Evaluating the behaviour of probiotic *Lactobacillus plantarum* 299v in non-dairy oat based yogurt using two different packaging materials

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Abstract

There is an upsurge of consumer interest in functional foods, especially probiotics. Alongside there is a global rise in the 'vegan' market. However, there is insufficient research and development in the field of non-dairy probiotic food formulations. Development of probiotic oat-based yogurt, called oatgurt that is manufactured by Oatly AB, was studied by incorporating *Lactobacillus plantarum* 299v strain, which maintains the products' vegan label. The two important factors that could affect probiotic bacterial strain viability and oatgurt's physicochemical properties include the step of strain incorporation and presence of oxygen. The probiotic strain was incorporated into two different food matrices; fresh oatgurt (incorporated before fermentation) and commercial oatgurt (incorporated after fermentation) maintained at 8°C in an incubator for 8 weeks. The effect of oxygen was evaluated by comparing polypropylene (PP) and glass as packaging materials for the two food matrices. In both food matrices, the viability of the strain in PP cups (~1 mm thickness), which has an oxygen transmission rate of 150-200 ml/m^2.day.atm, was similar to the viability obtained in glass jars, which is impermeable to oxygen. The presence of probiotic strain in oatgurt resulted in a gradual reduction in pH over time in both packaging materials. Glass had comparatively superior effect on maintaining oatgurt colour stability than PP ($p<0.05$), which was perceptible only after close observation even at week 8. The overall comparative analysis showed that PP cups could be effectively used as packaging units for probiotic oatgurt. Sensory evaluation and pilot scale experimentation of the resulting probiotic oatgurt remains necessary to confirm commercial product stability.

Keywords: oatgurt; *Lactobacillus plantarum* 299v; probiotic; polypropylene; glass.

Preface

‘Go basic!’ When I started this project my view of science and research always focused on finding innovative and alternative solutions. With a new food product to study, my entire attention was on finding the most exciting solution. As I started working on my project plan I soon realized when the basic questions were not answered, every other research, no matter how well done cannot show you the complete picture!

This is my attempt to answer a ‘basic’ question and further advance research on non-dairy probiotic food products.

I would like to dedicate my work to my mom, dad and brother.

Lund, June 2017

Divya Mohan

Acknowledgments

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I would like to dedicate this work to my FIPDes family who have enriched my experience away from home.

Lund, June 2017
Divya Mohan

Executive Summary

Introduction

Oatly AB, a Swedish food company founded in the 1990s, produces vegan milk-product alternatives made from oats. It caters to the appeal for veganism and vegan products, which is on a rise. This shift of food preference goes beyond a niche group who avoid animal meat for ethical purposes, towards consumers looking for a cleaner and healthier diet (Lea et al. 2006). With increased consumer awareness in recent years, food products are not only being consumed to satisfy hunger and basic nutritional requirements but also to enhance the quality of physical and mental wellbeing (Siro et al. 2008). This has paved way for the concept of 'functional foods', which include ingredients with additional health benefits or that can support specific body functions that conventional nutrition models do not address (Buttriss 2000; Menrad 2003). Probiotics represent a major segment of this functional food market (Granato et al. 2010).

Probiotics are live microorganisms that are natural inhabitants of the human gastrointestinal tract. The viability of probiotic strains in dairy products like yogurt has been studied extensively, however the research on non-dairy food matrices is limited. Among probiotic dairy products, yogurt has a wider consumer market (Siró et al. 2008) and hence, Oatly's spoonable oat based yogurt called 'oatgurt' was selected as the test product for the experiment (Figure 1). When probiotic strain is incorporated in certain quantities into food matrices and ingested, they can potentially improve the health of the host especially by contributing to intestinal microbial balance (FAO/WHO 2002; Grajek et al. 2004). The survivability and functionality of the probiotic culture is strain specific and depends on several factors including method of incorporation, temperature (Mokarram et al. 2009), pH, composition of the food matrix and level of available oxygen (Tripathi & Giri 2014).

Figure 1. Oatly's vanilla/blueberry oatgurt in polypropylene cup.

Objectives

This study aimed to investigate the behaviour of a probiotic strain obtained from Probi AB, *Lactobacillus plantarum* 299v (*L. plantarum* 299v), in Oatly's non-dairy oat-based yogurt called 'oatgurt'. The food product selected was Oatly's blueberry/vanilla oatgurt (Figure 1).

→ To study the viability of *L. plantarum* 299v in oatgurt

→ To evaluate the change in the physicochemical properties (pH and colour) of the probiotic oatgurt during storage.

→ To evaluate if incorporating *L. plantarum* 299v into oatgurt packed in polypropylene (PP) cups would be viable for Oatly

The two main influencing factors included how the strain was incorporated into the oatgurt and presence of oxygen in the packaging units.

Materials and Method

The viability of *L. plantarum* 299v (Probi AB, 2017) and changes in the oatgurts' physicochemical properties (pH and colour) was studied with respect to the two main influencing factors. Each factor had two levels that was analysed (Table 1).

Factor 1: Step of probiotic strain incorporation into the oatgurt

Fresh Oatgurt: *L. plantarum* 299v was added along with starter culture and fermented together. This was prepared without other additives like the stabilizers and fruit.

Commercial Oatgurt: *L. plantarum* 299v was added directly into ready-to-eat flavoured finished vanilla/blueberry oatgurt, post fermentation and cooling.

Factor 2: Presence of oxygen in packaging unit during storage

Polypropylene cups: The packaging unit currently used at Oatly are 1mm thickness polypropylene (PP) cups. They have an oxygen transmission rate (OTR) of around 150-200 $ml/m^2.day.atm$ (Buntinx et al. 2014). Thermosealable aluminium foil was used to seal the cup. Analysis of the gas in the headspace of sealed PP cups over time showed a gradually increase in O_2%.

Glass jars: To evaluate the effect of oxygen that is present in the PP cups, glass, which is impermeable to oxygen (Jayamanne & Adams 2004) was

used as a packaging unit for comparative analysis. Glass jars were sealed using heal sealed metal screw caps that maintained anaerobic condition inside the unit.

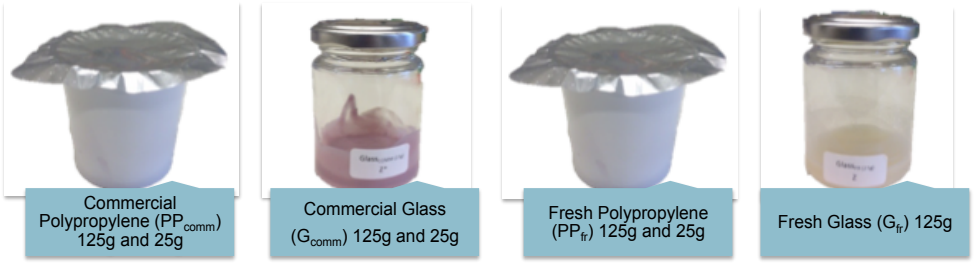

Figure 2 Manually packed commercial and fresh oatgurt samples in PP cups and glass jars.

The four samples were: Commercial oatgurt in PP cup, PP_{comm}; commercial oatgurt in glass jar, G_{comm}; fresh oatgurt in PP cup, PP_{fr}; and fresh oatgurt in glass jar, G_{fr}.The samples were prepared and packaged manually (Figure 2). They were stored at 8°C in a closed incubator with no light. Different tests (Table 1) were carried out for every sample in triplicates once a week for a period of eight weeks and analysed.

Table 1 Experimental design with two different packaging materials and food matrices.

Packaging Material / ***Food Matrix***	***Polypropylene***				***Glass***			
Commercial Oatgurt	Viability	pH	O_2	Colour	Viability	pH	O_2	Colour
Fresh Oatgurt**	Viability	pH	O_2	-	Viability	pH	O_2	-

*** prepared without additives (colour, flavour, stabilisers*

Viability of *L. plantarum* 299v: To study the viability of the four samples, standard spread plate method was used (NMKL 140). The samples at different dilutions were spread on solid MRS agar and incubated at 37°C for 48 hours followed by the enumeration of the colony forming units (cfu).

Oatgurt pH: The change in pH over time was evaluated to understand probiotic strain behaviour in all four samples.

Colour Stability: The colour stability was measured using Spectrophotometer-CM Food (Konica Minolta Colorimeter, Japan). L*a*b colour system was used and the change in visual perception, ΔE* was calculated.

Results and Discussion

As a food matrix, the oatgurt manufactured by Oatly AB appeared to be suitable for probiotic strain incorporation. It is rich in beta-glucan that is known to be a prebiotic (Mårtensson et al. 2002) and has a pH of 4.2 that most probiotic strains are tolerant to.

Viability: In all four samples, PP_{comm}, G_{comm}, PP_{fr} and G_{fr}, the strain viability from an initial concentration of $1.14\pm0.16*10^{8}$cfu/mL remained above recommended dosage of 10^{7} cfu/mL well after the storage period. There was however, a statistically significant difference in strain viability between fresh and commercial oatgurt from week 2 of analysis. The increased stress during fermentation during processing and presence of starter culture in the fresh oatgurt sample could explain the comparatively lower stability in viability of the probiotic strain in fresh sample. With the resulting data it was not possible to establish a significant effect that the packaging material had on the viability.

pH: The reduction in pH of the samples could mainly be attributed to the probiotic strain activity, as the pH of the control sample declined at a much slower rate (pH 4.26 to pH 4.01). There was a statistically significant reduction in pH over time in fresh and commercial samples; however, the effect of packaging material on the pH could not be established. Although the strain viability is not affected by the pH decline, there could be an influence on product flavour (taste). The fresh oatgurt had a comparatively low initial (start) pH and therefore the subsequent decline could have a bigger impact on product sensory properties.

Colour Stability: The change in colour in the vanilla/blueberry oatgurt specifically was a concern for the manufacturer. The presence of *L. plantarum* 299v in the product did not have an obvious affect on the product colour. The packaging material, however, seemed to have an impact on the change in colour, or change in visual perception (ΔE^*) of the product. The colour of week 0 sample was considered as the reference colour that was accepted by the manufacturer (desired value).

There was a steeper increase in total change in colour of oatgurt in PP compared to glass. In PP_{comm} after week 5, ΔE^* was above 2, indicating that the change in colour compared to desired reference would be perceptible at a glance. Till week 5 the change in colour in PP cups was only perceptible through close observation.

Conclusion and Future Recommendations

The study presents the possibility of commercially incorporating probiotic strain *L. plantarum* 299v into Oatly's oat-based yogurt (oatgurt) and packaging it in PP cups. Oatly's oatgurt was able to sustain *L. plantarum* 299v stably for over eight weeks when stored in cold chain. The current packaging material used for the commercially available non-probiotic oatgurt, PP, could be used for probiotic oatgurt as well since it sustains the viability of *L. plantarum* 299v over the incubation period well above recommended dosage. The colour of the oatgurt could be improved with a packaging material having higher oxygen barrier, however PP could ensure acceptable colour stability for atleast five weeks of storage.

Table 2 Overall conclusions of experimental results.

Packaging Material / ***Food Matrix***	***Polypropylene***				***Glass***			
Commercial Oatgurt	**Viability**	**pH**	**O_2**	**Colour**	**Viability**	**pH**	O_2	**Colour**
Fresh Oatgurt**	**Viability**	**pH**	**O_2**	-	**Viability**	**pH**	O_2	-

Note: Green – Favourable; Yellow – Acceptable; Red - Unacceptable

In order to further advance the development of this probiotic product, sensory analysis for appearance and change in flavour should be conducted. The organic acid profile due to the incorporation of *L. plantarum* 299v should be studied to understand its metabolic activity in this food matrix. Pilot scale experiments are required to ensure viability of the bacteria is not compromised because of other process and production related factors. With limited research on probiotic non-dairy food products, there is a need for several studies to establish concrete commercial product development strategies.

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List of acronyms and abbreviations

cfu	Colony Forming Unit
FCM	Flow Cytometry
G_{comm}	Commercial oatgurt in glass jars
G_{fr}	Fresh oatgurt in glass jars
GRAS	Generally regarded as safe
L. plantarum 299v	*Lactobacillus plantarum* 299v
MRS Agar	de Man, Rogosa and Sharpe Agar
OTR	Oxygen transmission rate
PCM	Plate Count Method
PP	Polypropylene
PP_{comm}	Commercial oatgurt in polypropylene cup
PP_{fr}	Fresh oatgurt in polypropylene cup
RT	Room temperature
TBC	Total Bacterial Count

1 Introduction

Oatly AB, a Swedish food company founded in the 1990s, produces vegan milk-product alternatives made from oats. It caters to the appeal for veganism and vegan products, which is on a rise. This shift of food preference goes beyond a niche group who avoid animal meat for ethical purposes, towards consumers looking for a cleaner and healthier diet (Lea et al. 2006). With increased consumer awareness in recent years, food products are not only being consumed to satisfy hunger and basic nutritional requirements but also to enhance the quality of physical and mental wellbeing (Siro et al. 2008). This has paved way for the concept of 'functional foods', which include ingredients with additional health benefits or that can support specific body functions that conventional nutrition models do not address (Buttriss 2000; Menrad 2003). Probiotics represent a major segment of this functional food market (Granato et al. 2010).

Probiotics are live microorganisms that are natural inhabitants of the human gastrointestinal tract. When incorporated in certain quantities into food matrices and ingested, they can potentially improve the health of the host especially by contributing to intestinal microbial balance (FAO/WHO 2002; Grajek et al. 2004). The survivability and functionality of the probiotic culture is strain specific and depends on several factors including method of incorporation, temperature (Mokarram et al. 2009), pH, composition of the food matrix and level of available oxygen (Tripathi & Giri 2014). The viability of probiotic strains in dairy products like yogurt has been studied extensively, however the research on non-dairy food matrices is limited.

Among probiotic dairy products, yogurt has a wider consumer market (Siró et al. 2008) and hence, Oatly's spoonable oat based yogurt called 'oatgurt' was selected as the test product for the experiment. The vanilla/ blueberry flavour was chosen as the manufacturers wanted to study the product colour stability over time. Due to lack of research on probiotic incorporation in oatgurt, two different steps at which the strain could be incorporated was studied (before fermentation and after fermentation). The effect of the presence of oxygen during storage can influence the viability and product physicochemical properties. The primary packaging unit currently used at Oatly, polypropylene cup, that has an oxygen transmission rate (OTR) of around 150-200 $mL/m^2.day.atm$ (Buntinx et al. 2014) was evaluated.

This is the packaging that is in direct contact with the product (Hellström & Saghir 2006). In order to evaluate the effect of oxygen, the change in viability of the strain should be compared with a material with no oxygen transmission. Glass, although impractical for commercial use, is impermeable to oxygen and easily available and was therefore selected for this comparative study. In addition to probiotic viability, the effect of permeability to oxygen of the packaging material on product physicochemical properties like pH and colour was analysed.

1.1 Purpose

This study aimed to investigate the behaviour of a probiotic strain obtained from Probi AB, *Lactobacillus plantarum* 299v (*L. plantarum* 299v), in Oatly's non-dairy oat-based yogurt called 'oatgurt'. The viability of the strain in an oatgurt and change in the physicochemical properties (pH and colour) of the oatgurt during storage was studied. The factors that could influence these aspects included how the strain was incorporated into the oatgurt and presence of oxygen in the packaging units. The food product selected was Oatly's blueberry/vanilla oatgurt (Figure 1.1b), which is sold as a twinpack (Figure 1.1a).

The process step when the probiotic strain is incorporated into the product may influence its behaviour. Of the two steps selected for probiotic incorporation, one was called the 'commercial' oatgurt sample in which *L. plantarum* 299v was added directly into ready-to-eat flavoured finished oatgurt, post fermentation. As an alternative, the probiotic strain was added along with starter culture and fermented together. This was called the 'fresh' oatgurt sample. In fresh oatgurt, the frozen probiotic strain may thrive better due to more time for adaptation during the fermentation process or its viability may suffer due to stress and competition due to the presence of the starter culture.

Figure 1.1 Oatly's twin pack blueberry/vanilla oatgurt. (a) 2 oatgurts cups sold as one twin pack unit with cardboard secondary packaging; (b) oatgurt packed in polypropylene cup (current primary packaging material).

The effect of the presence of oxygen in the packaging unit was evaluated comparing polypropylene (PP), which has an oxygen transmission rate (OTR) of 150-200 ml/m^2.day.atm and glass that allows no oxygen transmission (Jayamanne & Adams 2004a). If the current oatgurt packaging material (PP) is able to match the functionality of glass, Oatly can use PP without having to invest in a new packaging material even for their potential range of probiotic oatgurt.

To our knowledge there has been no study conducted to evaluate the viability and growth behaviour of any probiotic strain in oatgurt. The effect of the oxygen permeability into a product-packaging unit on the viability and behaviour of the probiotic strain *L. plantarum* 299v has also not been explored.

1.2 Limitations/Focus

The study focussed on the current primary packaging material (PP) and glass with respect to the oxygen transmission rates and not chemical structure or environmental considerations. Other physical parameters like strength and flexibility of the packaging materials were not considered as influencing factors. Other packaging levels were not considered for this study and therefore will not be discussed. The samples were stored in controlled temperature with no light exposure, eliminating the influence of those factors on the experimental results. The study was centred on the viability of probiotics in the oatgurt including the physicochemical changes like the pH and colour. The sensorial changes and consumer acceptance was not evaluated.

2 Literature Review

2.1 Functional Foods

More than 2,500 years ago Hippocratic stated “let food be thy medicine and medicine be thy food”, presenting the relationship between our diet and health (Spyropoulou et al. 2016). Foods or food ingredients that are able to provide health benefits beyond basic nutrition and increase consumer physical and mental wellbeing are defined as functional foods by the academia and food industries (Da Cruz et al. 2007; Menrad 2003). This concept was first introduced in Japan in the mid-1980s and since then there has been an upsurge of consumer interest in the health enhancing abilities of these food products (Hasler 1998). Functional food plays a role in promoting overall health and not towards curing diseases (Sanders 1998). Foods with such properties are becoming more readily accepted by the industry and can be used as marketing tools (Nematollahi et al. 2016). The labelling laws for functional foods depend on the country legislation. The label ‘probiotics’, for example, is allowed in certain countries including the United States but is banned in Europe by the European Food Safety Authority (Crane 2015).

Functional foods are associated with different mode of operations including vitamin and mineral fortification, cholesterol reduction, dietary fibre, probiotics, prebiotics and symbiotics, antioxidants, phytochemicals, and herbs and botanicals (Roberfroid 2000; Hironaka et al. 2011; Alzamora et al. 2005). In the European market, functional foods that improve the intestinal microbial balance and activity has been gaining a lot of interest (Alzamora et al. 2005). The microbial activity in the human gut is essential in maintaining metabolic and immune functions of the host (Cencic & Chingwaru 2010). The equilibrium of the gut microbiome can be affected by aging, type of food consumed, stress, use of antibiotics and other health conditions (Andrade et al. 2012). The increased consumption of processed food can further lower the chances of colonisation by certain types of bacteria in the gut. This imbalance can cause diarrhoea for the host, mainly affecting the elderly and also weaken the immunity of the host (Andrade et al. 2012). The main ways to sustain the population of viable bacteria in the gut is by ensuring consumption of sufficient daily nutrition without external stresses or by continuous ingestion of high viable population of probiotics (Mortazavian et al. 2012). It is not possible to permanently establish a probiotic organism in the gut and therefore multiple doses are required (Sanders 2008).

Probiotics have been defined in several ways over the past century. The definition used today is “probiotics are live microorganisms, administrated in certain quantities that confer health benefits to the host” (FAO/WHO 2002). The general attributes of a probiotic strain includes being naturally present in the hosts’ gastrointestinal tract, surviving the passage through the stomach and retaining its functionality in the intestine (Andrade et al. 2012). There are also some strains used as probiotics for humans that are not isolated from humans (Sanders 2008). Probiotic are generally consumed by adding the culture concentrate into a food, or is directly present in ready-to-eat food products, or via dietary supplements like powders, capsules or tablets. A widespread and efficient system to ingest probiotics is via commonly consumed food products (Mortazavian et al. 2012). With a present consumer trend of convenience and health, probiotic functional food products are being extensively consumed (Granato et al. 2010). Additionally, probiotics have been used for a long time as natural components in fermented foods and has a positive association with health among consumers (Forssten et al. 2011). Hence, probiotic food products have gained popularity in the market.

2.2 Non-Dairy Probiotic Food Products

Probiotic food products have been frequently cited as having health benefits including cholesterol level reduction, stimulating antibody production and phagocytic activity against pathogens in the gut and other tissues, anti-carcinogenic effect and reducing the symptom of Irritable Bowel Syndrome (IBS). The effect is strain specific (Fiorentini et al. 2011). Probiotics have been used mostly in dairy food products as they have proven to be efficient delivery vehicles for live bacteria (Andrade et al. 2012; De Vuyst 2000). Consumers are used to its presence in this type of product both in fluid milk and fermented dairy products (Pimentel et al. 2015). It also does not require a significant change in the manufacturing process or technology. There is a global rise of probiotic use, however, recently there is a reduction in dairy applications in Europe due to inflexibility in dairy regulatory laws and increasing dairy stock maintenance costs (Reid 2015). Moreover there is an evolving trend of vegetarianism and reduction in the consumption of dairy for ethical reasons. There is also a prevalence of lactose intolerance or allergy to milk proteins in many populations around the world (Nematollahi et al. 2016). Considering these factors, food producers are exploring non-dairy probiotic products that can be suitable alternatives that also offers variety in the market (Pimentel et al. 2015; Nematollahi et al. 2016). Fruit juices, desserts and cereal products have been found to be appropriate non-dairy media for incorporation of probiotics (Granato et al. 2010). Close to 78% of probiotic sales are through yogurt (Panthari & Kharkwal 2017), indicating that development of non-dairy yogurt alternatives could be a profitable option for the

food industry. The non-dairy probiotic yogurt products are mostly artisanal, generally small-scale and are mainly produced and sold in the USA (Table 2.1).

Table 2.1 Commercial non-dairy probiotic yogurts.

Product	*Country Manufactured*	*Company*	*Packaging Material*	*Culture(s)*	*Reference*
Dream™ Yogurt	USA	The Hain Celestial Group, Inc	PP	*L. casei, S. thermophiles, L. rhamnosus, L delbrueckii lactis and bulgaricus*	(’dreamplant based’ 2017)
Silk Dairy Free Yogurt	USA	Silk	PP	Live and active cultures.	('Silk Yogurts’ 2017)
Almond milk Artisanal Yogurt	USA	kite hill	Plastic-cardboard combination	*Bifidobacteria, L. acidophilus, S. thermophiles, L bulgaricus*	('kite hill Yogurt’ 2017)
Nancy’s Cultured Soy Yogurt	USA	Nancy’s	HDPE	*L. acidophilus, S. thermophiles, L bulgaricus, L. rhamnosus, L. casei, B. lactis*	('Nancy’s Yogurt’ 2017)
Original Hemp yogurt	USA	Living Harvest Tempt	PP	Active cultures	('Original hemp yogurt’ 2017)

2.3 Commercial Probiotic Strains

One of the initial steps in the process of developing non-dairy probiotic products is selecting a suitable probiotic strain (Figure 2.1). Probiotic strains that are used in the food industry should have well documented health benefits and possess certain technological properties. It includes being safe for consumption by the host, genetically stable, mass producible, be stable in the food product and not negatively influence product flavour (Forssten et al. 2011). Apart from these factors, the cost also influences the selection process of probiotic strains (Mortazavian et al. 2012). In a vegan food product it is essential that the strain be produced in dairy-free cultivation conditions (Figure 2.1). O’Soy vanilla, a soy based yogurt that used active culture that was cultivated on milk received huge consumer displeasure (’thevegblog’ 2007).

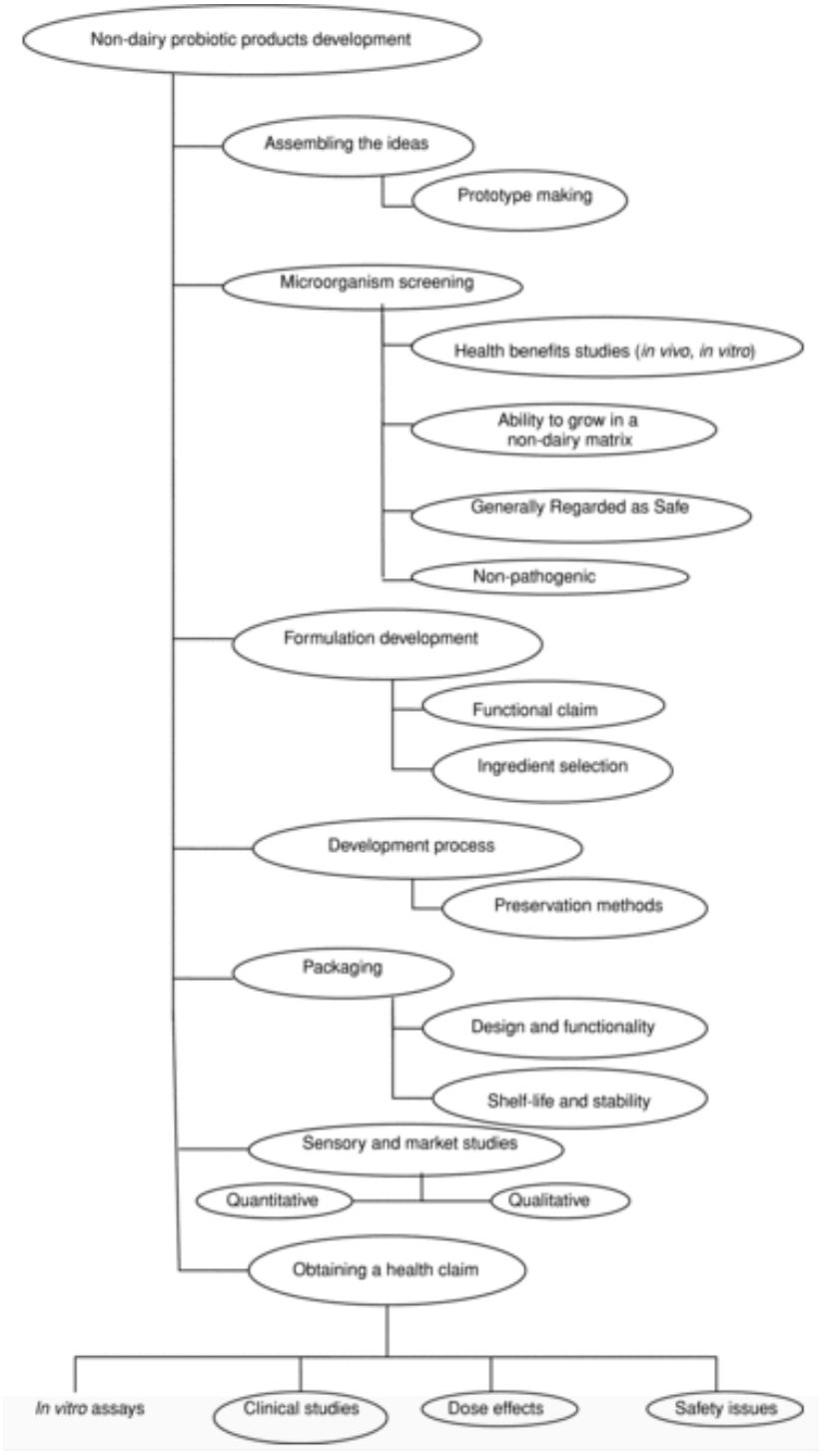


Figure 2.1 Non-dairy probiotic product development process (Granato et al. 2010)

The bacterial strains commonly used as probiotics in the food industry belong to *Lactobacillus* and *Bifidobacterium* genera (Andersson et al. 2001). Bacteria from both genera have been used for decades in food and are considered as GRAS (generally regarded as safe). Many of them are isolated from human faecal matter maximising their compatibility to the human intestine (Mortazavian et al. 2012). Probiotic strains of the *Lactobacillus* species are generally more robust and technologically compatible for usage in food production (Lee et al. 2009). They also demonstrate comparatively higher resistance to low pH, which is essential to survive the acidic environment of the stomach.

Viability is the number of live microbial cells per gram or mL of a food product. For a food product to be considered a probiotic or to have the stated/claimed health efficiency, the viability or minimum number of probiotic bacteria at the time of consumption (date of minimum durability) should be 10^7 colony forming units (cfu) per mL (or gram) of the product (Beena Divya et al. 2012). The recommended inoculation level is at least 10^8 or 10^9 cfu/ mL to account for the loss of bacteria that may occur till and after consumption of the product (Talwalkar et al. 2004). The survival of the bacterial strain(s) in the food matrix and throughout the time of storage is essential. The commonly used probiotics in the *Lactobacillus* genera are strains of *L. rhamnosus, L. rueteri, L. casei* and *L. acidophilus* (Cencic & Chingwaru 2010). Recently however, new strains with high stability and health benefits have been emerging both in different food matrices and as individual or mixed microbial cultures. All the benefits, chemical stability and adaptability to a food matrix is strain specific, hence choosing the appropriate probiotic strain is the first prerequisite for product development (Varga-Visi & Pápai 2015).

L. plantarum 299v, a versatile lactic acid bacterium patented by Probi AB, has been successfully used commercially in non-dairy food matrices ('ProViva' 2012). The *L. plantarum* 299v strain is found in environments that are protein rich. It is a rod shaped, gram positive, aerotolerant strain (Molin 2015). This strain is able to resist the low pH passage of the stomach and also the bile acids. It has also been found to be able to colonize the entire gastrointestinal tract (Nematollahi et al. 2016). It is a well documented and researched strain in a variety of food environments, with its complete genome sequenced (de Vries et al. 2006). It can ferment different types of carbohydrates, making it more adaptable (Molin 2015). There have been clinical trials positioning this probiotic as benefitting the host by reducing bloating, abdominal pains, improvement of the IBS symptoms and normalizing stool frequency ('Probi' 2016). A clinical study also demonstrated the ability of this strain to induce a pro-inflammatory response and increase immune alertness in intestinal epithelial cells (Cammarota et al. 2009). Considering all these factors, along with its vegan label, it is a suitable and promising choice for a new probiotic non-dairy formulation. Its natural environment is either anaerobic or microaerobic. In the absence of oxygen, *L. plantarum* produces D- and L-configurations of lactate. In aerobic conditions the lactate is further converted to acetate (Kleerebezem et al. 2003). The effect of this aerobic metabolism could

influence the flavour and other characteristics of the food product. Additionally, in aerobic conditions, at the early stages *L. plantarum* have reported to have growth stagnation (Stevens et al. 2008). It is documented to have maintained viability for about one month when refrigerated in fruit juices having pH <2.8-3.4 (Molin 2001).

2.4 Factors Affecting Viability of Probiotics (Oatly's Oatgurt as a delivery vehicle)

There are three main factors that influence the stability of incorporated probiotic strains in a food matrix: production factors, formulating factors (food matrix) and physical factors. First, the strain has to be able to sustain the production process and manufacturing conditions that the product goes through (Mortazavian et al. 2012). The stage at which the strain is incorporated into the product is detrimental. If the probiotic is added along with the starter culture, it should be able to remain functional throughout the fermentation process at both the incubation temperature and along the cooling rate (Andrade et al. 2012). The scale of production can also impact probiotic survival; therefore, choosing a robust strain is vital.

The matrix and product selected influences the probiotic stability, which are the formulating factors. The chemical composition of the food with respect to the carbon source available, nitrogen, minerals, growth promoters, inhibitors, salt and sugar content can alter the bacterial performance (Mortazavian & Cruz 2011; Granato et al. 2010). Including prebiotics in the matrix, for example, can aid the viability of the probiotic strain (Varga-Visi & Pápai 2015). It is important that the probiotic survives and is benefited from the food matrix, but does not affect its flavour adversely (Andrade et al. 2012). During strain selection and product formulation, the researcher should check if any ingredients of the product have an inhibitory activity on the probiotic strain.

Apart from sustaining the production process, the strain should also stay viable throughout the storage period till the time of consumption. This depends on the conditions of the food matrix the strain is added into. Oatly AB produces a non-dairy yogurt alternative called oatgurt that is available in different flavours ('Oatly' 2017). It is an oat-based product rich in beta-glucan. Beta-glucan is considered a prebiotic that aids the survival of probiotic bacterial strains (Mårtensson et al. 2002). The important physical factors of the matrix that could affect the probiotic strain include water activity, pH, oxygen content and temperature during storage (Heller 2001). The oatgurt has high water activity (>0.95), therefore, will have a short shelf life (4-6 weeks) (Forssten et al. 2011). The recommended storage condition of the oatgurt by the manufacturer is at 4-8°C. Such low temperatures tend to favour the survivability of probiotic strains and restrict post-acidification (Anadón et al. 2016). The temperature can be controlled as this product is

generally transported and stored in cold chain. The pH of oatgurt is around 4.27, and *L. plantarum* 299v is known to be resistant to even lower pH.

In the industry several approaches have been used to reduce the impact of O_2 on the probiotic strain viability. The effect of the presence of O_2 in the packaging unit and in the food matrix depends on the strain used. Addition of O_2-scavengers, encapsulation of the probiotics and varying production methods to lower O_2 incorporation are some of the techniques used (Macbean 2009). The packaging container used during storage can also be an important barrier to control the permeation of O_2 into the product. This can, hence, effect on the viability of the probiotic based on its characteristics (Talwalkar & Kailasapathy 2004).

2.5 Packaging for Probiotics

The important factors that the packaging of a probiotic product can impact include presence of oxygen and light (Varga-Visi & Pápai 2015). The selection of the packaging should be done keeping in mind shelf life and product stability (Figure 2.1). Since the oatgurt is stored after production for several weeks, it is liable to oxygen permeation (Talwalkar et al. 2004). The packaging material can limit this phenomenon. Oxygen permeation could lead to a negative effect on probiotic cells viability or change in its metabolic pathway and activity (Kleerebezem et al. 2003). It can also affect the physicochemical characteristics of the product. Studies have shown that adding probiotics did not significantly influence physicochemical properties like colour (Kailasapathy 2006), but the presence of oxygen could have an impact on it. The use of appropriate packaging, that acts as a barrier against the external environment, could ensure maintenance of physicochemical quality and bacterial viability throughout the shelf life (Da Cruz et al. 2007).

A challenge for most probiotic food product developers is maintaining the probiotic level during storage till the end of storage time to guarantee therapeutic results. Studies have shown that packaging material with lower oxygen permeability rates have higher stability in probiotic bacteria count (Da Cruz et al. 2007). The type and thickness of the packaging material are important features to be considered during selection. This further affects the gas and light permeability (Korbekandi et al. 2011). Most probiotic products commercially available are stored in plastic containers, which may be a problem because of high oxygen permeability depending on the probiotic and food matrix used (Pimentel et al. 2015). Conversely, glass containers have practically no gas permeability (Jayamanne & Adams 2004a) but poses other challenges in food production such as risk of breakage and high material and logistics cost (Da Cruz et al. 2007). The sealing technique used in glass jars, however, may allow permeability of gases. The screw cap is considered to be a closure with the best barrier to oxygen (Poças et al. 2010). Apart from the method of sealing, the packaging technique, that is if it

has been vacuum packed or involves a modified atmosphere packaging, can alter stability of the probiotic strain (Korbekandi et al. 2011). Comparing glass and plastic, which have contrasting oxygen permeability rates would be an effective method to understand the influence of the presence of oxygen on the viability of the probiotic strain. In previous studies comparing a lactic acid probiotic bacterial strain stability in dairy yogurt in glass and high density polyethylene (HDPE), it was found that glass bottles demonstrated the best results (Dave & Shah 1997). Another study with Bifidobacteria in fermented buffalo milk packaged in clay, plastic and glass jars demonstrated the comparative superiority of glass in sustaining the viability of the strain (Jayamanne & Adams 2004a). *L. plantarum* 299v is microaerobic and can switch to an aerobic metabolism, but the study of the dependence of its viability on packaging material (varying OTRs) has not been carried out. The change in behaviour that could result from this dependence when incorporated into a food matrix as well is not researched.

3 Research Methodology

The study was carried out to evaluate the influence of packaging materials with different oxygen transmission rates and incorporation methods on the survivability of probiotic *strain L. plantarum* 299v in non-dairy, spoonable blueberry/vanilla flavoured oat-based yogurt (oatgurt). Oatly AB is exploring the development of vegan, oat-based probiotic food products. Research on non-dairy food products as carriers for probiotic bacteria is limited compared to its dairy counterparts (Molin 2001). *L. plantarum* 299v (Probi AB 2017) was selected as the probiotic bacterial strain as it maintains the vegan label of oatgurt. It is also an extensively researched and documented probiotic strain.

The viability and behaviour of *L. plantarum* 299v (Probi AB, 2017) was studied by incorporating it at two alternative stages of processing and in two packaging materials with differing oxygen transmission rates. Additionally, the effect of the packaging material and its interaction with the product and outer atmosphere on commercial oatgurt colour stability was investigated. To understand the behaviour of the bacterial strain the product pH and gas in the headspace were monitored.

This work mainly shows the effect of using PP and glass as packaging materials on survival of *L. plantarum* 299v strain in Oatly's vanilla/blueberry oatgurt and on its physicochemical properties (colour, stability and pH).

The framework of the experimental tests was based on the literature studies. The aim was to obtain information about previous literature on different aspects of the study. Journals, books and company specification sheets were used. The focus was on:

i. Research on probiotic food products.
ii. Probiotic strain requirements in non-dairy products.
iii. Factors affecting survival of probiotic bacteria in food matrix.
iv. Probiotic food and packaging material interaction.
v. Experimental tests reported to evaluate the viability of probiotic strains in yogurt.
vi. Consumer acceptability to non-dairy probiotic foods.
vii. Influence of material oxygen transmission rate on food characteristics.

The application and product development specialist at Probi AB, Anna Andrys, was consulted prior designing the experimental plan. The results of the previous experiments carried out at Probi with the probiotic were discussed in order to avoid repetition and explore different approaches.

Since there is limited research on probiotic stability in non-dairy food matrices in different packaging material, it was difficult to correlate literature data with no practical evidence. Oatly has not yet developed a probiotic product; hence, experimental data was crucial for understanding the behaviour of the food matrix. The efficiency and viability of probiotics is specific to the strain and will vary depending on the product selected. With a detailed experimental design, several aspects of the research could be validated.

Laboratory experiments were performed to evaluate the performance of the two packaging materials (PP and glass) over a period of eight weeks in controlled temperature environment (Figure 3.1). The evaluation was done once a week for eight weeks as an extended shelf life analysis. The expected shelf life was 4-5 weeks. The microbiological behaviour and physicochemical parameters were tested and analysed. The trials were performed in triplicates to eliminate bias and experimental errors.

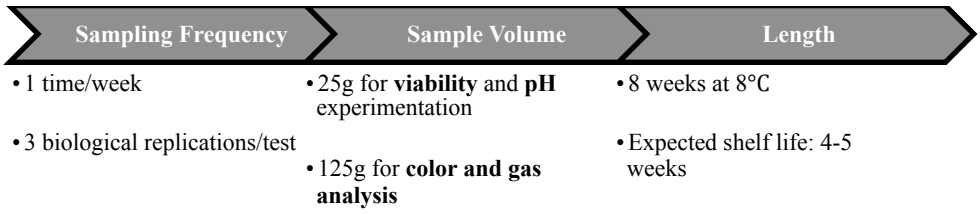


Figure 3.1. Conditions followed for experimental set-up and trials.

4 Materials and Methods

4.1 Pre-experimental Design

Viability test for probiotic freeze-dried powder: Sealed aluminium sachet with freeze-dried *L. plantarum* 299v powder was received from Probi AB. Freeze dried powder was suspended in sterile saline solution. Ten times serial dilutions of the sample were prepared and spread on de Man, Rogosa and Sharpe (MRS) agar plates (Merck, Germany) in duplicates using standard spread plate method (NMKL 140). The plates were incubated in 37°C and monitored to determine the time required for the colonies to develop. After 48 hours of incubation time the colonies on the plates were enumerated using plate count method (PCM) (Annex Figure B.1). The mean value for each dilution was multiplied by the respective dilution factor to determine the cfu/mL (Table 4.1).

For a product to be considered a probiotic it should have a probiotic strain concentration of atleast 10^7 cfu/mL of product. In the study the start concentration was around 10^8 cfu/mL and the change with time was evaluated. To reach 10^8 cfu of probiotic bacteria /mL of oatgurt sample, 0.2 g of freeze dried powder needed to be added to 1 litre (kg) of sample as predetermined at Probi AB and confirmed from the viability test.

Table 4.1 *L. plantarum* freeze dried powder viability results using PCM.

Dilution	***Plate 1 (cfu)***	***Plate 2 (cfu)***	***Mean (cfu/0.1 mL)***	***Mean (cfu/1 mL)***
10^5	884	1010	$947*10^5$	$0.9*10^9$
10^6	100	109	$105*10^6$	$1*10^9$
10^7	10	10	$10*10^7$	$1*10^9$
Mean	-			$1*10^9$

Glass jar sealing: 156 mL glass jars (56 mm diameter, 85 mm height) were received from Oatly AB as one of the packaging unit for analysis. The glass jars are impermeable to oxygen but the method of sealing/closing the jar could alter the condition inside the packaging unit. The glass jars were provided with heat seal metal caps (Annex Figure A.1). The glass jar was heat sealed in two different

ways. The first method involved heat-sealing the screw cap directly to the glass jar (Figure 4.1a) and the second had an additional layer of heat seal aluminium foil between the jar and cap (Figure 4,1b). The caps were heated directly after screwing it on to the jar using a clothes iron at the highest temperature setting (~150°C) (Schneider, 2010) and evaluated for different exposure times. Anaerotest® strip (Merck, Darmstadt, Germany), was used as an indicator for absence of O_2 in the sealed glass jar. The change of colour of the strip from blue to white indicated anaerobic environment in the jar (Figure 4.1a). The strip reversed colour back to blue within 20 minutes of exposure to aerobic condition. The glass jars that were directly heat-sealed for 10 seconds at ~150°C maintained anaerobic condition without exchange of O_2 with the atmosphere for eight weeks. There was a change in strip colour back to blue for the jar with the additional aluminium layer indicating non-hermeticity (Figure 4.1b). Therefore, all glass jars used in the experiments were hermetically sealed by screwing the metal cap on directly and exposing it to ~150°C (Schneider, 2010) for 10 seconds.

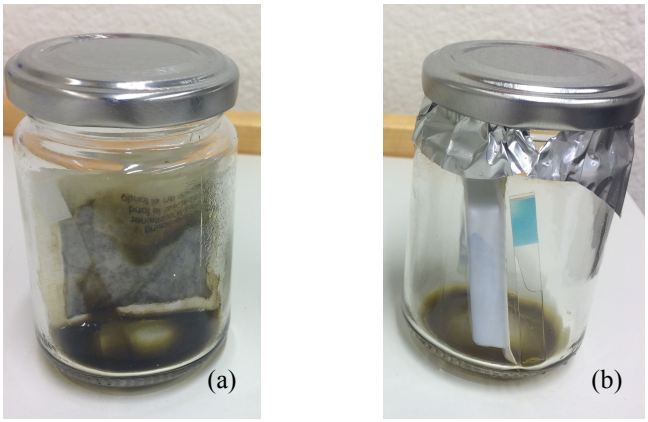


Figure 4.1 Anaerotest® strip (Merck, Darmstadt, Germany), indicator to evaluate anaerobicity inside the glass jar packaging. (a) Strip remaining white indicating anaerobic condition maintained in glass jar closed with heat sealed screw cap; (b) Colour of the strip reversed back to blue colour indicating aerobic condition in glass jar closed with screw cap having aluminium foil barrier.

PP cups sealing: 150 mL PP cups, having 1mm thickness are currently used as the packaging unit for the non-probiotic oatgurt at Oatly AB. These cups are closed using thermosealable aluminium foil (Annex Figure A.2). The foil thickness was 38±3.04 µm (Oatly AB, 2017). The cups and the foil were received from Oatly AB. To check for leakage, the cups were half filled with water and sealed using a clothes iron at the highest temperature setting (~150°C) (Schneider, 2010) and evaluated for different exposure times. The sealed cups were held upside down for 60 seconds to carry out leakage detection tests. After several trials the sealing condition was determined to be direct heating at ~150°C for 20 seconds.

4.2 Sample Preparation

Fresh Oatgurt Preparation and Packaging: 'Fresh' oatgurt was prepared in the product development laboratory at Probi AB, Sweden. Probi has standardized the method. 12 L of 0.5% fat pasteurised Oatly ecologic oat drink was added into a sterilized 12 L culture vessel (fermenter). Fermentation was carried out in a double jacketed fermenter with 0.52 g of commercially available yogurt starter culture used by Oatly (Danisco Yo-Mix 511 LY0300 DCV) along with 1 mL of defrosted *L. plantarum* 299v culture pellet (2.4 g of probiotic culture). The pH and temperature was monitored using probes. The resulting product, which did not contain the additives (stabilizer, fruit concentrate, sugars) present in the commercial oatgurt, was termed as 'fresh oatgurt' for this study. The fresh oatgurt was cooled to 4°C before packaging.

25 mL of the fresh oatgurt was added (by weighing) into PP cups and glass jars as the sample to be used for testing pH and viability. 125 mL of this fresh oatgurt was added into PP cups for headspace gas analysis. The cup/jar was partially closed with the foil or cap and flushed with nitrogen (N_2) gas using a syringe probe (Figure 4.2). The cup/jar was then sealed as described in the preparatory setup and stored at 8°C. The entire process was carried out in the sterile bench. Biological triplicates of 25 g and 125 g samples were prepared to test for 9 weeks (week 0 + 8 weeks). The sampling was performed once per week.

Commercial Oatgurt Packaging: The commercially available oatgurt (vanilla/blueberry flavour) was received from Oatly AB. 125g and 25g of oatgurt were added into both PP cups and glass jars.

The results obtained from the preparatory experiments resulted in 10^8 cfu/mL when 0.2 g of freeze-dried probiotic powder was suspended in 1 L (1000g) of saline. Therefore, 0.025 g of freeze-dried probiotic powder should be added to 125 g of the oatgurt sample. To simplify the process of adding a small quantity of probiotic to every sample packaging unit (PP cup and glass jar) a stock solution was prepared. 4 g of the probiotic powder was added to 160 mL of saline. The powder was suspended in saline by vortexing the tube till it completely dissolved. This stock solution was also plated using standard spread-plate method on MRS plates to verify the concentration of the probiotic bacteria (NMKL 140, 2007).

1 mL and 0.2 mL of the probiotic stock solution was pipetted into the 125g and 25g cups/jars respectively and stirred thoroughly. The cup/jar was partially closed with the foil or cap and flushed with nitrogen (N_2) gas using a syringe probe. The cup/jar was then sealed as described in the preparatory setup and stored at 8°C.

Control samples (without probiotic) were also prepared as described without the addition of the probiotic and sealed. The entire process was carried out in the sterile bench (Figure 4.3). Biological triplicates of 25 g and 125 g samples were prepared to test for 9 weeks (week 0 + 8 weeks) along with 9 control samples each. The sampling was done once per week.

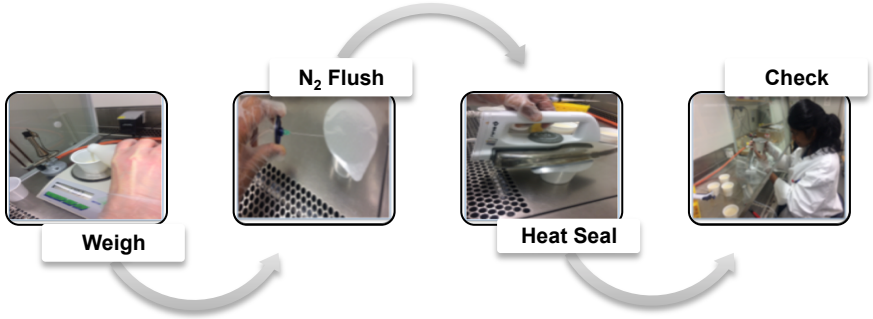


Figure 4.2 Process of packaging fresh oatgurt. 25 g and 125 g of fresh oatgurt was weighed and packed in PP cups. 25 mL fresh oatgurt was weighed and packed in glass jars.

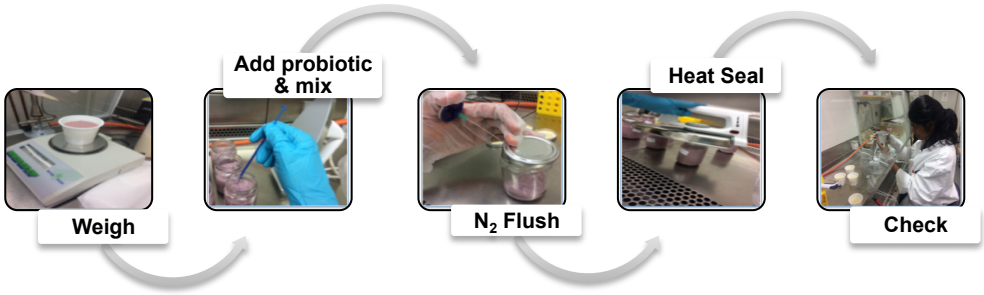


Figure 4.3 Process of packaging commercial oatgurt. 25 g and 125 g of commercial oatgurt was weighed and packed in PP cups and glass jars

Storage of Packaging Units: The temperature and relative humidity of the atmosphere a package is kept in can impact oxygen permeability (Da Cruz et al. 2007); therefore both PP cups and glass jars must be stored in the same conditions to effectively compare the results. The manufacturer expected to store the probiotic oatgurts at 4-6°C. All prepared samples were stored in 8°C cold incubator (Termaks, Norway). The recommended temperature for storage of oatgurt is a maximum of 8°C and this was selected for the study. The packaging units were not exposed to light, eliminating its influence in the study.

4.3 Experimental Design

The experiment was a 2^2 factorial design (Table 4.2). The two factors were the packaging material and stage of incorporation. The packaging materials used were PP and glass. The PP cup used was ~1mm thick with an OTR of around 150-200 mL/m^2.day.atm (Buntinx et al. 2014). The glass jars did not allow oxygen permeation. The experimental results from two different packaging materials could demonstrate the influence of the presence of oxygen inside the packaging unit on product characteristics. The method of incorporation included using commercial and fresh samples. The strain was incorporated during production by adding probiotic stain along with the oatgurt starter culture followed by fermentation; this was called 'fresh oatgurt' (Annex Figure A.5). The other method was to incorporate the strain after production to the ready-to-eat finished product post fermentation called 'commercial oatgurt' (Annex Figure A.4).

Table 4.2 Experimental design with two different packaging materials and food matrices.

Packaging Material / *Food Matrix*	*Polypropylene*				*Glass*			
Commercial Oatgurt	Viability	pH	O_2	Colour	Viability	pH	O_2	Colour
Fresh Oatgurt**	Viability	pH	O_2	-	Viability	pH	O_2	-

*** prepared without additives (colour, flavour, stabilisers*

The combination of sample used were: Commercial oatgurt in PP cup, PP_{comm}; commercial oatgurt in glass jar, G_{comm}; fresh oatgurt in PP cup, PP_{fr}; and fresh oatgurt in glass jar, G_{fr} (Figure 4.4). Commercial oatgurt without probiotic strain was prepared as control samples and packed in PP (PPC) and glass (GC).

Figure 4.4 Manually packed commercial and fresh oatgurt samples in PP cups and glass jars.

The viability of *L. plantarum* 299v and change in pH was tested in all the samples (25g of sample/ packaging unit). The headspace O_2% was measured in the PP cups (PP_{fr} and PP_{comm}) (125g of sample/ packaging unit). The anaerobicity of the glass jars was evaluated during the pre-experimental setup. The colour stability of the commercial oatgurt (125g of sample/ packaging unit) over time was measured (PP_{comm} and $G_{comm)}$, as the fresh oatgurt did not contain the colour pigment. All experiments were conducted in biological triplicates and once every week for 8 weeks.

As an additional study, the effect of temperature on colour commercial oatgurt control samples that were packaged at Oatly were stored at room temperature (RT) and analysed.

4.4 Probiotic Viability Test

4.4.1 **Plate count method (PCM).**

25 g of fresh and commercial oatgurt in sealed PP cups and glass jars were used as experimental samples. The four samples, PP_{comm}, G_{comm}, PP_{fr} and G_{fr} were analysed at each time point (once per week).

100µL of fresh oatgurt was pipetted into 900µL of saline in a 2mL eppendorf tube, followed by 10x serial dilution. The commercial product was denser and therefore was first diluted with 25 mL saline and mixed into a homogeneous mixture. 200µL of this mix was added to 800µL of saline for the first dilution. Transferring 100µL from the first dilution to 900µL of saline continued the 10x serial dilution of the commercial samples.

The 10^{-6}, 10^{-7} and 10^{-8} dilutions of the samples were plated using standard spread plate method. Biological triplicates of every sample and technical duplicates of each dilution were plated using standard spread plate method (NMKL 2007). The commercial samples were free of the starter culture, therefore plated on solid MRS media. The fresh oatgurt sample had to be plated on solid MRS media supplemented with vancomycin (Sigma Aldrich Co., St. Louis, Mo.), an antibiotic, to suppress the growth of starter culture colonies ('Probi' 2016). The plates were incubated at 37°C incubators for 48 hours and counted to determine the total colony forming units (cfu) per mL of sample.

4.4.2 **Flow Cytometry (FCM)**

The standard plate count techniques accounts only for the viable bacteria that are able to grow and form colonies. It was therefore possible to underestimate the

actual total bacterial count (TBC) (Cassoli et al. 2007). To determine if the results of PCM corresponds to the total viable cell count, a dual staining technique that detects membrane integrity was carried out with two fluorophores (Gatza et al. 2013). BD Accuri™ C6 personal flow cytometer was used for detection and measurement according to the Eawag (2013) method (Gatza et al. 2013). It is a comparatively rapid and powerful method that uses less resources (Cassoli et al. 2007). SYBR® Green I (Sigma Aldrich Co.) is a green fluorescent nucleic acid stain that enters live and dead cells (Stiefel et al. 2015) and is detected by one of the flow cytometer fluorescent detectors (FL1). Propidium iodide (PI) (Sigma Aldrich Co.) is a red fluorescent nucleic acid stain that is unable to penetrate intact plasma membranes (healthy cells). The FL3 detector in the flow cytometer measures the red light. PI was added to differentiate between live and dead/damaged cells. By co-staining the sample, the cells with both stains get detected by both detectors (Annex Figure B.3). These results were plotted (Annex figure B.3) and the instrument automatically quantified the cell viability.

A mixture of SYBR® Green I (1X final concentration) and PI (0.3mM final concentration) was added to the 100 times diluted oatgurt sample. Sample preparation for FCM was done by adding 6 µL (SYBR Green I + PI), 489 µL deionised water and 5 µL sample (100X) in 150 mL eppendorf tubes. The samples were incubated at 37°C for 10 minutes and analysed. The control commercial sample was run unstained in the instrument to detect the background. The four samples (PP_{comm}, G_{comm}, PP_{fr} and G_{fr}) at 10^{-2} dilutions in 1.5 mL eppendorf tubes were stained with the dual flurophores and analysed in a similar manner. The results obtained were analysed to compare it with PCM. The experiment was set up only at week 4 and week 8 as it took time to standardise the method.

4.5 pH (Acidity Analysis)

The sample used for the viability test (remaining) was also used to measure pH using a pH meter (Mettler-Toledo™ FE20 Benchtop, Switzerland). Based on the week 0 sample pH values, the pH meter was calibrated between pH 2 and 4 while measuring fresh oatgurt samples and between pH 4 and 7 for commercial oatgurt and control. Biological triplicates of all samples were measured once every week for 8 weeks.

4.6 Headspace Gas Analysis

The O_2 content in the headspace of sealed 125 mL PP_{comm} and PP_{fr} samples was determined using Dansensor CheckMate® 9900 O_2/CO_2 (PBI Dansensor A/S, Ringsted, Denmark). A small volume of the headspace gas was drawn through a sampling probe needle introduced to the PP cup by piercing the aluminium foil. This was an intrusive method to analyse headspace in the packaging unit. The instrument determined the O_2% and CO_2% in the withdrawn headspace gas. The measurement was done once a week for 8 weeks.

4.7 Colour Stability

The colour stability was measured using the portable Spectrophotometer-CM Food (Konica Minolta Colorimeter, Japan). The spectrophotometer was equipped with a MAV target mask with an 8 mm opening for wet and viscous samples. L*a*b system was selected at D65 illumination and a 10° standard observation angle (Konica Minolta, Japan): L* value is a measure of luminosity (between 0 for black and 100 for white), a* value is a measure for redness (positive value) or greenness (negative value) and b* value is a measure for yellowness (positive value) or blueness (negative value) (Valencia 2011) (Figure 4.5). Zero calibration by measuring the surrounding and white calibration using the supplied white calibration plate (Konica Minolta, Japan) was done at the beginning of sample measurements.

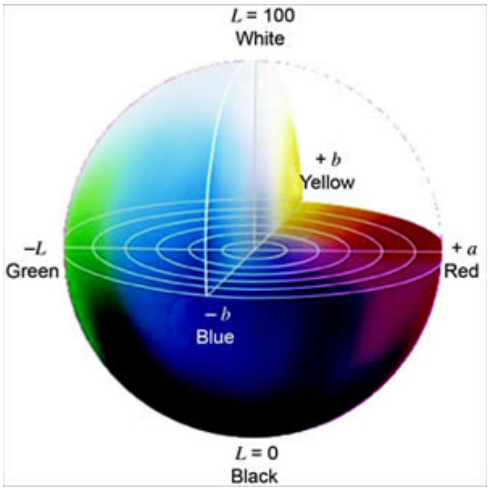


Figure 4.5 CIE LAB colour space used by the Spectrophotometer ('ColorCodeHEx' 2017)**.** This model was used to detect change in colour of the oatgurt samples using their L*, a* and b* values.

Commercial oatgurt samples stored in PP cup and glass jars were tested for colour stability once a week. 125 mL of the sample was transferred from the packaging unit to an opaque black plastic container, 5.5 cm in height and diameter (Annex Figure A.3). The portable instrument was held perpendicularly to the sample cup and the readings were carried out. The measurement was done thrice for each of the biological triplicates of every sample. The L*, a* and b* values were obtained and analysed using SpectraMagic™ NX, colour data software.

The total colour difference or change in visual perception, ΔE*, was calculated using the formulae (Equation 4.1) ('Konica Minolta' 2017).

$$\Delta E^* = [\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}]^{1/2} \quad (4.1)$$

ΔL*is the difference between the absolute L* value of the sample and a reference. Similarly Δa* and Δb* was calculated by finding the difference between the absolute a* and b* values of the sample and reference respectively. The ΔE* values of the probiotic and control samples were calculated with respect to two different reference values. For statistical comparison the pre-set target colour (L*= 87.99, a*=-3.06 and b*=15.13) configured in the instrument was used as the reference. These values indicated the colour of all samples including week 0 across incubation period with respect to the instrumental target coordinates. This was used to find if there was a variation in colour with time.

The other reference used was the average absolute coordinates values of the commercial control sample (week 0), which was considered as the 'desired' value (L*=58.55, a*=9.83 and b*= 0.07). ΔE* values of all samples with respect to this reference value was calculated to evaluate the deviation of the sample colour from desired colour over the eight weeks incubation period. The higher the value of ΔE* of the sample, more the deviation or difference in colour (Konica Minolta 2017). When ΔE* is <1, the change in colour is not perceptible through human eyes; when the value is between 1 and 2, the change in perceptible through close observation; and if the value is between 2 and 10, the change is perceptible in a glance (Schuessler 2016). The qualitative analysis of this difference was done by analysing the Δb* values of the samples compared to 'desired' value ($b^*_{sample} - b^*_{desired}$). An increase in the value of Δb* towards the positive scale indicated the sample to be less blue and more yellow compared to the selected reference sample.

A separate experiment was set up with commercial oatgurt in PP (control), stored at RT to evaluate the influence of temperature on colour. The evaluation was carried out only on weeks 2 and 4.

4.8 Statistical Analysis of Data

Statistical data analysis with 95% confidence interval was performed using the GraphPad software (Prism 7, 2017). The two factors were the matrices *L. plantarum* was incorporated into (fresh or commercial) and type of packaging (PP or glass). There were two levels within each factor that were compared against each other. Viability, pH and change in colour perception with respect to instrument target reference value were analysed using Analysis of Variance (ANOVA).

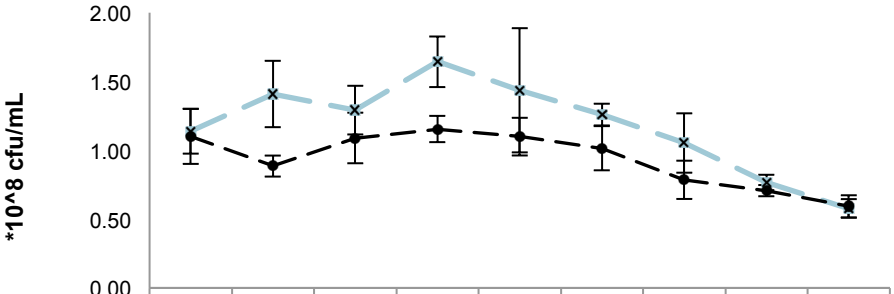
5 Results

The results obtained from eight weeks experimentation on fresh and commercial oatgurt in PP cups and glass jars (PP_{comm}, G_{comm}, PP_{fr} and G_{fr}) were documented and analysed.

5.1 Viability of *L. plantarum* 299v

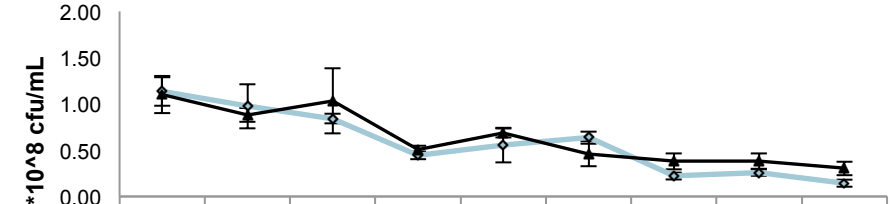
The samples that were plated using standard spread plate method and incubated at 37°C for 48 hours were enumerated. Each week the incubated MRS agar plates at different dilutions containing 10-300 colonies were selected and documented as cfu per mL of oatgurt sample. The viability of probiotic *L. plantarum* 299v inoculated into the commercial oatgurt in PP and glass remained well above the required recommended dosage (10^7 cfu/mL) (Figure 5.1). The average starting concentration at week 0 was 1.14±0.16 and 1.10±0.20 $*10^8$ cfu/mL of commercial oatgurt in PP and glass respectively. After eight weeks the concentration went down to 0.58±0.07 and 0.59±0.08 $*10^8$ cfu/mL commercial oatgurt in PP and glass respectively. There was a significant decrease in viability compared to week 0, only after seven weeks of incubation in both packaging materials ($p< 0.05$). In fresh oatgurt also the inoculated probiotic remained above recommended dosage but reduced significantly after two weeks of incubation ($p< 0.05$) compared to week 0. The average starting concentration at week 0 was 1.13±0.15 and 1.10±0.2 $*10^8$ cfu/mL of fresh oatgurt in PP and glass respectively. The starting concentrations were similar to that of the commercial oatgurt samples. After eight weeks the concentration reduced to 0.38±0.09 and 0.30±0.08 $*10^8$ cfu/mL of fresh oatgurt in PP and glass respectively.

It was not possible to establish a significant effect of the packaging material, and hence presence of O_2, on the viability of the probiotic strain. However, the stage of incorporation (food matrix) of *L. plantarum* did have a significant effect on viability ($p< 0.05$). The viability remained comparatively more stable when the probiotic strain was added to the finished commercial product (Figure 5.3).



	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
PPcomm	1.14	1.41	1.29	1.64	1.44	1.26	1.05	0.77	0.58
Gcomm	1.10	0.89	1.09	1.15	1.10	1.01	0.79	0.71	0.59

Figure 5.1 Viability of *L. plantarum* 299v in commercial oatgurt sample. The average probiotic cfu/mL of commercial oatgurt using PCM for samples incubated for eight weeks in PP cups and glass jars.



	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
PPfr	1.13	0.98	0.84	0.44	0.56	0.64	0.22	0.26	0.14
Gfr	1.10	0.88	1.03	0.51	0.69	0.46	0.38	0.38	0.30

Figure 5.2 Viability of *L. plantarum* 299v in fresh oatgurt sample. The average probiotic cfu/mL of fresh oatgurt using PCM for samples incubated for eight weeks in PP cups and glass jars.

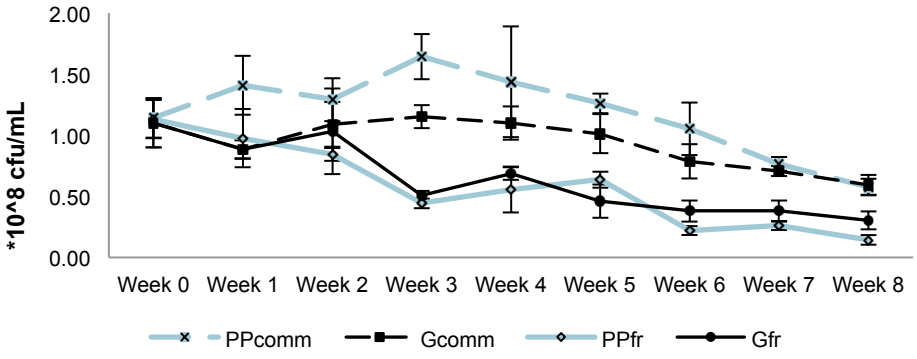


Figure 5.3 Viability of *L. plantarum* 299v in commercial and fresh oatgurt sample. The average probiotic cfu/mL of sample using PCM for samples incubated for eight weeks in PP cups and glass jars.

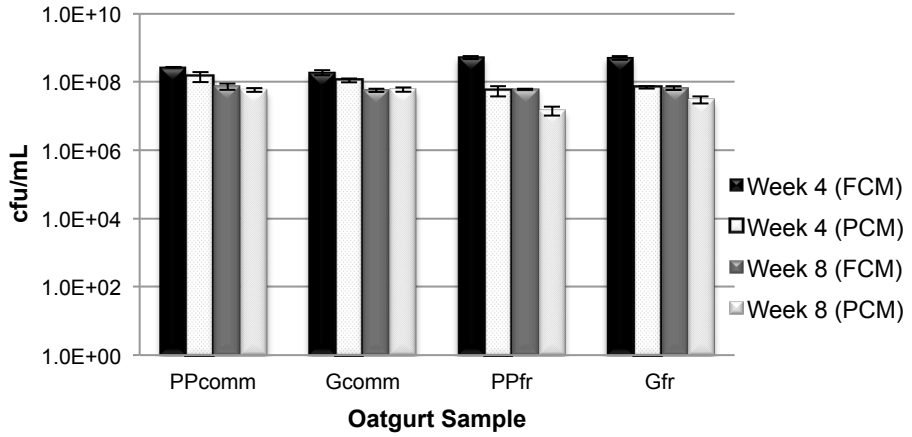
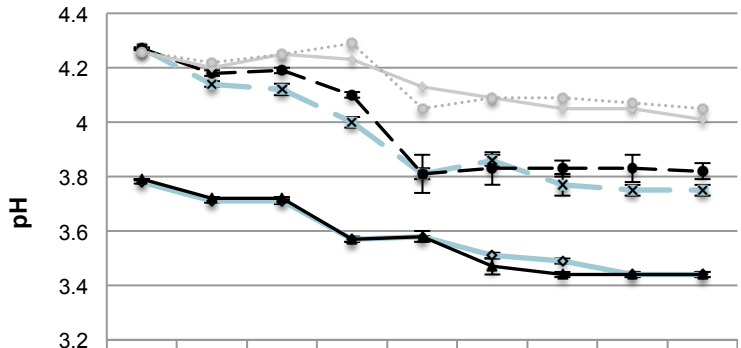


Figure 5.4 Comparing FCM and PCM results of *L. plantarum* viability on week 2 and week 4 for PP_{comm}, G_{comm}, PP_{fr} and G_{fr} samples.

The viability (live cells) was measured in all samples on week 4 and 8 using FCM and compared with the number of cfu/mL using PCM. The results of FCM did not deviate greatly from PCM (Figure 5.4). The FCM results were relatively higher than the PCM (Annex Table B.1), which could be accounted as the cells that were unable to form colonies.

5.2 pH

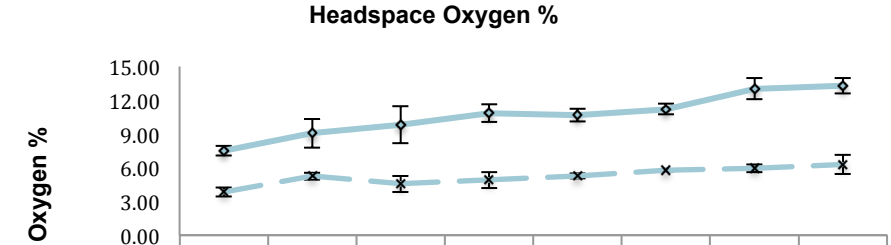
The pH values of the control, commercial oatgurt and fresh oatgurt were measured once a week over eight weeks of incubation. There was a significant difference ($p< 0.05$) between the pH of commercial product control samples and product samples containing probiotic right from week 1. This ascertained that the drop in pH was caused by the activity of the probiotic strain. In the commercial and fresh oatgurt it was not possible to establish a significant effect of packaging material on the pH change. There was a statistically significant difference between using glass and PP at week 3 and week 7 in the commercial oatgurt and week 5 and week 6 in fresh oatgurt. However, this was not sufficient data to ascertain a difference caused by packaging material. The time of storage had a significant effect ($p< 0.05$) from week 1 of incubation in all samples (PP_{comm}, G_{comm}, PP_{fr} and G_{fr}). There was a gradually decreasing trend in pH with time. The change in pH of the commercial product, PP_{comm} and G_{comm} was from pH 4.2±0.01 to pH 3.75±0.02 and 3.82±0.03 respectively. In the fresh product, PP_{fr} and G_{fr}, the pH declined from 3.78±0.01 to 3.44±0.01. The starting pH at week 0 for commercial and fresh samples were different, therefore there was an obvious significant difference with time based on method of incorporation (Figure 5.5).



	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
PPcomm	4.27	4.14	4.12	4	3.81	3.86	3.77	3.75	3.75
Gcomm	4.27	4.18	4.19	4.1	3.81	3.83	3.83	3.83	3.82
Control PPcomm	4.26	4.2	4.25	4.23	4.13	4.09	4.05	4.05	4.01
Control Gcomm	4.26	4.22	4.25	4.29	4.05	4.09	4.09	4.07	4.05
PPfr	3.78	3.71	3.71	3.57	3.58	3.51	3.49	3.44	3.44
Gfr	3.79	3.72	3.72	3.57	3.58	3.47	3.44	3.44	3.44

Figure 5.5 Sample pH measured over eight weeks of incubation. The control commercial samples (grey) were plotted against commercial probiotic sample to analyse the cause for change in pH.

5.3 O_2% in PP_{comm} and PP_{fr} Headspace



	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
PPcomm	3.84	5.25	4.56	4.90	5.27	5.76	5.94	6.28
PPfr	7.51	9.07	9.83	10.85	10.67	11.21	13.03	13.30

Figure 5.6 O_2% in PP cup headspace using Dansensor CheckMate® 9900 O_2/CO_2. The results were susceptible to errors as it was an intrusive method to measure headspace gas.

The O_2% in the headspace of the PP cups over 8 weeks of storage was analysed. The plotted graph (Figure 5.6) showed an increasing trend, which could be indicative of the permeability of the packaging material. The glass packages maintained close to an anaerobic environment that was determined during the pre-

experimental setup. The instrument used was intrusive and obtained results could be susceptible to a sub-optimal way of performing the measurements or due to handling error. Non-intrusive oxygen analysing methods might have provided more accurate outcome and the glass jar anaerobicity could also be asserted.

5.4 Colour Stability of Commercial Oatgurt

The commercial oatgurt colour was measured using a handheld spectrophotometer. It detected a peak at around 480 to 550 nm (Figure 5.7), which corresponds to the typical absorption bandwidth of anthocyanin (Routray & Orsat 2011). Anthocyanin is the naturally occurring blue colour pigment in blueberries. The absolute L*, a* and b* coordinate values of the samples from week 0 over eight weeks were measured. The change in visual perception, ΔE*, was calculated using the absolute coordinates and instrumental reference point (Figure 5.8). There was a significant difference between the ΔE* values of the oatgurt sample in PP and glass from week 2 ($p < 0.05$). Compared to week 0, a significant change was observed after week 2 in the PP cup and after week 6 in the glass jars. The interaction between the packaging material and time, therefore, had a significant effect on colour ($p < 0.05$).

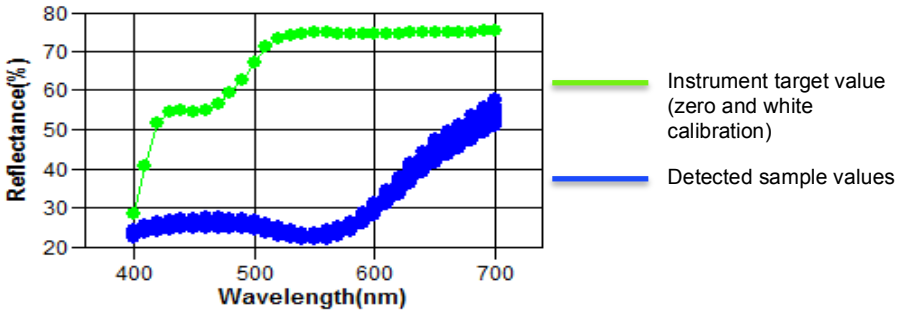


Figure 5.7 Detection spectrum for blueberry/vanilla oatgurt sample using Spectrophotometer. Peak detected at around 480 to 550 nm, corresponding to anthocyanin (blue colour pigment) absorption wavelength. .

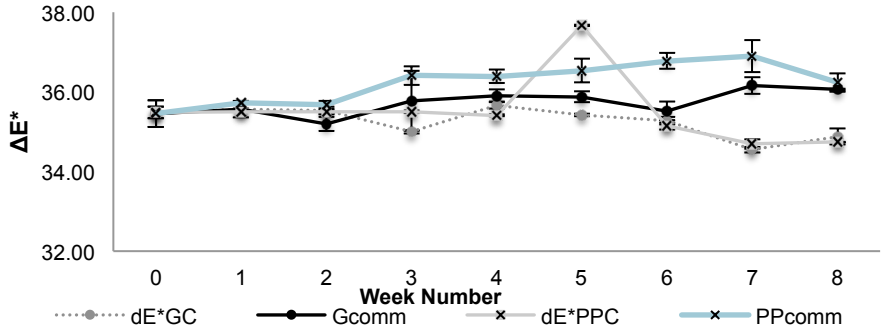


Figure 5.8 Change in visual perception of colour with respect to instrumental target reference over eight weeks of incubation at 8°C. The ΔE* was calculated using the target reference value (L*= 87.99, a*=-3.06 and b*=15.13) for control (PPC and GC) and probiotic commercial sample.

The presence of the probiotic strain, *L. plantarum* 299v, in the product did not seem to have an impact on the visual perception of colour. There was no significant difference in **ΔE*** values between the control sample and G_{comm} with probiotic strain till week 6 (Figure 5.8). In PP cups a significant difference after week 3 of incubation was observed between control and probiotic sample. A clear relation between the presence of the probiotic and change in **ΔE*** values could not be established with measured results.

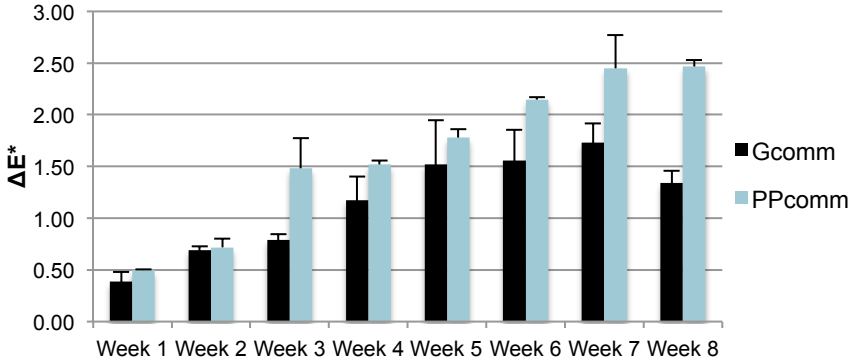


Figure 5.9 Deviation of sample colour from desired colour over eight weeks of incubation at 8°C. The change in visual perception was calculated using average colour coordinates of week 0 control sample referred to as the desired colour coordinates (L*=58.55, a*=9.83 and b*= 0.07) as the reference.

The average value of week 0 control colour coordinates was considered as the reference colour (Figure 5.11a) accepted by the manufacturer (desired value). The values measured for all samples were calculated using the 'desired' coordinates as the reference to compare deviation of the product colour from the desired colour. There was a steeper increase in total change in colour of oatgurt in PP compared to glass (Figure 5.9). The ΔE* value for G_{comm} remained below 2 even at week 8. In PP_{comm} after week 5 the value increases beyond 2, indicating that the change in

colour would be perceptible at a glance. Upto week 3 the change in colour is not perceptible through human eyes when in glass jar, however after week 2 it is perceptible for PP. The Δb* values (Table 5.1) of the samples increased and shifted towards more positive values indicating decrease in blue appearance and increase in yellowness of the product over time.

Table 5.1 Sample Δb* (blueness-yellowness indicator) values across time.

Incubation Period	PP_{comm}	G_{comm}
Week 1	-0.01±0.01	-0.03±0.13
Week 2	0.27±0.09	0.44±0.09
Week 3	0.18±0.20	0.31±0.08
Week 4	0.27±0.12	0.42±0.02
Week 5	0.07±0.20	0.25±0.13
Week 6	0.42±0.15	0.61±0.13
Week 7	0.71±0.13	0.81±0.07
Week 8	0.87±0.14	0.74±0.11

Note: Values calculated using 'desired' reference coordinate (Average b week 0); b^*_{sample}-$b^*_{desired}$.*

The additional study results showed the influence of temperature on colour. The evaluation was done on week 2 and week 4 of incubation at RT. The coordinates were calculated using 'desired' value as the reference. The deviation from desired value was calculated. The results were compared with the values of oatgurt stored at 8°C. The total colour difference was significantly higher, that was observed both in the instrumental values (Figure 5.10) and visual observation (Figure 5.11b). The colour right at week 2 is perceptible at a glance when the oatgurt is stored in RT.

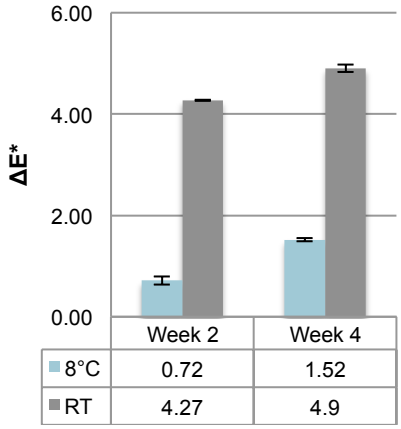


Figure 5.10 Deviation of sample colour incubated at 8°C.and RT at week 2 and week 4 from week 0 desired reference value.

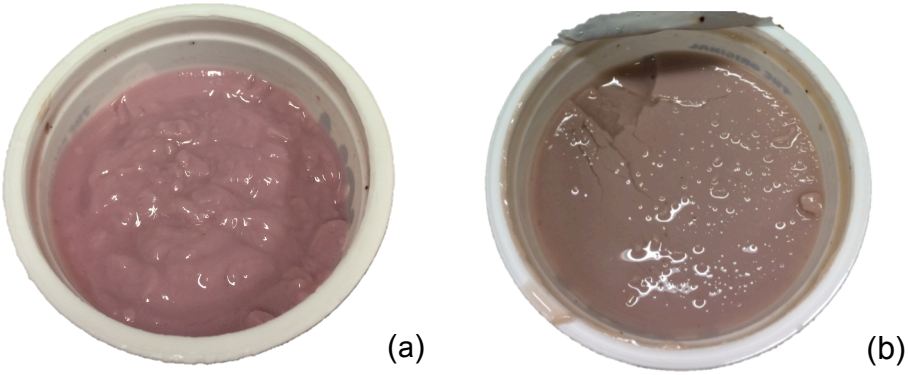


Figure 5.11 Visual appearance of commercial oatgurt sample. The samples analysed were (a) commercial control sample at week 0 stored at 8°C and (b) commercial control sample at week 2 stored at RT.

6 Discussion

As a food matrix, the oatgurt manufactured by Oatly AB appeared to be suitable for probiotic strain incorporation. It is rich in beta-glucan that is known to be a prebiotic (Mårtensson et al. 2002) and has a pH of 4.2 that most probiotic strains are tolerant to. Oatgurt is currently packed in ~1mm thick PP cups closed with thermosealable aluminium foil. The packaging unit could be susceptible to O_2 permeation due to the material properties. An increase in the O_2% in the headspace of the PP cups over time (Figure 5.5) filled with the commercial and fresh oatgurt samples (PP_{comm} and PP_{fr}) ascertained this occurrence. The impact of the presence of O_2 was not found to be adverse on the viability of selected probiotic strain, *L. plantarum* 299v (Figure 5.5). In all four samples, PP_{comm}, G_{comm}, PP_{fr} and G_{fr}, the strain viability from an initial concentration of $1.14\pm0.16*10^8$cfu/mL remained above recommended dosage of 10^7 cfu/mL well after the storage period. This was the required concentration for a food product to be considered a probiotic. There was however, a statistically significant difference in strain viability between fresh and commercial oatgurt from week 2 of analysis. The increased stress during fermentation during processing and presence of starter culture in the fresh oatgurt sample could explain the comparatively lower stability in viability of the probiotic strain in fresh sample. With the resulting data it was not possible to establish a significant effect that the packaging material had on the viability. The tolerance towards presence of O_2 could be explained by the microaerophilic nature of *L. plantarum* 299v. The FCM results did not deviate greatly from that of PCM, indicating the prospect of using FCM for future viability studies of *L. plantarum* 299v. This would significantly reduce the time and labour required to conduct conventional plate viability counts. The flow cytometer needed to be optimized for the oatgurt sample and *L. plantarum* 299v bacteria. This process took time; therefore, it was not possible to get results for week 0. This information could have improved the analysis by providing a reference to compare the week 4 and week 8 results.

The strain viability was also not influenced by the decrease in pH over time in both food matrices. This result agrees with the studies that show *L. plantarum* 299v stable in food matrix at pH as low as 2.8 (Molin 2001). The reduction in pH of the samples can mainly be attributed to the probiotic strain activity, as the pH of the control sample declined at a much slower rate (pH 4.26 to pH 4.01). There is a statistically significant reduction in the pH over time in fresh and commercial sample, however the effect of packaging material on the pH could not be established. Although the strain viability is not affected by the pH decline, there

could be an influence on product flavour (taste). The increasing acidity of fresh oatgurt that had a comparatively low initial (start) pH could have a bigger impact on product sensory properties. During commercial production, the fresh oatgurt would be supplemented with additives like stabilizers and sweeteners. This would potentially increase its pH, and could considerably reduce sensory impact. However, adding the probiotic strain at the end of the production line (commercial oatgurt sample) could potentially reduce the change required in the production and processing conditions. This could benefit the manufacturer during commercial production.

The change in colour in the vanilla/blueberry oatgurt specifically was a concern for the manufacturer. The packaging material seemed to have an impact on the change in colour, or change in visual perception (ΔE*) of the product. Statistically there was a significant difference from week 2 of incubation between the two packaging materials. The G_{comm} samples were comparatively more consistent with the week 0 ΔE* value. The change mostly occurred in the form of increasing Δb* values compared to the desired reference value. This increase in Δb* values of the sample meant that the product was becoming less blue and showing more yellowness according to the L*a*b* colour format. Visually it was perceived as changing from bluish-purple to a dull bluish-grey colour.

The presence of *L. plantarum* 299v in the product did not have an obvious affect on the product colour. The resulting colorimetry results comparing the control and oatgurt with probiotic was not sufficient to establish a relationship between the colour and presence of probiotic strain. On direct visual inspection, the difference in colour between sample stored in PP and glass was not obvious. However, there was a clear difference, both with the instrumental data and visual inspection, between the samples that were stored at 8°C and RT (21°C). The colour seemed to be greatly influenced by the temperature of the atmosphere compared to presence of oxygen that permeated through the packaging material. This change in colour could also be affected by growth of other organisms that are generally supressed at cold chain temperatures.

Although glass as a packaging material demonstrated better results in terms of colour stability over time, it is not suitable for commercial usage. Glass also allows light, which could also affect the product physicochemical properties. The influence of light, however, was controlled in this study. The weight and fragility of glass compared to PP, along with the high cost limits its application in the food industry. An alternative would be selecting a packaging material with improved oxygen barrier properties or adding an additional layer like ethylene vinyl alcohol (EVOH) to the current PP cup. This could reduce the change in colour perception, while being practical in terms of handling and logistics.

Using packaging with improved oxygen barrier properties compared to PP could improve blueberry/vanilla oatgurt colour positively (closer to desired value), but under controlled conditions (cold chain and no light) PP demonstrated results

similar to glass. The material used did not affect probiotic strain viability and pH in this study. The results obtained are specific for *L. plantarum* 299v and the incorporation methods that were used. Jayamanne & Adams (2004b) demonstrated glass to be superior while using bifidobacteria strain as the probiotic. A study with *Lactobacillus paracase*i conducted by Pimentel et al. (2015) also concluded glass to be better at maintaining viability of that particular strain. This indicates that the results are exceedingly dependent on the selected strain. However, similar to the results found in this study, Pimentel et al. (2015) concluded that the packaging material (plastic or glass) did not affect physiochemical properties (colour and pH) of probiotic apple juice. The results obtained from the study indicate that Oatly can continue using PP for their probiotic oatgurt.

7 Conclusion and Future Scope

The study presents the possibility of commercially incorporating probiotic strain *L. plantarum* 299v into Oatly's oat-based yogurt (oatgurt) and packaging it in PP cups. Oatly's oatgurt was able to sustain *L. plantarum* 299v stably for over eight weeks when stored in cold chain. The current packaging material used for the commercially available non-probiotic oatgurt, PP, could be used for probiotic oatgurt as well since it sustains the viability of *L. plantarum* 299v over the incubation period well above recommended dosage. Adding the strain to the finished oatgurt would be a more viable option for the manufacturer in terms of processing method. The viability and pH of the fresh oatgurt in this study is comparatively less favourable than commercial oatgurt. The colour of the oatgurt could be improved with a packaging material having higher oxygen barrier, however PP could ensure acceptable colour stability for atleast five weeks of storage.

Table 7.1 Overall conclusion of experimental results.

Packaging Material / *Food Matrix*	*Polypropylene*				*Glass*			
Commercial Oatgurt	**Viability**	**pH**	**O_2**	**Colour**	**Viability**	**pH**	O_2	**Colour**
Fresh Oatgurt**	**Viability**	**pH**	**O_2**	-	**Viability**	**pH**	O_2	-

Note: Green – Favourable; Yellow – Acceptable; Red - Unacceptable

In order to further advance the development of this probiotic product, sensory analysis for appearance and change in flavour should be conducted. The organic acid profile due to the incorporation of *L. plantarum* 299v should be studied to understand its metabolic activity in this food matrix. Pilot scale experiments are required to ensure viability of the bacteria is not compromised because of other process and production related factors. Literature study indicates oat-based medium to be well suitable for the incorporation of *L. plantarum* 299v, paving way for probiotic product development with other Oatly food products. With limited research on probiotic non-dairy food products, there is a need for several studies to establish concrete commercial product development strategies.

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Appendix A Material and Method

A.1 Packaging Materials

Figure A.1 Glass jar with heat seal metal cap.

Figure A.2 Polypropylene cups (current packaging at Oatly) with thermosealable aluminium foil.

A.2 Experimental Materials

A.2.1 Colorimeter Equipment

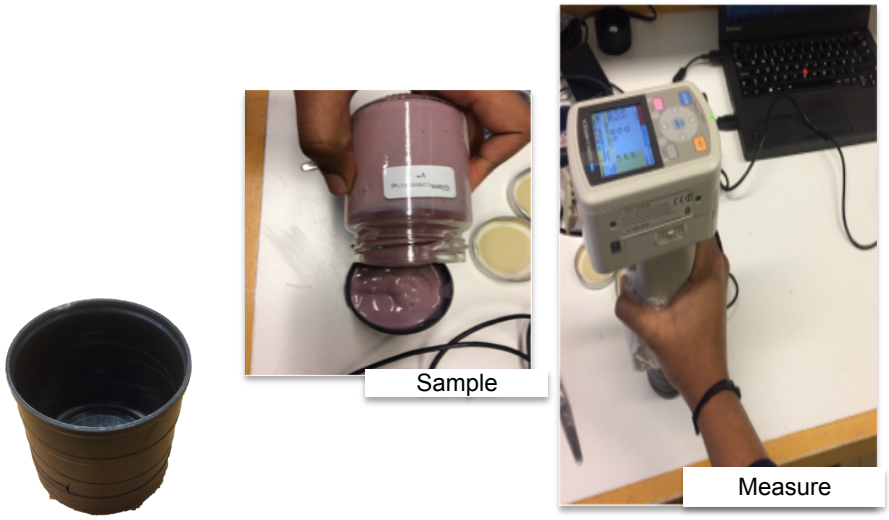


Figure A.3 Commercial oatgurt sample transferred into black plastic container for colorimeter measurement.

A.2.2 Steps of Incorporation

Figure A.4 Commercial oatgurt sample formulation and packaging process.

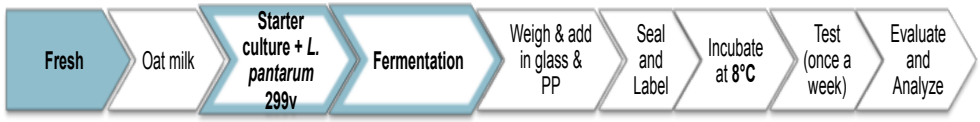


Figure A.5 Fresh oatgurt sample formulation and packaging process.

Appendix B Supplementary Results

B.1 Viability Analysis of Freeze Dried Probiotic Powder

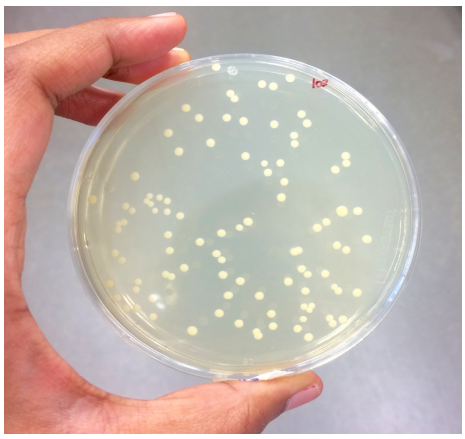


Figure B.1 10^6 dilution of freeze-dried probiotic powder stock plated using standard spread plate method on solid MRS agar. L. plantarum colonies are reported to be large, creamy and white-yellowish colour (Johansson et al. 1998)

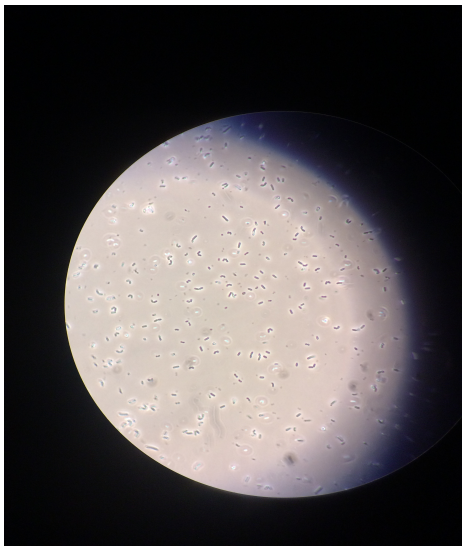


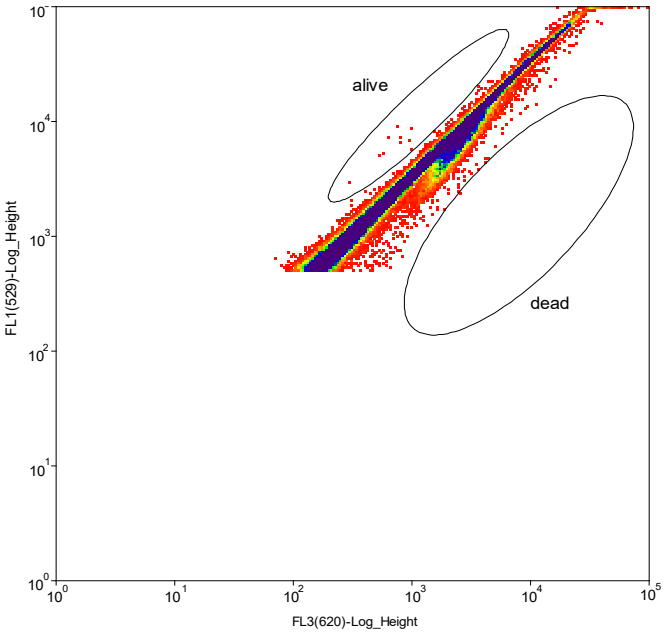
Figure B.2 Microscopic image of rod shaped *Lactobacillus plantarum* cells scooped from obtained colonies on MRS plate.

B.2 FCM Analysis Results

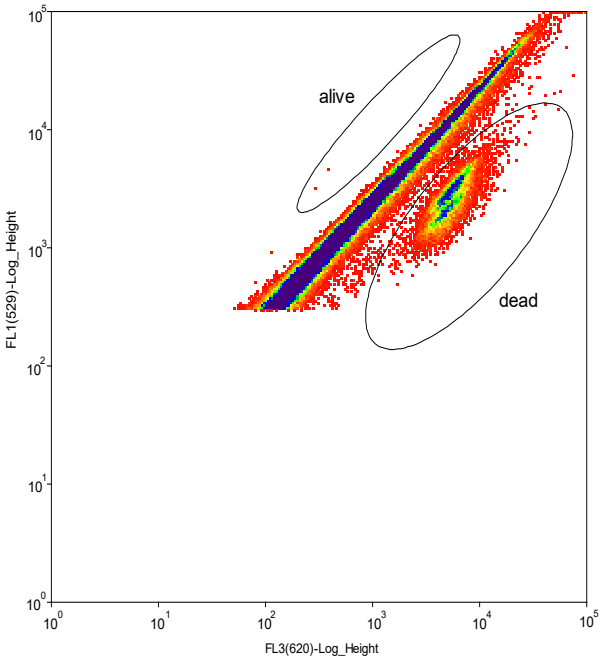
Table B.1 FCM and PCM results for viability of commercial and fresh oatgurt.

Sample	*Week 4*		*Week 8*	
	PCM	***FCM***	***PCM***	***FCM***
PP_{comm}	1.44E+08±4.52 E+07	2.66E+08±7. 7E+06	5.77E+07±6.81 E+06	7.31E+07±1.48 E+07
G_{comm}	1.10E+08±1.37 E+07	1.88E+08±3. 37E+07	5.93E+07±8.14 E+06	5.71E+07±6.48 E+06
PP_{fr}	5.57E+07±1.88 E+07	5.2E+08 ±5.7 E+07	1.43E+07±4.04 E+06	5.98E+07±1.69 E+06
G_{fr}	6.87E+07±5.13 E+06	5.06E+08±6. 7E+07	3.03E+07±7.51 E+06	6.57E+07±8.95 E+06

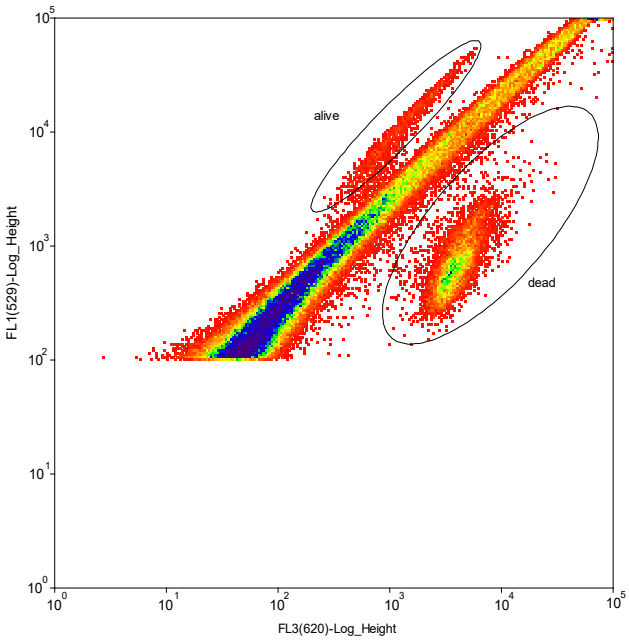
The graphs obtained from FCM demonstrated the events that were detected by the flow cytometer having the green or red fluorescent stains. Figure B.3a is the graph resulting from the unstained oatgurt sample. The background was defined using this sample. Figure B.3b is the control sample without probiotic. The resulting graph represents the dead region. The live region was defined using the probiotic stock solution. Figure B.3c, B.3d, B.3e and B.3f are the results of PPcomm, Gcomm, PP_{fr} and G_{fr} respectively. The coloured dots on the graph represent the detected events (cells). Red colour dot is a single event and as the number of events increase in the same position the increasing intensity is represented by different (colours red<orange<yellow<green<blue). Since it was not possible to analyse week 0 samples, this study using FCM is not complete. However, the results at week 4 and week 8 of the samples are comparable with PCM. This is a good indication of using this technique for quicker results.


alive
dead
FL1(529)-Log_Height
FL3(620)-Log_Height

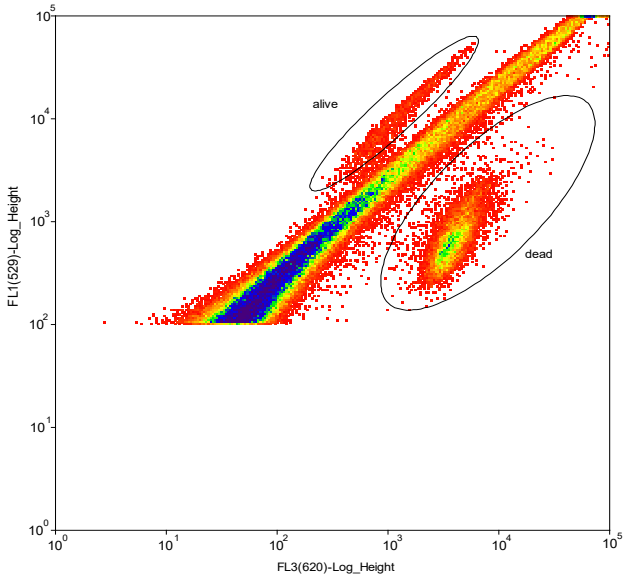
(a)


alive
dead
FL1(529)-Log_Height
FL3(620)-Log_Height

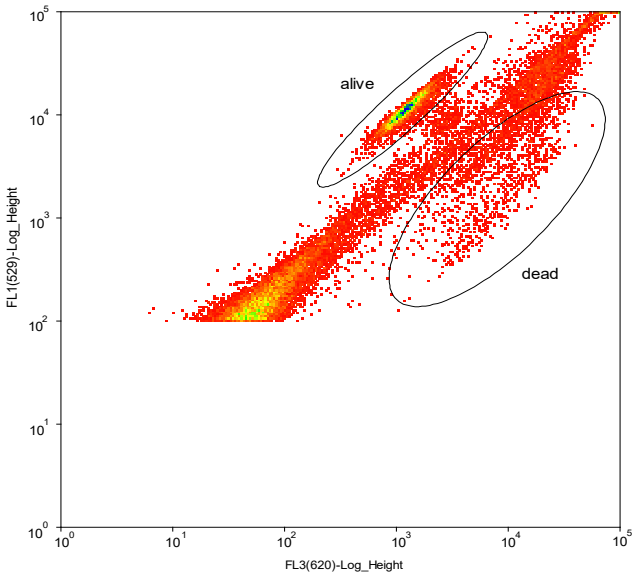
(b)


alive
dead
FL1(529)-Log_Height
FL3(620)-Log_Height

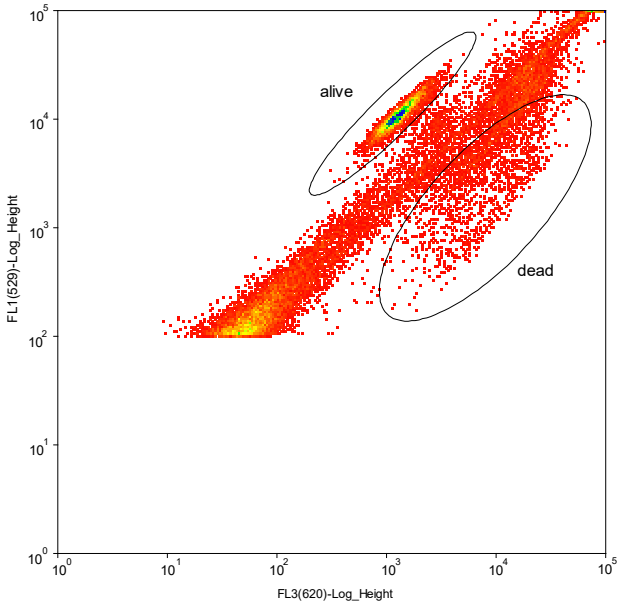
(c)


alive
dead
FL1(529)-Log_Height
FL3(620)-Log_Height

(d)



(e)



(f)

Figure B.3 Viability assessment results using FCM (a) Unstrained control oatgurt (background); (b) stained control oaygurt; (c) PP_{comm}; (d) G_{comm}; (e) PP_{fr} and (f)$G_{fr.}$ The different colours in the graph represent the intensity or number of events that occur in a particular position. The red dots are single events, while it changes to yellow, orange, green and blue with increasing number of events that occur in the same position.