhitzung deutlich unterschied: in Joghurt aus einer unerhitzten lupinen-basierten Milchalternative bildete sich lediglich eine lockere Formation von aggregierten Proteinen, die kaum als Netzwerk bezeichnet werden kann. Bei Verwendung einer pasteurisierten lupinen-basierten Milchalternative wurde ein lockeres Netzwerk mit einigen Verzweigungen gebildet, während sich in einer ultrahocherhitzten lupinen-basierten Milchalternative ein dichteres Netzwerk mit ungleichmäßig verteilten Hohlräumen verschiedener Größen bildete.

Diese Studien legen nahe, dass thermisch unbehandelte Lupinenproteine weniger Einfluss auf die Textur einer lupinen-basierten Joghurtalternative nehmen. Außerdem war die Fähigkeit unbehandelter Lupinenproteine mangelhaft, Wasser in die Poren des Netzwerkes einzubetten. Wurden die Lupinenproteinfraktionen im Verlauf der Erhitzung jedoch teilweise oder vollständig denaturiert, so konnten reaktive Seitengruppen durch die Auffaltung der Proteine freigelegt werden. Diese reaktiven Seitengruppen begünstigen Quervernetzungen wie Disulfid-Bindungen. Während der nachfolgenden Fermentation, scheint es, dass die Milchalternativen mit dem höheren Gehalt an aufgefalteten Lupinenproteinen mehr Verknüpfungen eingingen, da höhere viskose und elastische Module bei den Endprodukten gemessen werden konnten. Aus diesem Grund ist die Erhitzung der lupinen-basierten Milchalternativen unerlässlich, um hoch verzweigte Proteinnetzwerke der jeweiligen lupinen-basierten Joghurtalternativen zu erhalten, mit Texturen von hoher Festigkeit und einer geringen Anfälligkeit für Synärese.

Die Ergebnisse dieser Disseration belegen, dass Lupinenproteinisolate von *Lupinus angustifolius* cv. Boregine geeignete Substrate für die Herstellung von lupinen-basierten Joghurtalternativen darstellen, obwohl Lupinenproteine prinzipiell schlechtere Gelbildner als Sojaproteine sind. Diese Arbeit leistet einen Beitrag zur Aufklärung der Mechanismen bei der Modifikation der Textur in Joghurtalternativen und schafft damit die Grundlage für weitere Arbeiten mit anderen pflanzlichen Proteinquellen. Die gewonnenen Erkenntnisse dieser Arbeit finden bereits Anwendung: Die Prolupin GmbH vertreibt unter dem Namen „Made with LUVE“ seit 2015 in Deutschland die lupinen-basierte Joghurtalternative, die auf den Rezepturen und dem Herstellungsprozess dieser Arbeit basiert. Seit November 2017 sind Starterkulturen, die *Lactobacillus plantarum* und *Pediococcus pentosaceus* enthalten, kommerziell erhältlich, welche speziell für pflanzliche Milchersatzprodukte gedacht sind. Diese Stämme wurden im Zuge der Publikation Hickisch et al. 2016, die in dieser Arbeit enthalten ist, erstmalig als geeignete Starterkulturen für pflanzliche Milchersatzprodukte benannt.

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