

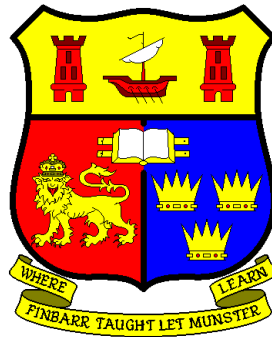
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Ollscoil na hÉireann

THE NATIONAL UNIVERSITY OF IRELAND

Coláiste na hOllscoile, Corcaigh
UNIVERSITY COLLEGE, CORK

SCHOOL OF FOOD AND NUTRITIONAL SCIENCES



**FUNCTIONAL APPLICATION OF LACTIC ACID BACTERIA
EXOPOLYSACCHARIDE IN COMPLEX FOOD SYSTEMS**

Thesis presented by

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MSc. Nutritional Science

Under the supervision of

Prof. Dr Elke K. Arendt

for the degree of

Doctor of Philosophy

(PhD in Food Science and Technology)

October 2015

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Declaration

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Signature:

Abstract

Lactic acid bacteria exopolysaccharides (LAB-EPS), in particular those formed from sucrose have the potential to improve food and beverage rheology and enhance their sensory properties potentially replacing or reducing expensive hydrocolloids currently used as improvers in food and beverage industries. Addition of sucrose not only enables EPS formation but also affects organic acid formation, thus influencing the sensory properties of the resulting food/beverage products.

The first part of the study the organoleptic modulation of barley malt derived wort fermented using *in situ* produced bacterial polysaccharides has been investigated. *Weissella cibaria* MG1 was capable to produce exopolysaccharides during sucrose-supplemented barley malt derived wort fermentation. Even though the strain dominated the (sucrose-supplemented) wort fermentation, it was found to produce EPS (14.4 g l⁻¹) with lower efficiency than in SucMRS (34.6 g l⁻¹). Higher maltose concentration in wort led to the increased formation of oligosaccharide (OS) at the expense of EPS. Additionally, small amounts of organic acids were formed and ethanol remained below 0.5% (v/v). *W. cibaria* MG1 fermented worts supplemented with 5 or 10% sucrose displayed a shear-thinning behaviour indicating the formation of polymers. This report showed how novel and nutritious LAB fermented wort-base beverage with prospects for further advancements can be formulated using tailored microbial cultures.

In the next step, the impact of exopolysaccharide-producing *Weissella cibaria* MG1 on the ability to improve rheological properties of fermented plant-based milk substitute plant based soy and quinoa grain was evaluated. *W. cibaria* MG1 grew well in soy milk, exceeding a cell count of log 8 cfu/g within 6 h of fermentation. The presence of *W. cibaria* MG1 led to a decrease in gelation and fermentation time. EPS isolated from soy yoghurts supplemented with sucrose were higher in molecular weight (1.1 x 10⁸ g/mol vs 6.6 x 10⁷ g/mol), and resulted in reduced gel stiffness (190 ± 2.89 Pa vs 244 ± 15.9 Pa). Soy yoghurts showed typical biopolymer gels structure and the network structure changed to larger pores and less cross-linking in the presence of sucrose and increasing molecular weight of the EPS.

In situ investigation of *Weissella cibaria* MG1 producing EPS on quinoa-based milk was performed. The production of quinoa milk, starting from wholemeal quinoa flour, was optimised to maximise EPS production. On doing that, enzymatic deconstruction of protein and carbohydrate components of quinoa milk was successfully achieved applying alpha-amylase and proteases treatments.

Fermented wholemeal quinoa milk using *Weissella cibaria* MG1 showed high viable cell counts (>10⁹ cfu/mL), a pH of 5.16, and significantly higher water holding capacity (WHC, 100 %), viscosity (≥ 0.5 Pa s) and exopolysaccharide (EPS) amount (40 mg/L) than the chemically acidified control. High EPS (dextran) concentration in quinoa milk caused earlier aggregation because more EPS occupy more space, and the chenopodin were forced to interact with each other.

Direct observation of microstructure in fermented quinoa milk indicated that the network structures of EPS-protein could improve the texture of fermented quinoa milk. Overall, *Weissella cibaria* MG1 showed favorable technology properties and

great potential for further possible application in the development of high viscosity fermented quinoa milk.

The last part of the study investigate the *ex-situ* LAB-EPS (dextran) application compared to other hydrocolloids as a novel food ingredient to compensate for low protein in biscuit and wholemeal wheat flour. Three hydrocolloids, xanthan gum, dextran and hydroxypropyl methylcellulose, were incorporated into bread recipes based on high-protein flours, low-protein flours and coarse wholemeal flour. Hydrocolloid levels of 0–5 % (flour basis) were used in bread recipes to test the water absorption. The quality parameters of dough (farinograph, extensograph, rheofermentometre) and bread (specific volume, crumb structure and staling profile) were determined. Results showed that xanthan had negative impact on the dough and bread quality characteristics. HPMC and dextran generally improved dough and bread quality and showed dosage dependence. Volume of low-protein flour breads were significantly improved by incorporation of 0.5 % of the latter two hydrocolloids. However, dextran outperformed HPMC regarding initial bread hardness and staling shelf life regardless the flour applied in the formulation.

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Dedicated to my son Tommaso and my husband Nicola

Chapter 1

Introduction

1.1 Introduction

Polysaccharides are industrially used as thickeners, stabilisers and gelling agents in food and beverage products. Despite the many sources of polysaccharides, the world market is dominated by plant and algal polysaccharides (e.g. starch, galactomannans, pectin, carrageenan and alginate) (Leung et al, 2006). This market was valued at >4 billion US\$ in 2008, with xanthan gum being the only significant bacterial EPS, accounting for 6% of the total market value (Seisun, 2009).

Polysaccharides derived from microorganisms, including bacteria, yeasts and moulds, still represent an unexploited market (Sutherland, 2001). EPS production from microorganisms has the following advantages: production in a matter of days compared to the 3–6 months in the case of plants; energy efficient, in the case of microalgae (production uses solar energy); possibility of utilising industrial wastes such as glycerol, whey, molasses, hydrocarbon residue and CO₂ as carbon substrates (Gassem et al., 1997; González López et al., 2009; Thompson and He, 2006) and the absence of competition with arable land (Donot et al., 2012). Furthermore, EPSs are naturally exuded by most microorganisms into the extracellular environment (Chen et al., 2006; Ravella et al., 2010; Staudt et al., 2004) facilitating their recovery. The main factors limiting EPS production by microorganisms are linked to the production costs. The main costs consist of purchasing substrate in certain cases and acquiring the infrastructure required for the up-stream, production and down-stream processes, which can include bioreactors and maintaining asepsis (Donot et al., 2012).

Based on their composition, lactic acid bacteria (LAB) EPS can be classified into homopolysaccharides (HoPS) and heteropolysaccharides (HePS). HoPS contain only one type of monosaccharide (e.g. glucans and fructans made of glucose and fructose, respectively), whereas HePS consist of several different monosaccharides. Since EPS biosynthesis is linked to the primary carbohydrate metabolism of the producing cells (Boels et al., 2001; Levander et al., 2002) its production is likely to take place during active sugar consumption, since it requires large numbers of activated nucleotide sugars, energy for building the repeating units, for transmembrane translocation and for polymerization (Jolly et al., 2002). However, EPS application is currently limited to dairy starter cultures and to date *in situ* EPS formation/application in plant-based fermented foods and beverages has not been described.

When produced within or added to food matrices EPS may have enormous and very different effects on the final properties of the food and beverage product. Among these, modification of water absorption in the food which can influence rheological properties. Glucan and fructans are basically tasteless and thus can serve as supplements in all kind of foods and beverages without affecting product taste. EPS can be produced in all groups of fermented foods and beverages making them targets for the optimisation of quality parameters by direct *in situ* or *ex situ* EPS application (Ullrich, 2009).

In situ produced EPS can enhance food and beverage structural and sensory properties without having to be mentioned on ingredient list. It does not have to be purchased and so does not cause additional cost when compared with conventional processes. However, *in situ* EPS production is dependent on different environmental

and biological factors such as pH, temperature, medium composition (carbon and nitrogen source), substrate concentration and inhibiting/limiting compounds and bacterial growth phase (De Vuyst and Degeest, 1999; Petry et al., 2000).

Fermentation process optimisation along with careful selection of starter cultures and their fermentation performance can result in a significant enhancement of EPS production and, in turn, of food and beverage properties. The *ex situ* EPS application may not deliver the same technological effects on food and beverage properties since *in situ* EPS production is a dynamic process progressing in parallel to fermentation. However, better control and reproducibility of EPS techno-functionality can be achieved when a known amount of pure EPS is added in the food system

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Chapter 2

Production, properties, and industrial food application of lactic acid bacteria derived exopolysaccharides: a review.

Emanuele Zannini, Deborah M. Waters, Aidan Coffey and Elke K. Arendt (2015) Submitted to *Applied Microbiology and Biotechnology*

2.1 Introduction

Lactic acid bacteria (LAB) have traditionally been associated with food and feed fermentations. Their uses around the world include improving the preservation, organoleptic characteristics and nutritional value of a large variety of food and beverage products. LAB are generally considered beneficial microorganisms with some strains known to have health-promoting (probiotic) attributes. However, other genera (*Streptococcus*, *Lactococcus*, *Enterococcus*, *Carnobacterium*) also contain species or strains that are recognised human and animal pathogens. A thorough understanding of the taxonomy, metabolism, and genetics of LAB is thus necessary to fully utilize the technological, nutritional, and health-promoting aspects of LAB while avoiding potential risks (Von Wright, A. and Axelsson, L, 2012). Several LAB produce polysaccharides that occur as cell wall constituents (peptidoglycan) or released from the cell. The latter are either permanently attached to the surface of the microbial cell in the form of capsules (capsular polysaccharide, CPS), or secreted into the environment as exopolysaccharides (EPS) in the form of a film (Chapot-Chartier, 2011).

The report of EPS formation by wine-spoiling LAB dates back to Pasteur (Pasteur, 1861), as cited by Leathers (2002). Orla-Jensen (1943) described EPS formation from sucrose by *Leuconostoc* spp., mesophilic lactobacilli, and pediococci and indicated the role of EPS formation in the spoilage of apple cider and beer.

Two phenotypes of EPS-producing strains exist. The “ropy” phenotype forms a long filament when an inoculation loop is placed onto into the EPS covered colony and then slowly withdrawn, while the “mucoid” phenotype strain appears as shiny and

smooth colonies growing on suitable agar plates. The roles of these EPS in their natural environment are complex and still unclear. However, among others, EPS is likely to play a role in cellular recognition (De Vuyst and Degeest, 1999; Looijesteijn et al., 2001) in exchange of genetic information and in the protection of the microbial cell integrity in an ecosystem against hostile environments (desiccation, osmotic stress, pH) and antimicrobial factors (bacteriophages, phagocytosis, predation by protozoa, metal ions, nisin, lysozyme, cleaning agents, ethanol and antibiotics) (Chapot-Chartier, 2011; Flemming and Wingender, 2010a; Ruas-Madiedo et al., 2002b).

EPS also have a key role in biofilm formation and surface adhesion enabling the colonisation of different environments (De Vuyst et al., 2001; Dertli et al., 2015; Flemming and Wingender, 2010b; Walter et al., 2008) (Table 2.1).

Since most EPS-producing bacteria are not capable of utilizing the EPS that they produce as a source of nutrients (Kim and Fogler, 1999), it is questionable that EPS serve as a food reserve (Cerning, 1990). Other species such as *Streptococcus mutans* and *Streptococcus sobrinus* can degrade the EPS dextran and *S. mutans* can also utilize oligosaccharides as a nutrient (Colby and Russell, 1997). Additionally, enzymatic EPS degradation was reported for *Streptococcus thermophilus* and *Lactobacillus rhamnosus* during prolonged fermentations (Degeest et al., 2002; Pham et al., 2000). EPS can also act as a substrate for other organisms in complex ecosystems. In this regard, Salazar et al, (2008) showed that EPS synthesized by intestinal bifidobacteria could act as fermentable substrates for microorganisms in

Table 2.1 Some of the roles ascribed to exopolysaccharides in biofilms^a

Process	Functional roles of exopolysaccharides to biofilms
Adhesion	Exopolysaccharides facilitate the initial steps in the colonization of surfaces (abiotic and biotic) and long-term attachment of biofilms
Bacterial cell aggregation	The bridging between cells is enabled by exopolysaccharides, allowing the temporarily immobilization of the bacterial population and the subsequent development of high cell densities and cell–cell recognition.
Water retention	Hydrophilic exopolysaccharides have high water retention ability thus maintaining a hydrated microenvironment around biofilm and this leading to the survival of desiccation in water-deficient environments
Cohesion of biofilms	Neutral and charged exopolysaccharides forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations), determining biofilm architecture, as well as allowing cell-cell communication.
Nutrient source	Exopolysaccharides could serves as source of carbon, nitrogen, and phosphorus containing compounds for utilization by the biofilm community.
Protective barrier	Exopolysaccharides confer resistance to non-specific and specific host defences during infection, confers tolerance to various antimicrobial agents, protects cyanobacterial nitrogenase from the harmful effects of oxygen, and offers protection against some phagocytic protozoa.
Sorption of organic Compounds and inorganic ions	Charged and hydrophobic exopolysaccharides mediates the accumulation of nutrients from the environment, sorption of xenobiotics and recalcitrant materials. They promote polysaccharide gel formation resulting in ion exchange, mineral formation and the accumulation of toxic metal ions (thus collectively contributing to environmental detoxification).
Binding of enzymes	Non-glycolytic extracellular enzyme interaction with exopolysaccharides leads to retention stabilization and accumulation.
Export of cell components	Lipopolysaccharides (isoprenoid glycosyl carrier lipids), which lipo-glyco conjugate, mediates the releases cellular material as a result of metabolic turnover.
Sink for excess energy	Exopolysaccharides store excess carbon under unbalanced carbon to nitrogen ratios

From Nwodo et al., (2012)

the human gut environment, promoting shifts in short chain fatty acids (SCFA) profiles and changes in relationships among intestinal microbial populations.

EPS producing LAB are industrially important microorganisms in the development of functional food products and are used as starter cultures or coadjutants to develop fermented foods such as yogurt, cheese and cereal based products (Badel et al., 2011; Jolly et al., 2002; Patel A, 2013; Ruas-Madiedo et al., 2002a; Tieking et al., 2003) because of their viscosity and mouth-feel enhancement properties. In contrast, alcoholic beverages such as beers, ciders and wines are spoiled by EPS-producing LAB. In wine, this spoilage can occur either during vinification or after bottling and leads to an alteration known as “ropiness” or “oilness,” characterized by a viscous, thick texture, and oily feel, which although not appreciably altering the taste, renders the products unpleasant to the palate causing considerable economic loss (Gindreau et al., 2001). More recently EPS were used as depollution agents and there was a growing interest in their biological functions such as antitumor, antioxidant or prebiotic activities (Liu et al., 2010)

2.2 Chemical composition of LAB-EPS

According to the chemical composition and biosynthesis mechanisms, LAB EPS are classified into two distinct groups: homopolysaccharides (HoPS) and heteropolysaccharides (HePS).

Homopolysaccharides

HoPS contain a single type of monosaccharides; either fructose or glucose, synthesised by extracellular glucansucrase activity using sucrose as the glycosyl (fructose or glucose) donor (Leemhuis et al., 2013; Van Hijum et al., 2006). Glucansucrases are able to use the energy of the osidic bond (also called glycosidic linkages) of sucrose to catalyse the transfer of a corresponding glycosyl moiety (Figure 2.1).

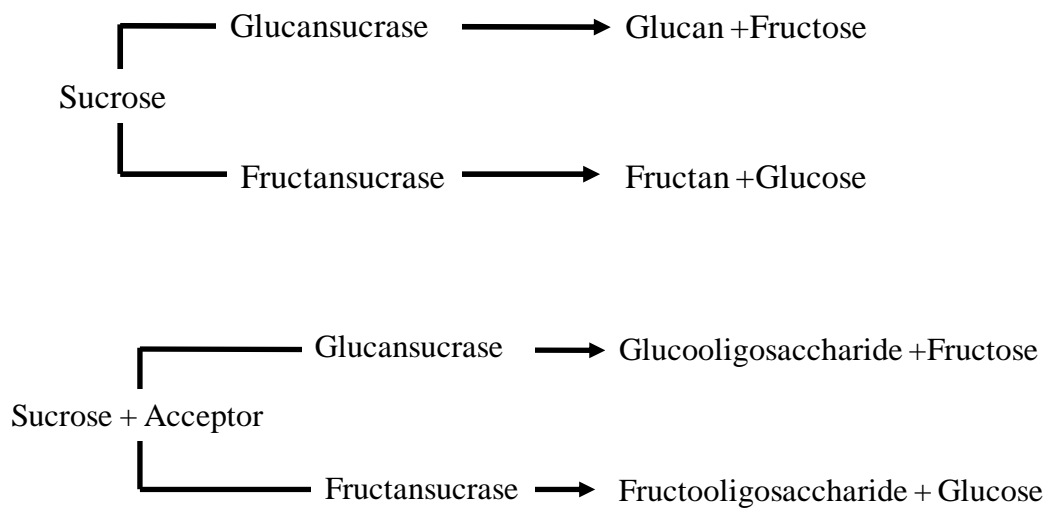


Figure 2.1 Biosynthetic pathways leading to EPS synthesis in LAB

The HoPS composed of glucose are further classified as α -D glucans (dextran, mutan, reuteran and alternan) and β -D glucans, whereas those containing fructose are fructans (levan and inulin-types) (Ruas-Madiedo and De Los Reyes-Gavilan, 2005). Glucans and fructans are found most frequently among the homo-polysaccharides and they are both applied as ingredient in the food industry (Anwar et al., 2010; Buchholz and Seibel, 2008).

Moreover, a fourth group of HoPS; polygalactans, composed of a pentameric repeating unit of galactose have also been described (Gruter et al., 1992a) (Table 2.2).

Table 2.2 Types of homopolysaccharides produced by lactic acid bacteria and their predominant linkages

HoPS	Main linkage ^a (branching linkage)	Species	References
α-Glucans			
Dextran	α -1 \rightarrow 6 (α -1 \rightarrow 3)	<i>Lactobacillus reuteri</i> , <i>Lactobacillus casei</i> , <i>Lactobacillus sakei</i> , <i>Lactobacillus fermentum</i> , <i>Lactobacillus parabuchneri</i> , <i>Lactobacillus</i> <i>curvatus</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i> , <i>Streptococcus mutans</i> , <i>Streptococcus</i> <i>downei</i> , <i>Streptococcus sobrinus</i> <i>Streptococcus</i> <i>salivarius</i> , <i>Streptococcus gordonii</i> <i>Weisella cibaria</i> , <i>Weisella confusa</i>	(Galle et al., 2010a; Korakli and Vogel, 2006a; Kralj et al., 2004a; Ruas-Madiedo et al., 2002b; Tiekling et al., 2005; Katina <i>et al.</i> , 2009)
Mutan	α -1 \rightarrow 3 (α -1 \rightarrow 6)	<i>Lactobacillus reuteri</i> , <i>Streptococcus mutans</i> <i>Streptococcus downei</i> , <i>Streptococcus sobrinus</i>	(Giffard et al., 1991; Kralj et al., 2004a; Ruas- Madiedo et al., 2002b)
Alternan	α -1 \rightarrow 3 (α -1 \rightarrow 6)	<i>Leuconostoc mesenteroides</i>	(Giffard et al., 1991; Korakli and Vogel, 2006a; Kralj et al., 2004a; Ruas-Madiedo et al., 2002b)
Reuteran	α -1 \rightarrow 4	<i>Lactobacillus reuteri</i>	(Kralj et al., 2004b)
Others	α -1 \rightarrow 2	<i>Leuconostoc mesenteroides</i> B-1355	(Smith et al., 1998)
β-Glucan			
β -glucan	β -1 \rightarrow 3	<i>Pediococcus damnosus</i> , <i>Pediococcus parvulus</i> , <i>Lactobacillus diolivorans</i> G77, <i>Lactobacillus</i> sp.	(Duenas-Chasco et al., 1998; Duenaschasco et al., 1997; Werning et al., 2006)
Fructans			
Levan	β -2 \rightarrow 6 (β -2 \rightarrow 1)	<i>Lactobacillus reuteri</i> , <i>Lactobacillus</i> <i>sanfranciscensis</i> , <i>Leuconostoc mesenteroides</i> , <i>Streptococcus sobrinus</i> , <i>Streptococcus salivarius</i>	(Giffard et al., 1993a; Korakli et al., 2000; Korakli and Vogel, 2006a; Ruas-Madiedo et al., 2002b)
Inulin-like	β -2 \rightarrow 1 (β -2 \rightarrow 6)	<i>Lactobacillus reuteri</i> , <i>Leuconostoc citreum</i> , <i>Streptococcus mutans</i>	(Olivares-Illana et al., 2003; Rosell and Birkhed, 1974; Van Hijum et al., 2001; Van Hijum et al., 2004)
Polygalactan	α -Gal (β -Gal)	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> H414	(Gruter et al., 1992b)

^a At least 50% of the respective linkage

HoPS have a main backbone structure with variable degrees of branching and linkage sites, which differ among bacterial strains (De Vuyst and Degeest, 1999; Monsan et al., 2001). HoPS have high molecular weights in the order of 10^6 Da (Ruas-Madiedo et al., 2002b). In this regard, the average molar mass of dextran (α -glucan) produced by *Leuconostoc mesenteroides* NRRL B-512 varies between $6.2 - 7.1 \times 10^6$ Da. Levans produced by several strains of *S. mutans* have a higher molar mass ($2.7 - 21.6 \times 10^6$ Da) and *S. mutans* JC2 synthesises a fructan inulin-like polysaccharide of 12.4×10^6 Da (Cerning, 1990). Concerning the carbon involved in the linkage, glucan are subdivided into dextran [α -1 \rightarrow 6 (α -1 \rightarrow 3)], mutan [[α -1 \rightarrow 3 (α -1 \rightarrow 6)], reuteran [α -1 \rightarrow 4], and alternan [α -1 \rightarrow 6 / α -1 \rightarrow 3)] while fructans are subdivided into levan [β -2 \rightarrow 6 (β -2 \rightarrow 1)] and inulin-like [β -2 \rightarrow 1 (β -2 \rightarrow 6)] (Table 2).

Dextran

The term dextran refers to a large group of α -glucans in which the main backbone chain consists of α -1 \rightarrow 6 glycosidic linkages. Dextran may also be branched through various secondary linkages such as α -1 \rightarrow 2, α -1 \rightarrow 3, and α -1 \rightarrow 4 (Monsan et al., 2001).

Pasteur was the first to describe the microbial origin of the gelification process observed in cane sugar syrup (Pasteur, 1861). This class of α -glucans was later assigned the name dextran due to its positive rotatory power (Monsan et al., 2001). In 1943, the corresponding enzyme was subsequently named dextransucrase (Hestrin et al., 1943). The synthesis of dextran from sucrose was recorded for *Leuconostoc mesenteroides* subsp. *mesenteroides*.

Nevertheless, the ability to synthesize dextran can be lost when serial refreshments are performed in media with increasing amount of salt (Harutoshi, 2013).

Kim et al., (2003) investigated the size distribution and the degree of branching of dextrans synthesized by *L. mesenteroides* B-512FMCM dextransucrase, using different processing parameters (sucrose concentration, 0.1–4.0 M, pH 4.5–6.0, and temperature 4–45 °C). They found that with increasing concentrations of sucrose, from 0.1 to 4.0 M, *Leuconostoc mesenteroides* B-512FMCM dextransucrase gave a decreasing amount of high-molecular weight dextran ($>10^6$ Da) with a concomitant increase in low-molecular weight dextran. The degree of branching increased from 5% for 0.1 M sucrose to 16.6% for 4.0 M sucrose while the temperature had very little effect on the size of the dextran, which was $>10^6$ Da, but it had a significant effect on the degree of branching, which was 4.8% at 4 °C and increased to 14.7% at 45 °C. Both the molecular weight and the degree of branching were not significantly affected by different pH values between 4.5 and 6.0.

Native dextrans, partially degraded dextrans, and their derivatives have immense commercial value in the pharmaceutical (lubricants and carriers), medical (antithrombotic, anticoagulant, and osmotic agents, and iron dextran for the treatment of anaemic deficiencies), and the food and chemical industries (adjuvant, emulsifier, carrier and stabilizer, preservative enzyme cryoprotective) (Toulmins H.A, et al, 1957; Aebischer et al., 2001; Bhavani et al., 2010; Goulas et al., 2004; Novak L. J. 1957; King and Speert, 2002; Pucci and Kunka, 1990; Bohn R. T., 1961; William and Joseph, 1959)

As a food ingredient, dextran was initially studied in the 1950's primarily as a thickener. Dextran has been listed as generally regarded as safe (GRAS) by the Food and Drug Administration (FDA) for use in animal feeds and medicines

(21CFR582.1275) and indirectly in human foods as food packaging materials (21CFR186.1275) (FDA, 2013). In 2001, the European Commission authorised the commercialisation of a *Leuconostoc mesenteroides* dextran preparation as a novel food ingredient in bakery applications up to level of 5% to improve the softness, crumb texture and loaf volume. According to the official decision statement, it is considered to have nutritional properties similar to starch (Byrne, 2001).

Alternan

Alternan is a branched glucan with a unique backbone structure of alternating α -1 \rightarrow 6 and α -1 \rightarrow 3-D-glucosidic linkages (Leathers et al., 2010). These types of linkages are thought to be responsible for its peculiar physical characteristic, that differ from commercial dextran, including high solubility, low viscosity and a remarkable resistance to microbial and enzymatic hydrolysis (Côté et al., 1997; Vandamme et al., 2002).

Leuconostoc mesenteroides NRRL B-1355 was first reported alternan-producing strain (Cote and Robyt, 1982). Two other strains of *Leuconostoc mesenteroides*, NRRL B-1501 and NRRL B-1498 are also known to produce alternan via alternansucrase; the enzyme that converts sucrose to alternan and fructose (Côté and Robyt, 1982; Jeanes et al., 1954). The gene that encodes alternansucrase also has been cloned and sequenced (Argüello-Morales et al., 2000).

While naturally occurring strains of *Leuconostoc mesenteroides* that produce alternan also produce dextran as a troublesome contaminant, genetically improved strains for production of alternan with little or no dextran synthesis have been

developed (Kim and Robyt, 1994; Leathers et al., 1997; Leathers et al., 1995; Smith et al., 1994). Alternan is a commercially exploited low viscosity bulking agent and carrier in foods and cosmetics. Extracellular alternanase depolymerises alternan to oligosaccharides. These alternan-derived oligosaccharides are used as low-glycemic sweetener in confectionaries (Leathers et al., 2003) and as prebiotics (Cote, 2009).

Reuteran

Reuteran is the name given to a specific α -glucan produced by the species *Lactobacillus reuteri* and is generally associated with fermented milk products. Reuteran is a glucan that contains ($\alpha 1 \rightarrow 4$) and ($\alpha 1 \rightarrow 6$) glycosidic bonds (Meng et al., 2004) with no repeating units present (Van Leeuwen et al., 2008). Two strains of *Lactobacillus reuteri* LB121 and ATCC 55730, were identified as reuteran producers and the corresponding enzyme reuteransucrase involved in the synthesis of this α -glucan has been characterised (Kralj et al., 2002). Reuteran, like other novel glucans, may play a role in the thickening of fermented dairy foods. Additionally, due to its water solubility, reuteran is used in bakery application also (Arendt et al., 2007)

Mutan

Mutan is a glucan synthesised through mutansucrase by various serotypes of *Streptococcus mutans*. Mutans contain mainly $\alpha 1 \rightarrow 3$ glycosidic bonds (Leemhuis et al., 2013) that are responsible for its insolubility in water. Mutan polysaccharides are

involved in the adhesion of oral flora microorganisms to the tooth surface forming a dental plaque (Hamada and Slade, 1980; Pleszczyńska et al., 2015).

β-glucan

(1,3)-β-D-Glucans from several bacteria and fungi constitute a group of naturally occurring polysaccharides with a main chain of (1,3)-linked β-glucopyranosyl units. It can be linear or branched with either (1,6)- or (1,2)-linked side chains of varying length and distribution.

Lactobacillus spp. G-77 secretes two exopolysaccharides when grown on glucose media. One of them was found to be a (1-3)-β-D-glucan, identical to that described for the EPS from *Pediococcus damnosus* 2.6 (Duenas-Chasco et al., 1998). The second a HoPS was a dextran-type polysaccharide with α-1,6 glycosyl linkages and α-1→2 branching of a single D-glucose unit.

When produced by beverage spoilage organisms, these β-glucans cause an undesirable thickening which has been reported during cider (Duenas Chasco et al., 1997) and wine (Gindreau et al., 2001) production leading to “oiliness” or “ropiness”. Conversely, (1,3)-β-D-glucans are considered as biological response modifiers and numerous publications describe their biological activities and therapeutic uses (Sletmoen and Stokke, 2008). Their biological effects are influenced by their degree of branching, chain length, and tertiary structure (Zhang et al., 2005).

Fructans

Microbial fructans are high molecular mass polymers, which consist of chains of fructosyl units connected through β -(2 \rightarrow 1) or β -(2 \rightarrow 6) linkages (Ernst et al., 1998); depending on the linkage type, they are called inulin and levan, respectively (Seibel and Buchholz, 2010). They are synthesised from sucrose by the activity of fructosyltransferases (FTF). Fructan production has been reported for the genera *Streptococcus*, *Leuconostoc*, *Lactobacillus* and *Weissella* (Monsan et al., 2001; Tiekink et al., 2003; Van Geel-Schutten et al., 1998).

The amount of fructan produced, type of linkages, molecular mass and degree of branching depend on the enzymes involved in the synthesis as well as on the sucrose concentration, the presence of acceptor molecules, and their concentrations. The enzymes catalysing the synthesis of these two polymers are inulosucrase and levansucrase, respectively. Most of the FTF characterised to date produce fructan of the levan type, and only a few were reported to produce inulin.

Levan

Levan is a non-toxic, biologically active, extracellular polysaccharide that can be produced by both plants and micro-organisms. It is a sugar polymer composed of fructose with 2,6-linkages (Melo et al., 2007).

Among LAB, levan is produced by strains from the oral flora, such as *Streptococcus salivarius*, *Streptococcus mutans* (Giffard et al., 1993b; Shiroza and Kuramitsu, 1988), and by strains of *Leuconostoc mesenteroides* NRRL B-512F, *Lactobacillus sanfranciscensis* LTH 2590 and *Lactobacillus reuteri* LB 121 (Patel et al., 2012)

Levan is also produced by non-LAB species like *Zymomonas mobilis*, *Gluconacetobacter diazotrophicus*, *Bacillus subtilis* and *Bacillus polymyxa* (Shih et al., 2010) or by enzymatic synthesis using sucrose as substrate (Donot et al., 2012).

To date, a few studies on this relatively new class of fructan have shown that oligosaccharides produced by *in vitro* partial acid hydrolysis of levan could be utilised by different pure cultures including *Bifidobacterium adolescentis*, *B. longum*, *B. breve*, *B. pseudocatenulatum*, *Lactobacillus plantarum* and *Pediococcus pentosaceus* (Kang et al., 2002; Marx et al., 2000).

Korakli et al., (2003a) studied the formation of levan and kestose during growth of *L. sanfranciscensis*, a typical LAB dominating traditionally prepared wheat and rye sourdoughs (Hammes, 1996). They observed that in static pH fermentation, the yield of levan increased with increasing sucrose concentrations and appreciable levels of 1-kestose were observed only when the sucrose concentration exceeded 50 g L⁻¹. When *Lactobacillus sanfranciscensis* LTH 2590 was used to ferment a wheat dough containing 60 g Kg⁻¹ of sucrose, the formation of more than 5 g Kg⁻¹ of levan was observed (Tieking and Ganzle, 2005a).

Levan is naturally present in various food products and thus, is regularly consumed in very small amounts by humans. However, until recently, it has been relatively ignored as a functional food ingredient due to its limited resources and very low content. Levan from *L. sanfranciscensis* LTH 2590 exhibits prebiotic effects (Korakli et al., 2003b) and has attracted attention for its antitumor properties (Yoo et al., 2004), cholesterol-lowering properties and application as an eco-friendly adhesive (Patel et al., 2012).

In the food industry, levan is used as a stabilizer, emulsifier, formulation aid, surface-finishing agent, encapsulating agent, and as a carrier of flavours and fragrances (Han, 1990a; Han and Clarke, 1990). The behaviour of levan in solution has also been studied (Kasapis et al., 1994a). Levan is valuable in terms of its water-holding capacity. At a concentration higher than 1% (w/v), a levan solution will display film-forming characteristics on a smooth surface. Levan solution (below 1%) can also be used in coatings or as bio thickener (De Vuyst et al., 2001) The enzymatic or chemical hydrolytic products of levan may be used in the food industry as sweeteners or dietary fibre, for example β -(2 \rightarrow 6)-linked fructofuranosyl oligosaccharides (Han, 1990a).

Inulin-type

Inulin-type EPS are fructans or fructooligosaccharides (FOS) containing β -(1 \rightarrow 2) osidic bonds. *Lactobacillus johnsonii* NCC 533 produces high molecular mass inulin from sucrose by using an inulosucrase enzyme. *Streptococcus mutans* strain JC2, *Leuconostoc citreum* CW28 and *Lactobacillus reuteri* 121 are some other LAB which produce inulins. These are not digestible and function as prebiotics in humans and animals.

Inulin and fructooligosaccharides were originally suggested as prebiotics that selectively stimulate bifidobacteria, intestinal microorganisms considered to be beneficial and that are extensively used as probiotics (Masco et al., 2005). Experimental evidence confirmed the bifidogenic effect of inulin and FOS (Gibson et al., 1995; Kruse et al., 1999; Wang and Gibson, 1993). Additionally, in the

intestine, inulin-type fructooligosaccharides can lead to butyrate increases through stimulation of synthesising microorganisms, which nourishes enterocytes, can prevent pathogenic adherence and decreases the pH in the lumen (Sartor, 2004). Inulin-type fructans may also be employed as vehicles for targeted drug delivery in treating colon cancer (Pool-Zobel, 2005).

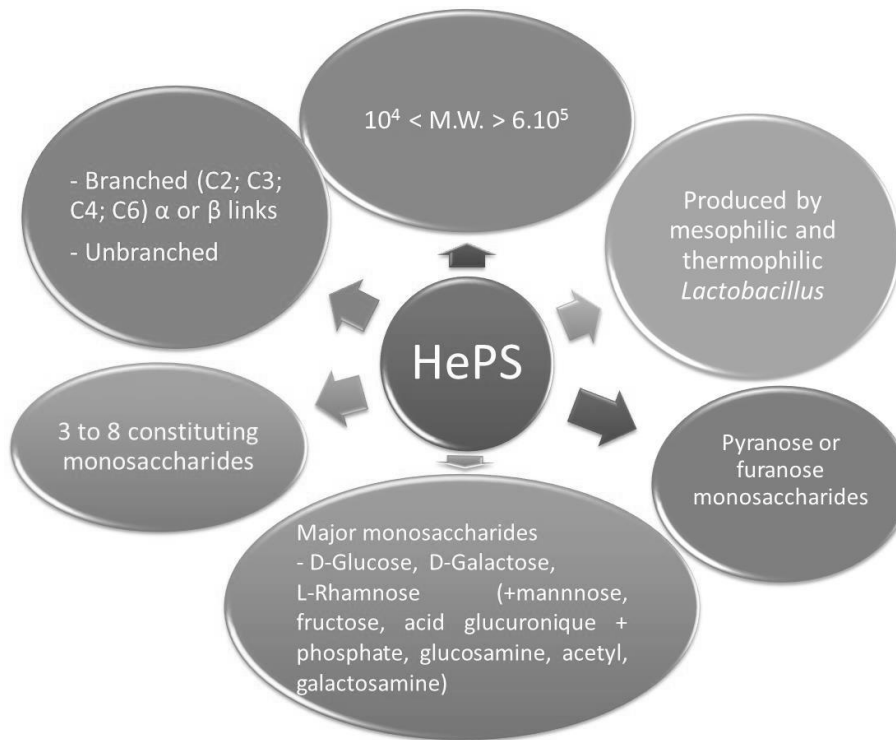
Besides its functionality as a prebiotic, inulin can also be used for its technological properties and it often delivers dual benefit: an improved organoleptic quality and a better-balanced nutritional compositions (Franck, 2002). In food production, inulin enhances the quality of the food products acting as texturizer, emulsion stabilizer and partial fat replacer. Inulin has a neutral bland taste, without any off-flavour or persistence. It combines easily with other food ingredients without altering delicate flavours. It is discreetly soluble in water (maximum 10% at room temperature) and brings a rather low viscosity (less than 2 mPa.s for a 5% w/w solution in water). Additionally, because of its gelling characteristics, inulin has a remarkable capacity to replace fat, particularly in products such as processed cheese, dairy spread cheese and butter like products (Franck, 2002).

Heteropolysaccharises

HePS are produced by a great variety of mesophilic (*Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *Lactobacillus casei*, *Lb. sake*, *Lb. rhamnosus*) and thermophilic (*Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus* and *Streptococcus thermophilus*) LAB. HePS are composed of a backbone of repeated subunits that are branched (at positions C2, C3, C4, or C6) or unbranched, and

consist of three to eight monosaccharides, (D-glucose, D-galactose and L-rhamnose) derivatives of monosaccharides (N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) or glucuronic acid (GlcA)) or substituted monosaccharides (such as phosphate, acetyl and glycerol) (De Vuyst and Degeest, 1999; Ruas-Madiedo et al., 2002b) (Figure 2.2).

Figure 2.2 Characteristics of heteropolysaccharides produced by *Lactobacillus* sp.



The thermophilic HePS producing LAB have recently received renewed interest, since they play a key role in the rheology, texture and body, and mouthfeel of fermented dairy drinks .HePS synthesis differs from HoPS synthesis since the precursor repeating units are formed intracellularly and isoprenoid glycosyl carried

lipids are involved in the process (Cerning, 1990). The repeating units are translocated across the membrane and subsequently polymerised extracellularly. Additionally, the biosynthesis and secretion of the LAB HePS occur at different phases of growth and the amount and type is regulated by fermentation conditions (De Vuyst and Degeest, 1999).

Structurally, HePS may beropy or mucoid. Under optimal culture condition a HePS yield of 0.15–0.6 g l⁻¹ may occur (Cerning, 1995). The molecular mass of these HePS polymers ranges between 1.0×10^4 and 6.0×10^6 Da (De Vuyst and Degeest, 1999). The producer strain, growth conditions (pH, temperature, incubation time, oxygen tension and turbidity) and medium composition (carbon, nitrogen sources and other nutrients) can influence the polymer yield and the monosaccharide subunits composition (De Vuyst and Degeest, 1999; Degeest et al., 2001; Grobben et al., 2000; Looijesteijn et al., 2000; Torino et al., 2000). HePS have a great variability in structures (Fig. 2.2).

The composition of the monosaccharide subunits and the structure of the repeating units are not species-specific, except in the case of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*. This species, isolated from a kefir grain, a fermented dairy food from the north Caucasus region, produces large amounts of water-soluble polysaccharides called kefiran. This consists of approximately equal proportions of glucose and galactose (Micheli et al., 1999) and is able to improve visco-elastic properties of acid milk gels. Additionally, kefiran is reported to have antimicrobial activity (Rodrigues et al., 2005) and the ability to significantly reduce blood pressure and the serum cholesterol levels (Maeda et al., 2004). Kefiran is reported to confer

protective immunity, maintain intestinal homeostasis, enhance IgA (immunoglobuline A) level at both the small and large intestine level and influence the systemic immunity through the release of cytokines into the blood (Lemieux et al., 2006; Mora-Gutierrez, 2014; Park et al., 2004; Piermaria et al., 2009; Stanton et al., 2014).

Application of LAB EPS in food industries

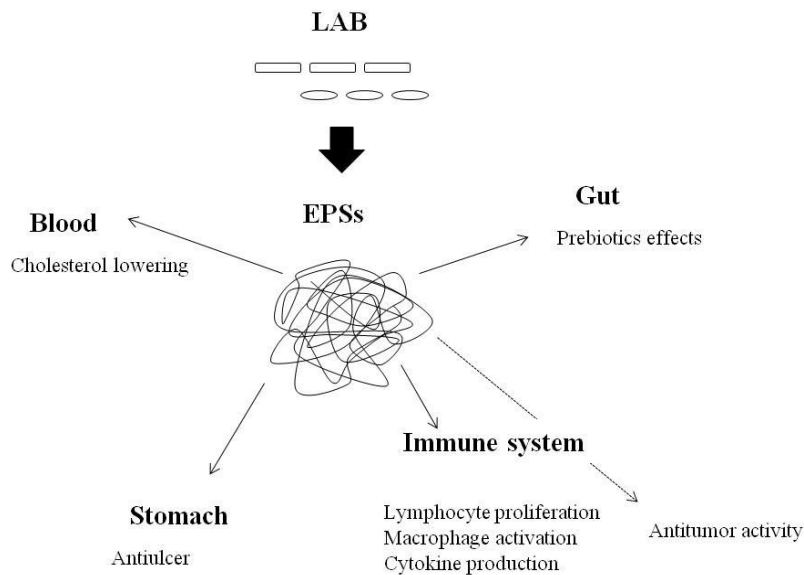
To date, polysaccharides recovered from plant, algae and animal sources (e.g. starch, galactomannans, pectin, carrageenan and alginate) are still the major contributors to the overall hydrocolloid market with xanthan gum being the only significant bacterial EPS, which accounts for 6% of the total market value (Imeson, 2010). This is mainly due to the higher prices of bacterial polysaccharides, which are a consequence of the high value of the carbon sources commonly used and of the associated downstream costs.

However, the regulatory status of safety associated with the majority of lactobacilli attracts a lot of industrial interest due to the simplified regulatory hurdles to application in food products. The genus contains GRAS (generally recognized as safe) or QPS (Qualified Presumption of Safety) bacteria, giving them exemption from risk for the application in the human health market. In this context, there is no need to eliminate biomass before the consumption of polysaccharide and low yield explain why the polysaccharide is seldom used as a purified additive.

Some bacterial EPS can directly replace polysaccharides extracted from plants (e.g. guar gum or pectin) or algae (e.g. carrageenan or alginate) in traditional applications

(Freitas et al., 2011), because of their improved physical properties. Conversely, other bacterial EPS possess unique properties that can launch a range of new commercial opportunities (e.g. bacterial cellulose or levan) (Kumar et al., 2007; Ullrich, 2009). Beside the technological properties, LAB EPS might also contribute to human health (Fig. 2.3) as prebiotics or due to antitumor, antiulcer, immunomodulating or cholesterol-lowering activities (De Vuyst and Degeest, 1999). However, no ESFA (European Food Safety Authority) or FDA health claims on LAB EPS have been approved.

Figure 2.3 Schematic representations of the possible health-promoting properties of LAB-EPSs.



In the dairy fermentation industry there is an increasing consumer interest for stirred yoghurt products with a smooth and creamy texture obtained by mild homogenization of the milk coagulum after fermentation. However, the homogenization strongly affects the rheology of the coagulum and facilitates undesirable serum separation (syneresis) since the network formed by the gel is broken. Several solutions were proposed to improve the texture of fermented milk products and reduce syneresis (Rohm and Kovac, 1994). In order to overcome this problem, a well-known technological solution is to improve the product quality by increasing milk solids such as fat, proteins (Rohm and Schmid, 1993) or sugars (sucrose, fructose), or by adding stabilizers such as pectin, starch, alginate, gelatin, when permitted by national legislation.

However, these approaches do not address an increasing consumer demand for products with low (or reduced) fat, low sugar, low cost and with as few food additives as possible. An answer to this challenge is to incubate the starter cultures at sub-optimal growth temperatures, which encourage EPS, especially HePS, production, and/or to take advantage of the EPS produced naturally by LAB used as starter culture in the fermentation. The success of EPS application in the dairy food industry is generally dictated by its ability to bind water, interact with proteins, and to increase the viscosity of the milk serum phase. EPS may act as texturizers and stabilizers, and consequently avoid the use of food additives (Duboc and Mollet, 2001).

Although the mechanism of the interactions between EPS and milk constituents in fermented dairy products is poorly understood, the viscosity and the charge of the

EPS determine largely the physical properties of the end product. EPS produced by LAB are taste-neutral, however since a fermented milk product becomes more viscous, its residence time in the mouth and time of contact with the palate and taste receptors is increased. As a result, taste perception is increased through an improved volatilization of the intrinsic yoghurt flavours. The benefits of EPS are detectable at extremely low concentrations. The aim is to obtain an appealing visual appearance (gloss) of a product, to prevent syneresis, to have a creamy and firm texture, and to give a pleasant mouth-feel (Duboc and Mollet, 2001).

The application of levan in beverages with particular emphasis on its solution properties (Kasapis et al., 1994b) have demonstrated that the viscosity of a levan solution is stable during heating and in sodium chloride. In addition, the viscosity of levan is influenced by acidic conditions (pH 2), but stable in the range of pH 4–10 (Kim, 1998).

Levan is useful in terms of its water-holding capacity (Table 3). At greater than 1% (w/v) concentration, a levan solution will show film-forming characteristics on an appropriate smooth surface. A low concentration levan solution (below 1%) can also be used in coatings.

Dextran is the collective term given to a group of bacterial polyglucan composed of chains of D-glucose units connected by α -(1→6) linkages. These polysaccharides are synthesized by a number of bacterial species. The synthesis occurs extracellularly and is catalysed by a species specific enzyme, dextransucrase. In the food industry dextran is currently used as thickener for jam and ice-cream. It

prevents crystallization of sugar, improves moisture retention and maintains flavour and appearance of various food items (Table 2.3).

The use of dextran in bread technology is not wide spread even allowing for the fact that its impact on bread quality (volume and texture) has been shown. A patented process has been developed to obtain a sourdough rich in dextran using *L. mesenteroides* spp (deposit number LMGP-16878) strain able to produce a sufficient amount of high molecular weight dextran (10^6 daltons) assuring a significant impact on bread volume (Lacaze et al., 2007; Vandamme et al., 2003). The sourdough obtained permits improved freshness, crumb structure, mouth feel and softness of all kinds of baked good from wheat rich dough products to rye sourdough breads.

Table 2.3 Food industrial application of functional exopolysaccharides and oligosaccharides from LAB

EPS	LAB producing EPS	Uses	References
Dextran	<i>Leuconostoc mesenteroides</i> NRRL B-512F	In <i>bakery products</i> improves softness, crumb texture, loaf volume and may compensate the low protein content of wheat flour.	(Mccurdy et al., 1994; Naessens et al., 2005; Vandamme, 2002; Vandamme, 1997; Wilham et al., 1959; Zannini et al., 2013; Katina <i>et al.</i> , 2009)
	<i>Leuconostoc mesenteroides</i> NRRL B-640	In <i>confectionary</i> dextran can be used as stabilizer for confectionery preventing crystallization, improves moisture retention, increases viscosity and maintain flavour. It use is also suggested in soft drinks, flavour extract, milk beverages and icing compositions.	
	<i>Leuonostoc mesenteroides</i> B-742		
	<i>Leuconostoc mesenteroides</i> B-1355		
	<i>Leuconostoc mesenteroides</i> B-1299		
	<i>Streptococcus mutans</i> 6715		
	<i>Weissella cibaria</i> MG1	In <i>ice cream</i> dextran can be used as stabilizers (2-4%) conferring beneficial properties on viscosity.	
		In <i>frozen and dried food</i> dextran can be used for stabilizing vacuum, air dried, and freeze-dried or frozen foods (fish products, meat, vegetables and cheese). Dextran can be also use as coating agent to protect food from oxidation and other chemical changes and also help to preserve texture and flavour.	
		In non-alcoholic <i>wort-based beverages</i> , dextran can provide desirable textural properties.	
Levan	<i>Streptococcus salivarius</i>	Levan can be uses as a source of di-fructofuranoses, fructose and fructooligosaccharides, as a stabilizer, an emulsifier, a formulation aid, surface-finishing agent, an encapsulating agent, a carrier of flavours, colour and fragrances and as fat substitute. Functional Chewing	(Han, 1990b; Jang et al., 2001; Park, 2007; Song et al., 2000; Van Geel-Schutten, 2006; Vijn and Smeekens,
	<i>Streptococcus mutans</i>		
	<i>Leuconostoc mesenteroides</i> NRRL, B-		

	512F		Gum Composition	1999; Vincent, 2005)
	<i>Lactobacillus sanfranciscensis</i> 2590	LTH	In <i>bakery products</i> levans can be used as bread improvers according to their ability to increase volume, retard staling and to improve texture and taste of breads	
	<i>Lactobacillus reuteri</i> LB 121			
Kefiran	<i>L. lactis</i> subsp. <i>lactis</i> <i>L. lactis</i> subsp. <i>cremoris</i> <i>L. mesenteroides</i> subsp. <i>dextranicum</i> <i>Streptococcus thermophilus</i>		In <i>fermented milk</i> likes Kefir, (traditional self-carbonated slightly alcoholic fermented milk from Eastern Europe). Kefiran is the “glue” of the grains and confers a slimy texture to the product by increasing the water binding reducing at the same time the water flow in the matrix space	(Duboc and Mollet, 2001)
Inulins	<i>Lactobacillus johnsonii</i> NCC 533 <i>Streptococcus mutans</i> JC2 <i>Leuconostoc citreum</i> CW28 <i>Lactobacillus reuteri</i> 121		Inulin can be used as a prebiotic, as a sugar replacer (especially in combination with high intensity sweeteners), as a fat replacer and texture modifier in low-fat dairy products improving the mouth-feel. Especially long-chain inulin addition to low-fat yoghurt resulted in enhanced creaminess. This effect also occurs in low-fat cheese, in yoghurt ice cream, in chocolate mousse and in custards.	(Cardarelli et al., 2008; Guggisberg et al., 2009; Guven et al., 2005; Hennesly et al., 2006; Kip et al., 2006; Koca and Metin, 2004; Paseephol et al., 2008; Tárrega and Costell, 2006)

2.3 Future trends

The molecular bases for structure-function relationships of EPS in food systems become clearer over the last decade. As this research continues, the food industry can expect to see more widespread application of EPS and EPS/LAB symbiotic cultures in ways that provide added value and innovation to food and beverage products for years to come. Additionally, the production of more than one EPS-type by single strains may have strong potential for development of future food applications as they potentially offer synergistic effects on texture (dextran, ropy CPS) and nutritional improvement (levan, inulin, OS). These single or multiple EPS-type producing LAB can also address the need for using natural additives to comply with food legislation that imposes severe constraints on materials that can be used. The LAB-EPS applications in food and beverages industries will be dependent on biotechnological developments from high yielding strains obtaining with all desirable properties linked to their use in food area. In this regard, future work needs to determine the functionality of individual strains and the derived polymers or oligosaccharides in order to enable their deliberate use in food and beverages applications. Furthermore, future academic research should be combined with industrial input to understand the technical shortfalls that EPS can address. However, this must be partnered with an understanding of the economic and regulatory restrictions imposed on the food and beverage industry by the EPS fermentation process and other competing technologies.

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Chapter 3

Objectives

3.1 Objectives

This thesis focuses on functional application *in situ* and *ex situ* of lactic acid bacteria exopolysaccharides, and in particular dextran, in complex food systems.

The first aim of the study was to evaluate the *in situ* potential EPS forming *Weissella* strain in wort-based substrate for developing innovative plant-based beverages

Secondly, to determine whether *in situ* HePS producing LAB have the potential as (adjunct) starter cultures for soy and wholemeal quinoa yoghurt production and, further to verify *in situ* HePS techno-functionality through yoghurt rheology. Thirdly, to investigate the influence of *ex-situ* LAB – dextran application, compared to other hydrocolloids as a novel food ingredient to compensate for low protein in biscuit and wholemeal wheat flour

Chapter 4

Barley malt wort fermentation by exopolysaccharide forming *Weissella cibaria* MG1 for the production of a novel beverage

Emanuele Zannini, Alexander Mauch, Sandra Galle, Michael Gänzle, Aidan Coffey, Elke K. Arendt, Josh P. Taylor and Deborah M. Waters (2013). *J Appl Microbiol* 115:1379-1387

4.1 Summary

The growing interest of governments and industry in developing healthy and natural alternative foods and beverages which will fulfil the consumer drive towards a healthy lifestyle and clean-label, natural diet has led to an increase into traditional lactic acid bacteria fermentation research. In particular, this research aims to address the organoleptic modulation of beverages using *in situ* produced bacterial polysaccharides. *Weissella cibaria* MG1 is capable of producing exopolysaccharides (dextran) and oligosaccharides (glucooligosaccharides) during sucrose-supplemented barley malt derived wort fermentation. Up to 36.4 g l⁻¹ of dextran was produced in an optimised system which improved the rheological profile of the resulting fermentate. Additionally, small amounts of organic acids were formed and ethanol remained below 0.5% (v/v), the threshold volume for a potential health claim designation. The results suggest that the cereal fermentate produced by *W. cibaria* MG1 could potentially be used for the production of a range of novel, nutritious and functional beverages. Using conventional raw materials and traditional processes, novel LAB-fermented beverages can be produced representing an innovative mechanism towards fulfilling the aim to decrease government and personal costs as well as potentially ameliorating consumer lifestyle regarding dietary-related disease.

4.2 Introduction

In recent years, there has been an increase in the number of novel foods (EFSA, 2010) designed to have specific health benefits and the largest sector includes fermented dairy drinks. However, the number of lactose intolerant individuals globally is around 20% and in some Asiatic (Japan, China) or African countries can reach up to 100% (Swagerty et al., 2002), but varies considerably geographically (Vesa et al., 2000). As such, there is a need for variety in this sector with cereal-based fermented beverages offering a suitable choice or alternative for the obligate consumer, self-diagnosed patients (Nicklas et al., 2009), and lifestyle customers who prefer to avoid dairy products, including those which are lactose-free, for other reasons. Other than beers, beverages such as: *kvass*, *bouza*, *chichi*, *mahewu*, represent traditional low or non-alcoholic drinks produced in many parts of the world, which are based on microbial fermentation of cereal extracts (Waters et al., 2014), with a generally acidic character due to the role of lactic acid bacteria (LAB) (Aloys and Angeline, 2009; Dlusskaya et al., 2008; Gadaga et al., 1999; Gotcheva et al., 2000; Muyanja et al., 2003; Prado et al., 2008). The production of these cereal beverages is usually based on spontaneous fermentations by indigenous microbiota with yeasts and LAB being the dominant microorganisms (Almeida et al., 2007; Gotcheva et al., 2000; Muyanja et al., 2003; Zorba et al., 2003).

Recently, as well as these traditional beverages, there is an increasing interest in the production of cereal-based beverages with defined starter-cultures for ease of control and reproducibility (Dlusskaya et al., 2008; Okafor et al., 1998; Zorba et al., 2003). Additionally, using a specific starter culture allows manipulation of the

functionality, texture, flavour and other characteristics of the final product (Blandino et al., 2003; Ganzle et al., 2009; Prado et al., 2008; Waters et al.). This approach potentially allows the development of novel products capable of imparting on the product characteristic organoleptic features due to the production of microbial metabolites which may include oligosaccharides (OS) and exopolysaccharides (EPS). Certain types of bioactive substances may also provide health benefits like prebiotics, which are defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” (Gibson et al., 2004; Roberfroid, 2007). Cereal associated LAB produce a large structural variety of EPS and OS from sucrose through the activity of glycosyltransferases (Tieking and Ganzle, 2005b). Homopolysaccharides are polymers composed of either glucose or fructose units, and are synthesised from sucrose through the action of extracellular glucosyltransferases or fructosyltransferases (glycosyltransferases), respectively (Korakli and Vogel, 2006b). In addition to EPS, glycosyltransferases synthesise OS by transferring glucose or fructose moieties to suitable acceptor carbohydrates, such as maltose (Ganzle et al., 2009).

Over the last two decades, comprehensive data has been generated regarding LAB-mediated polysaccharide production during sourdough fermentation (Brandt and Ganzle, 2006), with the primary focus on improving dough and bread quality, texture, flavour, and shelf-life (Galle et al., 2011). However, transferring the existing knowledge from sourdough technology into beverage production exhibits an interesting and promising approach for novel products, albeit with certain technological challenges to overcome.

The aim of this research is to enrich and revalorise highly nutritious barley malt extract using traditional LAB fermentation, resulting in a novel alcohol-free beverage base with desirable textural properties.

4.3 Material and Methods

Bacterial cultures and screening for exopolysaccharide and oligosaccharide production

Thirty-seven LAB isolates present in cereal environments were maintained as frozen stocks in 80% glycerol at -80°C . LAB were routinely grown on MRS-agar plates (Merck, Darmstadt, Germany) under microaerophilic conditions for 48 h at 30°C .

To screen for EPS, the LAB were grown on modified MRS agar (mMRS) (Meroth et al., 2003) plates (Galle et al., 2010b), supplemented with 10% sucrose (SucMRS). EPS positive isolates were defined as those displaying slimy colony morphology. To characterise EPS/OS production of the best producer strains in SucMRS broth, single colonies were picked from mMRS plates, subcultured once for 16 h in mMRS broth, and subsequently inoculated at 1% in SucMRS broth and incubated anaerobically at 30°C for 48 hrs.

Barley malt and mashing regime

Commercial malt, made from the barley variety *Sebastian*, which was harvested in 2008, was purchased from the Cork Malting Company and used in these trials. The design of a mashing regime contributing maximum amounts of sucrose and maltose in wort was based on a Response surface methodology (RSM) model as previously described (Mauch et al., 2011). The following mashing regime was

applied; 28.1 min at 62°C, 25.0 min at 72°C, and a mash off temperature of 78°C, with a heating rate of 1°C min⁻¹ between the individual rests.

Wort production and fermentation (beverage base production)

A pilot-scale (1,000 L) brewhouse was used for wort production and fermentation. Firstly, the malt was milled with a two-roller mill (0.4-mm distance between rollers) and 120 kg of grist was mashed into 540 L of water at 62°C. Wort was boiled for 1 h and adjusted to a final extract content of 9% (w/w) by adding hot water. Precipitates in hot wort were removed using a whirlpool. Hot wort was transferred into a 20 L stainless steel container. Sucrose was dissolved in the hot wort to achieve final concentrations of 5 and 10% (w/v). The wort was then cooled to 30°C in a tempered water bath.

Strains of *Weissella cibaria* MG1 were subcultured twice in MRS broth and inoculated to the wort to obtain a concentration of 10⁶ cfu ml⁻¹. LAB were inoculated into the cooled wort with subsequent fermentation at 30°C for 72 h. Samples were aseptically withdrawn at 0 h (after inoculation), 24 h, 48 h, and 72 h. LAB cell counts and pH were determined after wort inoculation and throughout fermentation. The number of colony forming units (cfu) was determined by standard microbiological plating assays. The characterisation of EPS and OS produced by the strains was described above.

EPS characterisation in sucrose-supplemented MRS and wort

EPS were isolated from SucMRS broth using chilled ethanol, with subsequent dialysis and freeze drying steps. The EPS composition, structure and molecular weight was analysed as previously described (Galle et al., 2010b). Briefly, EPS

monosaccharide composition was determined after acid hydrolysis and the linkage type was determined after digestion with dextranase and amyloglucosidase. The molecular weight was analysed with a Superdex 200 Column (GE Healthcare, Baie d'Urfe, Canada) with water as the solvent and a flow rate of 0.4 mL min^{-1} . Detection was by a Refractive Index Detector (RID).

Determination of oligosaccharides produced in MRS and wort

OS patterns of fermented MRS and wort both initially containing 10% sucrose were analysed with a CarbopacPA20 column (Dionex, Oakville, Canada) using water (A), 200 mM NaOH (B), and 1M Na-acetate (C) as solvents at a flow rate of 0.25 mL min^{-1} . The following gradient was used: 0 min 30.4% B, 1.3% C, 22 min 30.4% B, and 11.34% C, followed by washing and regeneration. OS were directly analysed from the cell free supernatant using maltose and panose as external standards for peak identification. Detection was by a Refractive Index Detector (RID).

Rheology

In advance of rheological analyses, fermented wort samples were centrifuged to remove LAB cells and any other particulate matter. The viscosities of these cell free supernatants (cfs) were measured on a controlled stress rheometer (Physica MCR 301, Anton Paar, Austria) using a cone-plate system at 20°C . Two ml of cfs sample was placed on the plate and the upper cone (diameter 74.997 mm) was lowered to 0.04 mm with removal of excess sample. Measurements were as a function of shear rate over a range of $10\text{--}1000 \text{ s}^{-1}$.

Determination of sugars and metabolites in wort

Wort extract was determined using an automated beer analyser (ServoChem, Tectator AB). For determination of sugars and organic acids, fermentation liquids were mixed in the ratio 1:1 with 7% perchloric acid and proteins were precipitated at 4°C overnight. Maltose, fructose, lactate, acetate, and ethanol were determined by high performance liquid chromatography (HPLC) using an Agilent 1200 HPLC system with a RID. A REZEX 8 μ 8% H organic acid column (300 x 7.8 mM, Phenomenex, USA) was used with 0.01N H₂SO₄ as the elution fluid, at a flow rate of 0.6 mL min⁻¹, at 65°C. Substrate and end product peaks were identified by comparison of their retention times with pure compound standards and their concentrations were also determined. Sucrose and glucose co-eluted and were not quantified separately.

Statistics

Experiments were performed in triplicate. The Excel Analysis ToolPak (Microsoft Corporation[®]) was used for statistical calculations. Data were checked for outliers by using Q test, and statistical analysis was performed with Minitab 16 software using one-way analysis of variances (ANOVA). All differences were considered as significant when $P < 0.05$. Multi-comparison of means was assessed by a Tuckey Post Hoc test.

4.5 Results

In vitro screening for EPS production by LAB

Thirty-seven LAB strains which had been previously isolated from cereal environments were screened for their ability to produce EPS on a suitable agar

media. Fourteen of the isolates showed slimy colony morphology (**Figure 4.1, A and B**) on SucMRS agar (**Table 4.1**) and were further investigated as EPS-positive strains (EPS+). These EPS+ isolates were belonging to the species *L. arizonensis*, *L. plantarum* and *W. cibaria*.

Figure 4.1 (A) EPS production by *W. cibaria* MG1, on 10% sucrose-supplemented MRS agar in the form of slimy colony morphology, and (B) no EPS production by the same strain in the absence of sucrose.

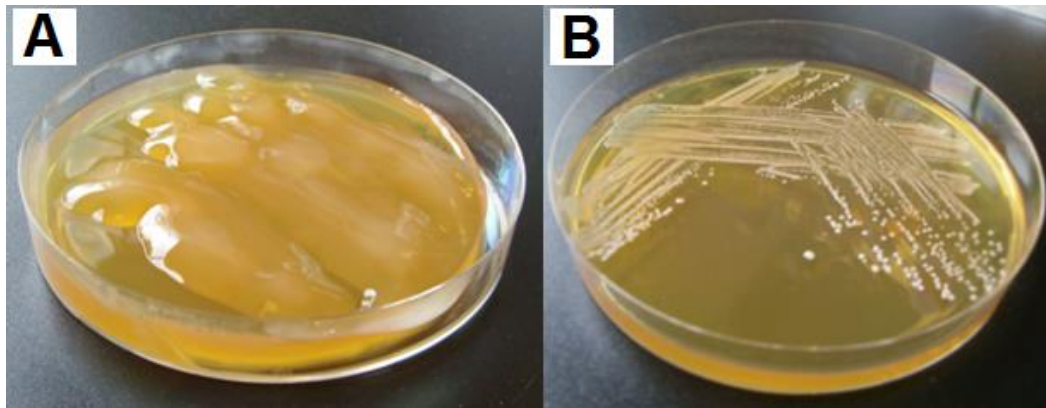


Table 4.1 Exopolysaccharide production by 37 LAB cereal isolates on SucMRS agar.

Species	EPS+	EPS-
<i>L. amylovorus</i>	-	FST2.11
<i>L. arizonensis</i>	F17, F8, F7, F6, F13, F12	F10, F2, F21, F22, F25, F26, F3, F34, F36, F4, F9
<i>L. coryniformis</i>	-	E12, E9
<i>L. fermentum</i>	-	F23, F31
<i>L. plantarum</i>	F5	FST 1.7, FST 1.9
<i>L. argentinum</i>	-	E11, E2, E4
<i>Pediococcus pentosaceus</i>	-	E6
<i>W. cibaria</i>	MG1, MG7, F33, F28, F27, F29, F27	E7

EPS+ and EPS- mean EPS detected (+) through slimy colony morphology after growth on MRS agar plates supplied with 100 g L⁻¹ sucrose, or not detected (-)

EPS characterisation in MRS and wort

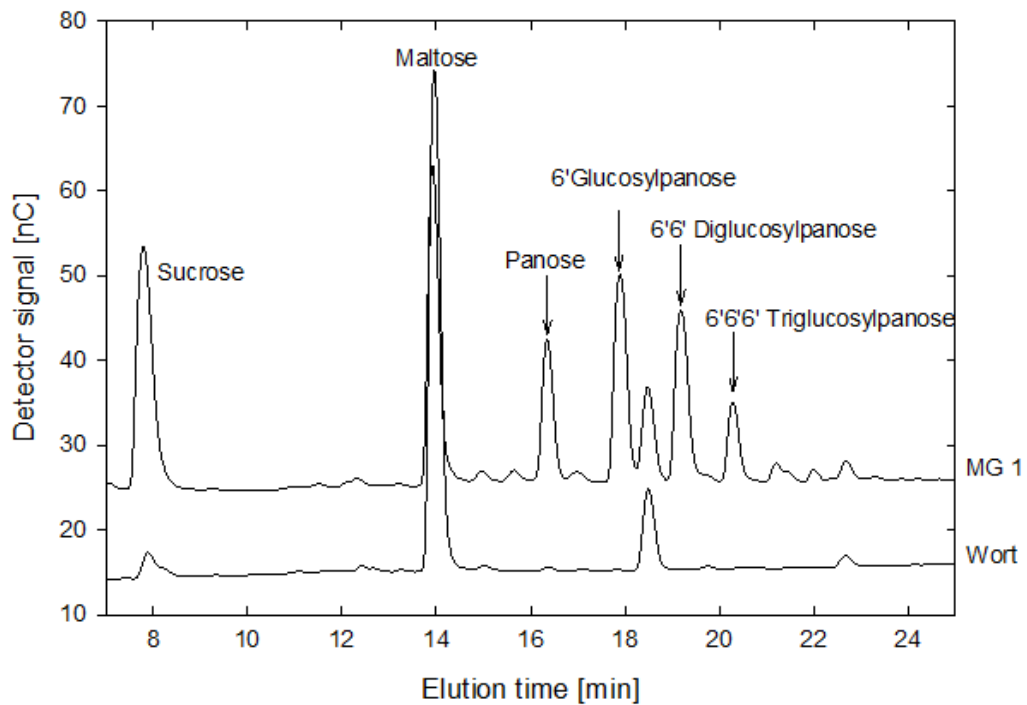
The 14 EPS+ isolates were cultured in SucMRS broth and the four strongest producer LAB strains were further analysed. These included the isolates: *W. cibaria* MG1, MG7, F28 and F33. The type of EPS (**Table 4.2**) and OS (**Figure 4.2**) produced by these strains were analysed after 72 h, however the levels produced remained unchanged after 48 h.

Table 4.2 Amounts of EPS (dextran, 5 x10⁶ – 4 x10⁷ Da) produced by the strongest LAB producer strains, all of which were from the *Weissella* genera

Substrate	Species	[g l ⁻¹]*
SucMRS	<i>W. cibaria</i> (MG1)	36.4 ± 0.6
	<i>W. cibaria</i> (MG7)	13.7 ± 1.8
	<i>W. cibaria</i> (F28)	13.4 ± 6.1
	<i>W. cibaria</i> (F33)	16.2 ± 1.8
Wort (0.3% sucrose)	<i>W. cibaria</i> (MG1)	1.4 ± 0.2
	<i>W. cibaria</i> (MG7)	1.4 ± 0.1
SucWort (5%)	<i>W. cibaria</i> (MG1)	8.6 ± 0.8
	<i>W. cibaria</i> (MG7)	7.2 ± 0.4
SucWort (10%)	<i>W. cibaria</i> (MG1)	14.4 ± 1.2
	<i>W. cibaria</i> (MG7)	10.6 ± 0.7

*Amount of EPS produced by the LAB isolate in 10% sucrose-supplemented media broth after 72 h, EPS means exopolysaccharide, SucMRS means sucrose-supplemented MRS broth, SucWort means sucrose-supplemented wort

Figure 4.2 Oligosaccharide-production patterns of wort containing 10% sucrose fermented with, *W. cibaria* MG1 (MG1) and unfermented wort control (Wort).



The EPS produced by all strains was dextran which has a glucan backbone consisting of α -1-6 linkages and the OS was a glucooligosaccharide (GOS), confirmed as previously reported (Galle et al., 2010b). Dextran was produced in highest amounts by *W. cibaria* MG1 and as such, this strain was further applied in wort fermentations.

Cell growth, pH, formation of metabolites and utilisation of sugars during wort fermentations

During wort fermentations, bacterial growth was monitored through determination of pH and cell counts. *W. cibaria* MG1 produced the highest amount of EPS amongst all strains in broth and wort fermentations, and was therefore applied for further analysis. In the non-sucrose supplemented wort

($c_{\text{sucrose}}=0.3\%$), *W. cibaria* MG1 grew from $\sim 10^6$ cfu ml⁻¹ to $\sim 2.0 \times 10^8$ cfu ml⁻¹ after 24 h of growth. After 72 h, the amount of viable cells in the wort decreased during fermentation ($6.4 \times 10^7 \pm 1.5 \times 10^7$ cfu ml⁻¹). A decreased cell count was also observed for *W. cibaria* MG1 upon sucrose supplementation at a final concentration of 5 or 10%. The pH of all worts dropped to 4.0 after 24 h of fermentation and this acidification profile was reflected in the lactic and acetic acids levels (**Table 4.3**).

Table 4.3 Formation of acetic and lactic acids, fructose, and ethanol, as well as utilisation of maltose during *W. cibaria* MG1 fermentation of worts containing different initial amounts of sucrose of approximately 0.3, 5, and 10%.

Product (g l ⁻¹)	Time (h)	Initial sucrose (% w/v)		
		0.3	5	10
Acetic acid	0	ND	ND	ND
	24	0.2	0.2	0.2
	48	0.2	0.3	0.3
	72	0.2	0.3	0.3
Lactic acid	0	ND	ND	ND
	24	1.7	1.8	1.8
	48	2.1	2.0	1.9
	72	2.3	2.3	2.0
Fructose	0	4	4	4
	24	6	30	54
	48	6	31	54
	72	6	31	55
Maltose	0	43	44	43
	24	40	28	23
	48	40	26	19
	72	39	25	19
Ethanol	0	ND	ND	ND
	24	0.80	0.66	0.64
	48	0.88	0.80	0.70
	72	1.02	0.88	0.72

ND means 'not detected'

These organic acids were primarily produced during the first 24 h of fermentation, particularly for the former metabolite. Generally, sucrose-

supplemented worts had slightly reduced lactic acid formation whilst acetic acid formation was independent of sucrose concentration. The formation of ethanol by the *W. cibaria* MG1 was negatively correlated with the initial level of sucrose and as such, the highest amounts were produced in non-supplemented worts.

Fructose formation by *W. cibaria* MG1 in wort was positively correlated with the initial concentration of sucrose present (**Table 4.3**), with the latter being depleted as fermentation progressed. An approximate conversion rate of 98% was found in 10% sucrose-supplemented wort and the use of maltose was greater in the 5 and 10% sucrose-supplemented worts by up to 34 and 47%, respectively (**Table 4.3**). The combined HPLC peak representing sucrose and glucose showed that the *W. cibaria* MG1 isolate used consumed more than 90% of these sugars after 72 h of fermentation in all worts (data not shown).

Formation of exopolysaccharides and oligosaccharides

The maximal EPS production during *W. cibaria* MG1 wort fermentations was exhibited in the 10% sucrose-supplemented sample (**Table 4.2**). Fermentation of sucrose non-supplemented wort resulted in the lowest EPS production levels (1.4 g l⁻¹).

The OS patterns of original and fermented worts are shown in **Figure 4.2**, but were not quantified due to the lack of commercially available standards. The fermented wort OS patterns were comparable to previous reports (Galle et al., 2010b) in *W. cibaria* MG1 fermented 10% sucrose-supplemented MRS broth. Panose was identified by external standards with subsequent peaks likely representing the oligosaccharides: 6'glucosylpannose, 6'6'diglucoylpannose, and higher oligosaccharides of the same series (**Figure 4.2**). Sucrose non-

supplemented *W. cibaria* MG1 fermented wort was used as a control and showed baseline levels of OS production.

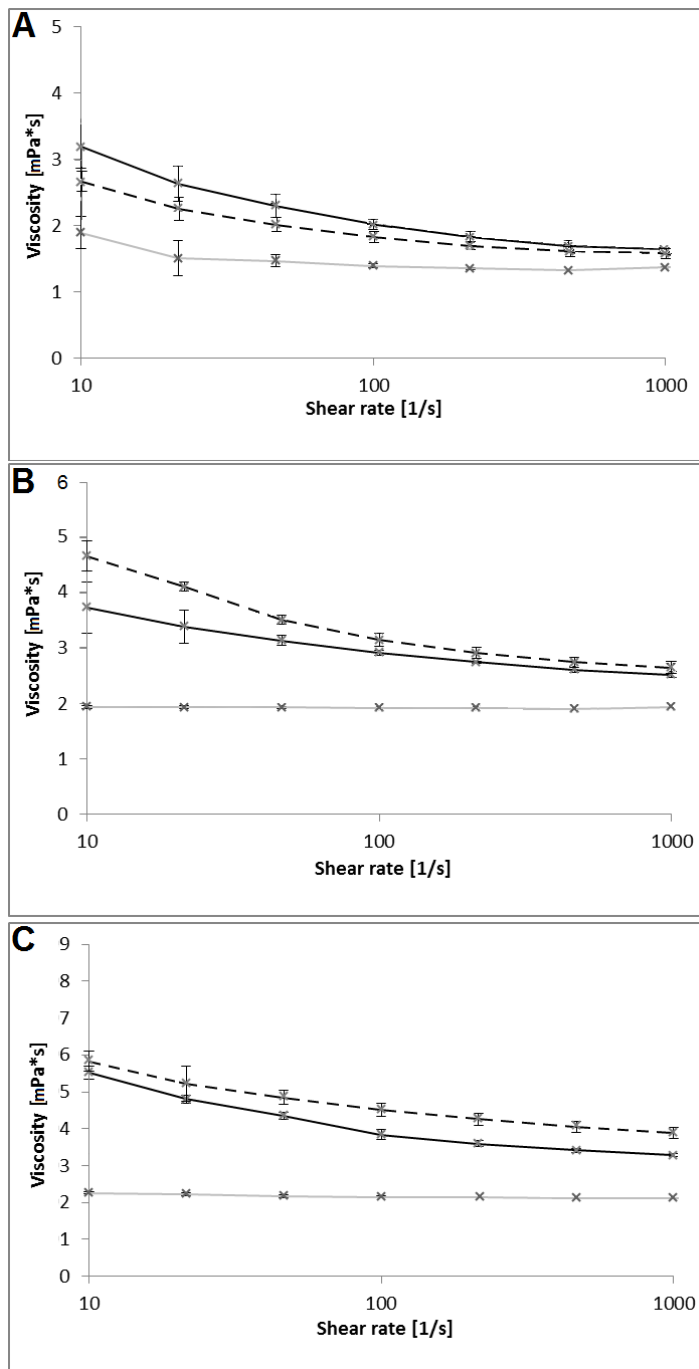


Figure 4.3 Apparent viscosities η as a function of shear rate of wort from 24 h *W. cibaria* MG1 fermentation (—), 72 h *W. cibaria* MG1 fermentation (- -), and unfermented wort (—), non-sucrose supplemented (A), 5% sucrose-supplemented (B), and 10% sucrose-supplemented (C).

Rheology of fermented worts

The apparent viscosities (η) as a function of the shear rate of the fermentation liquids of *W. cibaria* MG1 fermented (24 and 72 h) and unfermented wort, are shown in **Figure 4.3 (A-C)**. All fermented worts displayed a shear thinning behaviour with *W. cibaria* MG1 after 72 h fermentation exhibiting the highest viscosities, when the wort was supplemented with 5 or 10% sucrose (**Figure 4.3 B and C**). Sucrose un-supplemented unfermented wort exhibited a slight shear thinning behaviour (**Figure 4.3 A**) however, addition of sucrose increased the viscosities, and the worts developed Newtonian behaviour (**Figure 4.3 B and C**). Increasing initial concentrations of sucrose in worts fermented with *W. cibaria* led to increased differences in viscosities to the respective unfermented wort, at both at low and high shear rates, indicating the formation of polymers.

4.6 Discussion

LAB screened in this research originated from cereal or processed cereal environments. In total, 38% of the cereal isolates proved to be EPS producing strains, with all of these strains also producing OS. The importance of *in situ* production of EPS and OS through natural fermentation technologies lies in their functional applications regarding their textural amelioration abilities as well as potential health effects. Certain OS such as galacto-/ fructo-/ gluco-/ arabinoxylan- oligosaccharides are recognised as being useful as prebiotics (Grootaert et al., 2007; Rycroft et al., 2001), in the prevention of allergic diseases (Kukkonen et al., 2007), supporting the growth of gut-friendly microbial probiotics (Kondepudi et al., 2012), and aiding immunity through suppression of inflammatory markers (cytokines) (Bengmark, 2012; Macfarlane et al., 2009).

Additionally, the benefits of these EPS and OS producer strains combined with probiotic LAB could potentially lead to the production of a synbiotic, clean-label, cereal-based beverage (Grimoud et al., 2010).

W. cibaria MG1 dominated the (sucrose-supplemented) barley wort fermentation and produced 14 g l⁻¹ EPS. The high maltose concentration, compared to sucrose, in wort diverted dextransucrase activity from EPS production to formation of panose-oligoaccharides. Since the LAB analysed in this research originated from cereal or processed cereal environments, their suitability to dominate wort during fermentation was presumed. This theory was tested for the highest EPS-producer LAB, *W. cibaria* MG1. Even though the strain dominated the (sucrose-supplemented) wort fermentation, it was found to produce EPS with lower efficiency than in SucMRS. Higher maltose concentration in wort led to the increased formation of oligosaccharides at the expense of EPS production levels. LAB naturally produce EPS and OS, thus distinguishing them as microbes with potential for development of technologically functional ingredients which can be produced *in situ* to formulate alternative, non-alcoholic, cereal-based beverages. Additionally, diversifying the brewhouse product portfolio through incorporation of traditional fermentation technologies represents an economical approach to raw material revalorisation. This evolution of product conceptualisation allows brewers to implement these technologies in their existing plants, whilst concurrently continuing their regular brewing objectives, thus incurring no additional investment.

This study shows that the LAB *W. cibaria* MG1 can dominate a barley malt derived wort environment to produce up to 14.4 g l⁻¹ of the homoexopolysaccharide dextran, resulting in a novel and nutritious beverage

base with prospects for further advancements using tailored microbial cultures. The yield of EPS is dependent on sucrose concentration, the LAB strain used, and growth conditions, amongst others. Barley malt wort produced for beer production naturally contains between 0.3 – 6.0 g l⁻¹ of sucrose and 39 – 60 g l⁻¹ of maltose (Boulton and Quain, 2001), characteristics which can be manipulated through optimisation of the mashing program, however sucrose supplementation is still necessary to obtain sufficient yields of EPS. The barley malt invertase/glycansucrase enzymes function optimally at a lower temperatures than β -amylase maltogenesis (Narziß, 2004). As such, using an elevated temperature during the initial step of wort production will maximise saccharification. Thus, regardless of mash parameters, sucrose supplementation is necessary to allow efficient EPS and OS production by *W. cibaria* MG1 in wort.

As the ratio of sucrose to maltose increased, analyses of the *W. cibaria* MG1 wort fermentation revealed that EPS production was positively correlated. This effect was previously reported for a purified dextransucrase of *Leuconostoc mesenteroides* (Heincke et al., 1999; Paul et al., 1986). The depletion of maltose, a strong acceptor sugar for dextransucrase (Killey et al., 1955), in sucrose-supplemented worts indicates OS formation, as confirmed by the *W. cibaria* MG1 SEC-HPLC analyses. In the presence of maltose, *W. cibaria* MG1 is known to produce panose and higher OS of the panose series (Galle et al., 2010b), with the concomitant accumulation of fructose during fermentation indicating an almost complete conversion of sucrose. In keeping with previous observations (Galle et al., 2010b), *W. cibaria* MG1 did not reduce fructose to mannitol (data not shown).

During *W. cibaria* wort fermentation, due to a slightly negative correlation between lactic acid production and sucrose levels combined with the positive correlation with acetic acid production, the fermentation quotients decreased. These low acetate formation levels are beneficial in food processing due to the negative unpleasant flavour attributes associated with higher amounts (Galle et al., 2010b). Additionally, ethanol formation by the heterofermentative *W. cibaria* MG1 during wort fermentation was negatively correlated with sucrose content. Furthermore, as sucrose levels increase, one of its hydrolysis products (glucose not fructose) acts as building blocks for EPS production, whilst maltose concurrently acts as a an acceptor sugar for the glucansucrases catalysing the transferase reaction (Galle et al., 2010b).

The formation of EPS by *W. cibaria* MG1 significantly influenced the rheological behaviour of wort. Since dextran formed by *W. cibaria* MG1 solely consists of unbranched α -1,6 glycosidic linkages (Galle et al., 2011), it is linear in character. The levels of EPS formed were positively correlated with the initial sucrose concentration and EPS production continued throughout the complete fermentation time. Additionally, since the EPS remained stable after completion of fermentation, this dextran potentially represents a suitable viscosity-enhancer for beverages over their storage time.

Given the current government, industry, and consumer drive towards a healthy lifestyle, dietary amendments are a positive way to implement a widespread, effective, and simple societal reform. This can have a significant and positive repercussive effect on personal and government medical costs related to non-communicable diseases; such as cardiovascular, kidney, liver, certain cancers, and obesity-related chronic illnesses, particularly in Western developed

countries. However, these trends place substantial pressure on the food and beverage industries to be innovative by reformulating current, or reinventing novel products, in line with consumer expectations and regulatory authority stipulations. Thus, using conventional locally produced raw materials (such as cereal grain extracts) and traditional processes (such as fermentation and brewing), to produce a novel product (such as LAB-fermented beverages) represents an innovative yet technologically challenging mechanism towards fulfilling this aim. Therefore, producing a naturally fermented cereal-based beverage from wort easily accomplishes these requirements, with the capacity to produce tailor-made products addressing specific consumer requirements, such as; low-calorie, high vitamin and mineral, cholesterol-lowering, prebiotic and flavoursome beverage with desirable organoleptic attributes. Furthermore, the fermented beverage produced in the current work had ethanol levels less than 0.5% (v/v), the threshold volume for beverages with a health claim designation (Kreiszi et al., 2008). Additionally, the viscosity obtained using the *in situ* EPS production by LAB, also contributes to a shelf-stable desirable body in the final formulation. Finally, all these attributes can potentially be realised in a functional beverage using standard brewing equipment and widely available raw materials.

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Chapter 5

Impact of exopolysaccharide-producing *Weissella cibaria*

MG1 on the properties of soy yoghurt

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5.1 Summary

The fermentation performance of the exopolysaccharide (EPS) producing *Weissella cibaria* MG1 as an adjunct culture in soy milk yogurt production was investigated. Soy milk was fermented with a commercial yoghurt starter culture and *W. cibaria* MG1 which had been supplemented with varying sucrose levels, and the rheological impact on the final product was studied. *W. cibaria* MG1 grew well in soy milk, exceeding a cell count of log 8 cfu/g within 6 h of fermentation. The presence of *W. cibaria* MG1 led to a decrease in gelation and fermentation time. EPS isolated from soy yoghurts supplemented with sucrose were higher in molecular weight (1.1×10^8 g/mol Vs 6.6×10^7 g/mol), when compared to control, (no sucrose added) and resulted in reduced gel stiffness (190 ± 2.89 Pa vs 244 ± 15.9 Pa) and thus yoghurt quality. The rheology analysis highlighted that the fracture stress decreased and the network structure changed due to larger pores and less cross-linking in the presence of sucrose and increasing molecular weight of the EPS.

5.2 Introduction

The increasing demand for dairy alternatives initially arose out of necessities such as lactose intolerance and cow's milk protein allergy. Nowadays, the avoidance of dairy products forms part of particular lifestyles including vegetarian and vegan diets, health concerns associated with dairy consumption such as elevated cholesterol levels or antibiotic residues, and ethical considerations. Plant derived milk substitutes can be produced from a range of cereals, pseudocereals, beans and other grains including soy, almond, oat, rice, coconut and quinoa, which can be then processed further into yoghurt-type products (Mäkinen et al., 2013(Monitor 2005). However, the mouth-feel, texture and flavour is often significantly different from the traditional and widely accepted dairy-based yoghurt (Shirai et al. 1992; Cheng et al. 1990), thus, process optimisation has to be performed. In order to provide satisfactory product quality, several attempts have been made to improve the sensory properties by adding different ingredients such as calcium (Yazici et al. 1997), gelatine and lactose (Cheng et al. 1990), inulin, raffinose and glucose (Donkor et al. 2007), whey protein concentrate, non-fat dry milk (Lee et al. 1990), fructose, evaporated milk (Buono et al. 1990), hydrocolloids or by employing different (bio)processing tools such as the pre-treatment of soy milk with ultra-high-pressure homogenization (Cruz et al. 2009), microwave processing (Bhattacharya & Jena 2007) or through the use of germinated soybeans as the primary raw material (Yang & Li 2010). Since consumers increasingly demand and producers strive to provide products which are low in fat, sugar and additives, the use of exopolysaccharide (EPS) producing LAB as texturisers and stabilising tools represent an attractive and economical option (Duboc & Mollet 2001).

A recent trend is the application of cultures that possess at least one functional property contributing to the health properties and the texture of the fermented food. The role of EPS in food matrices has been extensively reviewed (De Vuyst & Degeest 1999; Jolly et al. 2002; Monsan et al. 2001; Ruas-Madiedo et al. 2002; Ricciardi & Clement 2000; Cerning 1990; Cerning 1995; Duboc & Mollet 2001)(Badel et al. 2011). The improvement of the rheological properties of foods by EPS is primarily due to their ability to bind water thereby reducing syneresis in dairy and dairy-type replacement products during storage. This causes altered viscosity of the serum phase, which leads to a modified taste perception. EPS are produced by a range of LAB and vary greatly in their composition, molecular weight, charge, spatial arrangement and flexibility (Duboc & Mollet 2001; Jolly et al. 2002). The impact of EPS on texture is not only based on the concentration of EPS (Gentès et al. 2013; Bouzar et al. 1997; Ruas-Madiedo et al. 2002) but also on the molecular weight (Faber et al. 1998), charge (van den Berg et al. 1995; Girard & Schaffer-Lequart 2007; Gentès et al. 2013), degree of branching and flexibility of the backbone (Tuinier et al. 2001; Gentès et al. 2013), monomer composition and its linkages (Laws & Marshall 2001). Milk protein-EPS interactions have been extensively studied (Ayala-Hernández et al. 2009; Girard & Schaffer-Lequart 2008; Gentès et al. 2013) but interactions of EPS with soy protein have rarely been investigated (Li et al. 2014). Generally, EPS can be classified into homopolysaccharides (HoPS), composed solely of either glucose or fructose units, and heteropolysaccharides (HePS), consisting of two or more different sugar moieties such as glucose, galactose, rhamnose and fructose (De Vuyst & Degeest 1999). HoPS, e.g. dextran, levan, reuteran and mutan, are synthesized by transglycosylases using sucrose as their sole substrate. Energy

supply for the polymerization is provided by the hydrolysis of the glycosidic bond of sucrose (Duboc & Mollet 2001; Monsan et al. 2001). In the dairy industry it is typically HePS-producing LAB that are used for fermentation (De Vuyst et al. 2001; De Vuyst & Degeest 1999). However, in this study the HoPS-producing strain *Weissella cibaria* MG1 is investigated for its impact on the rheological properties of a fermented soy product. To date, little attention has been paid to the role of EPS in plant-based food and beverage industries, except for a study focussed on LAB fermentation and EPS formation in an oat-based milk substitute (Mårtensson et al. 2000).

W. cibaria MG1 was isolated from sourdough by Galle et al. (2010) and identified as a hyper-producer of dextran with a molecular weight of 7.2×10^8 g/mol (Galle et al. 2012). Dextrans synthesized by *W. cibaria* are glucans connected by α -(1,6) glycosidic bonds (Galle et al. 2010) but may also contain small amounts of α -(1,3) linkages (Bounaix et al. 2009). In recent studies, *W. cibaria* MG1 was used in barley malt wort fermentation (Zannini et al. 2013) and sourdough fermentation made from wheat or gluten-free flours. Further, the impact on the bread quality due to *W. cibaria* MG1 EPS formation monitored at ultrastructure and macrostructure level has been investigated (Galle et al. 2012; Wolter, Hager., et al. 2014; Wolter, Hager, et al. 2014; Galle et al. 2012). In all of these studies *W. cibaria* MG1 dextran formation ameliorate the structural and sensory characteristics of the investigated food matrices.

The aim of the present investigation was to determine the impact of *W. cibaria* MG1 and its EPS on the quality parameters of set-style soy yoghurt fermented with commercial starter cultures. Microbial growth and primary metabolites of the EPS producer *W. cibaria* MG1 as well as its influence on the growth

performance of the commercial starter culture (CSC) and on the rheological properties of the final product have been investigated.

5.3 Material and methods

Substrates and Chemicals

Soy milk (Provamel, Alpro, Ghent, Belgium) was purchased from a local grocery store. The product contained 3.3 g protein, 2.4 g sugars, 1.9 g fat and 0.06 g sodium per 100 ml, and was sweetened with apple concentrate according to the package label. MRS and M17 broth were purchased from Oxoid (Basingstoke, UK), bacteriological agar and Ringer solution tablets were obtained from Merck (Darmstadt, Germany). Sucrose, lactose and standards for sugar, acid and EPS analysis were purchased from Sigma-Aldrich (St Louis, US). Bromocresol green and skimmed milk powder were obtained from BHD Chemicals Ltd (Poole, UK) and Dow Wolff Cellulosics (Bomlitz, Germany), respectively.

Microorganisms and growth conditions

Commercial starter culture YC-470, containing *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* (*S. thermophilus*), was provided by Chr Hansen (Hørsholm, Denmark). *W. cibaria* MG1 was obtained from the culture collection of the Cereal Science Laboratory of University College Cork, Ireland. *W. cibaria* MG1 has been identified earlier as hyper- producer of dextran (Galle et al. 2010). Working cultures from *W. cibaria* MG1 were prepared from a glycerol stock stored at -80°C by streaking it on MRS agar (pH 5.8) supplemented with 1 ml/l vitamins (Meroth et al. 2003) and 0.05 g/l bromocresol green (Dal Bello & Hertel 2006). Plates were incubated at

30°C for 48 h under anaerobic conditions. Ten ml MRS broth was inoculated with one single colony, incubated at the same conditions overnight, transferred to 1 l MRS broth and incubated for 24 h. The broth was centrifuged (5,000 rpm, 10 min, 4°C), the supernatant was discarded and the pellet transferred to sterile tubes (Sarstedt, Nümbrecht, Germany), containing sterile skimmed milk solution (10 % w/v). The mixture was then freeze-dried. The resulting *W. cibaria* MG1 powder had a concentration of 2.44×10^{10} cfu/g.

Soy yoghurt production

Six different types of soy yoghurt were produced. All soy yoghurts were inoculated with the CSC YC-470 I, and three of them were also co-fermented with *W. cibaria* MG1 (CM). To allow the EPS production by *W. cibaria* MG1, sucrose was added in three concentrations, 0, 5 and 10% (w/v). The sucrose contents are respectively indicated by numbers in sample abbreviations: C, CM, C5, CM5, C10 and CM10. The inoculum of the CSC was prepared following manufacture's instructions (for dairy yoghurt preparation) by activating 0.03 % YC-470 in 5 ml of soy milk. The inoculum was vortexed and let rest for about 30 min at room temperature. The inoculum of *W. cibaria* MG1 was prepared in the same way with 0.0035 % freeze dried powder. Before inoculation, soy milk was heat-treated at 90°C for 10 min, using a water bath under stirring condition (Lochner Labor + Technik GmbH, Berching, Germany), and brought to 37°C . Sucrose was mixed in the milks for yoghurt types C5, C10, CM5 and CM10, and the mixtures were inoculated with CSC and *W. cibaria* MG1 starter cultures. pH was monitored during fermentation, and once the pH had reached 4.5 – 4.6, the

samples were stored overnight at 4°C before analyses. The soy yoghurts were prepared and analysed in triplicate.

Cell counts

Samples were taken immediately after inoculation, and at the end of fermentation. They were serially diluted and incubated as follows: *S. thermophilus* on M17 agar with 5 g/l lactose at 43°C, *W. cibaria* MG1 on MRS agar at 30°C, and *L. bulgaricus* on MRS agar at 37°C (soy yoghurts without *W. cibaria* MG1) or 43°C (soy yoghurts with *W. cibaria* MG1). All plates were supplemented with 1 ml/l vitamins and 0.05 g/l bromocresol green and incubated under anaerobic conditions for 48 to 72 h. Cell counts were performed in triplicate from two independent serial dilutions. Plates in the range of 30 to 300 cfu/ plate were counted and used for subsequent calculations. The identity of starter cultures and MG1 was confirmed by colony morphology and metabolic patterns [1].

Acid production

The total titratable acidity (TTA) was determined by suspending 10 g of yoghurt in 90 ml distilled water, and titrating the sample with 0.1 N NaOH to a pH of 8.5 (Katina et al. 2006). After 3 min, the pH was readjusted to 8.5. The TTA was expressed as the amount NaOH (in ml) used for titration.

Acid profiles were analysed using an Infinity 1260 HPLC system equipped with a 300 x 7.7 mm Hi-Plex H column and a diode array detector (Agilent Technologies, Palo Alto, US). H₂SO₄ (4 mM) was used as an eluent with a flow rate of 0.5 ml/min at 65°C. Sample preparation was carried out as described by

Indyk, Edwards, & Woollard (Indyk et al. 1996). Sample (5 g) was mixed with 15 ml warm water in a 25 ml volumetric flask, 250 µl of Carrez reagent I and II were added sequentially while shaking gently and let rest for 20 min. The volume was adjusted to 25 ml, mixed and aliquots were centrifuged (10,000 g, 5 min, 4°C) and filtered (0.45 µm). Lactic acid, acetic acid and propionic acid were used as external standards.

Sugar profile

Samples were prepared for sugar analysis by Carrez clarification, as described in the previous paragraph. For the analysis, 750 µl sample was mixed with 250 µl I. Sugars were analysed with a 250 x 4.6 mm Supercosil LC-NH₂ (Sigma-Aldrich) column and a refractive index detector. Samples were eluted with I:H₂O (3:1, v/v) at a flow rate of 1 ml/min. Sugar concentrations were determined using glucose, fructose, maltose and sucrose as external standards. Standard calibration curves were prepared at 5 different concentrations and measured in duplicate always at the beginning and end of a sample set.

Analysis of EPS

Soy yoghurt samples (100 ± 0.5 g) were precipitated with 2 volumes of chilled 96 % (v/v) ethanol and stored at 4 °C for 16 h. Samples were centrifuged (12,000 g, 20 min, 15°C) and the supernatants discarded. The pellets were dissolved in distilled water, dialyzed (Av. Flat width 32 mm, MWCO 12,400, Sigma-Aldrich) against distilled water for 24 h and freeze dried. The powder was treated with protease (Neutrase 0.8 L, Novozymes) and precipitated overnight with 96 % (v/v) ethanol. After precipitation, two layers were formed.

The white top layer was removed from the light yellow bottom layer using a Pasteur pipette and centrifuged. The supernatant was discarded and the pellet was resolved in distilled water and centrifuged again. The supernatant, containing the polysaccharides, was then freeze dried. The EPS concentration was determined gravimetrically in duplicate by measuring the polymer dry mass after the freeze drying process. Size exclusion chromatography was performed using a PL aquagel-OH 60 column (Agilent Technologies) with a corresponding guard column and a refractive index detector. Water was used as eluent at a flow rate of 0.9 ml/min, and a temperature of 35°C. Dextran standards with molecular weights (MW) of 270,000; 670,000; 1,400,000 g/mol were used.

Rheology

Acidification curves were recorded under small amplitude oscillatory shear by fermenting the yoghurts in a controlled stress rheometer (Physica MCR 301; Anton Paar GmbH, Graz, Austria) equipped with a 25 mm concentric cylinder geometry (CC27-SN8085, $d = 0$). Soy milk was inoculated as described previously, and 12 g was transferred to the preheated cup (37 °C). The measurement was started 10 min after inoculation to allow the preparations to relax before analysis. Storage (G') and loss (G'') moduli were recorded at a frequency (f) of 1 Hz and a shear strain (γ) of 0.01 % (within the linear viscoelastic range). Simultaneously, the pH of a sub-sample that had been incubated at 37°C was monitored.

The measurement was stopped when desirable pH (4.5 – 4.6) was reached in the sub-sample, and the samples were subjected to a series of creep-recovery tests to determine the stress at fracture. A Shear stress (τ) was applied for 5 min,

followed by 5 min of recovery after the load had been removed. The applied stress ranged from 0.2 to 204.8 Pa, increasing the previous stress by two-fold. The maximum strains from the creep-recovery curves were plotted against applied stress, showing the yield point as a drastic change of slope.

Serum separation

Forty millilitres of inoculated soy milk were filled into sterile tubes, incubated at 37°C and stored at 4°C after a pH of 4.5 – 4.6 had been reached. The tubes were then centrifuged 220 g x 10 min at 4°C, and the supernatant was weighed (Keogh & O’Kennedy 1998). Serum separation (%) was expressed as the weight of supernatant in relation to the initial weight of the soy yoghurt sample.

Microscopy

Samples were prepared for scanning electron microscopy (SEM) by carefully cutting a small piece of soy yoghurt and immersing it in liquid nitrogen. The frozen samples were fractured and freeze dried immediately. Dry samples were mounted on SEM stubs (Agar Scientific, plain stubs 10 mm x 10 mm) with conductive carbon cement (Leit-C, Neubauer Chemikalien, Münster, Germany), and sputter coated with a 5 nm layer of gold/palladium (80:20) (Polaron E5150 Sputter Coating Unit, Quorum Technologies, Ringmer, UK). Samples were then examined with a JEOL Scanning Electron Microscope (JSM-5510, Jeol Ltd., Tokyo, Japan) at 5 kV and working distance of 15 nm.

Statistical analysis

Results are presented as average values with relevant standard deviations. Data were checked for outliers by using Q test, and statistical analysis was performed

with Minitab 16 software using one-way analysis of variances (ANOVA). All differences were considered as significant when $P < 0.05$. Multi-comparison of means was assessed by a Tuckey Post Hoc test.

5.4 Results

Cell growths in soy milk

The microbial growth of *L. bulgaricus*, *S. thermophilus* and *W. cibaria* MG1 during fermentation of soymilk is presented in Table 5.1. Although being marketed for cow's milk, *L. bulgaricus* and *S. thermophilus* starter cultures grew well in soy milk. According to the package of the CSC, the ratio of the bacteria was close to 1:1 but after inoculation, the cell numbers already exhibited a deviation of more than 10-fold with *S. thermophilus* being the better growing bacteria. The addition of the adjunct culture *W. cibaria* MG1 did not significantly influence the *L. bulgaricus*, *S. thermophilus* cell grow profile.

After fermentation, cell counts for *W. cibaria* MG1 and *S. thermophilus* increased by about 2 logs, while those for *L. bulgaricus* increased only by 1 to 1.5 logs. *W. cibaria* MG1 did not seem to have any further impact on the growth of the CSC, except for low cell counts of *L. bulgaricus* at the end of fermentation, especially when 10% sucrose was added in the system. The cell counts for *S. thermophilus* and *W. cibaria* MG1 did not show any dependency on the sucrose content.

Acidification and organic acids

Table 5.2 shows the average fermentation time for each soy yoghurt type.

Samples co-fermented with *W. cibaria* MG1 have shorter fermentation times (306 – 321 min) compared to controls (355 – 395 min).

Table 5.3. Cell counts (log cfu/g) of *L. bulgaricus*, *S. t hermophiles* and *W. cibaria* MG1 upon inoculation and after fermentation (~18 h stored at 4°C).

	<i>L. bulgaricus</i>		<i>S. thermophilus</i> (log cfu/g)		<i>W. cibaria</i> MG1	
	Inoculated	Fermented	Inoculated	Fermented	Inoculated	Fermented
C	5.00 ± 0.09	6.51 ± 0.22	6.41 ± 0.08	8.39 ± 0.17	-	-
C5	5.78 ± 0.07	7.24 ± 0.16	6.51 ± 0.21	8.32 ± 0.11	-	-
C10	5.07 ± 0.11	6.30 ± 0.20	6.64 ± 0.02	8.19 ± 0.21	-	-
CM	4.87 ± 0.10	6.06 ± 0.36	6.40 ± 0.03	8.26 ± 0.23	6.35 ± 0.01	8.22 ± 0.06
CM5	4.76 ± 0.10	6.18 ± 0.42	6.48 ± 0.02	8.61 ± 0.08	6.51 ± 0.05	8.18 ± 0.09
CM10	4.82 ± 0.12	5.73 ± 0.19	6.62 ± 0.15	8.64 ± 0.10	6.54 ± 0.10	8.31 ± 0.12

Mean values ± standard deviation

A positive correlation between sucrose level and fermentation time was observed. The acidification rate was higher for samples co-fermented with *W. cibaria* MG1 when compared to soy yoghurts fermented with the CSC only (data not shown). The highest acidification rate was observed between 2 to 4 hours of fermentation with a little delay in the soy yoghurt controls. On approaching pH 4.5, the acidification rate reduced (data not shown). The co-fermentation with *W. cibaria* (CM) resulted in a higher titratable acidity (TTA) compared to controls (5.43 Vs 4.99 ml 0.1 M NaOH). Increasing the sucrose content decreased the TTA (**Error! Reference source not found.**).

This was reflected as slightly lower, but not statistically different, lactic acid contents in sucrose-containing yoghurts. Acetic and propionic acid content in all fermented samples were below the limit of detection. Lactic acid content at the end of fermentation didn't differ significantly among the samples and was ranging from 50.02mM (sample C) to 45.6mM (sample CM10).

Table 5.4. Fermentation time, TTA, and changes in sugar levels during fermentation.

	Time to pH 4.4 (min)	TTA ^b (ml)	Fructose	Glucose (mg/g) ^c	Sucrose
C	355 ± 5.3 ^{AB}	4.99 ^A	0.45	-0.30	-3.84
C5	348 ± 27 ^{AB}	4.77 ^B	0.35	-0.33	1.75
C10	395 ± 5.7 ^{aA}	4.64 ^C	0.03	-0.95	-3.54
CM	306 ± 15 ^B	5.43 ^D	0.84	-2.22	-4.53
CM5	313 ± 9.2 ^{aB}	5.18 ^E	1.12	-1.97	-3.25
CM10	321 ± 7.0 ^B	4.95 ^A	1.68	-1.94	-8.86

Mean values ± standard deviation (n=3)

^a n=2

^b n=9 ; standard deviation less than 0,1 ml

Different superscript letters ABCDE in values of the same column indicate statistical differences (P < 0.05)

^c Positive values indicate assimilation, negative values utilization

Sugar utilization and assimilation during fermentation

During fermentation, sucrose and glucose contents decreased, and fructose contents increased in all samples (Table 5.2). The changes were more pronounced for soy yoghurts fermented with the CSC and *W. cibaria* MG1 (CM). Samples co-fermented with *W. cibaria* MG1 showed a higher extent of sucrose utilisation, particularly at 10% sucrose.

EPS formation during fermentation

The EPS contents in all soy yoghurts ranged from 1.79 for CM10 to 2.08 g/kg for C5 (Figure 5.1a). Size-exclusion chromatography analyses resulted in 4 peaks. The first 3 peaks were probably from EPS as the determined MWs were considerably higher than expected for naturally present polysaccharides and

showed similar trends to the dextran control samples. However, it cannot be excluded that some peaks are from polysaccharide naturally present in soy milk.

Rheology

The onset of structure formation of a gelling system can be observed as a rapid increase in the storage modulus (G'). This occurred 172-208 min after inoculation (Table 5.3). The gelation process was faster in yoghurts co-fermented with *W. cibaria* MG1: both the time of the onset of gelation (t_{onset}) and the time required for G' to plateau (t_{final}) were lower compared to the controls. Sucrose addition appeared to increase t_{final} in both systems. The measured gelation pH (pH_{gel}) ranged from 5.52 to 5.73. The presence of *W. cibaria* MG1 appeared to decrease pH_{gel} , but the differences were not significant ($P < 0.05$). Co-fermentation with *W. cibaria* MG1 led to the formation of weaker gels. The final G' of CM was 243.7 Pa, compared to 288.7 Pa of its control I. Sucrose addition decreased the gel strength in both systems. A series of creep-recovery tests were performed on yoghurts after acidification to determine the stress at which the structure fractures. In yoghurts with added sucrose, the fracture occurred while applying a 102.4 Pa, while the controls without sucrose resisted this stress (Figure 5.2a-b). The creep strains were higher in samples with *W. cibaria* MG1, and increased with an increasing sucrose concentration (Figure 5.2 c-d), indicating a weaker structure. Samples with sucrose also showed less elastic recovery. Observations were similar at lower shear stresses (not shown).

Syneresis

Serum separation ranged from 1.63 to 2.84 % for soy yoghurts prepared with the

CSC and from 1.35 to 2.50 % for soy yoghurts co-fermented with *W. cibaria* MG1 (Figure 5.3). Sucrose addition decreased serum separation by max. ~40% in both systems, but the presence of *W. cibaria* MG1 had only a minor influence.

Table 5.3 Time (t_{gel}) and pH (pH_{gel}) of gelation (G' > 1 Pa), total duration of fermentation (t_{ferm}) and final gel stiffness (G'_{ferm}).

	T _{onset} (min)	pH _{gel} (-)	t _{final} (min)	G' _{final} (Pa)
C	192.0 ± 5.57 ^A	5.67 ± 0.06 ^A	355.0 ± 5.29 ^{AB}	288.7 ± 32.6 ^A
C5	202.3 ± 9.29 ^A	5.58 ± 0.05 ^A	347.7 ± 27.2 ^{AB}	217.7 ± 18.0 ^{BC}
C10 ^a	208.0 ± 58.0 ^A	5.73 ± 0.18 ^A	395.0 ± 56.6 ^A	253.5 ± 7.78 ^{AB}
CM	172.0 ± 7.55 ^A	5.52 ± 0.08 ^A	306.0 ± 15.4 ^B	243.7 ± 15.9 ^{AB}
CM5 ^a	180.0 ± 5.66 ^A	5.60 ± 0.00 ^A	312.5 ± 9.19 ^B	199.5 ± 6.36 ^{BC}
CM10	175.3 ± 11.9 ^A	5.55 ± 0.07 ^A	321.0 ± 7.00 ^B	189.7 ± 2.89 ^C

Mean values ± standard deviation (n=3)

^a n=2

Different superscript letters ABC in values of the same column indicate statistical differences

Definitions:

tonset – time when G' exceeds 1 Pa

pHonset – pH at which this occurs

t_{final} – time at which G' plateaus

G'_{final} – max G'

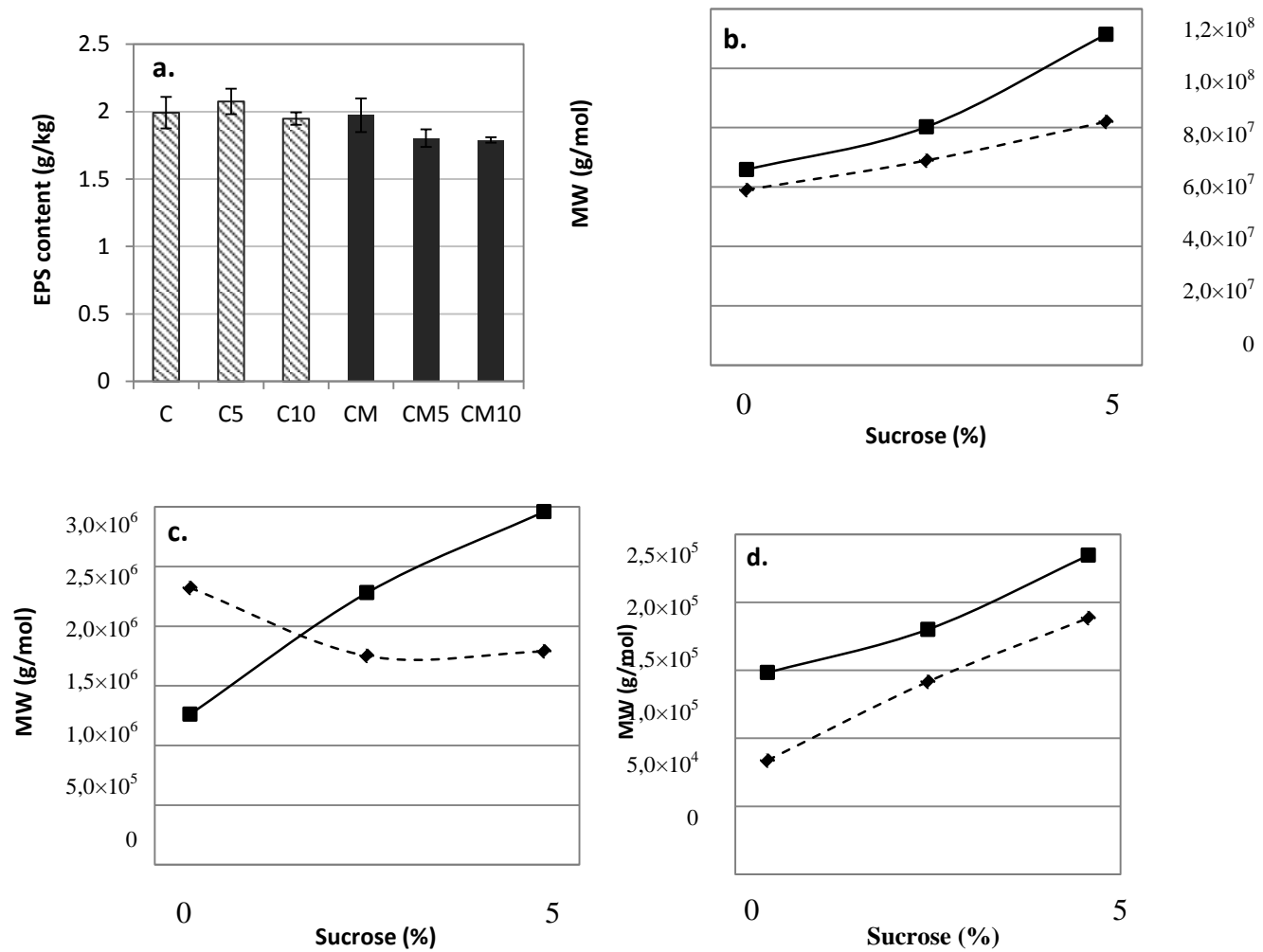


Figure 5.1 EPS concentration (a) and molecular weight (b-d) of soy yoghurts fermented with CMC (C; striped/dotted) and *W. cibaria* MG1 (CM, solid). Molecular weights calculated against a dextran standard curve from peaks 1 (b), 2 (c) and 3 (d).

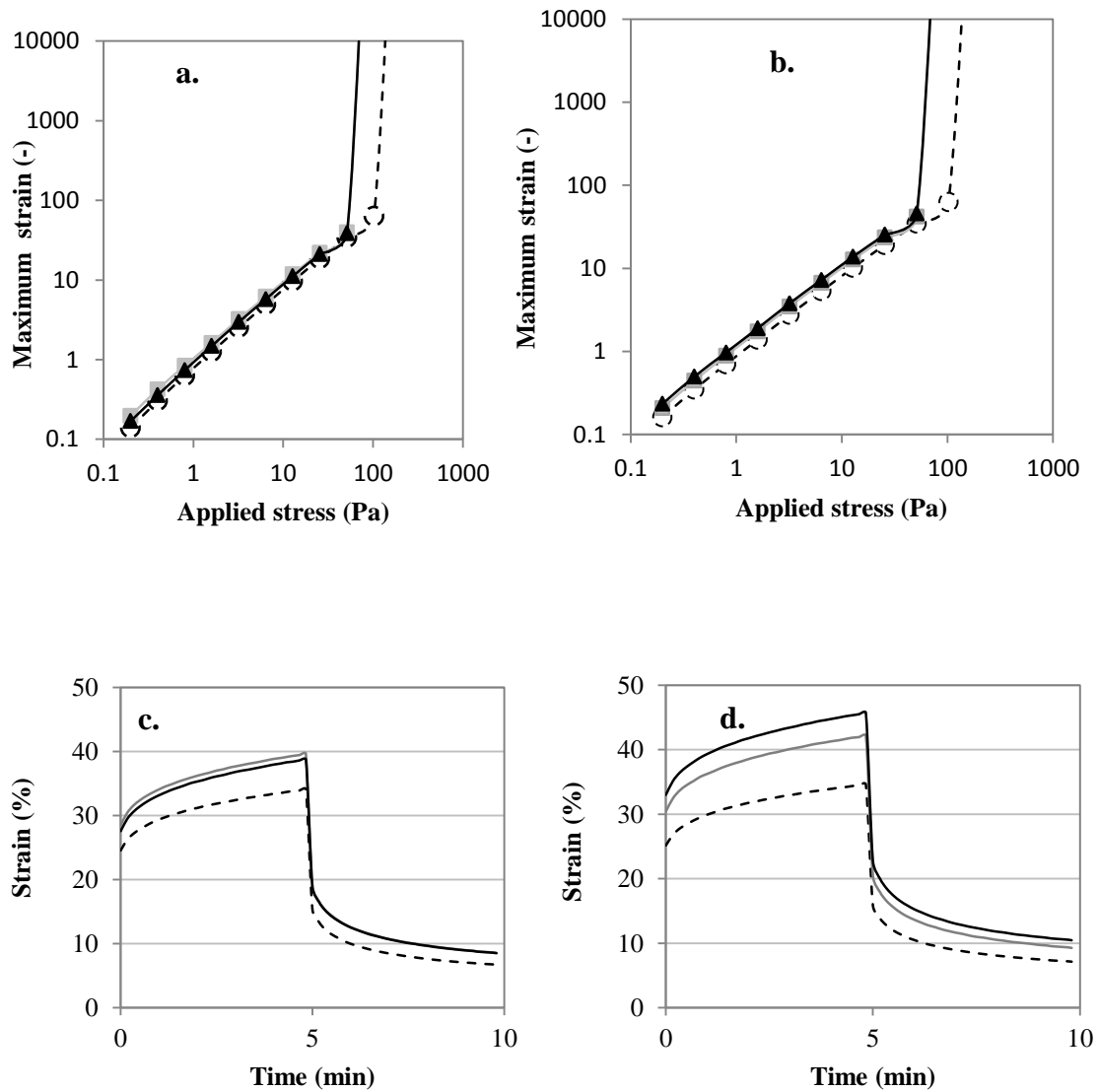


Figure 5.2 Maximum strain as a function of the applied stress (a-b) and creep recovery curves at 51.2 Pa (c-d) in soy yoghurts fermented with the CSC only (a and c) and in combination with *W. cibaria* MG1 (b and d) and the addition of 0 (dashed line), 5 (grey) or 10 (black) % of sucrose.

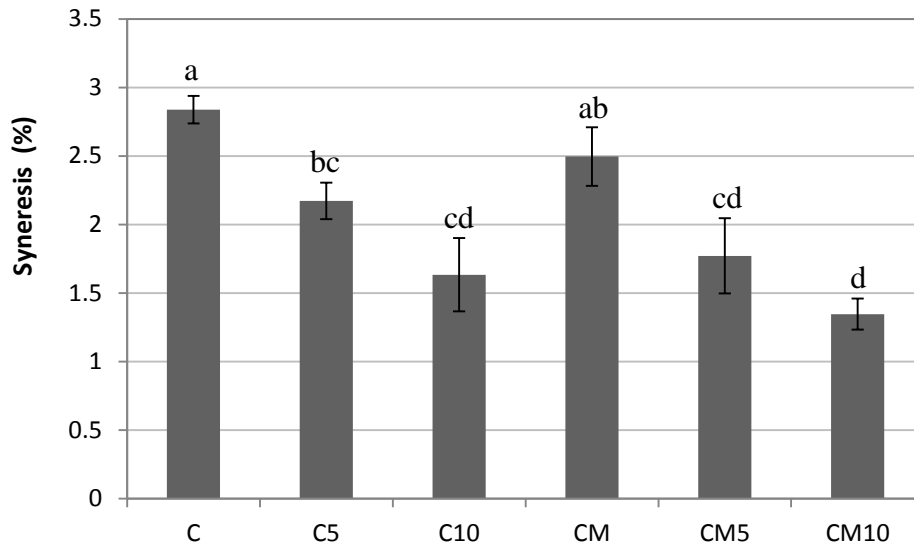


Figure 5.3 Syneresis of soy yoghurts prepared at different levels of sucrose (0, 5, 10 %) and a CSC and in combination with *W. cibaria* MG1. Different letters ABCDE indicate statistical differences ($P < 0.05$).

Microstructure

SEM micrographs are presented in Figures 5.4 and 5.5. Yoghurts without MG1 appeared to have a denser structure compared to their counterparts at all sucrose levels. Increasing sucrose levels led to the production EPS which contributed to an increase in gel pore.

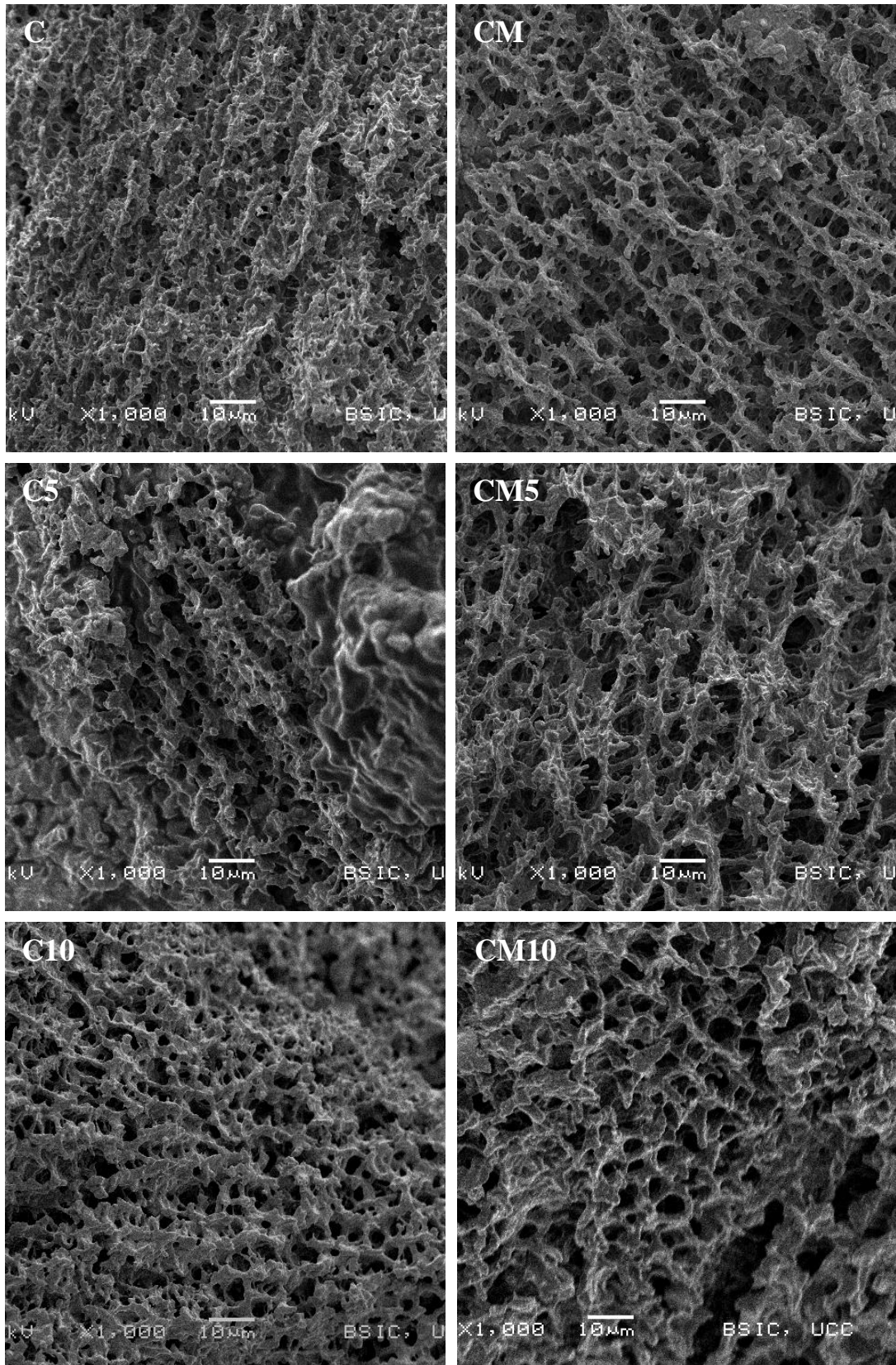


Figure 5.4 Scanning electron micrographs of soy yoghurt controls I and soy yoghurts fermented additionally with *W. cibaria* MG1 (CM) enriched with 0, 5 and 10 % sucrose at a magnification of 1 000.

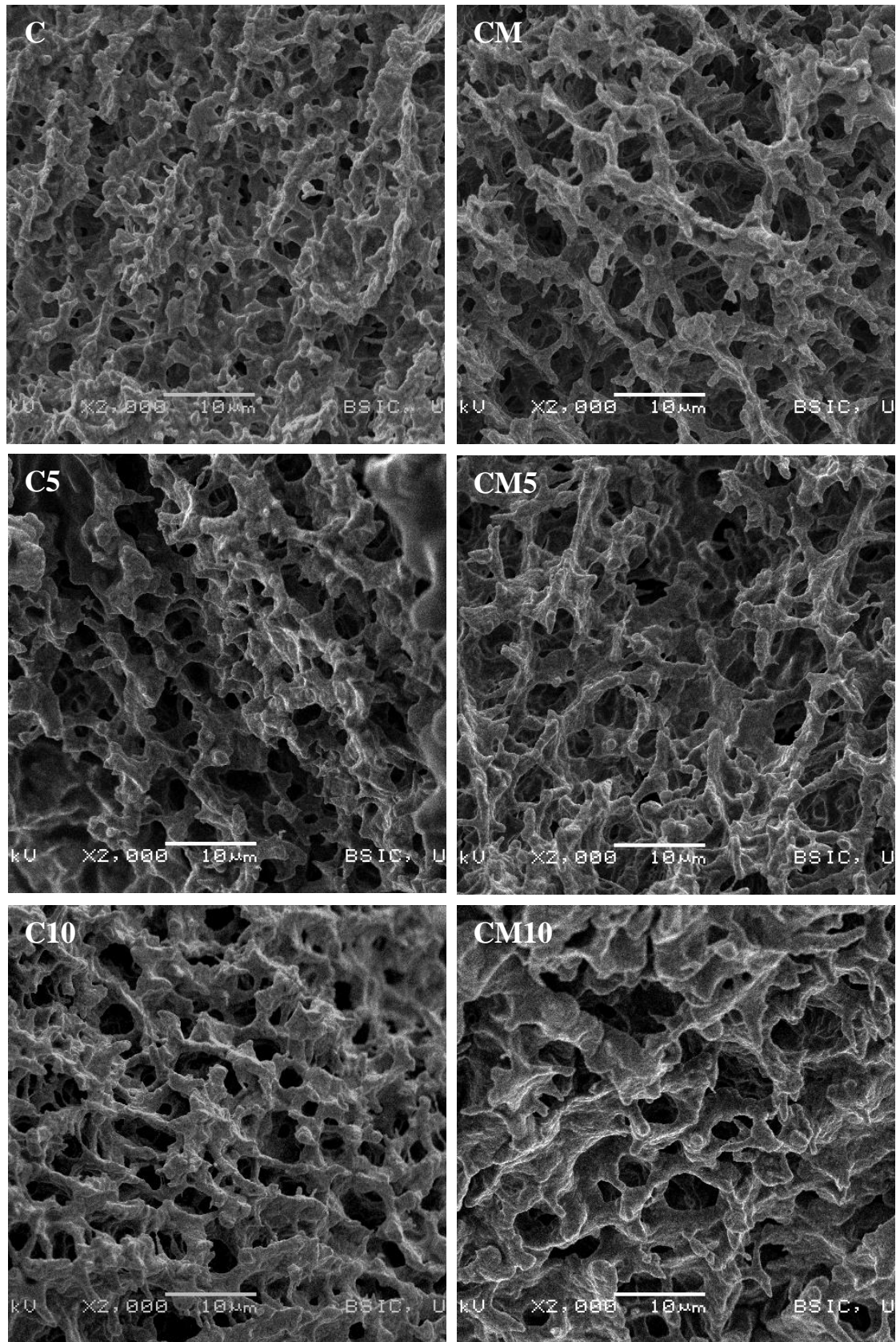


Figure 5.5 Scanning electron micrographs of soy yoghurt controls I and soy yoghurts additionally fermented with *W. cibaria* MG1 (CM) enriched with 0, 5 and 10 % sucrose at a magnification of 2 000

5.5 Discussion

In the present study, soy milk was fermented with a commercial yoghurt starter culture (CSC) and the exopolysaccharide (EPS) – producer *W. cibaria* MG1. Soy yoghurt samples fermented with the CSC and *W. cibaria* MG1 decreased the pH more effectively and reached the required pH (4.5 to 4.6) earlier than soy yoghurts fermented with CSC alone. *W. cibaria* MG1 was able to enhance the acidification rate of the CSC exerting, especially towards *S. thermophilus*, a growth stimulation effect superseding *L. bulgaricus* performance during symbiosis in a cow's milk yogurt (Purwandari & Vasiljevic 2009). Since *L. bulgaricus* itself is incapable of acidifying soy milk sufficiently at its initial pH (Mital & Steinkraus 1974; Shelef et al. 1988; Chumchuere & Robinson 1999; Wang et al. 2002), the addition of *W. cibaria* MG1 supports *S. thermophilus* especially at the beginning of fermentation. McGregor & White (1986) reported an increase in fermentation time and Kulmyrzaev et al. (2000) described a reduced gelation rate upon the addition of sucrose. The delay might be related to the increase in viscosity of the continuous phase, possibly impeding intermolecular interactions (Asylbek Kulmyrzaev et al. 2000). A tendency of an increase in fermentation time with increasing sucrose content was related to an increase of solid content (McGregor & White 1986). In this study, the presence of higher sucrose content (5 – 10%) may also lead the reduction of the water availability for microbial growth thus increasing the osmotic pressure of the system contributing to a delayed pH drop during fermentation. Additionally, the faster entry of *L. bulgaricus* into the lag phase growth can be explained by the drop in pH which does not favour its growth and by the inability of *L. bulgaricus* to use sucrose as carbon source (Wheater 1955; McGregor & White 1987). The

growth performance of *S. thermophilus* and *L. bulgaricus* in soy milk observed in our investigations has been confirmed by several authors [45, 47, 54, 45, 48, 54]. *W. cibaria* MG1 seems to be a suitable adjunct culture for yoghurt production. However, due to its heterofermentative metabolism the impact on the sensory attributes must be investigated. The increase in fructose content is caused by the partial inability of the microbes to metabolize fructose originating from sucrose hydrolysis (Gänzle et al. 2007). Only lactic acid was found in the soy yoghurts even though *W. cibaria* MG1 is a heterofermentative bacterium. As reported by Galle et al. (Galle et al. 2010), the strain lacks mannitol dehydrogenase which inhibits the conversion of fructose to mannitol and the concomitant formation of acetate, explaining why no acetic acid was detected. The results from HPLC and TTA indicate that acid production decreased with increasing sucrose content. Reduced acidification was also observed by McGregor & White (1986) and Bills et al. (1972). The latter attributed the reduced acidification rate to reduced cell counts which is not in accordance with the present results and those of McGregor & White (1986). On the other hand, Favaro Trindade et al. (2001) found an enhanced acidification rate when soy milk was supplemented with 2 % sucrose. The choice of LAB and the enrichment with certain carbohydrates for fermentation seem to be crucial for the microbial growth and its metabolism, thus, the comparison of results has to be done with caution.

The MW of the EPS produced by the CSC and *W. cibaria* MG1 are in the typical range for EPS ($1 \times 10^6 - 1 \times 10^8$) (van Hijum et al. 2006; Ruas-Madiedo et al. 2002). The addition of *W. cibaria* MG1 did not result in a higher EPS content but in a higher MW EPS when sucrose was present in the fermentation substrate.

During acid gelation, the gradual lowering of pH decreases the electrostatic repulsion between soluble protein aggregates. Larger aggregates start to form via hydrophobic interactions and hydrogen bonding, eventually forming a three-dimensional gel network (Puppo et al. 1995)(Ringgenberg et al. 2013). The major proteins in soy, glycinin and β -conglycinin both have the ability to form elastic networks (Kohyama et al. 1992). Their isoelectric points are both in the acidic range, at 5.2 for glycinin and 4.95 for β -conglycinin (Thiering et al. 2001). Reported values for the gel point of soy milk varies between 5.7 and 6.3 (Ringgenberg et al. 2013)(Grygorczyk & Corredig 2013)(Mäkinen et al. 2014)(Malaki Nik et al. 2011). These differences may be related to protein concentration, acidification rate, processing history and protein composition. In this study, gelation started at pH ca. 5.7 when soy milk was fermented with a yogurt culture, and at a slightly lower pH, 5.5-5.6 when co-fermented with MG1. In these yoghurts, the pH dropped more rapidly. The pH of gel point has been shown to decrease with increasing acidification rate in dairy systems and soy milk, possibly because is slower acidification allows more protein rearrangements to occur during the process, resulting in an destabilization and gel formation at a higher pH (Jacob et al. 2011)(Grygorczyk & Corredig 2013). Gel stiffness, measured as the storage modulus G' , is one of the most important characteristics in set-style yoghurt (Folkenberg et al. 2006). This depends on the number and strength protein-protein interactions, and the structure of the network (Folkenberg et al. 2006). The final G' decreased with increasing sucrose content with both starters. Also the stress at fracture and creep strains were lower in the presence of sucrose, indicating a weaker structure. This might be partly due to a dilution factor: less protein in the system yields less protein-protein interactions.

Sucrose also protects proteins against denaturation (Gu et al. 2009). A lower degree of denaturation results in less exposure of reactive groups, that consequently affects structure formation (Bryant & Julian McClements 1998). Co-fermentation with MG1 led to a gels with lower G' , and a more open gel structure with larger pores. The dextran produced by *W. cibaria* MG1 was identified by Galle et al. (Galle et al. 2010). It is a neutral, linear α -1,6 glucan chain with MW ranging from 5×10^6 to 3×10^9 g/mol (Sandra et al. 2012; Wolter, Hager, et al. 2014). The influence of a polysaccharide on a protein gel depends largely on its charge, molecular weight and structure. Anionic polysaccharides reinforce the protein network due to electrostatic protein-polysaccharide interactions, but neutral polysaccharides may decrease the strength due to phase separation that modifies the gel structure (Gentès et al. 2011; Ayala-Hernández et al. 2009; Girard & Schaffer-Lequart 2008). The decrease in G' might also be attributed to the phase separation between the proteins and the non-adsorbing EPS due to depletion forces which results in a modified gel structure (Tuinier et al. 2000; Gentès et al. 2011). This can occur above a certain concentration of non-adsorbing polysaccharides (Doublier et al. 2000). Hassan et al. (2003) observed large pores containing the EPS and thick strands of highly aggregated proteins using confocal laser scanning microscopy. EPS-negative gels appeared more homogenous, pores were smaller and evenly distributed and the strands were thinner all of this leads to a higher gel stiffness than gels formed in the presence of EPS. Additionally, EPS that cannot interact with proteins are concentrated in the continuous phase and are expected to inhibit interactions between protein aggregates (Hassan et al. 2003).

The water holding of yoghurt had a significant positive correlation with sucrose content. Generally, water holding can be improved by an increase in total solids content or by the addition of hydrocolloids (Tamime & Deeth 1980). Sucrose does not influence the extent of serum separation at least up to 6% (Calvo et al., 2002, Cinbas and Yazici, 2008). Water holding capacity is strongly dependant on the microstructure: increasing the coarseness and pore size of gels results in a loss of water holding capacity (Hermansson 1986). EPS can decrease serum separation of gelled systems by increasing the viscosity of the serum phase, binding hydration water, and in the case of anionic exopolysaccharides, strengthen the network by interacting with proteins (Hermansson 1986). More than gel microstructure, the ability of the EPS to decrease the separation of liquid is related to its properties (Gentès et al. 2013). Past studies show that a reduction in whey separation was observed when the EPS present were ropy or encapsulated (Wacher-Rodarte et al. 1993) (Amatayakul et al. 2006) (Hassan et al. 1996) or neutral and stiff (Gentès et al. 2011). In this study, the largest effect on water holding was observed in samples with EPS of the highest MW, but this effect cannot be related to EPS alone, as the presence of sucrose appeared to have a major influence. At the ultrastructure level, the soy yoghurt control (without sucrose addition) consisted of a fine, well cross-linked network with small and evenly distributed pores which might result in more protein-protein interactions and in turn in a higher G' (Renkema 2004). The addition of sucrose led to a different microstructure of the soy yoghurts. The network formed was coarser and the pore size increased possibly causing the lower G' . During gel formation typically rearrangements, i.e. the breakage and fusion of proteins to larger aggregates, occur which can result in an increase in pore size. Large pores

represent weak spots in the formed network and can further weaken the protein-protein interactions involved in the gel network (Lee et al. 2004).

5.6 Conclusion

In the present study, soy milk was fermented with a commercial dairy starter culture and *W. cibaria* MG1 previously characterised for its ability to produce high MW EPS. The latter proved to be a suitable adjunct starter culture due to satisfying growth, utilization of the sugars present and the enhancement of acidification rate. The growth and metabolic characteristics of *W. cibaria* MG1 considerably exceeded those of *L. bulgaricus*. Even though a heterofermentative bacterium, *W. cibaria* MG1 did not produce acetic acid which might have negatively influenced the sensory properties of the produced soy yoghurt. Serum separation and gel stiffness were markedly reduced upon the addition of sucrose and the presence of high molecular weight EPS. The texture seemed to have interdependency with the fermentation time, addition of sucrose and EPS produced. Since the molecular weight of the EPS and the amount of sucrose added correlated positively, the observed rheological properties could not be clearly attributed. Further trials involving *ex-situ* dextran application will improve, to a certain extent, the general understanding of the EPS role in soy – based yoghurt production. The *ex-situ* EPS application may not result in the delivery of the same technological effects on soy-yoghurt properties since the *in-situ* EPS production is a dynamic process progressing in parallel to fermentation.

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Chapter 6

Development of novel quinoa-based yoghurt fermented with dextran producer *Weisella cibaria* MG1

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(submitted)

6.1 Summary

The market for plant-based dairy-type products is growing, and quinoa offers an interesting raw material due to its high nutritional quality in terms of protein, minerals and vitamin contents. The aim of this study was to develop a novel beverage fermented with *Weissella cibaria* MG1 based on aqueous extracts of whole meal quinoa flour.

For the production of the quinoa based-milk a procedure for rice-milk production was adapted followed by a homogenisation step and ultra-high pressure treatment (180 MPa).

The protein digestibility of quinoa based-milk was improved by applying complex proteolytic enzymes able to increase protein solubility by 54.58 %. A bleaching step with calcium carbonate was successfully applied to improve colour and sensory properties of the final beverage. The growth and fermentation characteristics of *Weissella cibaria* MG1 including changes in viable cell counts, pH, titratable acidity, viscosity and EPS production at the end of fermentation were investigated. Fermented wholemeal quinoa milk using *Weissella cibaria* MG1 showed high viable cell counts ($>10^9$ cfu/mL), a pH of 5.16, and significantly higher water holding capacity (WHC, 100%), viscosity (0.57 mPa s) and exopolysaccharide (EPS) amount (40 mg/L) than the chemical acidified control. High EPS (dextran) concentration in quinoa milk caused earlier aggregation because more EPS occupy more space, and the chenopodin were forced to interact with each other. Direct observation of microstructure in fermented quinoa milk indicated that the network structures of EPS-protein could improve the texture of fermented quinoa milk. Overall, *Weissella cibaria* MG1 showed satisfactory technology properties and great potential for further possible application in the development of high viscosity fermented quinoa milk.

6.2 Introduction

Consumer demand for cow's milk alternatives has increased as a result of people being intolerant to cow's milk, including lactose intolerance and cow's milk allergy. While lactose intolerance is triggered by the consumption of lactose-containing foods and a deficiency of lactase to enable lactose digestion, the cause cow's milk allergy is an abnormal immune response to one or more milk proteins (Crittenden & Bennett 2005). Soy milk is the most common milk-substitute. However, 14% of the individuals who suffer from cow's milk allergy also have reactions to soy (Zeiger et al. 1999). Beside soy, other plants are used to process non-dairy milk products, including oat, almond, coconut, rice and quinoa and their market is increasing. Even though the acceptance and consumption of these plant-based milk substitutes ("plant-based milks") is rising, many of these products have sensory characteristics that do not match the consumer preference in western countries (Jago 2011, Mäkinen et al. 2014, Organic Monitor 2005). Furthermore, plant-based milks, based on rice and other types of plants, have low or no protein content and are therefore not suitable to replace cow's milk or even soy milk (Mäkinen et al. 2014). Therefore, the development of new milk-substitute products that cause no adverse effects in humans and that have better nutritional, sensory and technological properties is necessary.

In general, the dairy substitute market is still growing: Packaged Facts (2012) estimated the U.S. market for plant based milk substitutes to have a total value on USD 1.33 bn in 2011, which is expected to increase to USD 1.7 bn by 2016. Also the market for lactose-and dairy free products in general, estimated to be worth USD 3.6 bn in 2010, is growing in the U.S. and Western Europe. The figure includes lactose-free dairy products, but much of the growth has been attributed to soy milk like products (Leatherhead Food Research, 2011).

One grain with such potential is quinoa (*Chenopodium quinoa*). It has been declared as "one of humanity's most promising crops" by the FAO, and it has been considered as a potential crop for NASA's Controlled Ecological Life Support System (Arendt, Zannini 2013). Quinoa

is an ancient crop that originates from the Andean region in South America (Jacobsen 2003). It can grow from 1 m to 3 m tall and produces achenes as a fruit. These seeds are gluten-free, rich in starch and have a comparable chemical composition to cereal grains even though belong to the same botanic family of spinach or sugar beet. The protein content is remarkably higher than in true cereals, with literature values ranging from 12 to 23 % (Abugoch 2009, Arendt, Zannini 2013). Quinoa seeds also have significantly higher levels of minerals and some vitamins than true cereals (Arendt, Zannini 2013, Jacobsen 2003). The interesting nutritional quality of quinoa supports its inclusion in different food products. Many studies have however reported grassy and bitter off-flavours when used at high levels. Therefore, quinoa is usually used in only low concentrations (Lorenz, Coulter & Johnson 1995, Repo-Carrasco, Espinoza & Jacobsen 2003).

The main objective of this research is to prototype a novel quinoa-based fermented beverage employing complementing bio-theological food processes able to deliver quinoa-based beverages mimicking, as much as possible, bovine milk sensory attributes.

6.3 Materials and Methods

Organic quinoa grains were obtained from Infinity Foods Co-operative Ltd. Chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA) unless otherwise stated.

Commercial “Quinoa drink” was used as reference for the characterisation of the experimental wholemeal quinoa milk production. “Quinoa drink” is a commercial quinoa plant milk produced by EcoMil, with a labelled protein content of 1.5 % and was purchased from a local store in Ireland.

Weissella cibaria MG1 was obtained from the culture collection of the Cereal Science laboratory of University College Cork, Ireland. *W. cibaria* MG1 has been previously identified as dextran hyper-producer (Galle et al. 2010) Working cultures from *W. cibaria*

MG1 were prepared from glycerol stock stored at -80°C by streaking onto MRS agar (pH 5.8) (Meroth et al. 2003) containing 0.05 g/l bromocresol green (Dal Bello & Hertel 2006). Plates were incubated at 30°C for 48 h under anaerobic conditions. A single colony was inoculated into MRS broth and grown overnight at 30°C prior to use.

Quinoa milk production and characterisation

The procedure for the quinoa-based milk production was adapted from the flowchart of rice-milk processing (Mitchell, & Mitchell, 1998) summarised in Figure 6.1.

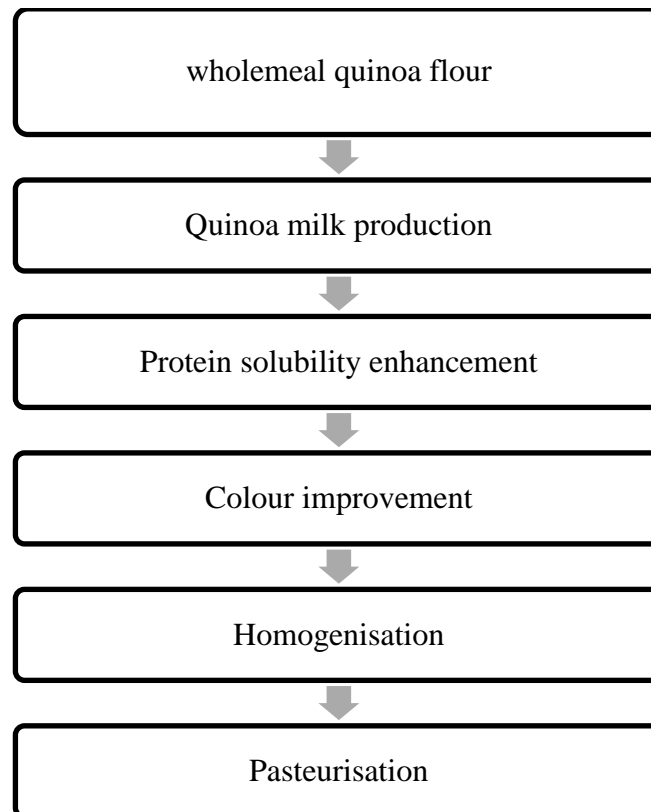


Figure 6.1 Process steps for the quinoa-based plant milk production

Fifty grams of wholemeal quinoa flour, milled using a hammer mill (Mod. EM50, AMA Magico, San Martino in Rio, Italy) with a disc size of 1.5 mm, was mixed with 350 g water (ratio 1:7). The resulting suspension was autoclaved in glass bottles for 15 min at 121°C to

allow starch cooking and gelatinization. The suspension was blended in a semi-industrial blender (Titanium Major KMM020, Kenwood, Havant, United Kingdom) at medium speed for 6 minutes. Heat stable alpha-amylase Hitempase 2XP (Kerry Group, Ireland), at a concentration of 0.5 % (w/w), was used for the dextrinization and liquefaction of starch. For starch degradation the suspension was heated to 50 °C for 20 min followed by 65 °C for 90 min. A conventional starch-iodine test was conducted to check the progress of starch breakdown. The suspension was left at 65 °C until the iodine test was negative followed by cooling to 25 °C and stirring for 40 min. The wholemeal quinoa flour suspension was mixed at medium speed for 6 min and bottled in previously sterilized 1 L glass bottles. High-pressure homogenisation treatments were carried out in a high pressure homogeniser (APV 1000, APV Homogenisers AS, Denmark) by applying 180 MPa.

Protein content and protein solubility

The quinoa wholemeal flour and the resulting wholemeal quinoa milk were analysed according to the Kjeldahl method (MEBAK 1.5.2.1). The total nitrogen content was converted into protein content, using factor 6.25. (Matissek, Steiner & Fischer 2014). In order to analyse the solubility of the proteins, samples were centrifuged at 3900 g for 5 minutes. The protein contents of the supernatants were analysed using the Bensadoun and Weinstein (1976) modification of the Lowry assay (Lowry et al. 1951). The precipitation steps were not needed and therefore omitted. A calibration curve of bovine serum albumin was used to determine the concentrations. The results were expressed as % (w/w) of protein content in the supernatant of total protein content.

Salt concentration

To enhance the protein solubility, the impact of different salt concentration, proteases and pH were evaluated. Salt solutions with NaCl concentration of 0, 0.025 and 0.05 mol/L was used in place of water for quinoa milk production using the procedure described above.

Protease treatment

The salt concentration with the highest protein solubility was chosen for the investigation of protease treatment. Two different proteases were tested to increase the protein solubility. Rustom et al. (1993) reported that papain increased the protein yield of peanut and soy milks. Purified papain preparation (Profix 100L, Kerry Group, Ireland) and a complex proteolytic enzyme system, obtained from different microbial strains and plant species (Bioprotease PF50, Kerry Group, Ireland) were used. The recommended dosage (Profix 100L: 4 g/HL, Bioprotease PF50: 1 kg/tonnes) as well as a tenth and 10-fold of the recommended concentration were used. Samples were prepared in 50 mL tubes and heated in a shaking water bath at the enzyme's optimum temperature (Profix 100L: 60°C; Bioprotease PF50: 50°C) for one hour. The protein solubility was analysed as described above. For the best performing protease and concentration, different reaction times was tested. Samples were taken every 30 min until the end of reaction (3 h). At each time point protein solubility was measured. After choosing the best protease and reaction conditions, the effect of pH on protease performance was investigated.

pH

The pH influence on protein solubility was determined by adjusting the pH from the original pH value up to 8.0 in 0.5 pH increments using 0.1-1 mol/L HCl or NaOH, performed at 20 °C using a pH-meter (SevenGo Mettler-Toledo Ag, Switzerland). The samples were incubated at

4 °C overnight. In the case of a pH shift, it was re-adjusted before measuring protein content and protein solubility.

Bleaching of the quinoa-based milk

To improve the colour of the wholemeal quinoa milk, bleaching agents were applied. Four food bleaching agents that are commercially used in food industries were chosen. The oxidative enzymes BioGox, a glucose oxidase, and Bioperoxidase P Conc, a peroxidase, were provided from Kerry Group, Ireland. The enzymes were applied at the recommended concentration of 70 mg/kg (BioGox) and 25 mg/kg (Bioperoxidase P Conc), and at double and half the recommended concentration. Forty grams of sample were inoculated with the recommended enzymes dosage in a shaking water bath at 35 °C (BioGox) and 50 °C (Bioperoxidase P Conc) for one hour.

The bleaching effects of calcium carbonate (Kerry Group, Ireland) and titanium were also investigated. Both bleaching compounds were individually mixed into 40 g of quinoa-based milk sample by shaking in a 50 mL tube for 2 min. Considering that the average calcium content in cow's milk is 112 mg/100g (FAO & WHO 2002), whereas the calcium content in MW quinoa milk was 14 mg/100g, 245 mg/100g of calcium carbonate (98 mg/100g calcium), was chosen to achieve the same value as cow's milk. A tenth, 10-fold and 100-fold of that concentration were used also. Titanium dioxide was also evaluated for its bleaching properties. 0.10 µg/mL titanium was added to the beverage and mixed into the milk as described for the calcium carbonate. For comparison a tenth and 10-fold of that concentration were also tested. After the bleaching treatments, the colour of the resulting samples (CIE L*a*b* colour system) was determined with a Chroma Meter (Minolta CR-300, Osaka, Japan). Colour of samples were characterized according to whiteness index (WI) as defined in Equation 1.

Equation 1 Whiteness index

$$WI=100-\sqrt{(100-L^*)^2+a*2+b*2)}$$

Wholemeal quinoa yoghurt production

WM quinoa yoghurt was produced by inoculating pasteurised (110 °C for 10 min) wholemeal quinoa milk with 1×10^7 CFU/mL of *Weissella cibaria* MG1. To enable dextran production by *W. cibaria* MG1, sucrose was added in concentrations of 10% (w/v). The inoculum of *W. cibaria* MG1 was prepared as follows. An overnight culture was enumerated by performing a viable plate count in duplicate. For yogurt production, the desired number of cells were pelleted, washed once in Ringer solution and re-suspended in quinoa milk.

The MG1 inoculum, prepared as describe above, was added to the quinoa milks pre-warmed to 30°C. During fermentation samples were taken at 0, and 24 h for bacterial enumeration on MRS5 agar plates. After 24 h fermentation the samples were cooled to 4°C and stored overnight before analyses. The wholemeal quinoa yoghurts were analysed in triplicate. Chemically acidified WM quinoa milk, supplemented with the same concentration of both sucrose and organic acids as measured by HPLC to be present at the end of an MG1-fermented wholemeal quinoa milk fermentation, was used as a control.

Technological, microbial, chemical and ultra-structural properties of wholemeal quinoa yoghurt

Total titratable acidity

The total titratable acidity (TTA) was determined by suspending 5 g of yoghurt in 45 mL distilled water, and titrating the sample with 0.1 N NaOH to a pH of 8.5 (Katina et al. 2006). After 3 min, the pH was readjusted to 8.5. The TTA was expressed as the number of millilitres of NaOH used for titration.

Acid and sugar profiles

Acid profiles were analysed using an Infinity 1260 HPLC system equipped with a 300x7.7 mm Hi-Plex H column and a PL Hi-Plex Guard column with a diode array detector (Agilent Technologies, Palo Alto, US). Sulphuric acid (0.005 mM) was used as an eluent with a flow rate of 0.5 mL/min at 65°C. Samples were clarified with 7% perchloric acid solution overnight (16 h, 4°C), centrifuged (1'000 rpm, 5 min), and filtered (0.2 µm pore size filter). Lactic acid and acetic acid were used as external standards.

Sugar profiles were analysed with a 250x4.6 mm Supercosil LC-NH₂ (Sigma-Aldrich) column and a refractive index detector. Samples were eluted with ACN: H₂O (75:25, v/v) at a flow rate of 1 mL/min.

Sugar concentrations were determined using maltose and sucrose as external standards. Sample preparation was carried out as follows: Sample (2 g) was mixed with 7 mL water in a 10 mL volumetric flask, 250 µl of Carrez reagent I and II were added sequentially while shaking gently and allowed to rest for 20 min. The volume was adjusted to 10 mL, mixed and aliquots were centrifuged (10000 g, 5 min, 4 °C) and filtered (0.2 µm pore size filter). For analysis, 750 µl sample was mixed with 250 µl ACN.

Quantification of dextran EPS

At the end of fermentation, the EPS was precipitated with 3 volumes of chilled 96% (v/v) ethanol. After standing overnight at 4 °C, the resultant precipitate was collected by centrifugation at 20900 g for 20 min at 4 °C,

For removal of proteins, TCA was added at a final concentration of 20% and the suspension was further incubated for 2 h at 4°C under gentle agitation. Precipitated proteins were removed by centrifugation at 13,000 × g for 20 min at 4°C, and supernatants were collected.

Two volumes of cold ethanol were added to the supernatants for EPS precipitation, and the centrifugation process was followed as described above.

The re-suspended EPS were dialyzed against distilled water at 4 °C for 2 to 3 days, and freeze-dried. The dry weight of the freeze-dried EPS measured and the yield calculated per litre of culture.

Rheological properties of wholemeal quinoa yoghurt

Viscosity measurements of yoghurt samples were made using a controlled stress rheometer (MCR301, Anton Paar GmbH, Austria) equipped with a parallel plate geometry (diameter 50 mm). Prior to analysis the samples were gently stirred with a spatula to achieve a homogenous mixture. The samples were then pre-sheared at 200 s⁻¹ for 1 min to remove any residual structure (Vasiljevic, Kealy, & Mishra, 2007), allowed to equilibrate for 1 min, sheared from 0.01 to 200 s⁻¹ over 2 min, held at 200 s⁻¹ for 1 min and sheared from 200 to 0.01 s⁻¹ over 2 min. All tests were performed at 20 °C.

Determination of water holding capacity (WHC) of wholemeal quinoa yoghurt

The water holding capacity of wholemeal quinoa yoghurt was analysed using slight modification of the centrifugation method previously reported by (Keogh & O’Kennedy 1998). Forty millilitres of fermented quinoa yogurt was centrifuged 220 g x 10 min at 4°C, and the supernatant was weighed. The supernatant was collected, weighed, and water holding capacity (WHC) was calculated according to the following equation:

$$\text{WHC (\%)} = (1 - W1/W2) \times 100$$

where: W1 =weight of supernatant after centrifugation (g); W2 = fermented quinoa milk weight (g). All measurements were carried out in triplicate.

The weight of the drained liquid was recorded and related to the initial weight of WM quinoa yoghurt.

Microscopy

The wholemeal quinoa yoghurt ultrastructure was evaluated by scanning electron microscopy (SEM). Samples were prepared by freezing an aliquot of sample to -80°C and freeze drying. Dry samples were carefully cut and mounted on SEM stubs (Agar Scientific, plain stubs 10 mm x 10 mm) with conductive carbon cement (Leit-C, Neubauer Chemikalien, Münster, Germany), and sputter coated with a 5 nm layer of gold/palladium (80:20) (Polaron E5150 Sputter Coating Unit, Quorum Technologies, Ringmer, UK). Samples were then examined with a JEOL Scanning Electron Microscope (JSM-5510, Jeol Ltd., Tokyo, Japan) at 5 kV.

Statistical analyses

All analyses were carried out at least in triplicate. Means were compared using one-way analysis of variance (ANOVA) and Tukey's post hoc-test using Statistica 12 (StatSoft, Tulsa, OK, USA). The level of significance was determined at $p < 0.05$.

6.4 Results

Protein content and protein solubility of wholemeal quinoa milk

The protein contents of the wholemeal quinoa flour and produced milk were 13.28 g/100g and 1.59 ± 0.02 g/100g, respectively. The commercial quinoa drink used as reference showed a protein concentration of 0.23 ± 0.01 g/100g. The protein solubility for the untreated wholemeal quinoa milk samples and for the commercial quinoa drink was 18.06 % and 24.27 %, respectively. The solubility was increased significantly only with the addition of 0.05 mol/L salt solution (Table 1).

However, since the sensory properties in preliminary tests (data not shown) were negatively affected by this high salt content, the addition of 0.025 mol/L salt was chosen because of its positive effect on the sensory properties of the wholemeal quinoa milk sample (data not

shown), even though the protein solubility was not improved. Bioprotease PF50 was able to provide the highest protein solubility followed by Profix 100L.

Even though a dosage of 10 kg/tonnes of Bioprotease PF50 yielded a significantly higher solubility, the recommended dosage of 1 kg/tonnes was chosen since a lower dosage is more cost effective and an over dosage should be avoided.

Furthermore, the pH and reaction time of the enzyme was observed. The results for the incubation time showed that the protein solubility increased slowly over time. Indeed, a reaction time longer than 0.5 h was able to increase the protein solubility of 6.8%.

Therefore 0.5 h was chosen as a reaction time able to significantly improve quinoa protein solubility. The outcome obtained from the pH trials showed that the enzyme activity is not significantly influenced by the pH within the range investigated (Table 6.1). Considering all these findings, no pH adjustment was considered for wholemeal quinoa milk production.

The protein solubility of the enzymatically treated wholemeal quinoa milk was significantly higher than those recorded for the commercial quinoa drink.

Table 6.1 Protein solubility of untreated and treated wholemeal quinoa milk samples (WM) compared to the values of commercial quinoa drink.

Sample	Protein solubility [%]
Untreated	18.06±0.34 ^p
Salt 0.025 mol/L	18.67±0.21 ^p
Salt 0.05 mol/L	25.87±0.26 ^k
Profix 100L 0.4 g/HL	25.9±0.00 ^{no}
Profix 100L 4 g/HL	27.01±0.23 ^{mno}
Profix 100L 40 g/HL	51.66±0 ⁱ
Bioprotease PF50 0.1 kg/tonnes	33.59±0.75 ^{kl}
Bioprotease PF50 1k g/tonnes	54.58±1.70 ^{hi}
Bioprotease PF50 10 kg/tonnes	57.08±0.80 ^h
Bioprotease PF50 1 kg/tonnes 0.5h	64.26±0.49 ^{efg}
Bioprotease PF50 1 kg/tonnes 1.0h	67.25±0.00 ^{bcd}
Bioprotease PF50 1 kg/tonnes 1.5h	68.44±00 ^{bcd}
Bioprotease PF50 1kg/tonnes 2.0h	68.67±00 ^{bcd}
Bioprotease PF50 1 kg/tonnes 2.5h	68.99±0.60 ^{bc}
Bioprotease PF50 1 kg/tonnes 3h	70.65±0.72 ^b
Bioprotease PF50 1 kg/tonnes pH 6.11/6.29	68.41±1.24 ^{bcd}
Bioprotease PF50 1 kg/tonnes pH 6.51/6.53	65.17±0.70 ^{def}
Bioprotease PF50 1 kg/tonnes pH 7.01/7.01	63.47±1.05 ^{efg}
Bioprotease PF50 1 kg/tonnes pH 7.51/7.45	65.79±0.00 ^{cdef}
Bioprotease PF50 1kg/tonnes pH 8.01/8.03	67.1±0.00 ^{bcdef}
Commercial quinoa drink	24.27±0.92 ^o

Different letters in same column indicates significant differences between treatments in 95% of confidence

Efficacy of bleaching agents on wholemeal quinoa milk

The whiteness indices (WI) obtained from wholemeal quinoa-based plant milk samples treated with different whitening agents are shown in Table 6.2. The bleaching enzymes did not increase the WI but reduced it even below the WI recorded for the untreated wholemeal quinoa milk sample. Titanium dioxide had a negligible impact on the WI and significantly increase the WI only at the highest concentration. On the contrary, calcium carbonate showed a significant impact even at lower concentrations (Table 6.2). For wholemeal quinoa milk production the highest dosage of calcium carbonate was chosen.

Table 6.2 Effect of bleaching agents on protease treated WM quinoa-based milk samples

Bleaching agent	Concentration	Whiteness index
Untreated		51.68±0.49 ^{fg*}
Bioperoxidase P Conc	1.25 mg/100g	48.63±0.4 ^{ij}
Bioperoxidase P Conc	2.5 mg/100g	49.53±0.4 ^{hi}
Bioperoxidase P Conc	5 mg/100g	50.66±0.25 ^{gh}
BioGox	3.5 mg/100g	47.14±1.37 ^j
BioGox	7 mg/100g	48.24±1.47 ^{ij}
BioGox	14 mg/100g	48.81±1.54 ⁱ
Calcium carbonate	123 mg/100g	54.31±0.52 ^{cd}
Calcium carbonate	245 mg/100g	54.26±0.99 ^{cd}
Calcium carbonate	490 mg/100g	55.24±0.2 ^{bc}
Calcium carbonate	2450 mg/100g	62.12±0.2 ^a
Titanium dioxide	9.8 µg/100g	52.59±1.48 ^{fg}
Titanium dioxide	19.6 µg/100g	52.06±1.05 ^{ef}
Titanium dioxide	98 µg/100g	53.53±1.63 ^{de}

*Different letters in same column indicates significant differences between treatments in 95% of confidence.

Physicochemical characteristics and microstructure of wholemeal quinoa fermented EPS producer Weisella cibaria MG1

The values of viable cell counts, pH, titratable acidity, WHC, sugar and acid profile, viscosity and EPS production of fermented wholemeal quinoa milk with EPS producer *Weisella cibaria* MG1 are presented in Table 6.3.

Table 6.3 Physicochemical characteristics of *W. cibaria* MG1 fermented wholemeal quinoa milk

Parameters	Time (h)	
	0	24
Cell counts (cfu/mL)	1.11±0.06	1.31±0.3
pH	6.28±0.02	5.18±0.01
TTA (mL of 0.1 N NaOH)	0.2±0.01	2.8±0.00
WHC (%)	38.15±0.12	100±0.00
Sugar profile		
Maltose	31.00±0.06	0.0±0.00
Sucrose	110.00±0.02	0.0±0.00
Acid profile		
Lactic	0.0±0.00	17.46±0.18
Acetic	0.0±0.00	0.0±0.00
EPS amount (mg/L)	0.0±0.00	40.00±0.6
Viscosity (Pa*s)	0.04±0.00	0.57±0.02

The results showed that wholemeal quinoa milk facilitated the growth of *W. cibaria* MG1.

After fermentation, the fermented wholemeal quinoa milk had pH values of 5.18.

The titratable acidity values had similar trends than that of pH values.

Furthermore, the fermented wholemeal quinoa milk had significantly ($P < 0.05$) higher apparent viscosity (0.57Pa s) than the original unfermented wholemeal quinoa milk, which was agreed with the apparent WHC measurements.

The WHC of fermented wholemeal quinoa milk was significantly ($P < 0.05$) higher than that its control essentially due to the hyper production of EPS from the strain MG1. The differences in WHC of the fermented wholemeal quinoa milk is attributed to the impressive amount of EPS (dextran) amounts produced. Only lactic acid was produced as main metabolite during wholemeal quinoa milk fermentation.

Microscopy

Scanning electron microscopy (SEM) is a useful tool for providing information on microstructure of food products, which assists researchers in understanding factors affecting functional, sensory, and physical properties.

Fig. 6.2 shows microstructure of wholemeal quinoa yoghurt made with EPS-producing *W. cibaria* MG1 and supplemented with 10% sucrose. The control was made by chemically acidification of the wholemeal quinoa milk using the amount of lactic acid detected at the end of MG1 fermentation.

The EPS molecules appear as thin layer and clearly interact not only with protein network but also with all other wholemeal quinoa milk constituent.

This may explain the viscosifying effect that the presence of EPS has in the fermented wholemeal quinoa milk (Figure 6 – h). The fermented quinoa milk made using EPS-producing culture MG1 had small and evenly distributed pores (Figure 6.2 e).

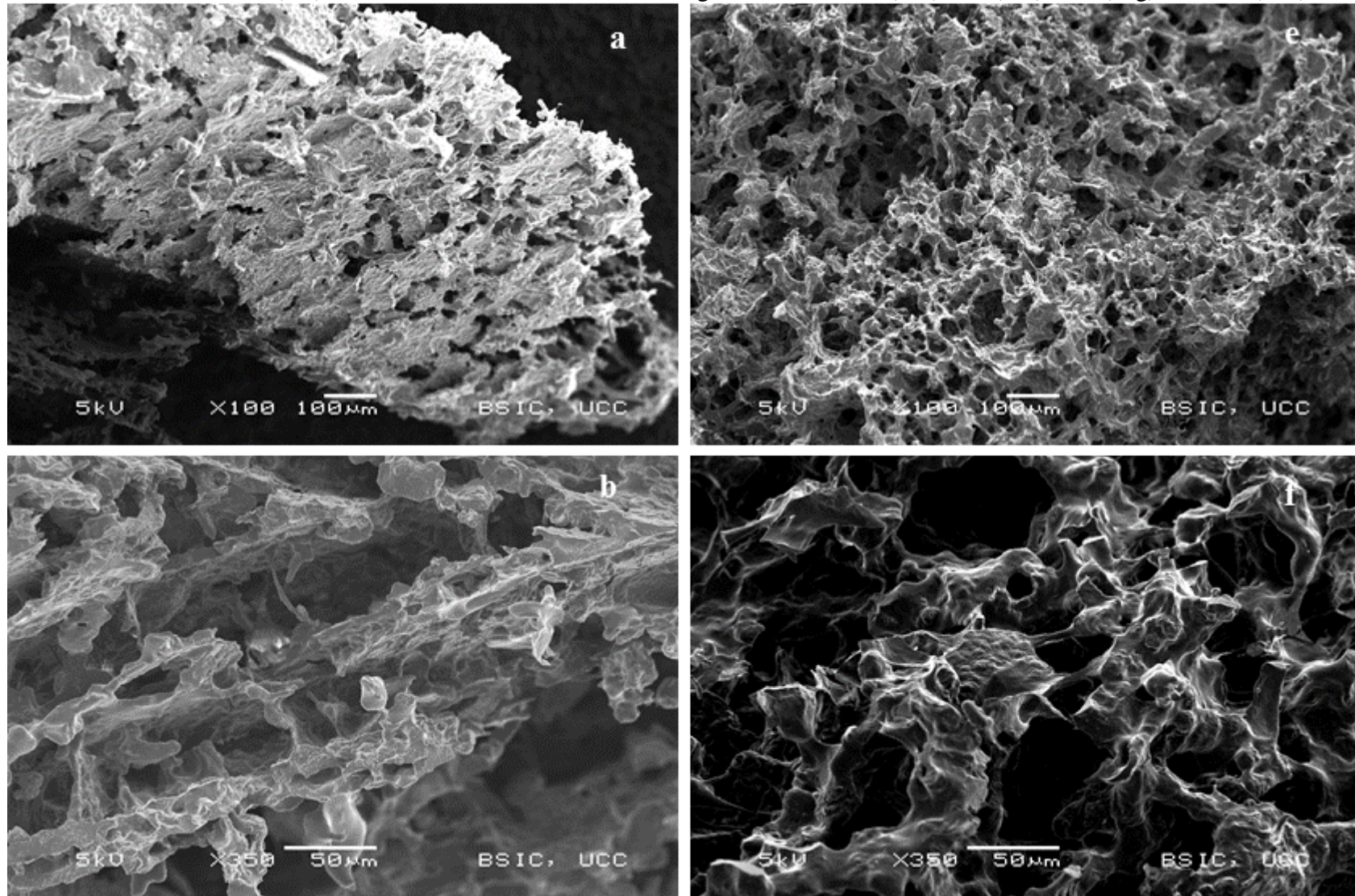
The results of Figure 6.2 (e-h) were associated with the presence of EPS, and EPS led to this network structure.

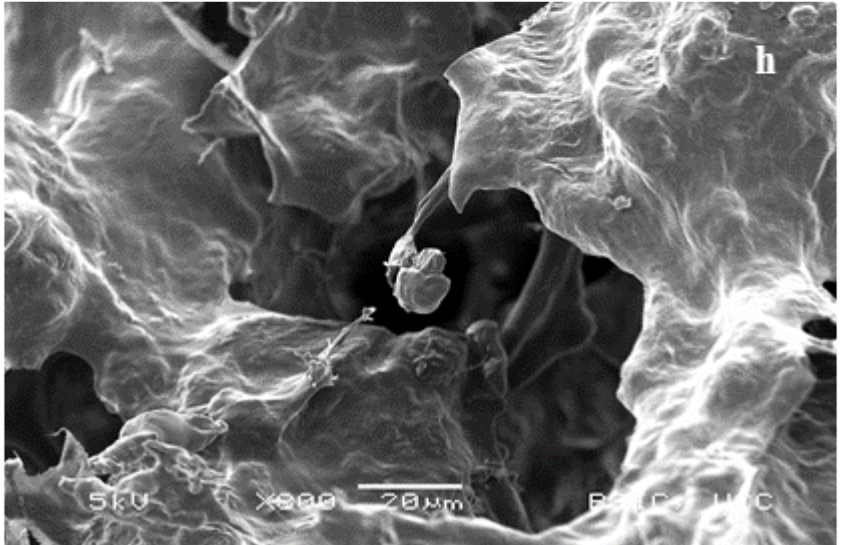
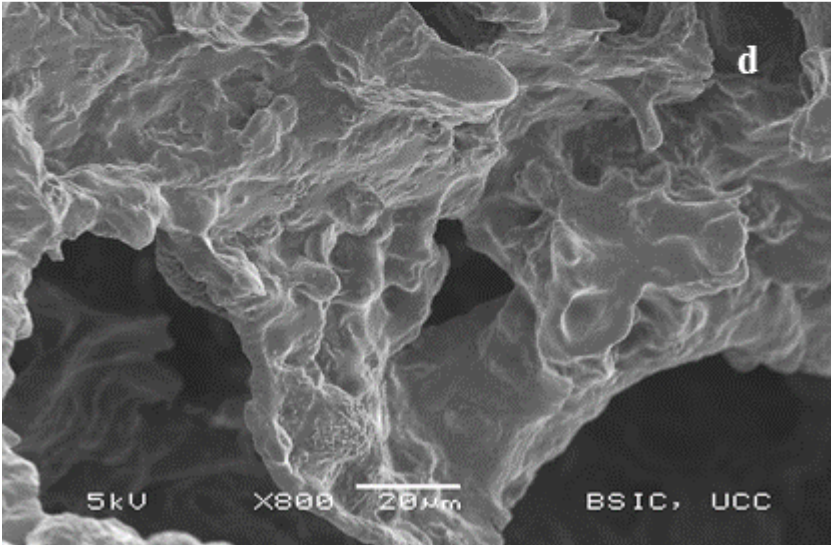
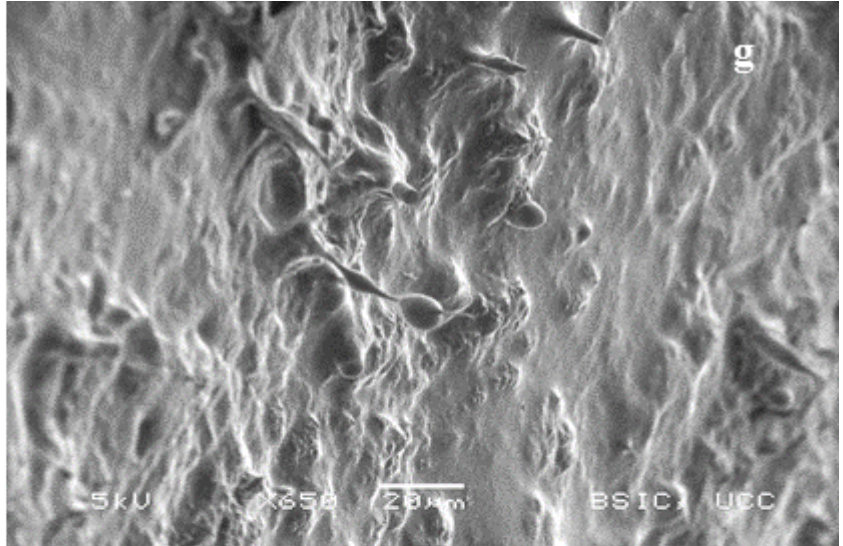
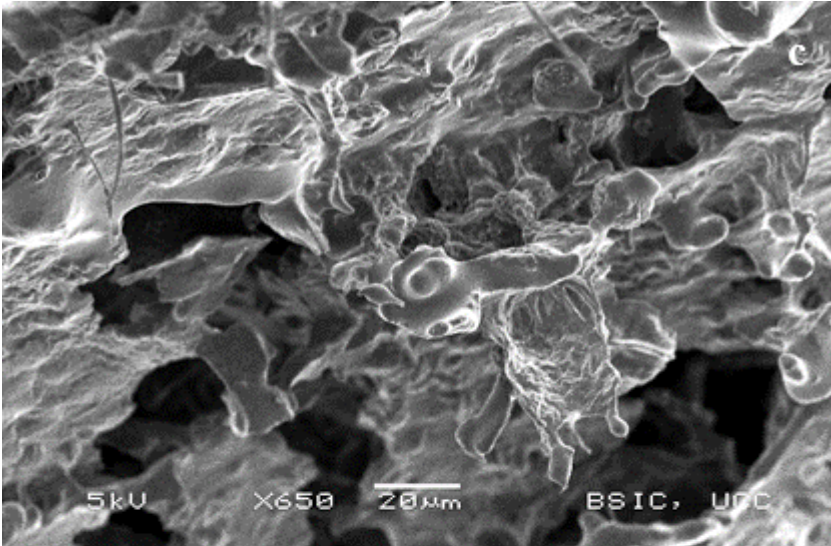
Additionally, protein agglomerates in quinoa yoghurt made with EPS-producing *W. cibaria* MG1 (Fig. 6.2 e-h) appeared to be more fused than those observed in the control (Fig. 6.2a-d) in which agglomerates junctions were more apparent.

SEM micrographs showed that a compact well-defined network was observed in chemically acidified wholemeal quinoa (Fig. 6.2 a-d). Conversely, an open structure was observed in wholemeal quinoa fermented with the EPS-producing *W. cibaria* MG1. The EPS also partially or completely filled pores within the structure (Fig. 6.2 e-h).

An open structure in yogurt and cheese made with EPS-producing cultures was also previously observed by Hassan et al., (1995) and Bhaskaracharya, et al., (2000).

Figure 6.2. Scanning electron micrographs of wholemeal quinoa chemically acidified yoghurt controls (a-d) and wholemeal quinoa yoghurts fermented with *W. cibaria* MG1 (e-h) enriched with 10 % sucrose at a magnification of 100 (a, e) 350 (b, f), 650 (c, g) and 800 (d, h)





6.5 Discussion

Due to of the new consumers' trends (organic, biodynamic, personalized food, allergen-free, ready to eat etc.), food and beverage industries are forced to explore different raw materials, or to use new technologies/ food processes with the aim to develop new food and beverages products. Quinoa (*Chenopodium quinoa*) is an Andean grain highly appreciated because of its nutritional properties (Vega et., 2010, Repo-Carrasco et al., 2003, Bhargava et al., 2006, Oshodi, 1999) representing an ideal substrate for satisfying consumer's requests for healthy and nutritious foods. However, because of its organoleptic characteristic, quinoa needs to undergo different food processing steps before meeting the Western world consumer's preferences.

The present work was aiming to develop novel quinoa-based yoghurt fermented with dextran producer *Weissella cibaria* MG1.

The first adjustments to improve quinoa protein solubility in the wholemeal quinoa milk were achieved with salt since the major storage proteins are globulins, containing a legumin type (11S) named chenopodin (37% of total protein) and a 2S type protein (35% of total protein) (Brinegar & Goundan, 1993, Brinegar *et al.*, 1996).The solubility of quinoa proteins showed a trend comparable to the described soy proteins and salt interactions. Jiang, et al. (2010) analysed the solubility characteristics of the soy protein glycinin, which is an 11S type as well as a function of pH and salt. According to their findings, the solubility of proteins were low (under 20 %) for pH values ranging from 4-6 in a water solution. The protein solubility in a 0.1 mol/L NaCl solution was increased to more than 60 % at a pH of 6 but stayed low for pH between 4 and 5.

Both glucose oxidase and peroxidase, commonly used in several products like bread or in the paper industry as bleaching agents (Kornbrust *et al.*, 2012, Farooq *et al.*, 2013), were used to enhance the WI of the wholemeal quinoa milk. Both oxidizes glucose in the presence of oxygen to gluconic acid. During this reaction hydrogen peroxide is produced which is capable of acting as a bleaching agent by decomposing colouring composites. The gluconic

acid on the other hand acts as a chelating agent for metal ions and could be able to stabilize plant-based milks (Kornbrust *et al.*, 2012). The enzymatic strategy for improving the WI wholemeal quinoa milk colour didn't provide significant results. This could be the reason that the colouring pigments are actually not water-soluble but lipophilic. Stauffer (1991) reported this issue for bleaching flour.

The evaluation of suitable lactic acid bacteria for fermenting a non-dairy food product is a crucial step in the development of new products within the non-dairy "field. In this research activity the capacity of *W. cibaria* MG1 to ferment wholemeal quinoa milk and its ability to hyper-produce EPS (dextran) has been shown. Different from heterofermentative lactobacilli and *Leuconostoc* spp., *W. cibaria* MG1. do not harbour mannitol dehydrogenase, and thus do not produce notable amounts of acetate when sucrose is present (Galle *et al.*, 2010) with positive repercussions on the organoleptic attributes. The viable counts and pH reduction were comparable to those in a medium based on cow milk (Mozzi *et al.*, 1995). This shows that the wholemeal quinoa milk substrate contains components suitable for supporting LAB growth. However, it seems that *W. cibaria* MG1 didn't express its entire fermentation capability (acid production), as indicated by the low TTA values, when compared to other LAB fermented plant-based milks (Martensson *et al.*, 2000, Li *et al.*, 2014) even though it reach a high cell counts at the end of fermentation. This may be due to the fact that the extreme viscous environment developed by the extracellular enzyme dextransucrase, counteract the nutrient availability and nutrient uptake by the microbial cells compromising thus, the exploitation of the full fermentation performance of the LAB strain.

Differently from the control sample, whole meal quinoa milk fermented with *W. cibaria* MG1 did not exhibit any phase separation. The differences in WHC of the samples are mainly attributed to the EPS over production ensured by *W. cibaria* MG1.

Interactions of EPS with proteins are very important in fermented milk and soymilk because of their effects on the microstructure and texture of these foods (Folkenberg *et al.*, 2005, Ferragut *et al.*, 2009). However, in wholemeal quinoa milk fermented with *W. cibaria* MG1, the quinoa protein micelle matrix has been extensively hydrolysed by proteases. So the exceptional viscosity recorded at the end of fermentation, comparable to commercial cow's yoghurt, is essentially due the production of high molecular weight EPS rather than an intimate interaction with the protein fraction. In a previous study (Li *et al.*, 2012) has been proven that there was a positive correlation between the viscosity and high molecular weight EPS production with pure and mixed cultures

Schellhaass and Morris (1985) observed that yogurts made with ropy cultures (EPS production) had higher viscosity values than yogurts made with non-ropy cultures. Because of EPS produced by LAB interacts with the free water in the gel-like structure, the texture of yogurt was improved (De Vuysi & Degeest, 1999; Hassan, Frank, Schimidth, & Shalabi, 1996a, 1996b, 2002; Rohm & Kovac, 1994; Skriver, Roemer, & Qvist, 1993; Tegatz & Morris, 1990; Vlahopoulou & Bell, 1993).

Properties of polysaccharides in solution are related to their molecular structure and microstructure (Muller, 1990). For instance, the high branching xanthan gum stimulates conformational disorder and interferes with ordered assemblies and network formation leading to great solubility and highly viscous solutions (Sutherland, 2001; Daas, Grolle, van Vliet, Schols, & de Jongh, 2002).

Structurally, dextran is an α -1,6 linked glucose-based polysaccharide with various combinations of α -1,2, α -1,3 and/or α -1,4 branching leading to a globular formation which is dense and can exist as a spherical suspension in a complex matrix, (Ioan *et al.*, 2000). These properties aid macromolecule mobility resulting in low viscosity (Zannini *et al.*, 2014) mimicking Newtonian behaviour (Ribotta *et al.*, 2005). However, as a high-molecular weight

sorbent, LAB EPS (dextran) showed more binding-sites than that of low molecular weight oligosaccharide enhancing thus, the water absorption (Zhang et al., 2009) from the aqueous extracts of whole meal quinoa flour.

The increased viscosity upon MG1 fermentation in sucrose-enriched substrate is in accordance with other previous studies (Galle et al, 2010, Zannini et al., 2012). However, the recorded EPS amount detected in different matrices such as sourdough, barley-based wort by MG1 fermentation never reached the 40g/L production detected in wholemeal quinoa milk produced in this study. Rühmkorf et al., (2012) confirm this author's finding indicating quinoa as one of the ideal substrate for LAB EPS production able to ensure an EPS-yield of 20.63 g/Kg of sourdough., This clearly demonstrates that quinoa represents an excellent substrate for allowing the full expression of LAB metabolic performance. This is essentially due to the exceptional amino acid composition that characterizes quinoa grain along with its high buffer capacity (Arendt et al.2013).

6.6 Conclusion

The present study has evaluated the fermentation characteristics and microstructure of wholemeal quinoa milk with a starter cultures of *W. cibaria* MG1. The research activity also involved the analysis of viable cell count, pH, titratable acidity, viscosity and EPS production during storage. The research outcomes showed that *W. cibaria* MG1 can grow well in wholemeal quinoa milk positively characterising the resulting yoghurt with high viability ($>10^9$ cfu/mL), viscosity (> 0.5 Pa s) and amounts of EPS (40 g/mL). Generally, *W. cibaria* MG1 showed satisfactory technological properties and great potential for further possible application in the development of high viscosity fermented wholemeal quinoa yoghurt. Further work on the structural characteristics of EPS from the fermented wholemeal quinoa milk and their potential functional and sensory properties is in progress.

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Chapter 7

The application of dextran as novel food ingredients to compensate for low protein in wheat flour

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7.1 Abstract

Wheat is primarily used for bread-making. However, fungal diseases, grain moisture at harvest, and low protein contents, strongly influence the quality of the wheat flour, thus creating challenges for traders, millers and commercial bakers who struggle to produce consistently high-quality products. Replacing functionality and economic losses associated with using low-protein/wholemeal flours for bread making purposes are addressed here.

Three hydrocolloids, xanthan gum, dextran and hydroxypropylmethylcellulose (HPMC), were incorporated into bread recipes based on high protein flours, low-protein, flour and coarse wholemeal flour. Hydrocolloid levels of 0 to 5% (flour basis) were used in bread recipes to test the water absorption. The dough and bread qualities parameters were determined.

Results showed that xanthan had negative impact on the dough and bread quality characteristics. HPMC and dextran generally improved dough and bread quality and showed dosage dependence. Volume of low-protein flour breads were significantly improved by incorporation of 0.5% of the latter two hydrocolloids. However, dextran outperformed HPMC regarding initial bread hardness and staling shelf-life regardless the flour applied in the formulation.

7.2 Introduction

Wheat is one of the major grains in the diet of a vast proportion of the world's population. Currently, about 95% of global wheat produced is for bread with most of the remaining finding use in pastas (Arendt and Zannini, 2013). However, environmental conditions, crop management practices during grain development, and post-harvest control strongly influence the quality of the wheat flour obtained (Mikhaylenko et al., 2000). This creates challenges for traders, millers and the commercial bakers who struggle to produce consistently high-quality products using the resulting wheat flour (Altenbach et al., 2002). Particularly, northern Europe's generally temperate climate can have adverse effects on the crop. These negative attributes include diseases (especially those of fungal origin), grain moisture at harvest, and low protein contents, which collectively impede premium pricing of wheat grain for milling purposes (flour production) (Teagasc, 2007).

Additionally, other factors relating to the bread-making performance of flour also lead to variation in the bakers' purchase prices. One such parameter is water absorption which is directly positively correlated with bread yield. Furthermore, both the colour and ash levels of the flours, which are both products of the bran content, also exert a pivotal role in the bread-making performance of the flour (Campbell et al., 2003).

Wheat flour popularity as a raw material for staple baked goods arises from the properties of the final baked products. These attributes are gained through the formation of a viscoelastic dough during processing and gluten development, as well as gluten interactions with starch and other wheat flour components. Flour with high protein contents, specifically gluten, is better suited to bread-making

resulting in a final baked product with a high volume and fine open crumb structure (Cauvain and Young, 2008). The quality of the dough matrix relies on sufficient gluten development (gas retention and oven-spring), due to the quality and quantity of gliadin and glutenin subunits, their ability to absorb water, and interactions between dough constituents (Correa et al., 2010).

In the case of low-protein wheat flours, such as those often produced in northern European countries, it is necessary to increase the protein (gluten) content before incorporation into bread-making processes. To ensure standardisation of flour protein contents, it may be blended with high-protein raw materials resulting in a reliance on the availability of imported wheat and either domestic or external government policies, thus incurring price dependency issues (Hayta and Çakmakli, 2001). Another approach involves flour fortification with vital wheat gluten which may be obtained as a by-product of wheat fuel crop saccharification (Campbell et al., 2003) or as a starch industry co-product (Day et al., 2006). However, these solutions are economically challenging to the milling industry and rely on a number of factors external to the control of the domestic industry. These include import constraints due to privatised sales and competition (Chand, 2007), environmental conditions (Xu et al., 2008), competition with non-food application of wheat crops (Koutinas et al., 2008), or delivery schedules.

As an alternative, weak (low-protein) wheat flour bread-making functionality can be increased using a range of processing aids such as; oxidising agents like L-ascorbic acid or potassium bromate (Wieser, 2003), enzymes like tyrosinases and peroxidases (Joye et al., 2009), emulsifiers including diacetyl tartaric acid ester of monoglycerides (Orthoefer, 2008), and hydrocolloids such as gums and fibres or their modified derivatives (Mikuš et al., 2013). Current consumer and food

industry trends are leading towards natural dough improvers such as clean-label enzymes and hydrocolloids in place of chemical additives. As such, in this study, the functionality of both natural (xanthan and dextran) and modified (hydroxymethylpropylcellulose) hydrocolloids in various strength wheat flour (high-protein, low-protein and coarse wholemeal flours) recipes was evaluated, with the aim to implement a cost-effective solution to address the problems associated with domestically available weak flours. Noteworthy, xanthan and HMPC, natural and chemically-modified, respectively, are widely used hydrocolloids and serve as benchmark dough improvers alongside dextran, which is an EU approved novel food ingredient whose potential in this role has not yet been fully characterised.

7.3 Materials and methods

Raw materials

Three different wheat flours, bakers' flour (high-protein content) (composition: carbohydrate 75.3 g/100g , protein 12.1 g/100g, fat 3.0 g/100g, fibre 3.1 g/100g, moisture 13.23 %), biscuit flour (low-protein content) (composition: carbohydrate 68.5 g/100g, protein 9.9 g/100g, fat 1.4 g/100g, fibre 3.0 g/100g, moisture 12.63%), and wholemeal flour, (Odlums, Portarlinton, Ireland) (composition: carbohydrate 64.0 g/100g , protein > 9.0 g/100g, fat 2.2 g/100g, fibre 9.0 g/100g, moisture 13.19%), were used in this study. Moisture contents of the flours were determined according to the AACC Method 44-15.02. Vivapur® Hydroxypropylmethylcellulose K4M (HPMC) (J. Rettenmaier and Söhne, Rosenberg, Germany), KeltrolF Xanthan gum (C.P. Kelco, USA), and high molecular weight ($5 \times 10^6 - 4 \times 10^7$ Da) dextran (Bio-e.r.g., Jesi, Italy) were used as

hydrocolloids in this study. Salt (Salt Union, Cheshire, UK), and dried yeast (Mauripan, Burns Philip Food Ltd., UK) were also incorporated into the bread-making recipes.

Hydrocolloid sample preparation and rheology

To analyse each hydrocolloid, 0.2 g was dissolved in 1 mL isopropanol at room temperature, and was subsequently added to 20 mL water in the rheometer cup, using a concentric-cylinder system (Anton Paar, Austria). The viscosity of each sample was measured over 10 min. at 20°C, using a constant shear rate of 500 sec⁻¹.

Preparative dough analyses

Farinograph (constant flour method) and extensograph characteristics were determined according to the AACC Methods, 54-21 and 54-10, respectively. The following parameters were determined using a Brabender-farinograph (Duisberg, Germany); water absorption, percentage of water required to yield dough consistency of 500 BU (Brabender Units), dough development time (DDT, time to reach maximum consistency), stability (length of time for which dough consistency is at 500 BU), mixing tolerance index (MTI, consistency difference between maximum peak height and peak height recorded 5 min. later), and elasticity (band width of the curve at the maximum consistency). After 50 mm stretching, the Brabender extensograph was used to measure the resistance to constant deformation (R_{50}), extensibility (E), and R_{50}/E ratio.

The Chopin rheofermentometre (Villeneuve-la-Garenne, France) was used to measure dough volume, as well as dough gas production and loss. Displacement

of a 1,500 g weight by the rising dough (300 g) was measured over 3h and was directly related to the volume of gas produced, thereby allowing calculation of the dough gas-retention capabilities. Dough was prepared as for the baking studies (reported below).

Bread making and analyses

On a 100% flour basis, the dough recipe contained water (calculated from farinograph, reported in Figure 2), and 2.0 g each of salt and yeast, 1.5 g sugar, 3.0 g fat, 0.5 g ascorbic acid, and 0.1 g Sodium Stearoyl Lactylate (SSL) supplemented with 0 (control), 0.5, 1.0, 2.5 or 5.0% of dextran, xanthan gum or HPMC. The dough was prepared by weighing out the dry ingredients, excluding yeast which was incorporated into the water at 30°C and subsequently added to the other ingredients. Everything was combined in a mixer (Kenwood Chef Classic KM336) at speed I for 60 s followed by scraping down the sides of the bowl and at speed II for 90 s.

After mixing, the dough was rested in a proofer (Koma, Koeltechnische, The Netherlands) at 30 °C and 85% relative humidity for 15 min before it was divided into 450 g portions and placed in non-stick baking tins (454 g tins, Sasa U.K., Middex, U.K.). The dough was then proofed for 60 min under the same conditions and baked immediately in a pre-heated deck oven (MIWE condo oven, MIWE Michael Wenz GmbH, Arnstein, Germany) at 220 °C top and bottom heat for 22 min. The oven was steamed (1,000 ml) before loading and again on loading the bread. For staling experiments, loaves were packed into plastic bags 120 min after baking, when adequately cooled, which were sealed and subsequently stored at room temperature for two and five days.

Bread analyses

After cooling, volume of the loaves was measured using a VolScan Profiler (Stable Micro Systems, Surrey, UK) and specific volume was calculated by dividing the loaf volume by its mass. Additionally, bake loss was determined (subtracting loaf weight from pre-baked dough weight) and calculated as a percentage using the VolScan software. Texture analysis was performed 120 min (day 1), 2 days and 5 days after baking using texture profile analysis (TPA) tests with a TA-XT2i texture analyser (Stable Micro Systems, Surrey, UK) equipped with a 25 kg load-cell and a 20 mm aluminium cylindrical probe. The settings used were a test speed of 2 mm sec⁻¹ with a trigger force of 20 g to compress the middle of the breadcrumb to 40 % of its original height. Results were analysed using Texture Expert 1.17 software (Stable Micro Systems). Values for hardness, springiness, chewiness and staling were calculated using the software. Crumb structure was evaluated by image analysis using C-cell Imaging System and associated software (Calibre Control International Ltd., UK). The parameters used were cells/cm² (number of cells/slice area), wall thickness and net cell elongation (degree of overall elongation).

Statistics

All determinations were performed in triplicate and the average result is presented. The Excel Analysis ToolPak (Microsoft Corporation[®]) was used for statistical calculations. Data were checked for outliers by using Q test, and statistical analysis was performed with Minitab 16 software using one-way analysis of variances (ANOVA). All differences were considered as significant

when $P < 0.05$. Multi-comparison of means was assessed by a Tuckey Post Hoc test.

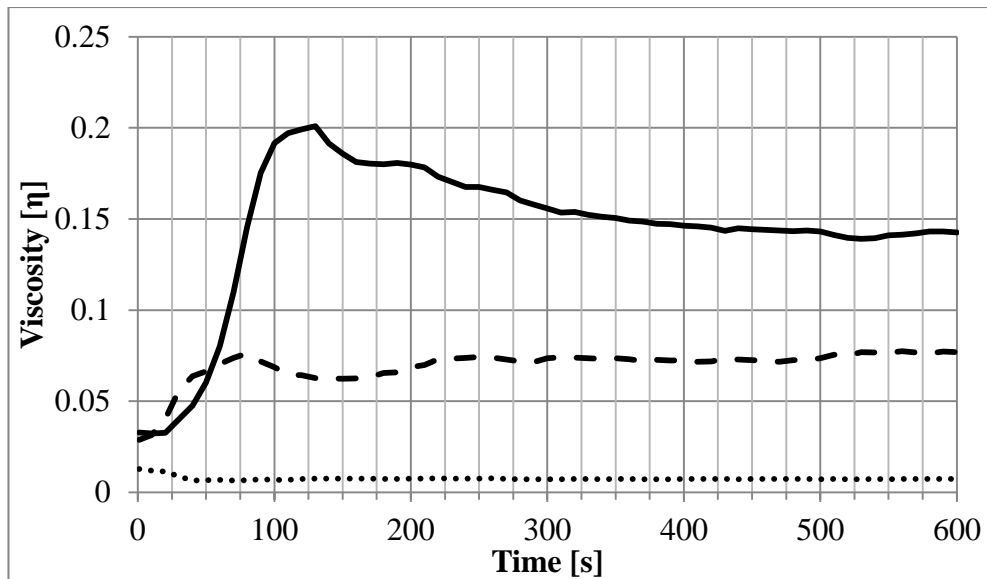
7.4 Results and discussion

In the baking industry, various baking aids, preservatives, and taste enhancers are added to the bread-making formulations. This ensures that natural seasonal variations in the raw ingredients do not result in an unpredictable end product which causes problems regarding processability and end product consumer acceptance. The potential of hydrocolloids to address these problems is of growing importance. Their effects on dough matrices and in the baked bread can be associated with two main molecular functionalities; (i) the modification of water retention, and (ii) interactions with other dough constituents as gluten, non-gluten proteins, fibres and starch, which have a combined influence on the dough development or structural network during the bread-making process.

Hydrocolloid viscosity

Amongst the three hydrocolloids studied, xanthan has the highest viscosity followed by HPMC and dextran (Figure 1). The latter shows a constant viscosity profile over the 10 min. of analyses under constant shear rate (500 s^{-1}). After hydration, a similar behaviour was observed for dextran but at a lower overall viscosity than HPMC. Additionally, the hydration rate of dextran was accelerated when compared to HPMC and particularly xanthan (Figure 1).

Figure 7.1 Viscosities over time under constant shear rate (500 s^{-1}) for dextran (dotted line), xanthan (solid line) and HPMC (dashed line).

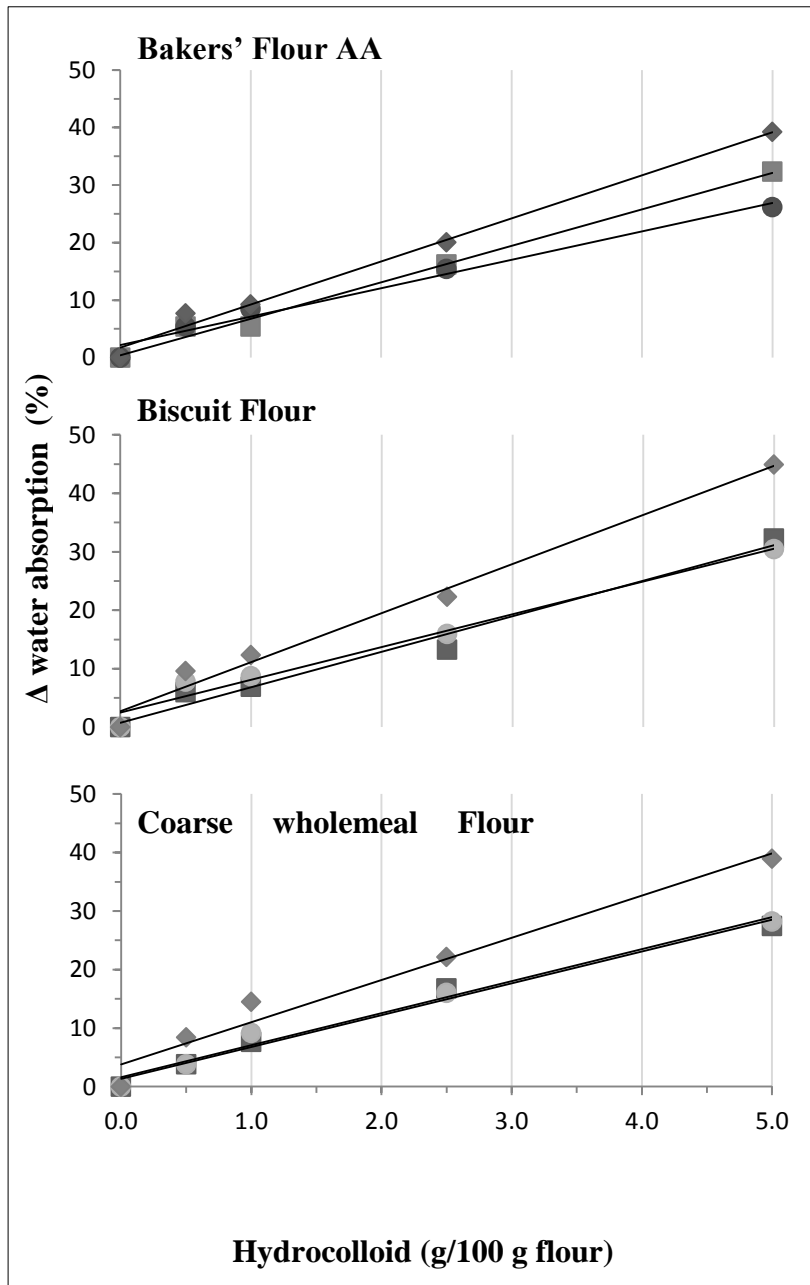


Structurally, dextran is an α -1,6 linked glucose-based polysaccharide with various combinations of α -1,2, α -1,3 and/or α -1,4 branching leading to a globular formation which is dense and can exist as a spherical suspension in a complex matrix, such as dough (Ioan et al., 2000). These properties aid macromolecule mobility and low viscosity (Figure 1) mimicking Newtonian behaviour (Ribotta et al., 2005). However, after the initial hydration of xanthan (110 sec.) the slope of the viscosity curve decreased slowly over time until a plateau was reached after approximately 440 sec. of measurement. The unfolding of the xanthan macromolecular structure under shear stress resulted in its high viscosity, thus potentially limiting the mobility of the solution and leading to a low-density, high-volume pseudoplastic-type behaviour (Linden and Lorient, 1999). The viscosity of HPMC was intermediate between xanthan and dextran, likely due to its water-binding properties discussed below (*Hydrocolloid performance in dough system* section).

Hydrocolloid performance in dough system

Water absorption was positively correlated with hydrocolloid addition in a linear manner ($R^2 \geq 0.97$), regardless of whether HPMC, xanthan, or dextran, were used, in each of the three flours analysed by the farinograph (Figure 2). Of the hydrocolloids tested, HPMC absorbed the greatest volume of water followed by dextran and xanthan, respectively, though the latter two were indistinguishable in biscuit and wholemeal flour analyses. For HPMC, the water binding capacity was greater due to this hydrocolloids' polar hydroxyl group structure which promoted increased water interactions through hydrogen bonding (Rosell et al., 2001; Shalini and Laxmi, 2007). Irrespective of the flour type, increasing levels of hydrocolloid resulted in a higher dough development time (DDT), due to the increased time needed for gluten hydration, except for dextran in biscuit flour whose DDT was unaffected. In biscuit flour, due to the low-protein content, dough development is dependent on the hydrocolloid used rather than solely on the gluten development. Additionally, since dextran displayed relatively rapid hydration (Figure 1), the lag period is eliminated and the time necessary to reach a dough consistency of 500 BU is significantly reduced. Even though dough development is heavily dependent on the type of hydrocolloid used, at lower dosages (0 to 1.0%) there is minimal difference between the hydrocolloids and their relationships with dough development time (Figure 3 A). Furthermore, dough stability is negatively correlated with hydrocolloid addition with the exception of the biscuit flour where increasing the dosage generally tended to improve stability, the only exception was dextran which had little dose-dependent effect (Figure 3 B).

Figure 7. 2 Change in water absorption of formulation dependant on flour/hydrocolloid ratio, as measured by the farinograph. Hydrocolloid used included dextran (■), xanthan (●) and HPMC (◆). The R² value for all plots ≥ 0.97 .

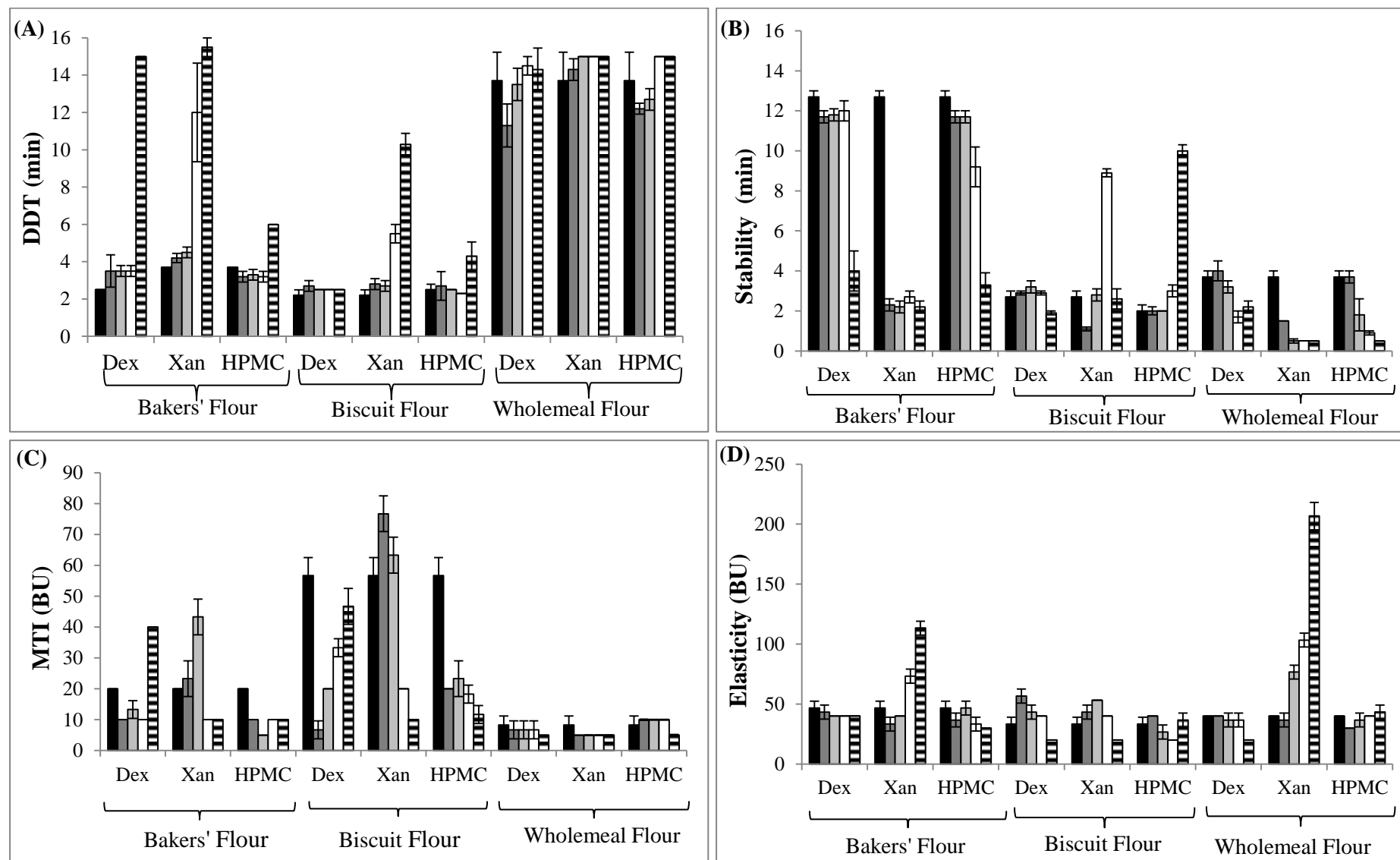


A strong gluten network ensures dough stability and in general, it is likely that the disruption of this matrix in the high-protein flours (Bakers' and Wholemeal flours) by increased hydrocolloid addition is responsible for the negative dose-

response correlation. However, in the low-protein biscuit flour the electrostatic interactions of both xanthan and HPMC with the proteins supporting the weak gluten network (Collar et al., 1999; Collar and Armero, 1996), thus explain the observed positive correlation between dough stability and hydrocolloid dose, up to a certain hydrocolloid-specific level of saturation (Figure 3 B).

The mixing tolerance index (MTI) of the wholemeal dough was unaffected by addition of any of the hydrocolloids at all dosage levels tested due to the disruptive interaction of fibre with the gluten network (Wang et al., 2002). However, bakers' flour MTI was generally negatively correlated with hydrocolloid addition, with dextran proving the exception at lower addition levels (Figure 3 C). Furthermore, biscuit flour dough MTI were negatively correlated with the addition of xanthan (at low doses) but conversely, was positively correlated with HPMC addition and dextran addition at lower dosages (Figure 3 C). The increased stability of the dough (lower MTI value) can be attributed to the strengthening effect of hydrocolloid addition (Rosell et al., 2001), in agreement with the dough stability data (Figure 3 B). Dough elasticity measurements revealed that xanthan addition had a negative effect for bakers' and wholemeal flour dough, but had the opposite impact on biscuit flour at most levels of addition. This is regarded as a negative attribute as the dough viscoelastic ratio is altered when compared to the control samples. Dextran and HPMC generally do not have a strong influence on this parameter relative to xanthan (Figure 3 D), thus maintaining viscoelastic dough with properties which are similar to the control dough.

Figure 7. 3 Farinograph analyses of bakers, biscuit and wholemeal flours with dextran (dex), xanthan (xan) and HPMC illustrating: (A) dough development time (DDT); (B) dough stability, (C) mixing tolerance index (MTI), and (D) dough elasticity for the control (black bar), 0.5% hydrocolloid dosage (dark grey bar), 1.0% hydrocolloid dosage (light grey bar), 2.5% hydrocolloid dosage (white bar), and 5.0% hydrocolloid dosage (striped bar).



The R_{50}/E ratio, directly related to the flour functionality (gluten strength and dough extensibility), was generally not affected when dextran and HPMC were included in bakers' and biscuit flours, independent of the concentration used (data not shown). This low R_{50}/E ratio is desirable in a dough system as it promotes good oven-spring resulting from strong gas retention properties in agreement with HPMC performance in rheofermentometer trials (Figure 4) and previous studies (Rosell et al., 2001). Conversely, in wholemeal dough both dextran and HPMC slightly and significantly increase the R_{50}/E ratio (data not shown), respectively, mainly due to a reduction in elasticity. Regardless the type of flour investigated, higher additions of xanthan resulted in a significant R_{50}/E ratio increase due combined increased R_{50} and decreased E .

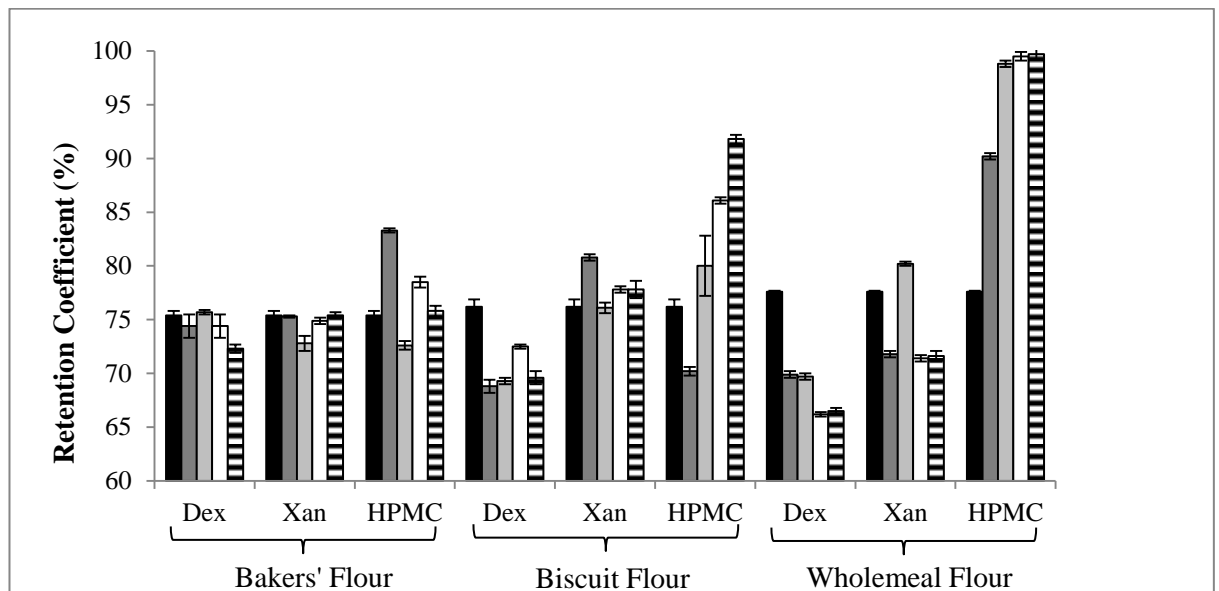
Gas retention capabilities (dough development)

Regarding the gas behaviour, the retention coefficient describes the dough ability to be stretched into thin membranes, and in turn, is associated with protein network quality. Gas retention during fermentation is indicative of the dough development potential and the tested flours all behaved similarly (Figure 4). In bakers' flour, there is no significant impact coming from the incorporation of dextran or xanthan, generally. However, generally when HPMC is used in this system, the retention coefficient of the dough increases inversely from the lowest level of incorporation (excluding the 1% HPMC formulation) (Figure 4).

In the biscuit flour system, dextran incorporation decreases the dough retention coefficient, whilst xanthan has no statistical impact on this parameter with the exception of 0.5% incorporation which results in improved dough development.

HPMC inclusion in the biscuit and wholemeal flour formulations was positively correlated with the retention coefficient and thus improved dough development.

Figure 7.4 Rheofermentometer analyses of bakers, biscuit and wholemeal flour dough with dextran (dex), xanthan (xan) and HPMC illustrating gas retention coefficient for the control (black bar), 0.5% hydrocolloid dosage (dark grey bar), 1.0% hydrocolloid dosage (light grey bar), 2.5% hydrocolloid dosage (white bar), and 5.0% hydrocolloid dosage (striped bar).



However, in wholemeal flour dough, both dextran and xanthan had a negative affect at most dosage levels. Overall, HPMC showed the biggest improvement on the quality of wholemeal flour dough than any of the other hydrocolloid-flour combination.

These results support the use of HPMC as an improver of gas retention during fermentation. As previously reported by Guarda et al. (2004), a possible explanation is that HPMC gives more stability at the gas-dough interface during proofing and confers additional strength to the gas cells during baking, thus

increasing the gas retention leading to greater volume. HPMC surface activity tends to stabilise the dough foam through dispersal of bubbles. This results in an even distribution of smaller gas cells which, in addition to increasing viscosity, confers structural stability particularly at the gas-liquid interface where an elastic micro-gel is formed (Schober, 2009). Conversely, the dough volume was decreased when microbial hydrocolloids, xanthan and dextran, with relatively lower water absorption properties (than HPMC) were used. The authors hypothesise that in the case of xanthan, its anionic properties likely interfere with the positively charged proteins, thus impeding the role of the otherwise elastic gas-retaining gluten film. Conversely, the large polymeric distribution of dextran may contribute a physically disruptive barrier to the gas-liquid interface and overall dough system, thus limiting free expansion during fermentation.

Bread analyses

Irrespective of the flour used, specific volumes of the breads were generally negatively correlated with, or did not affect, bread volume when any hydrocolloid was incorporated into the formulations. However, using 0.5% dextran or HPMC resulted in significantly increased specific loaf volumes for biscuit flour formulations (Figure 5D). These results suggest that xanthan, due to its strong gluten-hydrocolloid interactions, may limit dough extension. However, in the case of HPMC this strengthening property leads to stabilisation of the gas cell without compromising elasticity and expansion potential, thus resulting in a higher loaf volume (Barcenas and Rosell, 2005). Conversely, at higher addition levels dextran does not retain as much gas as HPMC but it also does not limit dough expansion due to a lack of electrostatic or ionic interaction with the gluten

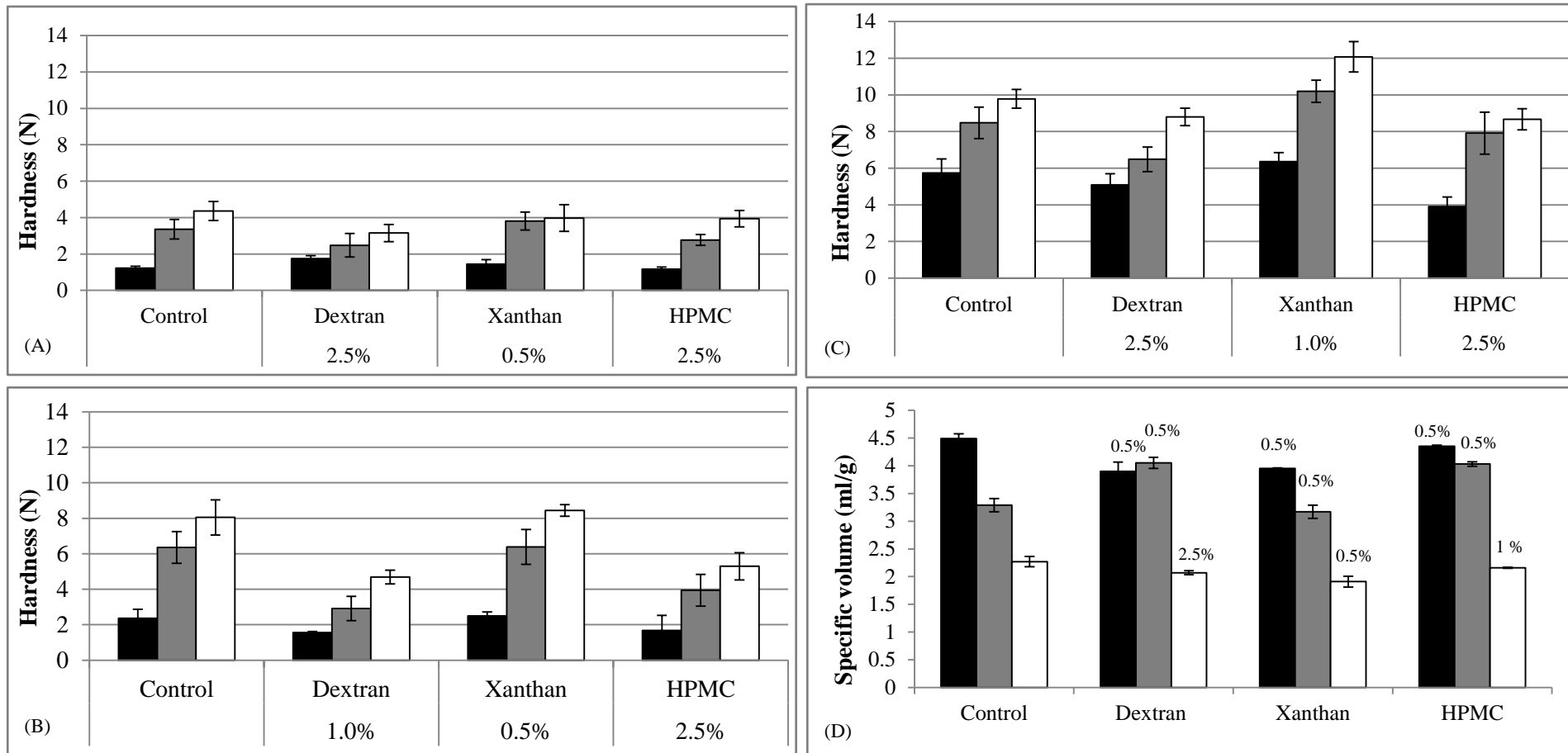
network, thereby allowing higher final bread volume. Dextran has previously been hypothesised to increase loaf volume by increasing the water-binding capacity of the dough and influence formation of the gluten through H-bonding or steric interactions (Ross et al., 1992). At optimal dosage levels (0.5%) of HMPMC or dextran, the hydrocolloid performances were very similar or indistinguishable over most parameters analysed (DDT, dough stability, MTI, elasticity, R_{50}/E and specific volume).

From a textural perspective (Figure 5A-C), there is a trend towards a reduction in initial loaf hardness when dextran (1.0%) and HPMPC (2.5%) are incorporated into the formulation; but the differences are not statistically relevant. However, the staling profile of the loaves over two- and five-day storage periods show that the incorporation of dextran or HPMPC results in a softer loaf than the control. The textural amelioration of breads due to HPMPC incorporation has been previously published (Rosell et al., 2001) and are likely due to the high fermentation gas retention (Figure 4), better water absorption (Figure 2) and redistribution of dough matrix components (gas cells and starch) before baking commences (Guarda et al., 2004; Schober, 2009). The successful application of dextran as a bread texture improver has been reported using an *in situ* sourdough application (Lacaze et al., 2007). However, its functionality as a bread-making ingredient has been less frequently explored, due to its novel food ingredient status (Van Geel-Schutten et al., 2006). Conversely, xanthan performs poorly from initial loaf hardness and staling viewpoints, likely due to a cell wall thickening effect (Rosell et al., 2001).

Using any of the three flours investigated in this study, crumb structure was generally unaffected by hydrocolloid addition when considering number and area of cells.

Figure 7.5 Hardness, staling and specific volume of wheat breads¹

¹The initial hardness (black bar) and staling values on days 2 (grey bar) and 5 (white bar) of breads made using; (A) bakers' flour, (B) biscuit flour and (C) wholemeal flour, incorporating the optimal hydrocolloid level (shown below bars) and the control breads with no hydrocolloids added. (D): the specific volumes of breads made using bakers' (black bar), biscuit (grey bar) or wholemeal flour (white bar), incorporating the optimal hydrocolloid level (shown above bars) and the control breads with no hydrocolloids added



However, when using bakers' flour formulations, the wall thickness of the crumb was significantly decreased upon addition of any hydrocolloid to the same extent regardless of the hydrocolloid used or its dosage (0.5 to 5.0%) (results not shown). When biscuit flour formulations were considered, cell wall thickness was also significantly reduced by HPMC addition to the same extent regardless of dosage (1.0 to 5.0%) (results not shown). For wholemeal flour dough, incorporation of any hydrocolloid at a dosage level of 0.5 or 1.0% resulted in a significantly thinner cell wall. In general, dextran displayed a trend towards a positive dose-response with wall thicknesses like HPMC; however, xanthan functioned in the opposite manner (results not shown).

4 Conclusion

In general, xanthan always performed relatively poorly in all wheat flour formulations, which may be related to its higher viscosity or its slower water absorption rate than either dextran or HPMC. As such, the incorporation of dextran or HPMC at 0.5 % dosage levels is a viable option to increase the specific volume in refined (bakers' and biscuit) wheat flours. However, dextran outperformed HPMC regarding bread initial hardness and staling shelf life at the lowest dosage level for biscuit flour or at 2.5 % for both hydrocolloids using bakers' or wholemeal flours. Additionally, the incorporation of clean label dextran as a novel food ingredient is the preferred hydrocolloid option.

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Chapter 8

General Discussion

8.1 General discussion

Given the current government, industry, and consumer drive towards a healthy lifestyle, dietary amendments are a positive way to implement a widespread, effective, and simple societal reform. This can have a significant and positive repercussive effect on personal and government medical costs related to non-communicable diseases particularly in Western developed countries. However, these trends place substantial pressure on the food and beverage industries to be innovative by reformulating current, or reinventing novel products, in line with consumer expectations and regulatory authority stipulations.

The fermentative metabolism of lactic acid bacteria (LAB) is the origin of a wide variety of compounds imparting flavor, texture, rheological properties, preservative characteristics and health beneficial attributes of lactic acid fermented foods and beverages (Pederson, 1971, Salminen, 2004).

LAB-Exopolysaccharides (EPSs) are polymers synthesised and release into the environment during fermentation in relatively substantial amounts when compared to cell size (Patel et al. 2010). Although microbial EPSs produced by non-food-grade bacteria such as gellan from *Sphingomonas elodea*, curdlan from *Alcaligenes faecalis*, and xanthan from *Xanthomonas campestris* are being widely applied as thickening, gelling and stabilizing ingredients in food industry, the consumers' opinion and country legislations restrict the use of genetically modified organisms (GMOs) for human food applications. Therefore, the developments of polysaccharides from LAB with GRAS (Generally Recognised as Safe) or QPS (Qualified Presumption of Safety) status hold high importance (Patel et al. 2010).

In fermented food and beverage two different strategies of application of EPS are possible: Either the *in situ* production by selected starter/adjunct cultures during the fermentation or the

ex situ production and addition of pure and defined amount of EPS at define time point of food process.

Food matrices fermented by functional LAB represents an elegant approach for modulating aroma, flavour and texture properties. EPS producing LAB are typical functional starter cultures because of their contribution to the consistency and rheology of fermented food products (De Vuyst et al. 2003). They can modify the flow characteristics of fluids, stabilize suspensions, flocculate particles, encapsulate materials and produce emulsions (Charchoghlyan & Park 2013).

In the first part of this thesis, *in situ* functional application of *Weisella cibaria* MG1 producing EPS has been investigated in barley wort, soy and quinoa-based beverages. *W. cibaria* MG1 was isolated from sourdough and identified as a hyper-producer of dextran with a molecular weight of 7.2×10^8 g/mol. Dextrans synthesized by *W. cibaria* are glucans connected by α -(1,6) glycosidic bonds.

W. cibaria MG1 dominated the (sucrose-supplemented) barley wort fermentation and produced up to 14.4 g l^{-1} of the dextran. The high maltose concentration, compared to sucrose, in wort diverted dextransucrase activity from EPS production to formation of panose-oligoaccharides. Even though the strain dominated the (sucrose-supplemented) wort fermentation, it was found to produce EPS with lower efficiency than in (sucrose-supplemented) MRS. When an efficient chain ending acceptor, such as maltose or isomaltose, is present in high concentration, dextransucrase will catalyze the synthesis of α -glucopoligosaccharides (Robyt, 1978) (panose and higher OS of the panose series) at the expense of EPS production levels.

The high fermentation quotient observed during *W. cibaria* wort fermentation prevents any negative unpleasant flavour attributes associated with high amount of acetate. The formation of EPS by *W. cibaria* MG1 significantly influenced the rheological behaviour of wort. The

levels of EPS formed were positively correlated with the initial sucrose concentration and EPS production continued throughout the complete fermentation time. Additionally, since the EPS remained stable after completion of fermentation, this dextran potentially represents a suitable viscosity-enhancer for beverages over their storage time. All these attributes can potentially be realised in a functional beverage using standard brewing equipment and widely available raw materials.

Many EPS-producing LAB are used in dairy products (Mende et al. 2013) while, there is little information on the use of EPS-producing LAB strains as functional starters for fermented plant-based milk manufacturing. Therefore, *in situ* application of dextran forming *W. cibaria* MG1 on soy-based and quinoa based milk was shown in Chapter 5 and 6, respectively.

Soy milk was fermented with a commercial yoghurt starter culture (CSC) and the EPS – producer *W. cibaria* MG1. The latter was able to enhance the acidification rate of the CSC exerting, especially towards *S. thermophilus*, a growth stimulation effect superseding *L. bulgaricus* performance during symbiosis in a cow's milk yogurt. *W. cibaria* MG1 proved to be a suitable adjunct starter culture due to satisfying growth, exceeding a cell count of log 8 cfu/g within 6 h of fermentation, utilization of the present sugars and the enhancement of acidification rate. Serum separation and gel stiffness were markedly reduced upon the addition of sucrose and the presence of high molecular weight EPS. The presence of *W. cibaria* MG1 led to a decrease in gelation and fermentation time. EPS isolated from soy yoghurts supplemented with sucrose were higher in molecular weight (1.1×10^8 g/mol Vs 6.6×10^7 g/mol), when compared to control, (no sucrose added) and resulted in reduced gel stiffness and thus yoghurt quality. The rheology analysis highlighted that the fracture stress decreased and the network structure changed due to larger pores and less cross-linking in the presence of sucrose and increasing molecular weight of the EPS.

In chapter, 6 *in situ* investigation of *Weissella cibaria* MG1 producing EPS on quinoa-based milk was performed. The production of quinoa milk, starting from wholemeal quinoa flour, was optimised to develop the most suitable substrate for maximising *in situ* *Weissella cibaria* EPS productions. On doing that, enzymatic destructurement of protein and carbohydrate components of quinoa milk was successfully achieved applying alpha-amylase and proteases treatments.

The growth and fermentation characteristics of *Weissella cibaria* MG1 including changes in viable cell counts, pH, titratable acidity, apparent viscosity and EPS production during and at the end of fermentation were investigated. Fermented wholemeal quinoa milk using *Weissella cibaria* MG1 showed high viable cell counts ($>10^9$ cfu/mL), a pH of 5.16, and significantly higher water holding capacity (WHC, 100 %), viscosity (> 0.5 Pa s) and exopolysaccharide (EPS) amount (40 mg/L) than the chemical acidified control. High EPS (dextran) concentration in quinoa milk caused earlier aggregation because more EPS occupy more space, and the chenopodin were forced to interact with each other. These proteins have their isoelectric point between pH 5.0 and 6.5 (Mäkinen et al., 2015), where they exhibit minimum solubility and their coagulation takes place.

Direct observation of microstructure in fermented quinoa milk indicated that the network structures of EPS-protein could improve the texture of fermented quinoa milk. Overall, *Weissella cibaria* MG1 showed satisfactory technology properties and great potential for further possible application in the development of high viscosity fermented quinoa milk.

LAB naturally produce EPS, thus distinguishing them as microbes with potential for development of technologically functional ingredients which can be produced *in situ* to formulate alternative plant-based beverages.

However, in all the experimental work described in the previous chapters, the amount and effects of EPS synthesized *in situ* vary considerably and depend, among others, on substrate,

fermentation conditions, and strain's metabolic activity. Thus, even though the *in-situ* LAB-EPS production satisfy the consumers demand for more natural and fewer additives in food, the reproducibility of the EPS techno-functional performance on the final food matrix can be compromised by different environmental and fermentation parameter linked to the EPS bio production.

Further trials involving *ex-situ* dextran application will improve, to a certain extent, the general understanding of the EPS role in plant-based beverage/yoghurt production. The *ex-situ* EPS application may not result in delivery of the same technological effects on plant-based beverage/yoghurt properties since the *in-situ* EPS production is a dynamic process progressing in parallel to fermentation.

The positive effect of *in-situ* application of MG1 producing EPS has been also extensively documented in different cereal-based products such as wheat and gluten-free sourdough breads (Galle and Arendt, 2014; Galle et al., 2014; Galle et al., 2012; Wolter et al., 2014). However, LAB EPS's bread making performance it has been always influenced by the balance between the amount of EPS produced and LAB fermentation metabolism (lactate and acetate production) that can negatively influence the EPS yield and counteract the beneficial EPS effects (Kaditzky & Vogel, 2008).

Tieking et al., (2003) showed that the addition of dextran to a level of 5 g/kg flour positively affected the viscoelastic properties of wheat dough and the volumes of the corresponding bread. In other to challenge further the techno-functional quality of LAB-EPS (dextran) on wheat system, in chapter 7 has been presented experiments where, for the first time, purified LAB dextran, have been added (*ex situ*) to various strength wheat flour (high-protein, low-protein and coarse wholemeal flours) recipes and its impact on the bread qualities was evaluated and challenged against the common food grade hydrocolloids xanthan and hydroxyl propyl methyl cellulose (HPMC). By using model bread recipes it was possible to

demonstrate that the effects of LAB-EPS (dextran) on bread quality depends on EPS concentration. Results showed that xanthan had negative impact on the dough and bread quality characteristics. HMPC and dextran generally improved dough and bread quality and showed dosage dependence. Volume of low-protein flour breads were significantly improved by incorporation of 0.5% of the latter two hydrocolloids. However, dextran outperformed HPMC regarding initial bread hardness and staling shelf-life regardless the flour applied in the formulation.

Beside the effect on bread properties, the addition of EPS as pure substance shows specific advantage such as a strict control of the EPS influence on dough and bread quality, possibility to optimisation the amount of EPS to be added as well as the time point of addition.

The results of this PhD work clearly shows that *in situ* and *ex-situ* EPS formed during different matrices fermentation from sucrose can be successfully applied to improve the rheological performance of plant-based beverage/food products opening interesting scenario of EPS bio-applications in food and beverage industries.

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Appendix

Publications and Presentations

Peer reviewed Publications

1. Zannini, E., Mauch, A., Galle, S., Gänzle, M., Coffey, A., Arendt, E.K., Taylor, J.P., and Waters, D.M. (2013). Barley malt wort fermentation by exopolysaccharide-forming *Weissella cibaria* MG1 for the production of a novel beverage. *Journal of Applied Microbiology* 115:1379-1387.
2. Zannini, E., Waters, D.M., Arendt E. K. (2014). The application of dextran as novel food ingredients to compensate for low protein in wheat flour. *Eur Food Res Technol* 238:763-771.
3. Zannini, E., Waters, D.M., Coffey, A, Arendt E. K. (2015). Production, properties, and industrial food application of lactic acid bacteria derived exopolysaccharides: a review. *Applied Microbiology and Biotechnology* (submitted)
4. Zannini, E., Mäkinen O. E., Waters, D.M., Arendt E. K., (2015). Impact of exopolysaccharide-producing *Weissella cibaria* MG1 on the properties of soy yoghurt. *Journal of Applied Microbiology* (submitted)
5. Zannini, E., Lyunch K., Jeske S., Waters, D.M., Arendt E. K., (2015). Development of novel quinoa-based yoghurt fermented with dextran producer *Weissella cibaria* MG1. *Food microbiology* (submitted)