CO-ENCAPSULATION OF OMEGA-3 FATTY ACIDS AND PROBIOTIC BACTERIA THROUGH COMPLEX COACERVATION

By

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ABSTRACT

The research described in this thesis investigated the microencapsulation of omega-3 oil and probiotic bacteria together in a protein-polysaccharide complex coacervate matrix. The synergistic or competitive interactions between the probiotic bacteria and omega-3 fatty acids when packaged in a single microcapsule was determined including how best to utilise such interaction to achieve improved oxidative stability of omega-3 fatty acid and better survival of the probiotic bacteria.

Encapsulation and co-encapsulation of tuna oil (O) and *Lactobacillus casei* 431 (P) as models of omega-3 and probiotic bacteria, respectively, were carried out and the works is described in this thesis in five distinct sections. (1) The optimisation of the complex coacervation process between whey protein isolate (WPI) and gum Arabic (GA). (2) Microencapsulation of tuna oil (O) in WPI-GA complex coacervates followed by spray and freeze drying to produce microcapsules (WPI-O-GA). (3) Microencapsulation of probiotic bacteria *L. casei* 431 (P) in WPI-GA complex coacervates followed by spray and freeze drying to produce microcapsules (WPI-P-GA). (4) Co-encapsulation of omega-3 oil and *L. casei* 431 together in WPI-GA coacervate matrix followed by spray and freeze drying to produce co-microcapsules (WPI-P-O-GA). (5) In-vitro digestion evaluation of co-microcapsules and microcapsules to indicate bioavailability.

The viability of *L. casei* was significantly higher in WPI-P-O-GA co-microcapsules than in WPI-P-GA microcapsules in both spray and freeze dried microcapsules. The oxidative stability of tuna oil was significantly higher in spray dried co-capsules. Also, co-microencapsulation increased the survivability of *L. casei* during simulated digestion. There was no significant influence observed on the release properties of omega-3 oil due to co-microencapsulation. However, the total omega-3 fatty acids in the released oil during in-vitro digestion were found to be higher, when co-microencapsulated. Hence, co-microencapsulation was shown to protect the *L. casei* and deliver both viable cells and omega-3 oil to human intestine without any significant adverse effect on their functionality and properties.

STATEMENT OF AUTHORSHIP

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

During this thesis, four first authored papers were published. These papers form the basis for chapters 3, 4, 5 and 6. Two more manuscript (Chapter 2 and 7) have been submitted and it is currently undergoing peer-review. In all, I designed the research questions and formulated research methodologies in collaboration with my supervisors. I carried out the experiments and measurements and wrote the first draft for each manuscript which was then edited and improved in collaboration with my supervisors as reflected in the authorship.

Signed: _____	Signed: _____
Dated: _____	Dated: _____
Divya Eratte	Professor Kim Dowling
Candidate	Principle Supervisor

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DEDICATION

I dedicate this thesis to my beloved husband and son. Without their patience, understanding and support, the completion of this work would not have been in time.

PUBLICATIONS FROM THIS WORK

Most of the chapters (Chapter 2 to 7) of this thesis have been published or submitted to the peer reviewed publications. These publications including the one which is undergoing peer review process are given below.

Eratte, D., Wang, B., Dowling, K., Barrow, C. J., & Adhikari, B. P. (2014). Complex coacervation with whey protein isolate and gum Arabic for the microencapsulation of omega-3 rich tuna oil. *Food Funct. 5*(11), 2743-2750. (This paper is presented as Chapter 3 in this thesis).

Eratte, D., McKnight, S., Gengenbach, T. R., Dowling, K., Barrow, C. J., & Adhikari, B. P. (2015). Co-encapsulation and characterisation of omega-3 fatty acids and probiotic bacteria in whey protein isolate–gum Arabic complex coacervates. *Journal of Functional Foods*, *19*, 882-892. (This paper is presented as Chapter 4 in this thesis and received Jack Kefford Award 2016 for the best research journal paper in Food Science).

This research work has made news in UK media:

Better together? Co-encapsulation study shows potential for stable omega-3 and probiotic mix.

(http://www.nutraingredients.com/Research/Omega-3-and-probiotic-mixture-may-have-benefits).

Functional formulation: The latest science on nutrient delivery in foods and drinks. (http://www.nutraingredients.com/Research/Functional-formulation-The-latest-science-on-nutrient-delivery-in-foods-and-drinks)

Eratte, D., Gengenbach, T. R., Dowling, K., Barrow, C. J., & Adhikari, B. (2016). Survival, oxidative stability, and surface characteristics of spray dried co-microcapsules containing omega-3 fatty acids and probiotic bacteria. *Drying Technology*, *34*(16), 1926-1935. (This paper is presented as Chapter 5 in this thesis).

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Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. (2017). In-vitro digestion of probiotic bacteria and omega-3 oil co-microencapsulated in whey protein isolate-gum Arabic complex coacervates. *Food Chemistry*, *227*, 129-136. (This paper is presented as Chapter 7 in this thesis).

Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. (2016). Recent advances in the microencapsulation of omega-3 oil and probiotic bacteria through complex coacervation: A review (Submitted to *Trends in Food Science & Technology* and under peer-review). (This paper is presented as Chapter 2 in this thesis).

INTERNATIONAL AND NATIONAL CONFERENCE PROCEEDINGS

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. An innovation in food science: Co-encapsulation of omega-3 oil and probiotic bacteria. Oral presentation at Food, Nutrition & Analytical Chemistry (FNAC) Group Student Symposium, RACI, Melbourne, Australia, 1 September 2016. **(Received Best Oral Presentation Award).**

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. In-vitro digestion of co-microencapsulated probiotic bacteria and omega-3 oil. Oral presentation at Federation University Australia Annual Research Conference, Ballarat, Australia, 21 July 2016. **(Received Best Oral Presentation Award).**

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. Effect of spray drying on the physico-chemical and surface properties of co-microcapsules containing omega-3 oil and probiotic bacteria *L. casei*. Oral presentation at IFT Quality Assurance Division Undergraduate & Graduate Virtual Competition, Chicago, USA, 18 November 2015. **(Received Best Oral Presentation Award).**

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. Omega-3 fatty acids and probiotic bacteria: Icing on the cake. Three minute thesis competition at Federation University Australia, Ballarat, Australia, 23 September 2015. **(Runner-up).**

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. An innovation: Co-encapsulation of omega-3 oil and probiotic bacteria using complex coacervates as shell materials. Malcolm Bird Commemorative Award Oral Presentation at 48th AIFST Annual Convention, Sydney, 11-13 Aug 2015 **(Received Malcolm Bird Commemorative Award 2015).**

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. Co-encapsulated microcapsules containing omega-3 rich tuna oil and probiotic bacteria *L. casei 431* in whey protein isolate- gum Arabic coacervate matrix through spray drying. Poster presentation at 48th AIFST Annual Convention, Sydney, 11-13 Aug 2015 **(Received Best Student Poster Award).**

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. Microencapsulation of *Lactobacillus casei* in whey protein isolate – gum Arabic complex coacervates and their potential applications in food industry. Oral presentation at 48th AIFST Annual Convention, Sydney, 11-13 Aug 2015 **(Received Young Professional in Science Competition Award)**

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. Co-encapsulation of omega-3 fatty acids and probiotic bacteria in whey protein isolate-gum Arabic complex coacervate. Oral presentation at AIFST Summer School, Melbourne, Australia, 28-30 Jan 2015.

- Eratte, D., Wang, B., Dowling, K., Barrow, C. J., & Adhikari, B. P. Stabilisation of omega-3 coacervates microcapsules by different drying methods. Poster presentation at 47th AIFST Annual Convection, Melbourne, 22-25 June, 2014.

- Eratte, D., Wang, B., Dowling, K., Barrow, C. J., & Adhikari, B. P. Comparison of spray and freeze dried tuna microcapsules protected with whey protein isolate-gum Arabic complex coacervate. Oral presentation at 47th AIFST Annual Convection, Melbourne, 22-25 June, 2014. (**Received Young Scientist Australia Talent Award**).

- Eratte, D., Wang, B., Dowling, K., Barrow, C. J., & Adhikari, B. P. Microencapsulation of omega-3 oil using complex coacervate of whey protein isolate-gum Arabic. Oral presentation at AIFST Summer School, Brisbane, Australia, 4- 7 Feb 2014.

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. Development of composite microcapsule consisting omega-3 fatty acids and probiotics in the powdered form through complex coacervation and spray drying- A review. Poster presented at Australian Society for Medical Research (ASMR) National Scientific Conference, Ballarat, 18 November 2013.

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. Complex coacervation of whey protein isolate- gum Arabic. Oral presentation at University of Ballarat Annual Research Conference, Ballarat, 14 November 2013.

- Eratte, D., Dowling, K., Barrow, C. J., & Adhikari, B. P. Formation, stability, characterisation of tuna oil emulsion stabilized by whey protein isolate. Poster presentation at 2 nd International Food Structures, Digestion and Health Conference, Melbourne, Australia, 21-23 October 2013**. (Received Best Poster Award)**

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AV	p-Anisidine value
a_w	Water activity
C	Carbon
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FAO	Food and Agriculture Organization
FD	Freeze dried
GA	Gum Arabic
HPLC	High performance liquid chromatography
L	Lactobacillus
ME	Microencapsulation efficiency.
N	Nitrogen
$\eta_{thermal}$	Thermal efficiency of spray drying process.
O	Oil
OSI	Oxidative stability index
P	Probiotic bacteria
pH	potential of hydrogen
PUFAs	polyunsaturated fatty acids
PV	Peroxide value
SD	Spray dried
SEM	Scanning electron microscopy

SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid.
SO	Surface oil
SSF	Simulated Salivary Fluid
TO	Total oil
TOTOX	Total oxidation value
WHO	World Health Organization
WPI	Whey protein isolate
XRD	X-ray diffraction
ANOVA	Analysis of variance
IEP	Isoelectric point
CLSM	Confocal laser scanning microscopy
MPa	Mega Pascal

Chapter 1

Introduction

1.1. Background

Consumers expect health benefits and disease prevention, beyond basic nutrition, from their diet, and this requires the development of foods with specific physiological function (Hilliam, 1996). Accordingly, functional foods have become an important category within the food industry (Stanton et al., 2001). Novel and innovative nutraceuticals and functional food products are being designed and manufactured to fulfil this growing demand (Earle, 1997; Jones et al., 2007). Two of the most widely used bioactive ingredients used in functional foods due to their unique health benefits are probiotic bacteria (Nazzaro et al., 2012) and omega-3 fatty acids (Verbeke et al., 2005; Verbeke et al., 2009) (Table 1.1). Microencapsulation technology has been successfully used to incorporate these bioactive ingredients in food matrices. Microencapsulation can be defined as the technology of packaging solid, liquid and gaseous materials in small capsules (micro level) that release their content at controlled rates over a prolonged period of time with suitable designs of wall materials and processing conditions.

Probiotic bacteria are a group of bacteria which are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Many studies report on the incorporation of probiotic bacteria in a range of food products (Farnworth et al., 2006). However, a major challenge in relation to the application of probiotic cultures in functional foods is the maintenance of viability during processing and host's natural barrier such as gastrointestinal transit (De Prisco et al., 2016).

Microencapsulation technologies are a promising prospect for introducing viable probiotic bacteria in foods because the encapsulation matrix can provide a certain degree of protection against physical and chemical stressors until it reaches the gastrointestinal tracts (Mattila-Sandholm et al., 2002, Del Piano et al., 2011).

Table 1.1: Health benefits of omega-3 fatty acids and probiotic bacteria

Bioactive ingredients	**Health benefits**	**References**
Omega- 3 fatty acids	Reduce the risk of cardiovascular diseases, decrease the overall mortality rate, minimize mortality owing to myocardial infarction	Bucher et al., 2002; Wen et al., 2013
	Immunomodulatory and anti-inflammatory effect	Wall et al., 2010; Tousoulis et al., 2014
	Reduce the risk of cancer	Rossmeisl et al., 2014; Berquin et al., 2008
	Help brain development and overall function	McNamara et al., 2006;
	Ease depression	Su et al., 2014
	Prevention and curing of Alzheimer's disease	Luchtman et al., 2013
Probiotic bacteria	Improve gastrointestinal health	Gorbach, 2000
	Reduce infection and improve immune response	Nayak, 2010
	Help in the treatment of diarrhoea	Wanke et al., 2013
	Prevention of eczema	Abrahamsson et al., 2007
	Help and improve good functionality of brain	Davari et al., 2013
	Anti-cancer effect	de LeBlanc et al., 2010

Omega-3 fatty acids are defined as polyunsaturated fatty acids (PUFAs) with their first carbon-carbon double bond present at the third carbon from the terminal methyl group and they are considered as essential fatty acids (Shahidi, 2015) and hence it is essential to take omega-3 fatty acids through food (Kris-Etherton et al., 2003). The increased consumption of omega-3 rich foods is hindered by their poor compatibility in water-based products and susceptibility to oxidative degradation due to their polyunsaturated nature, which can lead to off-flavours, off-odours, and loss of bioactivity (Barrow et al., 2007). Therefore, stabilisation of omega-3 rich oils using suitable microencapsulation technology is essential for successful incorporation of omega-3 rich oils in a broad spectrum of food products and beverages (Augustin et al., 2015). An increased trend of enriching food products with omega-3 fatty acids has been observed in the last few decades specifically for food products such as milk, bread, and pasta (Augustin et al., 2015).

Complex coacervation is a liquid-liquid phase separation phenomenon that occurs when electrostatically opposite charged biopolymers are brought together under certain specific conditions. Barrow et al (2007) reported that it is one of the most effective methods of microencapsulating omega-3 fatty acids rich oils, primarily using gelatine as the wall material. Broadening the range of suitable wall material for the microencapsulation is important for expanding the scope and applicability of this important technique. Hence, this thesis explores the process of complex coacervate formation between cationic whey protein isolate and anionic gum Arabic. WPI is prepared from whey which is a by-product of cheese making and it has been widely used in the food processing industry due to its nutritional qualities and unique physio-chemical properties such as emulsion stabilization and gel formation (Adhikari et al., 2007). GA is a complex polysaccharide which shows a

negative charge above pH 2.2 and it is one of the most commonly used stabilizers in the food and pharmaceutical industries (Nie et al., 2013).

A review of current literature suggested that the microencapsulation of omega-3 fatty acids and probiotic bacteria has, so far, been carried out individually (Barrow et al., 2009; Oliveria et al., 2007; Arslan et al., 2015; Gómez-Mascaraque et al., 2016). Co-encapsulation of omega-3 fatty acids and probiotic bacteria in a single matrix has not been described. Halwani et al. (2008) reported that co-encapsulation of more than one core materials in an encapsulation system can enhance the bioactivity of individual components. Co-encapsulation has been widely used in pharmaceutical delivery systems; however, the co-encapsulation of more than one bioactive component with different characteristics such as omega-3 oil (hydrophobic) and probiotic bacteria (hydrophilic) is challenging, particularly using complex coacervation.

Probiotic bacteria and omega-3 oils are known as unstable entities. When probiotic bacteria and omega-3 fatty acids are co-encapsulated in a single product, there may be synergistic and mutual stabilisation effect as well as increased health benefits. Interactions of these two inherently unstable entities when they are packaged together in a single microcapsule have to be studied to a considerable detail. Such interactions and resulting effects on the stability of either one or both entities can depend on the processing parameters against which the capsule formation process have to be optimised. A comparison of efficacy of complex coacervation followed by spray drying and complex coacervation followed by freeze drying must also be systematically assessed as these two drying processes are used industrially to produce food and pharmaceutical powders. This is

essential in quantifying the effect of the drying process on the particle formation process of wet microcapsules to enhance the storage life and to broaden the industrial applicability.

This work aims at investigating the fundamental science underpinning microencapsulation using complex coacervation to optimise the formation of composite microcapsules containing omega-3 fatty acids and probiotic bacteria. This work further aims at investigating the process of formation of solid microcapsule from liquid microcapsule using both spray and freeze drying. The advances made in this study on the fundamentals of synergistic interaction between the probiotic bacteria and the omega-3 fatty acids and the effect of such interactions on the efficacy of microencapsulation (payload, encapsulation efficiency, oxidative stability etc.) will provide the technological basis for food industries to develop and manufacture composite liquid and/or solid microcapsules containing live probiotic bacterial cells and omega-3 fatty acids.

In this context, this project involves investigation of the underpinning science of four research components. The first component involves the microencapsulation of omega-3 rich tuna oil in WPI-GA complex coacervates followed by spray and freeze drying. The second component involves the microencapsulation of probiotic bacteria *Lactobacillus casei (L. casei*) in WPI-GA complex coacervates followed by spray and freeze drying. The third research component involves microencapsulation of the omega-3 rich tuna oil together with probiotic bacteria in a single WPI-GA complex coacervate microcapsule followed by spray and freeze drying. The release and digestion of the co-encapsulated tuna oil and survival of probiotic bacteria in simulated intestinal digestion are studied in the final section of this research.

1.2. Research Hypotheses

The research hypotheses that underlines this thesis is as follow: "Synergistic interaction occurs between the probiotic bacteria and omega-3 fatty acids in a single microcapsule to achieve better stability of omega-3 fatty acids and better survival of the probiotic bacteria."

This thesis addresses the following research questions to test the research hypotheses systematically: (1) How can the process parameters be optimised to produce WPI-GA complex coacervates? (2) What is the best way to encapsulate omega-3 fatty acids in WPI-GA complex coacervate? (3) How can the probiotic bacteria and omega-3 fatty acids be best co-encapsulated in WPI-GA complex coacervate? (4) Finally, can multicore complex coacervates be used to better microencapsulate and stabilise probiotic bacteria and omega-3 fatty acids in a single complex coacervate until it reaches the human gastrointestinal tracts?

1.3. Research Objectives

This work involves understanding the synergistic or competitive interactions between probiotic bacteria and omega-3 fatty acids when packaged in a single microcapsule and how best to utilise such interaction to achieve improved stability of omega-3 fatty acid and longer survival of the probiotic bacteria.

The specific objectives of the project are:

1. To encapsulate omega-3 fatty acids in whey protein isolate-gum Arabic complex coacervate including developing the optimal complex coacervation process parameters.
2. To encapsulate probiotic bacteria in whey protein isolate-gum Arabic complex coacervate including optimising the encapsulation process parameters.

3. To encapsulate omega-3 fatty acids and probiotic bacteria in a single (complex coacervate) microcapsule including optimising the encapsulation process parameters and determining stability of oil and survival of probiotic bacteria during storage.

4. To conduct the comparative in vitro digestion studies on all the above microcapsules.

1.4. Outline of Thesis

This thesis is organized into eight chapters as outlined below. Six chapters (Chapter 2 to Chapter 7) have been peer reviewed as research manuscripts. The research described in Chapter 3 to Chapter 6 has been published while the contents compiled in Chapter 2 and Chapter 7 are currently undergoing peer review as manuscripts (Table 1.2).

Chapter 1: This chapter provides the background of the thesis and presents the updated status of science and gaps in knowledge in the area covered by the thesis. The rationale behind the research theme and the research questions which this thesis addresses are also documented. The research hypotheses, objectives of this project and approaches to the work are documented in this chapter.

Chapter 2: This chapter critically reviews the literature relevant to the thesis and covers fundamental concepts of complex coacervation process. It also covers microencapsulation and characterization of the individual ingredients, omega-3 fatty acids and probiotic bacteria, through complex coacervation. The bioavailability of these bioactive ingredients from the microcapsules has also been reviewed. Furthermore, recent advances on the co-encapsulation of different bioactive ingredients and current technological hurdles associated with this technology are also reviewed.

Chapter 3: This chapter documents the optimisation of the complex coacervation process between WPI and GA. The electrostatic complexation between two biopolymers as a function of different WPI to GA ratios, pH and morphology, was studied using turbidometric, zetapotential and confocal measurements. The microencapsulation of omega-3 oil was carried out with optimised WPI-GA complex coacervates. The two drying processes freeze drying and spray drying were used to produce powdered microcapsules. Microcapsules formed though there two processes were compared for surface oil, total oil, microencapsulation efficiency and oxidative stability.

Chapter 4: This chapter documents the detailed protocol for the microencapsulation of probiotic bacteria *L. casei* in WPI-GA complex coacervates as well as the co-encapsulation of omega-3 oil and probiotic bacteria in WPI-GA complex coacervates. The two drying processes freeze drying and spray drying were used to produce powdered microcapsules. The physico-chemical and surface characteristics of microcapsules were studied.

Chapter 5: Optimisation of spray drying to determine the best inlet and outlet air temperatures to produce co-microcapsules containing omega-3 oil and probiotic bacteria were investigated. Three sets of inlet and outlet air temperatures were used in nine combinations to produce powdered co-microcapsule. The viability of probiotic bacteria (*L. casei*), oxidative stability of omega-3 oil, surface oil, oil microencapsulation efficiency, surface elemental composition and morphology of the powdered samples were investigated and the results described in this chapter.

Chapter 6: The main hypothesis of this thesis has been tested in this chapter. The interaction between probiotic bacteria and omega-3 oil in co-microcapsules, particularly in terms of oxidative stability of omega-3 oil and vitality/viability of probiotic bacteria and any synergistic outcome, were measured and possible mechanisms for synergy are

discussed. The effect of storage temperature and time on the survival and fermentation activity of *L. casei* and oxidative stability of tuna oil in the microcapsules/co-microcapsules were determined.

Chapter 7: The release behaviour of co-microencapsulated omega-3 oil and viability of co-microencapsulated probiotic bacteria were studied to understand the applicability of complex coacervation-based co-capsules as a controlled release delivery system. The *in-vitro* digestibility of co-microcapsules (WPI-P-O-GA) and microcapsules (WPI-P-GA and WPI-O-GA) on sequential exposure to simulated salivary, gastric and intestinal fluids were examined.

Chapter 8: In this chapter, key conclusions drawn from current study are summarised. This chapter details the contributions made by this study to the field of microencapsulation and the advancement made in the co-encapsulation concept in food science. This chapter also provides future research directions based on the findings obtained and experience gained in this study.

Table 1.2: Publication summary of chapters

Chapter number	Title of the paper	Journal	Status	Contribution of candidate (%)
2	Recent advances in the microencapsulation of omega-3 oil and probiotic bacteria through complex coacervation: A review	Trends in food science and technology	Submitted	80
3	Complex coacervation with whey protein isolate and gum Arabic for the microencapsulation of omega-3 rich tuna oil	Food and Function	Published	80
4	Co-encapsulation and characterization of omega-3 fatty acids and probiotic bacteria in whey protein isolate-gum Arabic complex coacervates	Journal of Functional Foods	Published	80
5	Survival, oxidative stability and surface characteristics of spray dried co-microcapsules containing omega-3 fatty acids and probiotic bacteria	Drying Technology	Published	80
6	Survival and fermentation activity of probiotic bacteria and oxidative stability of omega-3 oil in co-microcapsules during storage	Journal of Functional Foods	Published	80
7	In-vitro digestion of co-microencapsulated probiotic bacteria and omega-3 oil in whey protein isolate-gum Arabic complex coacervates	Food Chemistry	Published	80

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Chapter 2

Recent advances in the microencapsulation of omega-3 oil and probiotic bacteria through complex coacervation: A review

Abstract

Background: Functional foods are one of the fastest growing sectors of the food industry. The development of functional foods comprising omega-3 fatty acids and probiotic bacteria, through complex coacervation process is an emerging area of research and product development.

Scope and approach: We reviewed relevant literature concerning the use of complex coacervation in microencapsulation, focusing primarily on the inclusion of probiotic bacteria and omega-3 oils into a single delivery format. This review covers advantages and disadvantages of the complex coacervation process to microencapsulate bioactive ingredients, viability of probiotic bacteria and oxidative stability of omega-3 oil during the complex coacervation process, the bioaccessibility of omega-3 oil and probiotic bacteria during simulated gastrointestinal conditions and in-vivo testings.

Key results and conclusions: The review describes the advantages of co-encapsulation using complex coacervation followed by spray drying. It also describes the technological hurdles that need to be resolved for further development of industrial applications of co-microencapsulation of probiotic bacteria and omega-3 lipids. The co-encapsulation concept has been widely used in pharmaceutical delivery systems, but is a relatively new concept in food ingredient stabilisation and delivery.

Key words: Functional foods, co-microencapsulation, omega-3 oil, probiotic bacteria, complex coacervation

2.1. Introduction

There has been a substantial increase in the demand for delivering bioactive ingredients through everyday foods over the last few decades, particularly due to the increased awareness of healthy life and the impact of food on health (Goldberg, 2012). Consequently, the functional food industry has become a rapidly growing sector of the food industry (Pulz et al., 2004). Novel and innovative nutraceuticals and functional food products are being designed and manufactured by incorporating different bioactive ingredients to fulfil growing consumer demand (Neethirajan et al., 2011).

The industrial production of functional foods requires the addition of stable bioactive ingredients (Champagne et al., 2007). Incorporation of bioactive ingredients to produce functional foods presents numerous challenges, particularly with respect to the stability of the bioactive compounds during processing and storage. It is also essential to prevent undesirable interactions with the carrier food matrix and to ensure their stability without losing functionality until they reach the human gastrointestinal tract and finally release the bioactive ingredients to the targeted site (Champagne et al., 2007). Microencapsulation of bioactive ingredients could address some of these requirements (Desai et al., 2005).

Microencapsulation technology has been defined as the technology of packaging solid, liquid and gaseous materials in small capsules that release their contents at controlled rates over prolonged periods of time (Champagne et al., 2007). Microencapsulation technology is commonly used in pharmaceutical and food industries (Mastromatteo et al., 2010). Shahidi et al (1993) proposed six significant reasons for applying microencapsulation in

food industry: to decrease any undesired reaction of core material with environmental factors such as heat, moisture, air, and light; to reduce or prevent the loss of the core material to outside the environment; to promote easier handling; to minimize or prevent the release of the core material until encountering the right stimulus; to mask the taste and odour of the core; and finally to dilute the core material when it is intended to use in very small amounts and to separate components in a mixture that would otherwise react with each another.

According to Gouin (2004), microencapsulation technology is not only used to produce value added food products but also used to produce entirely new and stable functional food components with desirable properties. This technology helps imparting improved controlled release properties. The applicability of a microencapsulation process in the food industry depends on the cost of the product given the pressure to keep the price low without compromising its health benefits (Gouin, 2004; Desai et al., 2005). Therefore, the factors that needed to be considered in the design of any functional food ingredient through microencapsulation in industrial scale are the cost of production, applicability in a wide range of products and ease of scale-up.

Two of the leading bioactive ingredients used in functional food products are probiotic bacteria (de Pinho Ferreira Guine et al., 2012) and omega-3 fatty acids (Kim et al., 2006). The functional foods developed from probiotic bacteria and omega-3 fatty acids have proven health benefits (Nazzaro et al., 2012; Wildman et al., 2016). Over the last few decades, a large number of food products have been fortified with probiotic bacteria and omega-3 oil, as illustrated in Table 2.1

Table 2.1: Food products fortified with omega-3 fatty acids and probiotic bacteria

Bioactive ingredients	Source of bioactive ingredient	Food category	Commercial product name	Manufacturer/Country
Probiotic bacteria	*L. casei immunitas* (DN-114001)	Yogurt	DanActiva	Dannon (France)
	L. ramnosus GG (ATCC 53103)	Infant formula	Mead Johnson Nutrition (Enfamil Nutramigen)	Valio (Finland)
	Lactobacillus plantarum (LP299V®)	Juice drink	GoodBelly probiotic juice drink, Probi's probiotic drink	GoodBelly (USA)
	L. acidophilus NCFM (ATCC SD5221)	Infant formula	Heinz Nuture Toddler	DuPont Danisco
	Danisco probiotic strains-*Howaru Dophilus* and *Howaru Bifidioacidophilus*	Chocolate	Attune Foods Probiotic Bar	Attunefoods (USA)
	Lactobacillus casei Shirota	Fermented milk drink	Yakult	Yakult (Japan)
	Bifidobacteria	Baby formula	Good Start Natural Cultures	Nestle (Canada)
	S. salivarius BLIS K12	Chewing gum	Probioticgum	CulturedCare (Canada)
	Bifidobacterium longum BB536	Milk	Caldus milk	Morinaga Milk Industry Co. Ltd. (Japan)
	Lactobacillus plantarum (LP299V®)	Fruit drink	ProViva	Dannon (France)
Omega-3 fatty acids	_	Bakery products	F Plus fortified biscuits	Cuetara (Spain)
	Tuna oil	Bread	Tip Top® The One	Coles (Australia)
	_	Eggs	DHA Gold eggs	Omega Tech (USA)
	_	Spread	Blue Gaio	Arla (Denmark)
	Menhaden oil	Buttery spread	SmartBalance OmegaPlus	GFA Brands Ltd.(USA)
	Flax seed oil	Dairy	Fortified skimmed milk	Natrel (Canada)

Probiotic bacteria are a group of bacteria which are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). Some of the health benefits associated with probiotic bacteria are alleviation of unwanted gastric and abdominal symptoms (Gomi et al., 2015), an improved immune response (Bogsan et al., 2014), reduction of total cholesterol (Ejtahed et al., 2011), and enhancement of intestinal microbiota (Wang et al., 2012). Probiotic bacteria require protection from metabolic inactivation due to their sensitivity to environmental factors such as heat, oxygen and humidity (Peighambardoust et al., 2011). There are several criteria that probiotic bacteria must satisfy, if they are to contribute in a positive way to human health, including their viability during manufacturing, storage and delivery. It is essential that the probiotic bacteria survive during the passage through the upper gastrointestinal tract in order to preserve their ability to function in the gut environment (De Prisco et al., 2016). Microencapsulation improves survivability of probiotic bacteria during processing as well as during the passage through the gastrointestinal tract (Champagne et al., 2007).

Omega-3 fatty acids provide well established and clinically proven health benefits to humans (Surette, 2008). Omega-3 fatty acids are defined as polyunsaturated fatty acids (PUFAs) with their first carbon-carbon double bond present at the third carbon from the terminal methyl group (Qi et al., 2004). The main bioactive omega-3 fatty acids are cis-5,8,11,15,17-eicosapentaenoic acid (EPA) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), as shown in Fig. 2.1. They are considered physiologically essential fatty acids and have numerous physiological roles (Barrow et al., 2013). Omega-3 fatty acids are recommended for decreasing the risks of cardiovascular disorders, Alzheimer's disease,

immune response disorders, diabetes and cancer (Luchtman et al., 2013; Wen et al., 2014; McNamara et al., 2006; Rossmeisl et al., 2014). There is a high commercial need for omega-3 fatty acids; for example, the estimated global demand for omega-3 ingredients was worth USD 1595 million in 2010 and is estimated to exceed USD 4000 million in 2018 (Transparency Market Research, 2012). Fish oil usually contains a higher proportion of omega-3 fatty acids than seed oils and algal oil (Rubio-Rodríguez et al., 2010). However, oil containing high amount of omega-3 fatty acids are highly sensitive to oxidation due to their polyunsaturated nature, which not only results into loss of nutritive value but also develops off-flavours (Karlsdottir et al., 2014). When encapsulated, the omega-3 fatty acids are more stable in food matrix (Smith et al., 2015).

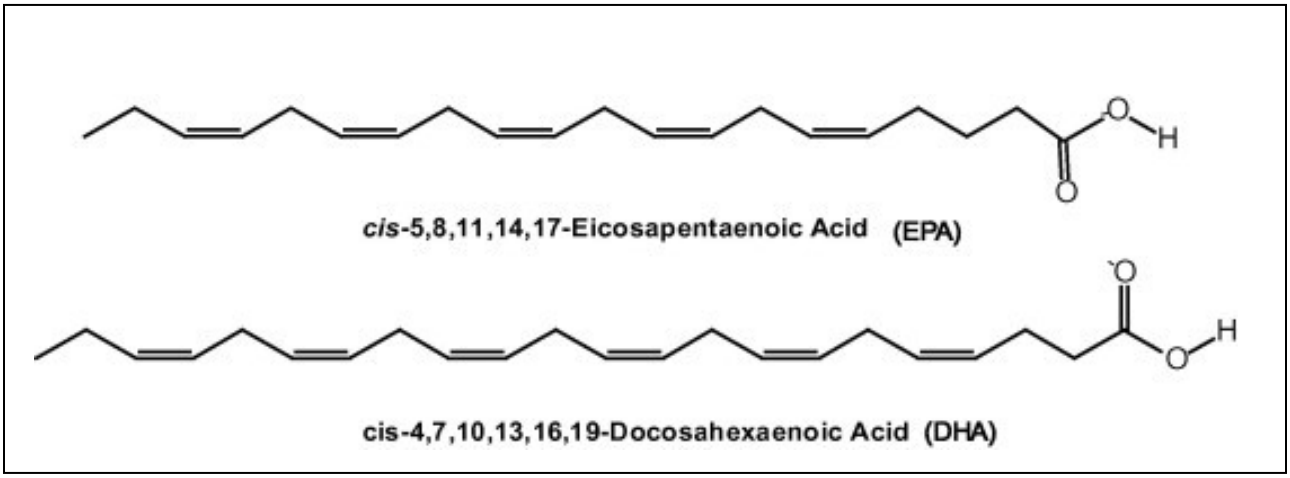

Fig. 2.1: Chemical structure of EPA and DHA. (Adapted from Kralovec et al., 2012)

There are several microencapsulation methods that have been used or at least studied for microencapsulation of omega-3 oil and probiotic bacteria. These methods include complex coacervation (Barrow et al., 2009, Liu et al., 2010, Oliveria et al., 2007 and Conto et al., 2013), spray drying (Quispe-Condori et al., 2011; Arslan et al., 2015), emulsion followed by spray granulation, fluidized bed drying, freeze drying (Anwar et al., 2011),

electrospraying (Coghetto et al., 2016; Gómez-Mascaraque et al., 2016). This review focuses on complex coacervation microencapsulation techniques as applied to encapsulate omega-3 oil and probiotic bacteria.

This review considers the emerging field of co-encapsulation in the food industry. To date, published reviews address microencapsulation of either probiotic bacteria or omega-3 fatty acids. Within this review, the complex coacervation method of microencapsulation will be discussed followed by an overview of research works reported on microencapsulation of omega-3 oil, probiotic bacteria and their co-encapsulation using complex coacervation. A brief overview of the digestion of probiotic cells and omega-3 fatty acids in the gastro-intestine system in-vitro and in-vivo models will then be presented. To close, future trends in the microencapsulation field will be discussed with reference to implication for the food industry.

2.2. The complex coacervation process of microencapsulation

A brief overview of complex coacervation process of microencapsulation is presented in this section. The electrostatic interaction between oppositely charged biopolymers results into the formation of soluble complexes, which further aggregate to decrease the free energy of the system until their size and surface properties render them insoluble. Subsequently, a liquid–liquid phase separation occurs which is known as complex coacervation (Schmitt et al., 2011). De Kruif et al. (2004) defined complex coacervation as a liquid–liquid phase separation phenomenon that occurs when electrostatically opposite charged biopolymers (protein/polysaccharides) are brought together under certain specific conditions. Some of the important and specific conditions influencing this process are pH,

ionic strength, polymers concentration, ratio of biopolymers, molecular weight of biopolymers, temperature, and the degree of homogenization (Fig. 2.2). Many authors have endeavoured to optimize the complex coacervation parameters between protein-polysaccharide pairs (Eratte et al., 2015, Kaushik et al., 2016; Timilsena et al., 2016; Wang et al., 2013; Schmitt et al., 2011).

There has been strong interest over the last thirty years to develop different protein-polysaccharide complex coacervates to microencapsulate unstable bioactive ingredients for industrial applications (Turgeon et al., 2007). The microcapsules that use complex coacervates as shell material generally have diameters ranging from nanometres to few millimetres. Depending on the size of the capsules, they can be classified as microcapsules (micron size) and nanocapsules (submicron size). Typical particle diameters of microcapsules are in the range of 1 μm to 1000 μm; whereas nanocapsules typically have diameters < 0.2 μm. Desired properties for these biopolymeric complex coacervate based capsules are biocompatibility, nontoxicity and biodegradability and controlled release capability (Kaushik et al., 2015). Furthermore, complex coacervation is a well-adapted and benign method since neither any organic solvents nor extreme reaction conditions are required. Frequently used proteins to produce complex coacervates are gelatine, albumin, whey protein, beta-lactoglobulin and various plant proteins. Common polysaccharides are gum Arabic, chitosan, pectin, alginates, xanthan gum, carrageenan, and carboxymethyl cellulose. Recently, plant gums such as flaxseed gum (Kaushik et al., 2016) and chia seed gum (Timilsena et al., 2016) have also been used to successfully encapsulate flaxseed oil and chia seed oil, respectively.

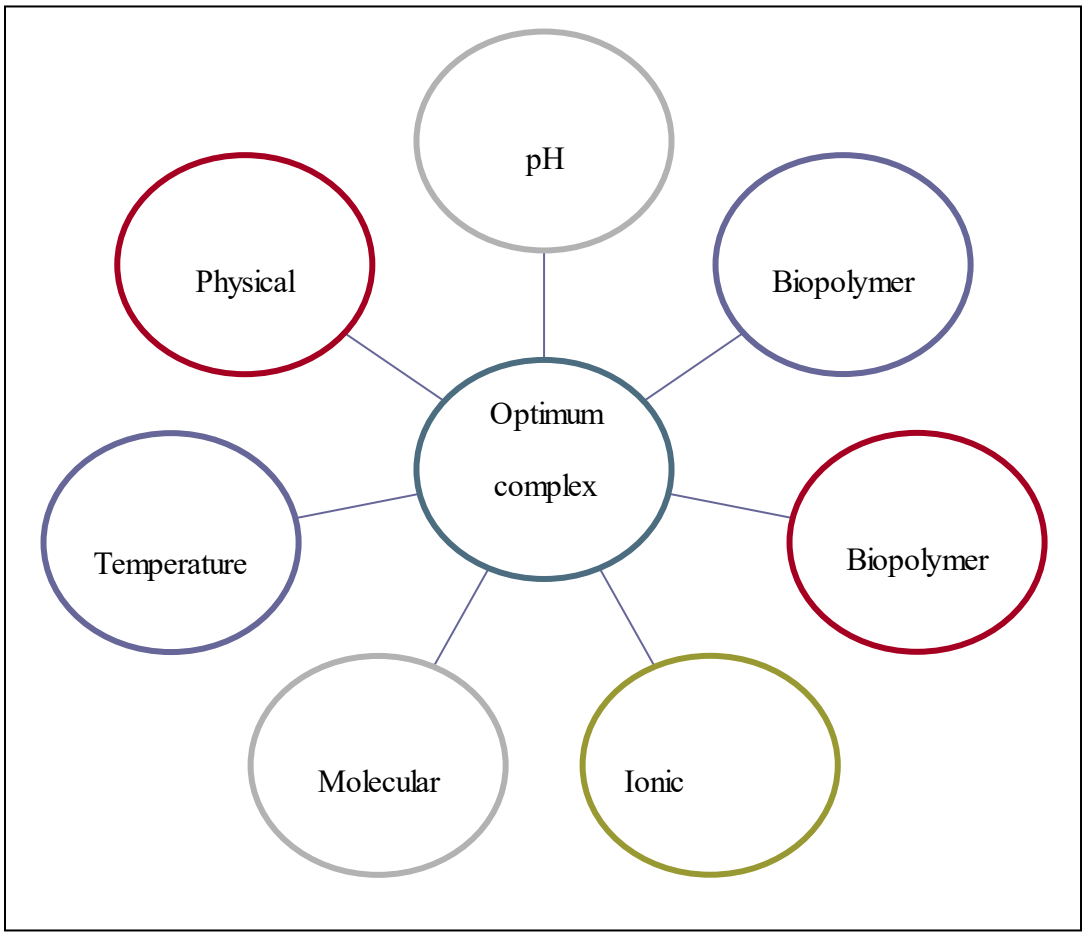

Fig. 2.2: Factors affecting the protein-polysaccharide complex coacervation process

2.3. Microencapsulation of omega-3 oil through complex coacervation

Microencapsulation of omega-3 oil through complex coacervation presents advantages and disadvantages of this technology for the food industry. In-vitro and in-vivo digestion studies on microencapsulated omega-3 oil must also be considered to determine the appropriateness of this methodology.

2.3.1. Complex coacervation to microencapsulate omega-3 oil

Omega-3 polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have clinically proven health benefits (Simopoulos, 1991).

However, the daily consumption of these fatty acids is generally too low, a fact attributed for the risk of some chronic diseases in humans (Kaushik et al., 2015). Hence, increased intake of omega-3 fatty acids is highly recommended, particularly in Western countries with a low intake. An approach to increasing consumption, without making major changes to eating habits, is to enrich frequently consumed and common food products with omega-3 fatty acids. However, omega-3 oils are very unstable due to their polyunsaturated nature and become rapidly oxidized leading to the formation of primary and secondary oxidation products such as peroxides, aldehydes and ketones. These oxidation products not only cause unpleasant odour and taste but also degrade the functional values of the omega-3 oils (Decker et al., 2010). The use of antioxidants can delay lipid oxidation (Logani et al., 1980). However, the availability of food grade antioxidants is quite limited. For example, cinnamic and benzoic acid derivatives, oregano, rosemary, parsley, flavonoids and phenolic compounds have been used to increase the stability of omega-3 oils against oxidation (Espinosa et al., 2015). However, the use of antioxidants alone is generally not adequate to enable sensory stabilisation of these oils for addition to many foods and beverages (Kralovec et al., 2012). Moreover, the antioxidant mechanisms in multi-component and multiphase food systems are complex and hence it is difficult to predict the behaviour and efficacy of an antioxidant in preventing lipid oxidation in food materials (Jacobsen et al., 2008). Therefore, the application of emulsifiers (proteins or/and polysaccharides) in combination with antioxidants has been studied to increase the stability of omega-3 oils (Baik et al., 2004). Microencapsulation of omega-3 oils using emulsification followed by spray drying has been successfully used to increase the shelf-life of this type of ingredients (Barrow et al., 2007). It has been reported that this technology can be successfully used in large variety of foods such as infant formulas and

bread mixes (Schrooyenet al., 2001), cream filled sandwich cookies (Borneo et al., 2007), and instant foods such as soups, cocoa drinks, potato puree, milk products etc. (Kolanowski et al., 2007).

Barrow et al. (2007) reported that complex coacervation is one of the most effective methods for microencapsulating omega-3 fatty acids and delivering them into foods. The complex coacervates using gelatine-gum Arabic to microencapsulate omega-3 oil has been commercialised by Ocean Nutrition Canada (Barrow et al., 2007). The complex coacervation-based microencapsulation produces agglomerates/assembles of micron sized single cores by primary (inner) as well as secondary (outer) shells and impart greater mechanical and oxidative stability to omega-3 oils (Barrow et al., 2009; Wang et al., 2014; Eratte et al., 2014). The complex coacervation-based oil microencapsulation process generally involves three key steps (Wang et al., 2014). The first step involves preparation of a stable oil-in-water emulsion. The second step is the complex coacervation step in which the emulsified oil droplets are coated by protein-polysaccharide complex coacervate layer. This step involves optimization of pH, protein-to-polysaccharide ratio and the size of the oil droplets. The final step usually involves introducing crosslinkers and lowering of temperature so that a robust primary and secondary shells are formed and aggregates of fully coated oils droplets are produced. Enzymatic or chemical crosslinking of the shell matrix is carried out and the microcapsules are hardened in this step so that the shell material redissolve easily with mechanical forces. Microcapsules formed by this method have shown excellent controlled release characteristics (Jun-xia et al., 2011).

The liquid microcapsule obtained from the complex coacervation process is dried using suitable drying methods such as spray drying or freeze drying. However, this step of the process is to remove the water and not to form the microcapsule. This is a major difference between microencapsulation using spray drying and microencapsulation by complex coacervation followed by spray drying. In complex coacervation, the size and type of particle formed is controlled during the wet process and not by the drying process. Microcapsules produced through complex coacervation followed by spray drying or freeze drying possess higher payload and improved stability compared with spray dried or freeze dried microcapsules prepared through simple emulsion (Barrow et al., 2007).

Table 2.2 summaries work to date on the microencapsulation of omega-3 oil using complex coacervation followed by drying. Gelatine-gum Arabic is the most commonly used protein-polysaccharide combination in the complex coacervation process. The variation of charge density of gelatin with pH and its unique gelling behavior makes it particularly suitable for complex coacervation process compared to other proteins. Moreover, the coiled structure of gelatin maintains sufficient charge density on its chains to avoid precipitation irrespective of the anionic compound used. On the other hand, gum Arabic also has a coiled configuration which preserves the charges and allows considerable quantity of water to be occluded between the chains to avoid precipitation (Ducel et al., 2004). The food industry sees the development of new protein-polysaccharide complex coacervates as shell materials that does not involve gelatine as desirable. For this, the complex coacervation process of each protein-polysaccharide combinations has to be optimized. Interestingly, many studies have characterised the physiochemical properties of

complex coacervates; however, their performance in food formulations have received very little attention (Table 2).

Table 2.2: Complex coacervation to microencapsulate omega-3 oil.

Omega-3 oil	**Wall materials**	**Drying techniques**	**Oxidative stability**	**Incorporation into foods**	**References**
Eicosapentaenoic acid ethyl ester	Gelatine and acacia	Spray drying	Increased	No	Lamprecht et al., 2001
Fish oil	Gelatine and polyphosphate	Spray drying	Not tested	No	Barrow et al., 2009
Omega-3 marine lipids	Whey protein isolate and gum Arabic	Not applied	Not tested	No	Zhang et al., 2009
Flax seed oil	Gelatine and gum Arabic	Freeze drying	Increased	No	Liu et al., 2010
Microalgal oil	Gelatine and gum Arabic	Oven dried	Not tested	No	Zhang et al., 2012
Fish oil ethyl ester	Soy protein isolate and gum Arabic	Freeze drying	Increased	No	de Conto et al., 2013
Cod liver oil	Sodium caseinate and gum Arabic	Freeze drying	Freeze drying	Commercial mixed fruit juice	Ilyasoglu et al., 2014
Tuna oil	Gelatine and sodium hexametaphosphate	Freeze drying	Increased	No	Wang et al., 2014
Tuna oil	Whey protein isolate and gum Arabic	Freeze drying and spray drying	Increased	No	Eratte et al., 2014
Linseed oil	Gelatine and gum Arabic	Not dried	Not tested	No	Ach et al., 2015
Stearidonic acid soybean oil	Gelatine and gum Arabic	Freeze drying	Increased	No	Ifeduba et al., 2016
Flax seed oil	Flax seed protein and gum	Spray drying and freeze drying	Increased	No	Kaushik et al., 2016
Chia seed oil	Chia seed protein and gum	Spray drying and freeze drying	Increased	No	Yakindra et al., 2016

Astaxanthin-containing lipid extract from shrimp waste	Gelatine and cashew gum	Freeze drying	Increased	Plain yogurt	Gomez-Estaca et al., 2016

Compared to conventional microencapsulation methods such as spray drying of oil-in-water emulsion, this "multicore" complex coacervation process achieves greater than 50% payload. This process also produces a thicker outer shell compared to the emulsion method thereby decreasing the surface oil and improving the oxidative stability of omega-3 oil. The complex coacervate-based shells provide microcapsules with good sensory properties and storage stability (Barrow et al., 2007; Wang et al., 2014). This process helps the production of microcapsules in the desired size range (1 to 1000μm) and successfully prevents the migration of oil to the particle surface and gives greater protection to the encapsulated omega-3 oil (Kaushik et al., 2014). The higher payload in microcapsules obtained by complex coacervation followed by spray drying makes it more cost effective for delivering of omega-3 oils (Kralovec et al., 2012).

There are few limitations with the complex coacervation technology. The protein-polysaccharides complex coacervates formed are stable on a very narrow range of pH, ionic strength and temperature. Therefore, reaching the correct endpoint and stabilizing the coacervate formed before cross-linking requires careful monitoring of the process (Zhang et al., 2009; Kaushik et al., 2014). As stated above, gelatine is ubiquitously used by industry as wall material to produce complex coacervates. This protein is not suitable for the vegetarian population and has poor sensory properties in some food applications (Kralovec et al., 2012). Alternative shell materials are required for use in the complex

coacervation process; however, the development of alternative protein-polysaccharide complex coacervates requires rigorous optimisation (De Kruif et al., 2004).

2.3.2. Bioaccessibility of EPA and DHA from omega-3 microcapsules

Bioaccessibility has been defined as the fraction of an ingested nutrient that is released from the food matrix and is available for absorption in the gut after digestion (Ilyasoglu et al., 2014). The relationships or links between food properties, human digestion processes, and final health outcomes have to be established to design a successful functional food. The initial assessment of any new microencapsulated product to test these relationships is to use in-vitro tests. This is because the in-vitro test incurs less cost, is rapid and avoids the need for ethics approval (Polli, 2008; Minekus et al., 2014). These in-vitro studies enable the food industry to design foods to modulate lipid digestion and absorption within the human gastrointestinal tract using simple simulated tests (Zhang et al., 2015). Therefore, it is useful to test the stability/survival and release behaviour of omega-3 and probiotic from these microcapsules during salivary and gastrointestinal transit prior to proceeding to full-fledge in-vivo trials.

The digestion and absorption of dietary omega-3 lipids in the human gastrointestinal tract are complex processes involving numerous physicochemical and biochemical events (Fave et al., 2004). Extensive reviews on fat digestion in gastrointestinal tracts are available (Embleton et al., 2007; Singh et al., 2009). In humans, the first step of fat digestion takes place in the stomach. Gastric lipases secreted in the gastric juice hydrolyse oils under acidic conditions (Ilyasoglu et al., 2014). Subsequently, the digestion of fat is completed in the small intestine, where absorption of lipolytic products occurs. The digestion of encapsulated oils is activated after adsorption of lipase to the oil–water interface of

emulsified oil droplets (Ilyasoglu et al., 2014). It has been reported that the digestion of oil-in-water emulsions is influenced by several factors: (a) size of the oil droplet; (b) the nature of the interfacial layer (Mun et al., 2007); (c) the physical state of the lipid; (d) the presence of other ingredients in the emulsion systems such as fibers and polysaccharides; and (e) the composition of digestion fluids (Chung et al., 2011). A substantial body of literature exists on various aspects of in-vitro digestion studies omega-3 oil emulsions (Klinkesorn et al., 2009; Farvin et al., 2010; Christophersen et al., 2016); however, only limited information exists on the in-vitro evaluation of oil release from the solid microencapsulated omega-3 oil. Ilyasoglu et al. (2014) reported the bioaccessibility of EPA, DHA and EPA + DHA as 56.16, 36.25 and 47.37 percent, respectively, from solid microencapsulated cod liver oil in sodium caseinate-gum Arabic complex coacervates. The authors suggested three main reasons for this low bioaccessibility values. Firstly, the lamination of fish oil with two interfacial complex coacervate layers might have led to a decrease in lipid digestibility. Secondly, the protein moiety of gum Arabic can strongly interact with the oil-water interface, possibly blocking the available interface thereby preventing the release of the non-esterified fatty acids generated at the droplet surface. Finally, some of the fish oil could reach the colon without lipolysis. It has been reported by Shen et al. (2011) that lipolysis of microencapsulated omega-3 oil powder during in-vitro digestion can be affected by the food matrix, its structure (e.g., solid versus liquid), and also by its composition. For example, after sequential exposure to simulated gastric and intestinal fluids, much higher extents of lipolysis of both EPA and DHA were obtained. For example, 73.2-78.6% of EPA and DHA were lipolyzed in neat microencapsulated tuna oil powder, fortified orange juice, and yogurt and 60.3- 64.0% were in the fortified cereal bar. The authors concluded that fortified cereal bar contained the highest content of fat and

polysaccharide (e.g., dietary fibers) and had the largest relative oil droplet size exposed to simulated intestinal fluids and thus having the least bioaccessibility of EPA and DHA. The complexity of all the above factors are the technological hurdles that need to be resolved for the introduction of omega-3 oil in wide range of daily life food matrices.

The microencapsulated bioactive ingredients will be beneficial, only if these are bioavailable and hence, testing the bioequivalence of encapsulated bioactive ingredients from microcapsules by conducting in-vivo studies are of prime importance. Barrow et al. (2007) reported a comparative human bioavailability study of microencapsulated omega-3 fish oil and standard fish-oil soft-gel capsules and concluded that the fish oil microencapsulated using complex coacervation is bioavailable and bioequivalent to dietary supplementation with soft-gel capsules. On the contrary, Raatz et al. (2009) reported that there was increased absorption of omega-3 fatty acids, EPA and DHA and a reduction in the omega-6/omega-3 fatty acid ratio as evidenced by changes in plasma phospholipid fatty acids (PLFA) composition from a preparation of emulsified fish oil compared to the fish oil capsules as well as parent oil in human trials. Arterburn et al. (2007) reported that two different algal oil capsule supplements and an algal oil-fortified snack bar represent bioequivalent and safe sources of DHA in human trial. Patten et al., (2009) reported that the nature of the protein–carbohydrate encapsulation matrix used to prepare spray-dried fish oil powders influenced the digestibility of the encapsulated oil in the gastrointestinal tract of rats.

A detailed understanding of the digestion of long chain polyunsaturated omega-3 fatty acids in different microencapsulated oil formulations and food matrices during in-vitro and

in-vivo digestion is lacking. Overall, research into factors that determine the choice of functional food is limited, and no studies to date have investigated the specific factors affecting the use of foods enriched with omega-3 fatty acids thorough clinical human studies to support the health claims, which are some of the spaces for future studies.

2.4. Microencapsulation of probiotic bacteria through complex coacervation

In this section we discuss the necessity for encapsulating probiotic bacteria, review probiotic microcapsules produced explicitly through complex coacervation, and provide a brief overview of in-vitro and in-vivo studies on encapsulated probiotic bacteria.

2.4.1. Complex coacervation to microencapsulate probiotic bacteria

Probiotic bacteria have been used as pharmaceutical products and as nutraceuticals and functional food ingredients (Martin et al., 2015). It has been generally accepted that the viability of probiotic bacteria in a food matrix should be more than 10^6 cfu/g, in order to impart effective health benefits (FAO/ WHO, 2002). There are various aspects that affect the viability of these bacteria in food matrices, irrespective of strains and genus (Burgain et al., 2011). These factors include physical and chemical properties of food products such as moisture content, acidity, temperature, buffering capacity in addition to the processing and storage conditions such as shear, temperature, humidity, and atmosphere (Martin et al., 2015). As the ultimate objective is to deliver these bacteria through daily life foods and absorb into the human body, the food matrices in which probiotic bacteria are incorporated should support them to survive through the gastrointestinal tract and regulate the colonization of the gastrointestinal tract. Hence, the selection of suitable food systems to deliver viable probiotic bacteria to the targeted site is an important consideration in

developing functional probiotic foods (Martin et al., 2015). Microencapsulation is a promising and development approach that has been shown to improve the viability of probiotic bacteria in some food matrices. In fact, numerous food products containing encapsulated probiotic bacteria have been introduced and accepted by consumers (Burgain et al., 2011).

Different methodologies and various wall materials have been tested for the microencapsulation of probiotic bacteria (Burgain et al., 2011; Kent et al., 2014; Tripathi et al., 2014; Martin et al., 2015; De Prisco et al., 2016). Here, we summarize the studies on the microencapsulation of probiotic bacteria, particularly by complex coacervation and subsequent drying and incorporation into food products. Ribeiro et al., (2014) reported that complex coacervation based microencapsulation does not expose microorganisms to as harmful condition as other prevailing techniques. Moreover, complex coacervation is particularly useful for the microencapsulation of probiotic bacteria that are required to be released when exposed to higher pH in the large intestine (Rathore et al., 2013). Table 2.3 summarizes the significant work on complex coacervation to microencapsulate probiotic bacteria.

The probiotic bacterial microcapsules produced through complex coacervation are usually dried so that they can be easily handled and stored. Drying methods include spouted bed drying, vacuum drying, freeze drying and spray drying (Cook et al., 2012). Spray drying is commonly used in the food industry since it is fast, economical and has a high throughput and yield (Keshani et al., 2015). It is also a mild technique compared to other convective air drying methods because of its very short product residence time (Kim et al., 2008).

Spray drying is 4-7 times less expensive than freeze drying (Klinkesorn et al., 2006). However, the viability of heat sensitive food components such as probiotic bacteria through spray drying can be a major challenge (Ghandi et al., 2012). Since spray drying is cost effective, scalable and efficient, many studies have investigated optimising spray drying and product formulations of probiotics towards increasing viability and minimising activity losses (Knorr, 1998; Tripathi et al., 2014; Eratte et al., 2016).

Table 2.3: Complex coacervation to microencapsulate probiotic bacteria.

Probiotic bacteria	**Wall materials**	**Drying techniques**	**Viability**	**Incorporation into foods**	**References**
Bifidobacterium lactis and *Lactobacillus acidophilus*	Casein/pectin	Spray drying	Preserved viability during drying and simulated gastric conditions	No	Oliveira et al., 2007
Bifidobacterium lactis and *Lactobacillus acidophilus*	Casein/pectin	Spouted bed drying	Greater viability at a storage temperature of 7°C, but did not protect during simulated gastrointestinal conditions	No	Oliveira et al., 2007
Lactobacillus	Gelatine/polyphosphate	Not dried	Preserved viability at storage temperature 10° C	No	Liu et al., 2009
Lactobacillus rhamnosus	Pectin/whey protein	Freeze drying	Preserved viability during simulated gastrointestinal conditions	No	Gerez et al., 2012
Lactobacillus acidophilus	Pectin/whey protein	Not dried	Did not confer additional protection during simulated gastrointestinal conditions.	No	Gebara et al., 2013

Lactobacillus acidophilus	Pectin/casein	Freeze drying	Preserved viability when stored under refrigerated conditions, but did not protect during simulated gastrointestinal conditions	Buffalo milk yogurt	Shoji et al., 2013
Lactobacillus plantarum	Whey protein isolate/ κ-carrageenan	Not dried	Greater viability during simulated gastrointestinal conditions	No	Hernández-Rodríguez et al., 2014
L. acidophilus	Pectin/whey protein	Not dried	Preserved viability on refrigerated storage for 35 days	Yogurt	Ribeiro et al., 2014
Lactobacillus casei	Whey protein isolate/gum Arabic	Spray drying and freeze drying	Greater viability when freeze dried	No	Eratte et al., 2015

A large number of articles have been published on the development of probiotic microcapsules and their application in food. However, this needs further and continuing investigations because many of the encapsulation technologies have practical difficulties for the food industry. For example, both intrinsic (culture selection, state of growth, subcellular injuries by heat or osmotic stress) and extrinsic factors (composition of food matrices, pH value, oxygen level, food manufacturing conditions) are important for the retention of desired viability of probiotic bacterial cells in the final end product. Establishing relationships between any of these factors to bacterial viability is challenging. Also, each food matrix poses different and new challenges to different strains of probiotic bacteria, and hence generalization is problematic. In addition, the monitoring of cell viability and vitality along the entire food production chain, including storage is still challenging due to the time and resource consuming microbial assays. Further, the assessment of metabolism and fermentation activity of the microencapsulated probiotic bacteria require further investigation (De Prisco et al., 2016). Finally, a need for more stringent quality control in the preparation of probiotic formulations is required (Martin et

al., 2015). Current probiotic functional foods available through microencapsulation strategy are primarily focused on milk based products, leaving more technically challenging foods such as bakery, meat products, and vegetable based products to be developed.

2.4.2. In-vitro and in-vivo studies on probiotic microcapsules

The resistance of various probiotic bacteria microencapsulated through complex coacervation, to simultaneous changes in pH and in the presence of salivary and gastro-intestinal (GI) enzymes has been examined. For example, Oliveira et al., (2007) studied the influence of casein/pectin complex coacervates for the protection of *B. lactis* and *L. acidophilus* in simulated GI conditions. In this study the authors concluded that complex coacervation, followed by spouted bed drying was adequate for microencapsulation of *L. acidophilus* during storage conditions at 7° C. However, this method was not efficient in protecting these microorganisms at pH values similar to those found in the human stomach. In another study, the same authors found that casein/pectin complex coacervate imparted better protection to both *B. lactis* and *L. acidophilus,* when microencapsulated in complex coacervate followed by spray drying during simulated GI conditions. Thus, the selection of the downstream drying process after complex coacervation is important in determining the performance of probiotic microcapsules (viability/vitality) during storage and gastrointestinal delivery. Possible cellular damage during the drying process can result in lower resistance of the encapsulated microorganisms to acidic conditions. Ghandi et al., (2013) reported that upstream processing conditions during spray drying such as the mode of atomization, rate of shear and presence or absence of antioxidants in the dryer feed affected the viability and vitality of dried bacterial cells.

Gebare et al. (2013) produced *Lactobacillus acidophilus* microcapsules in whey protein/pectin complex coacervates. This microencapsulation system conferred greater protective effect to *Lactobacillus acidophilus* as compared to the free cells. However, the whey protein/pectin did not confer additional protection to probiotic cells when exposed to simulated gastrointestinal conditions. In contrast, Gerez et al. (2012) found an improvement in the survival of probiotic bacteria, when they were microencapsulated in pectin particles coated with whey protein, after exposure to gastric conditions (pH 1.2), compared to free cells. Thus, this lack of confirming results make it difficult to select the most appropriate encapsulation process, parameters and wall materials, particularly for delivering viable microbial cells to the targeted sites (Liserre et al. 2007).

In vivo testing of probiotics microencapsulated using complex coacervation is limited. Rodents are the most commonly used laboratory animals used in probiotics research, although only a limited number of studies have been published. Wurth et al. (2015) found neither sodium caseinate (SC) nor newly developed SGF-resistant fat SC (FSC) capsules could increase two *Lactobacillus* strain's survival in the murine gastrointestinal system after 3 or 24 h of oral uptake, even though both showed good in-vitro effects. In contrast, Liu et al., 2016 reported that grafted alginate-sodium polyacrylate microcapsules enhanced the viability of probiotic *Lactobacillus plantarum* in the small and large intestinal fluids in both in-vitro and in-vivo analysis tests in rats. In-vitro evaluation followed by in-vivo studies on bioavailability of probiotic bacteria will enhance more insight of the applicability of the process to food industry.

2.5. Progress in the co-encapsulation of omega-3 oil and probiotic bacteria

The co-encapsulation concept has been widely used in pharmaceutical delivery systems. Co-encapsulation of more than one bioactive components with different characteristics is a challenging and is a relatively new concept, particularly for nutritional purposes. Recently, Wang et al., (2015) reported that complex coacervates of gelatine and sodium hexametaphosphate could effectively microencapsulate tuna oil fortified with the multiple functional lipophilic ingredients, vitamin A, D_3, E, K_2, curcumin and coenzyme Q_{10}. Further, it was reported that complex coacervates of whey protein isolate-gum Arabic could successfully microencapsulate lipophilic omega-3 oil and liphophobic probiotic bacteria (Eratte et al., 2015). A detailed illustration of the co-encapsulation process and produced structures is shown in Fig. 2.3A and Fig. 2.3B, respectively (Eratte et al., 2015). These studies show that microencapsulation using complex coacervation is suitable for stabilizing multiple bioactive lipophilic and liphophobic food ingredients. In-vivo testing is needed to confirm the simulated studies. Food companies are under pressure to develop functional foods with higher nutritional value, lower dose of synthetic preservatives and better organoleptic features within a budget. Co-encapsulation of multiple unstable ingredients decreases processing costs per ingredient, improving efficiency, but can also result in a more stable or improved product, as observed for the co-encapsulation of omega-3 and probiotics.

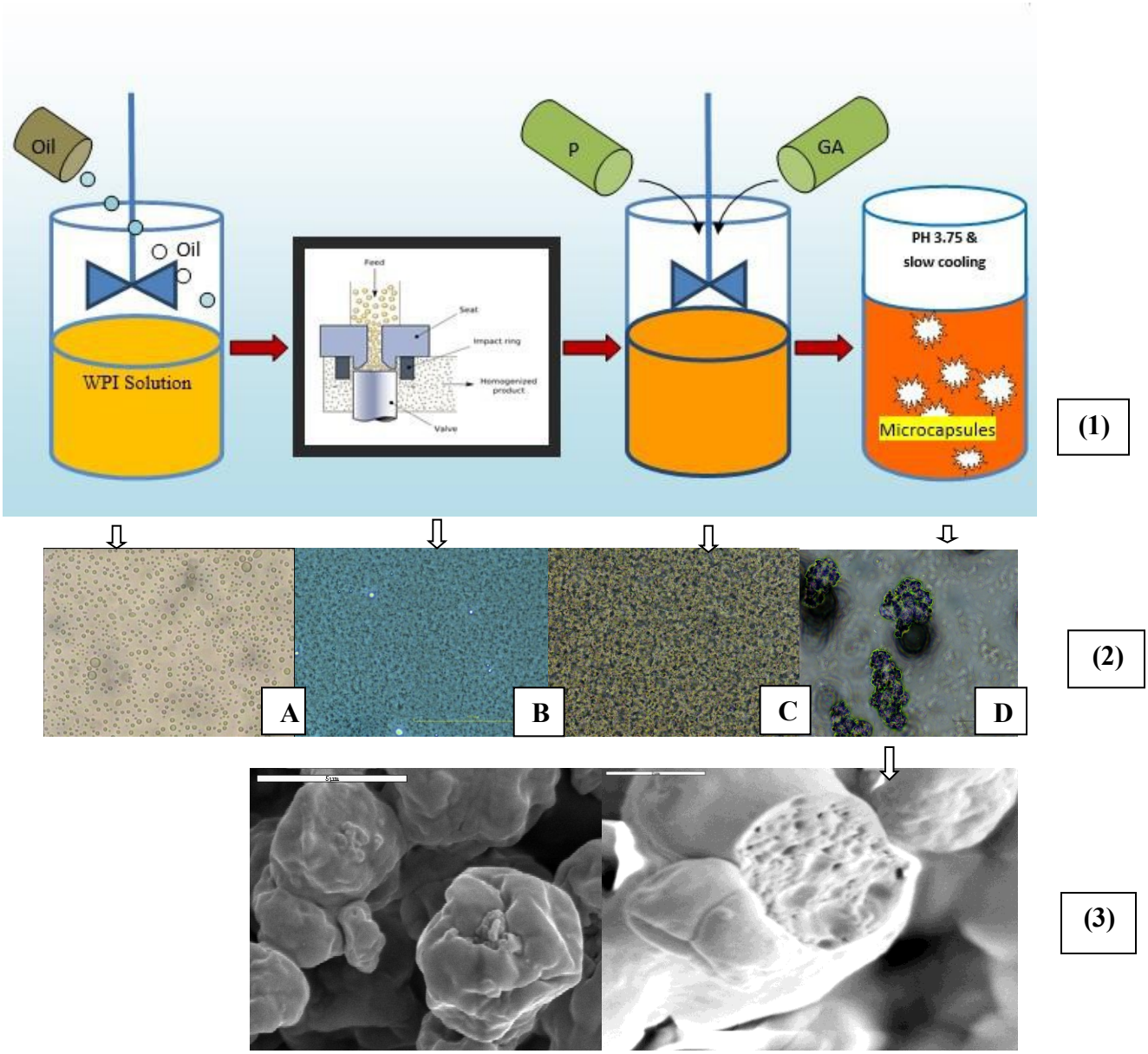

Fig. 2.3A: 1) Schematic representation of co-encapsulation of omega-3 oil (O) and probiotic bacteria (P) in whey protein isolate (WPI)-gum Arabic (GA) complex coacervates; (2) Optical micrographs of WPI-P-O-GA co-capsules formation: (A) Coarse tuna oil emulsion i in WPI; (B) Microfluidized tuna oil emulsion in WPI; (C) Complex mixture of bacterial cells and tuna oil in WPI-GA solution; (D) WPI-P-O-GA microcapsules formed at optimized conditions; (3) Spray dried WPI-P-O-GA microcapsules. Scale bar 5 µm for the spray dried microcapsules images

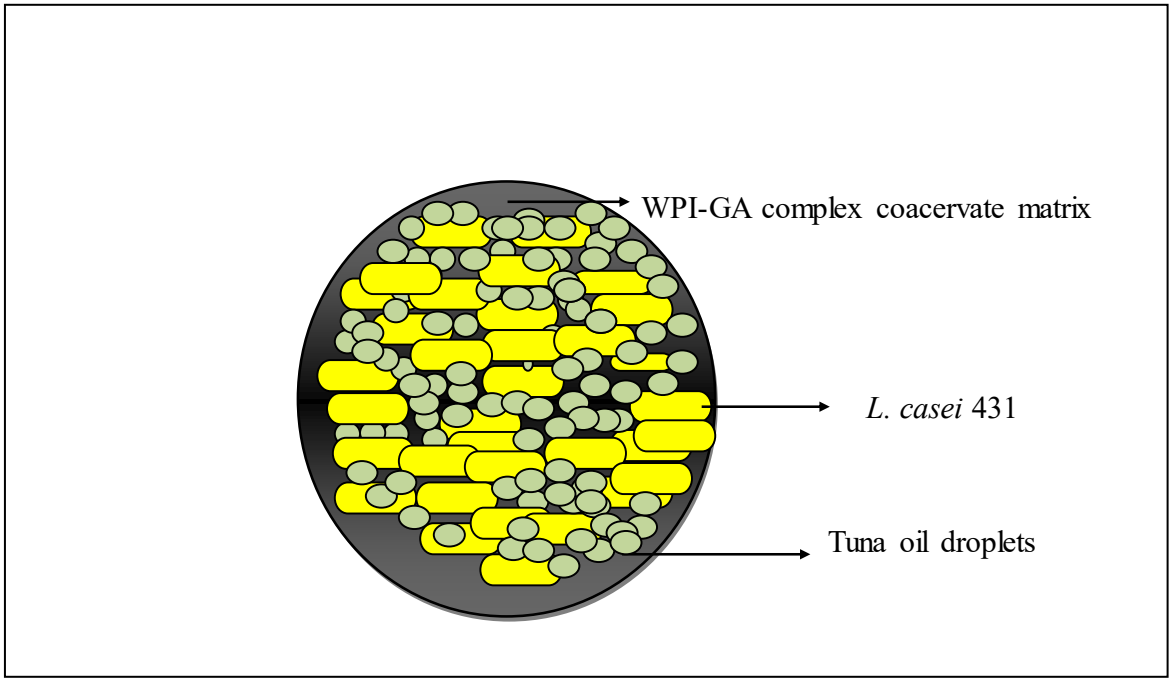

Fig. 2.3B: Graphical representation of WPI-P-O-GA microcapsules inferred from SEM analysis. (Adapted from Eratte et al., 2015).

2.6. Conclusions and future directions

Microencapsulation of omega-3 oils and probiotic bacteria are technically and commercially viable for the delivery of these bioactive ingredients into foods. However, testing of compatibility of these microcapsules in a range of food and beverage products is required to determine impact on the shelf-life and sensory properties of these products. At present, there exist several functional foods fortified with either omega-3 oils or probiotic bacteria, but not both. There is commercial need of co-encapsulation of multiple ingredients within a single microcapsules, due to decreased cost and improved product quality. Complex coacervation has been shown to be a useful method for the co-encapsulation of multiple unstable ingredients.

Complex coacervation followed by spray drying has shown to produce co-microcapsules of omega-3 oil and probiotic bacteria with improved oxidative stability and cell viability. The mechanism for improved performance over ingredients encapsulated individually is unclear, although the effect appears to be a synergistically beneficial to both active ingredients. Future work could focus on more specifically controlling the release of bioactive ingredients from these capsules, for targeted delivery including enteric delivery to the intestine. Currently, the majority of published data contains no information on the nature and mechanism of release of bioactive ingredients from co-microcapsules. Although in-vitro systems provide useful bioavailability information, additional animal and human bioavailability trials are needed to determine the effectiveness of bioactive release, particularly for microcapsules containing multiple bioactive ingredients.

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Chapter 3

Complex coacervation with whey protein isolate and gum Arabic for the microencapsulation of omega-3 rich tuna oil

Abstract

Tuna oil rich in omega-3 fatty acids was microencapsulated in whey protein isolate (WPI)-gum Arabic (GA) complex coacervates, and subsequently dried using spray and freeze drying to produce solid microcapsules. The oxidative stability, oil microencapsulation efficiency, surface oil and morphology of these solid microcapsules were determined. The complex coacervation process between WPI and GA was optimised in terms of pH, and WPI-to-GA ratio, using zeta potential, turbidity, and morphology of the microcapsules. The optimum pH and WPI-to-GA ratio for complex coacervation was found to be 3.75 and 3:1, respectively. The spray dried solid microcapsules had better stability against oxidation, higher oil microencapsulation efficiency and lower surface oil content compared to the freeze dried microcapsules. The surface of the spray dried microcapsules did not show microscopic pores while the surface of the freeze dried microcapsules was more porous. This study suggests that solid microcapsules of omega-3 rich oils can be produced using WPI-GA complex coacervates followed by spray drying and these microcapsules can be quite stable against oxidation. These microcapsules can have many potential applications in the functional food and nutraceuticals industry.

Keywords: Omega-3 fatty acids, Microencapsulation, Complex Coacervation, Whey protein isolate, Gum Arabic, Spray drying, Freeze drying

3.1. Introduction

The health benefits associated with the consumption of omega-3 fatty acids are well known, particularly for maintaining normal brain function (McNamara et al., 2006), reducing the risk of cancer (Rose et al., 1999) and preventing cardiovascular disease (Bucher et al., 2002). The lower actual versus recommended daily intake (RDI) of omega-3 fatty acids in many countries has been the major driving force for the development of functional foods and nutraceuticals containing eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3). Fish oil is the major source of EPA and DHA for fortified foods and nutritional supplements (Kris-Etherton et al., 2003; Dey et al., 2012). However, fish oils are highly susceptible to oxidation due to the highly unsaturated structure of long-chain omega-3 fatty acids. Oxidation of polyunsaturated fatty acids (PUFAs) produces peroxides and other harmful secondary oxidation products, which ultimately decreases the nutritional value of omega-3 fatty acids and negatively impacts the sensory properties (Hsieh et al., 1989; Frankel, 1989). Hence, the need for omega-3 rich oils to be microencapsulated in suitable wall materials to preserve their nutritional and organoleptic quality is ascertained (Heinzelmann et al., 1999; Velasco et al., 2003).

Complex coacervation is a liquid-liquid phase separation phenomenon that occurs when electrostatically opposite charged biopolymers are brought together under certain specific conditions (Ducel et al., 2004). Barrow et al (2007) reported that it is one of the most effective methods of microencapsulating omega-3 fatty acids rich oils, primarily using gelatine as the wall material. Broadening the range of suitable wall material for the microencapsulation of omega-3 fatty acids is important for expanding the scope and applicability of this important technique. Hence in the present study we explored the

process of complex coacervate formation between cationic whey protein isolate and anionic gum Arabic to microencapsulate tuna oil which is rich in omega-3 fatty acids. WPI is prepared from whey which is a by-product of cheese making and it has been widely used in the food processing industry due to its nutritional qualities and unique physio-chemical properties such as emulsion stabilization and gel formation (de Wit et al., 1998). As a natural protein, WPI exhibits positive charge below its isoelectric point (IEP). WPI is effective in lowering interfacial tension at the oil-water interface, quite promptly migrates to the interface from bulk solution, and possesses good skin forming ability when it is dried (Adhikari et al., 2007). GA is a composite edible polysaccharide which shows a negative charge above pH 2.2 (Butstraen et al., 2014). It is one of the most commonly used stabilizers in the food and pharmaceutical industries (Nie et al., 2013). GA mainly consists of six carbohydrates moieties and less than 2% proteins (Islam et al., 1997; Garti et al., 2001). Hence, complex coacervation between WPI and GA can occur by adjusting the pH and the WPI-to-GA ratio of their mixture.

However, there are only limited studies on the microencapsulation of oils using WPI-GA complex coacervates. Weinbreck et al (2004) encapsulated sunflower oil, lemon and orange oil flavours with WPI-GA coacervates. However, this work did not proceed to produce dried microcapsules and was confined in producing liquid microcapsules. Zhang et al (2009) investigated the encapsulation of fish oil using WPI-GA complex coacervates. This work focussed mainly on studying the effect of different GA and WPI types in forming complex coacervates and microcapsules. So far, the optimization of complex coacervation process between WPI and GA aiming to better microencapsulate omega-3 rich oils has not been systematically studied.

Dried microcapsules produced through complex coacervation could offer omega-3 rich oils better shelf-life and wider applicability (Ponsart et al., 1996). Both spray and freeze drying are commonly used in the food industry to produce powder or solid microcapsules. However, the application of different drying techniques can influence the stability and other characteristics of powdered microcapsules even at the same optimal complexation and emulsification conditions. For example, the freeze dried products are more porous due to the sublimation while spray dried powders containing biopolymers are usually irregular and contain surface folds (Anandharamakrishnan et al., 2010; Gharsalloui et al., 2007). However, the effect of spray and freeze drying processes on the microencapsulation efficiency, surface oil and oxidative stability of solid microcapsules produced using WPI-GA complex coacervates is poorly understood.

In this context, there were three key objectives in this study. Firstly, to determine the optimum process parameters at which complex coacervation between WPI and GA can occur. Secondly, to produce and characterise the liquid microcapsules of omega-3 rich tuna oil produced by using these WPI-GA complex coacervates as the shell of the microcapsules. Finally, to produce and characterise spray and freeze dried solid microcapsules in terms of oxidative stability, microencapsulation efficiency, and surface oil content and morphological analysis through scanning electron microscopic (SEM) examinations.

3.2. Materials and methods

3.2.1. Materials

Whey protein isolate (WPI 895^{TM} -93.9% protein by dry weight basis) was donated by Fonterra Cooperative, New Zealand. Gum Arabic was purchased from Sigma-Aldrich Ltd (New South Wales, Australia). Tuna oil (HiDHA), containing 39.03% omega-3 fatty acids (Table 3.1), was a gift from NuMega Ingredients Ltd. (Victoria, Australia) and stored at 4 ^{o} C until use. All other chemicals were purchased from Sigma–Aldrich Australia (New South Wales, Australia) and were of analytical grade and used without further purification.

Table 3.1: Omega-3 fatty acids composition of tuna oil

Omega-3 fatty acids	Percentage (%)
16:3w3	0.97
18:3w3 ALA	0.40
18:4w3	0.60
20:4w3	0.46
20:5w3 EPA	5.98
22:5w3	1.20
22:6w3 DHA	29.42
Sum Omega-3 –PUFAS	39.03

NuMega Ingredients Ltd. (Victoria, Australia)

3.2.2. Optimization of the complex coacervation process

The zeta potential, yield of complex coacervates and turbidity data were used to determine the optimum pH and WPI-to-GA ratio for the formation of complex coacervates.

3.2.2.1. Optimization of pH

Zeta potential values as a function of pH were used to determine the optimal pH range at which complex coacervation between WPI and GA was formed. Zeta potential values for WPI (1.5% w/w) and GA (0.5% w/w) were measured in the pH range of 3.0 -7.0 at 25° C by using a zetasizer (Zetasizer NanoZS 90, Malvern Instruments Ltd. Worcestershire, WR14 1XZ, UK), which determines the electrophoretic mobility and then calculates the zeta potential using appropriate conversion equations (Espinosa-Andrews et al., 2013; Yuan et al., 2014).

The absorbance of the mixture of WPI (0.1%) and GA (0.03%) was measured within 3.0-5.0 pH range using a UV spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at 750 nm. The absorbance (or turbidity) of WPI (0.1%, w/w), GA (0.03%, w/w), and the mixture of WPI and GA at different ratios, were measured at 750 nm. The pH value at which the maximum absorbance (turbidity) was observed was considered the optimal pH for complex coacervation between WPI and GA.

3.2.2.2. Optimization of the WPI-to-GA ratio

The turbidities of various mixtures of WPI and GA at different ratios (1:1, 3:1, 3:2, 4:1 and 5:1) were determined using the light absorbance of these mixtures at 750 nm using a UV spectrophotometer as detailed above.

The optimum WPI-to-GA ratio which resulted in the highest coacervation yield was also measured. For this purpose, aqueous dispersions containing WPI and GA at the above mentioned ratios were prepared at ambient temperature and the pH of these dispersions was adjusted to the optimum value (Section 3.2.2.1). These pH adjusted dispersions were allowed to stand for 5 h to facilitate the precipitation of the gel-like complex coacervates.

Then, the coacervates were carefully separated and dried at 105° C until a constant mass was reached. The coacervate yield was then calculated by using equation (1) given below.

$$\text{Coacervate yield } (\%) = \frac{\text{Mass of the dried coacervates (g)}}{\text{Total mass of WPI+GA used (g)}} \quad (1)$$

3.2.3. Confocal laser scanning microscopic (CLSM) analysis

The microstructure of tuna oil microencapsulated with WPI-GA using complex coacervation at various pH was observed using a CLSM (Eclipse Ti, Nikon, Japan). WPI and GA were covalently labelled with fluorescein 5-isothiocyanate (FITC) and rhodamine B-isothiocyanate (RITC), respectively. Tuna oil was physically labelled with Bodipy-X-Azide. Briefly, FITC solution (10 mg/100 ml ethanol) and RITC solution (10 mg/ 100 ml water) were prepared separately. Then 50 ml of FITC solution and 50 ml of RITC solution were used for the preparation of WPI (3%) and GA (1%) solutions and the microencapsulation procedure was carried out as per Section 2.4. Covalent labelling of WPI and GA was done in order to visualize these biopolymers in the mixture. A lens with 40 X magnification and a laser with an excitation wavelength of 645 nm (for Bodipy-X-Azide), 488 nm (for FITC) and 561nm (for RITC) were used.

3.2.4. Microencapsulation of tuna oil

Firstly, 250 ml WPI solution (3%, w/w) was prepared at ambient temperature and 15 g of tuna oil was dispersed in this solution. The mixture was stirred using a mechanical stirrer (IKA® RW 20 digital overhead stirrer, Germany) at 800 rpm for 10 min and was further homogenized using a microfluidizer at 45 MPa for 3 passes (M110L, Microfluidics, Newton, USA) to produce an O/W emulsion. Then 250 ml GA solution (1%, w/w) was

added drop wise into this O/W emulsion and was stirred at 800 rpm. The pH of this emulsion was then adjusted to 3.75 by adding 1% citric acid drop wise in order to induce electrostatic interaction between WPI and GA. The microencapsulation procedure was carried out at 25° C, followed by cooling to 5° C at a rate of 5° C/h using a programmable water bath (PolyScience, Niles, Illinois, USA). A microscope (Eclipse 80 i, Nikon, Japan) was used to obtain optical images of the coacervates microcapsules. The morphology of the microcapsules was captured as a function of pH and temperature. Finally, the microcapsules were dried to produce solid or powder microcapsules.

3.2.5. Drying of coacervate microcapsules

A portion of suspension containing microcapsules produced as per Section 3.2.4 was spray dried (Mini spray dryer B-290, BÜCHI Labortechnik, Switzerland) using inlet and outlet temperatures of 180° C and 80±3 ° C, respectively. The powdered microcapsules were collected and stored in an air tight desiccator for further characterization.

The second portion of the liquid microcapsules was frozen at -20° C overnight and was freeze dried (Christ Alpha 2-4LD, Osterode, Germany). The temperature of the ice condenser was set at -50^0 C and the vacuum pressure was set to 0.04 mbar. The frozen samples were dried for 30 hrs and the dried product was collected, pulverized and stored in an air tight desiccator for further tests.

3.2.6. Physicochemical properties of the microcapsules

3.2.6.1. Oxidative stability

Accelerated oxidation tests were carried out for the liquid oil and the solid or powdered microcapsules using a Rancimat (model 743, Metrohm, Herisau, Switzerland) (Mathaus,

1996). Four ml tuna oil or 1.5 g dried microcapsule powder was heated at 90° C under purified air (flow rate of 20L/h). Briefly, when the oxidation of oil takes place, the conductivity of Milli-Q water in the collection chamber increases due to the entrapment of the volatile products (formic acid) and this increase is plotted by the accompanied software (Rancimat Control, version 1.1,Metrohm, Herisau, Switzerland). Then the *OSI* value of the samples is graphically determined by locating tangential intersection point on experimental data as described by Läubli et al, (1986).The induction time (at which the conductivity of sample increases sharply due to oxidation) of the test sample was recorded and used as the oxidative stability index (*OSI*). Analyses were performed in duplicate.

3.2.6.2. Microencapsulation efficiency

Microencapsulation efficiency was calculated by measuring the surface oil (solvent extractable) and total oil of the microcapsules. Surface oil was determined by the washing method described by Liu et al (2010) with slight modification. Three grams of dried microcapsule sample was dispersed in 30 mL isohexane and this was shaken at 225rpm for 5 minutes on an orbital shaker (Stuart SSL-1, Carl Roth, Karlsruhe, Germany). The slurry was then filtered through filter paper (Whatman, 5µm) and the solid particles caught on the filter were further washed three times with 10 ml of isohexane in each wash. The filtrate was dried under nitrogen followed by drying at 100° C for 1h in an oven. The sample dried in this way was placed in a fume hood overnight to remove the residual solvent. The surface oil content was then measured gravimetrically.

The total oil content in the dried microcapsules was determined by an acid digestion method using 4N HCl. Three grams of powdered microcapsule sample was dispersed in 30 ml of 4N HCl and shaken at 225 rpm for 15 minutes on an orbital shaker (Stuart SSL-1, Carl Roth, Karlsruhe, Germany) in order to dissolve the shell materials. Fifteen ml of

isohexane was added to this mixture and then shaken for 18 h at ambient temperature to extract the oil. The mixture was centrifuged at 24,471 g at 20°C for 30 minutes. The hexane phase containing the dissolved oil was collected and dried by nitrogen under fume hood. This partially dried sample was further dried at 100° C in an oven and then placed under a fume hood to remove the residual solvent. The oil content was then determined gravimetrically.

The percent surface oil (SO), total oil (TO) and microencapsulation efficiency (ME) were calculated using equations (2), (3) and (4), respectively.

$$SO = \frac{w_s}{w_m} \times 100\% \quad (2)$$

$$TO = \frac{w_t}{w_m} \times 100\% \quad (3)$$

$$ME = \frac{w_t - w_s}{w_t} \times 100\% \quad (4)$$

where w_t and w_s are the mass values (g) of total and surface oil of the microcapsules and w_m is the mass (g) of the microcapsules.

3.2.7. Surface morphology of the solid microcapsules

A Scanning Electron Microscopy (JEOL JSM6300 SEM, Tokyo, Japan) was used to acquire the morphology of dried microcapsules. Samples were lightly gold sputter coated (Sputter coater, Agar Aids, England) for 45 seconds and imaged under scanning electron microscope operated at 7kV and low beam current.

3.2.8. Statistical Analysis

All measurements were performed at least in triplicates and the results are reported as mean± standard deviation. The SPSS statistical package (Version 21, Lead Technologies, USA) was used for the analysis of variance (ANOVA) to determine whether or not significant difference existed between two mean values. The confidence level of 95% ($p<0.05$) was used.

3.3. Results and discussion

3.3.1. Optimal parameters for complex coacervation between WPI and GA

3.3.1.1. Optimal pH for complex coacervation

The zeta potentials of WPI and GA within the pH range of 3.0 - 7.0 are presented in Fig. 3.1A. The zeta potentials of WPI within this pH range varied from positive (16.80 mV at pH 3.0) to negative (-20.21 mV at pH 7.0). The isoelectric point (IEP) at which the zeta potential becomes zero was found to be 4.4, which is in agreement with previous reports (Chen et al., 2012).

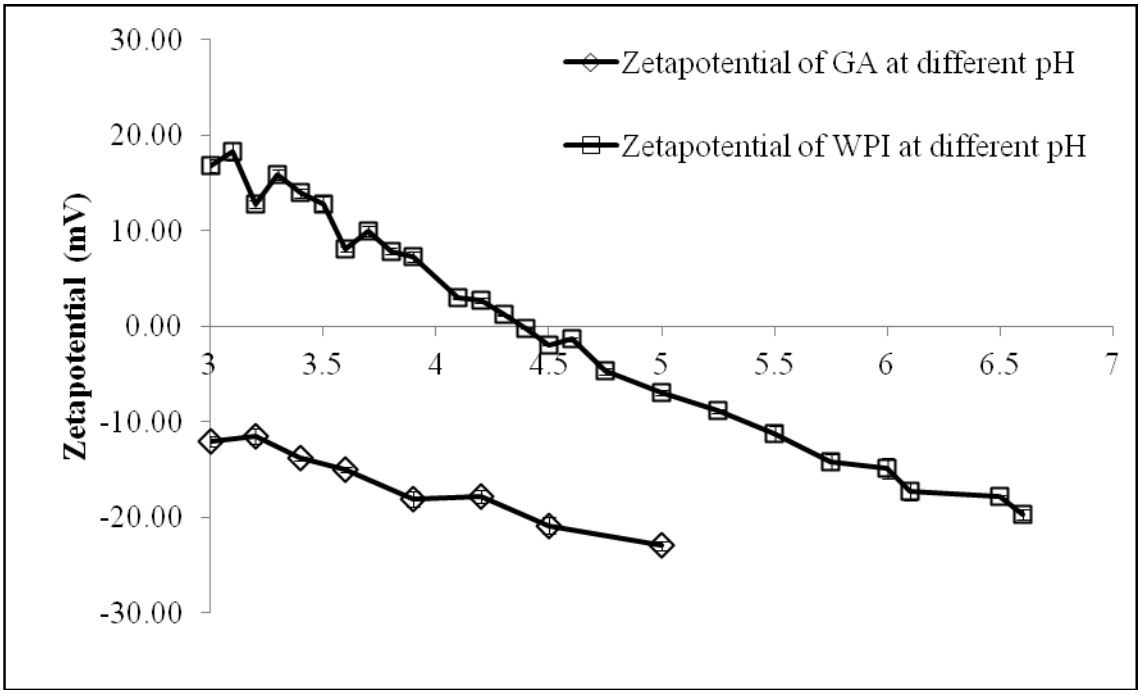


Fig. 3.1A: Effect of pH on zetapotential of WPI and GA dispersions.

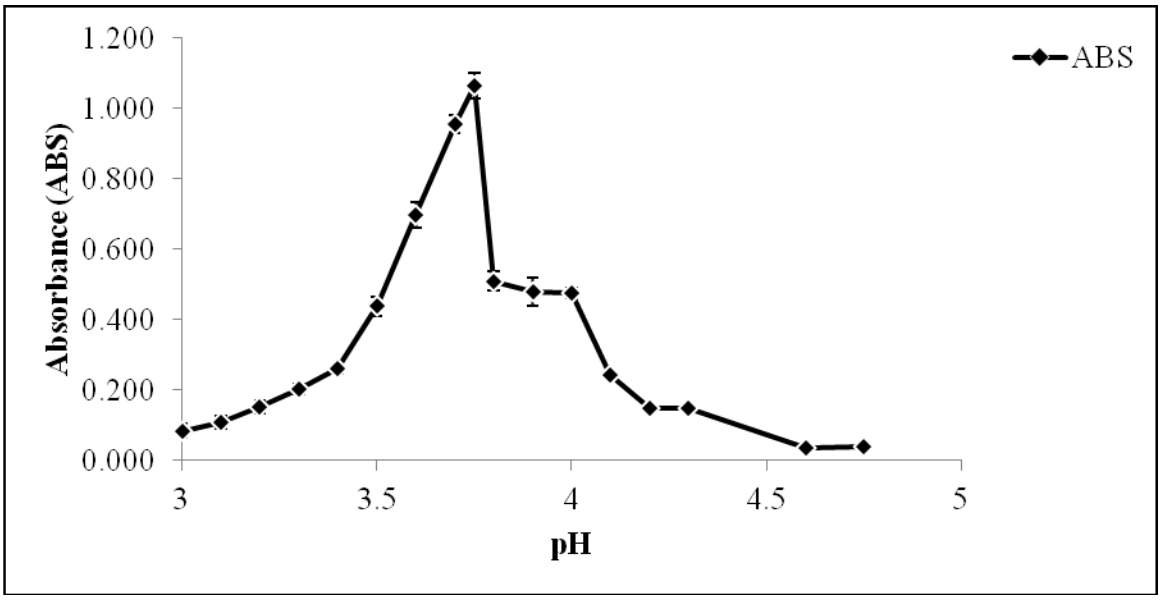


Fig. 3.1B: Turbidity values of WPI and GA mixed dispersions at different pH values at WPI to GA ratio = 3:1.

The zeta potential of GA is always negative independently of pH due to carboxylate groups being the only charged functionalities present in its globular-like random coil structure (Dickinson, 2003). If at least one of the macromolecules in a mixture is not a strong polyelectrolyte, then coacervation is likely to occur (de Kruif et al., 2004). Therefore, it can be inferred from the Fig. 3.1A that the pH at which complex coacervation between WPI and GA will occur is at or below pH 4.4.

The turbidity test (Section 3.2.2.1) was carried out within the pH range of 3.0 -5.0 in order to locate the optimum pH value for complex coacervate formation. The absorbance values of the WPI and GA mixed dispersions are presented in Fig. 3.1B. As can be seen from this figure, formation of dense complex coacervates occurred within the pH range and formation of the complex coacervates was greatest at pH 3.75. Hence this pH value was chosen as the optimum pH for complex coacervate formation.

3.3.1.2. Optimal WPI-to-GA ratio for complex coacervation

The ratio of protein to polysaccharide in the mixture influences the charge balance of polyions and consequently their complexation behaviour (Ye, 2008). Based on the work described in the previous section (Section 3.3.1.1) we used the optimum pH of 3.75 to study the binding between WPI and GA. As can be seen from Fig. 3.2A, the highest absorbance value was observed at a WPI-to-GA ratio of 3:1, which is due to the highest level of turbidity caused by the electrostatic interaction between WPI and to corroborate the data, the yield of complex coacervates was measured (Section 3.2.2.2) at different WPI-to-GA ratios and the data is presented in Fig. 3.2B.

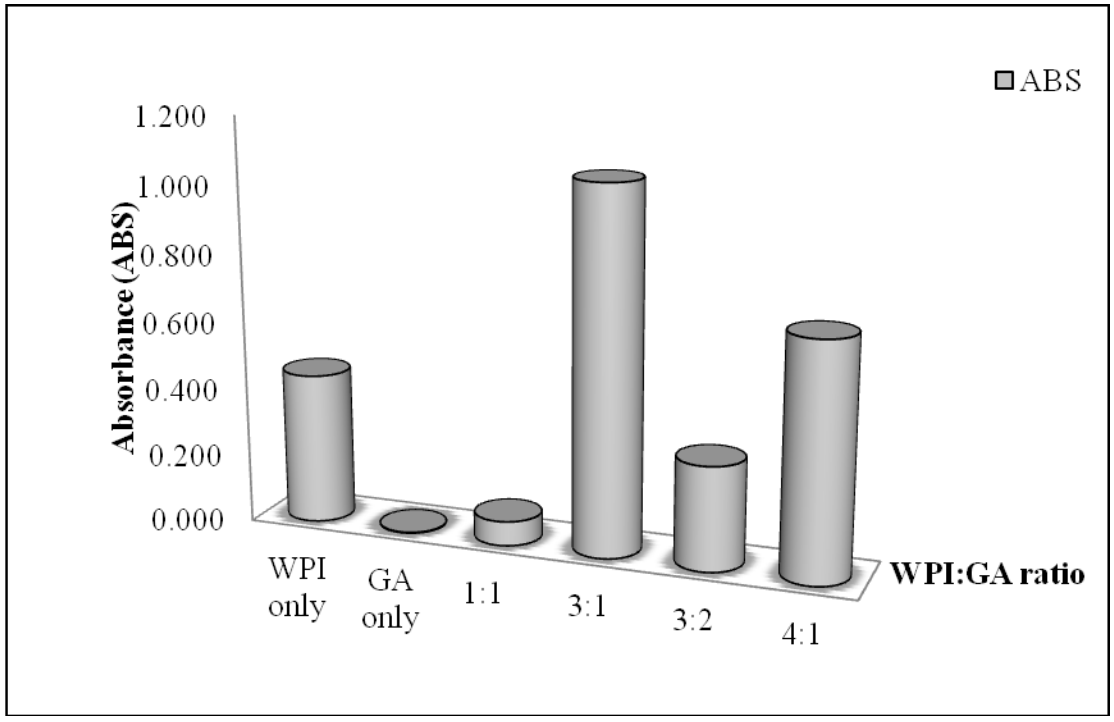


Fig. 3.2A: Turbidity values at different WPI to GA ratios.

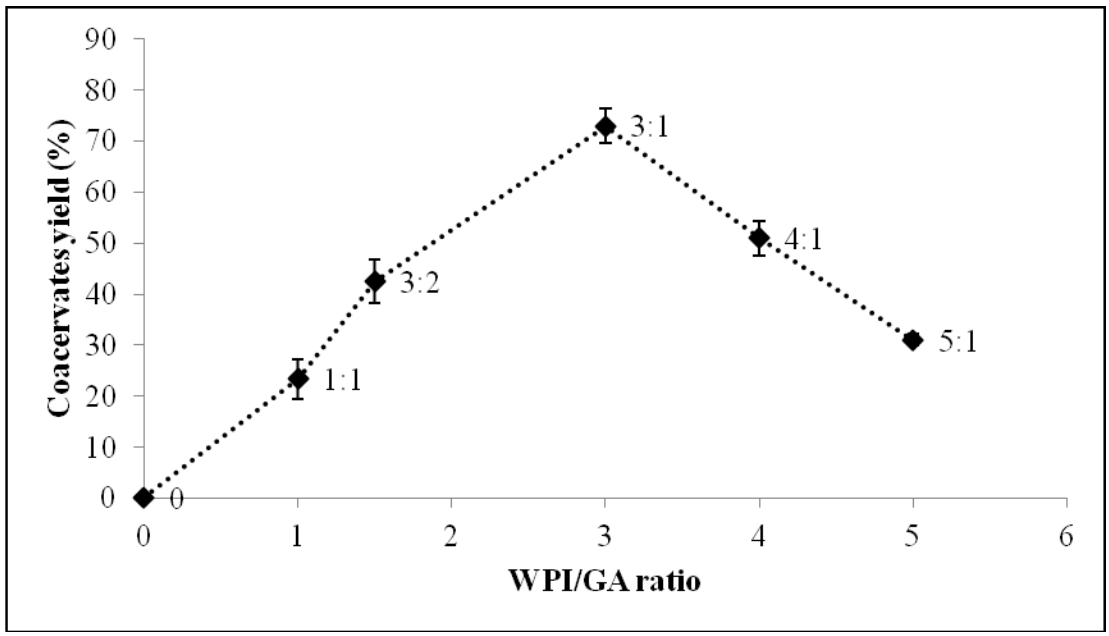


Fig. 3.2B: Yield of complex coacervates at different WPI to GA ratios.

The highest coacervate yield of 71.26 % was obtained at the WPI-to-GA ratio of 3:1, which corroborates the turbidity data (Fig. 3.3.2A). Other WPI-to-GA ratios produced

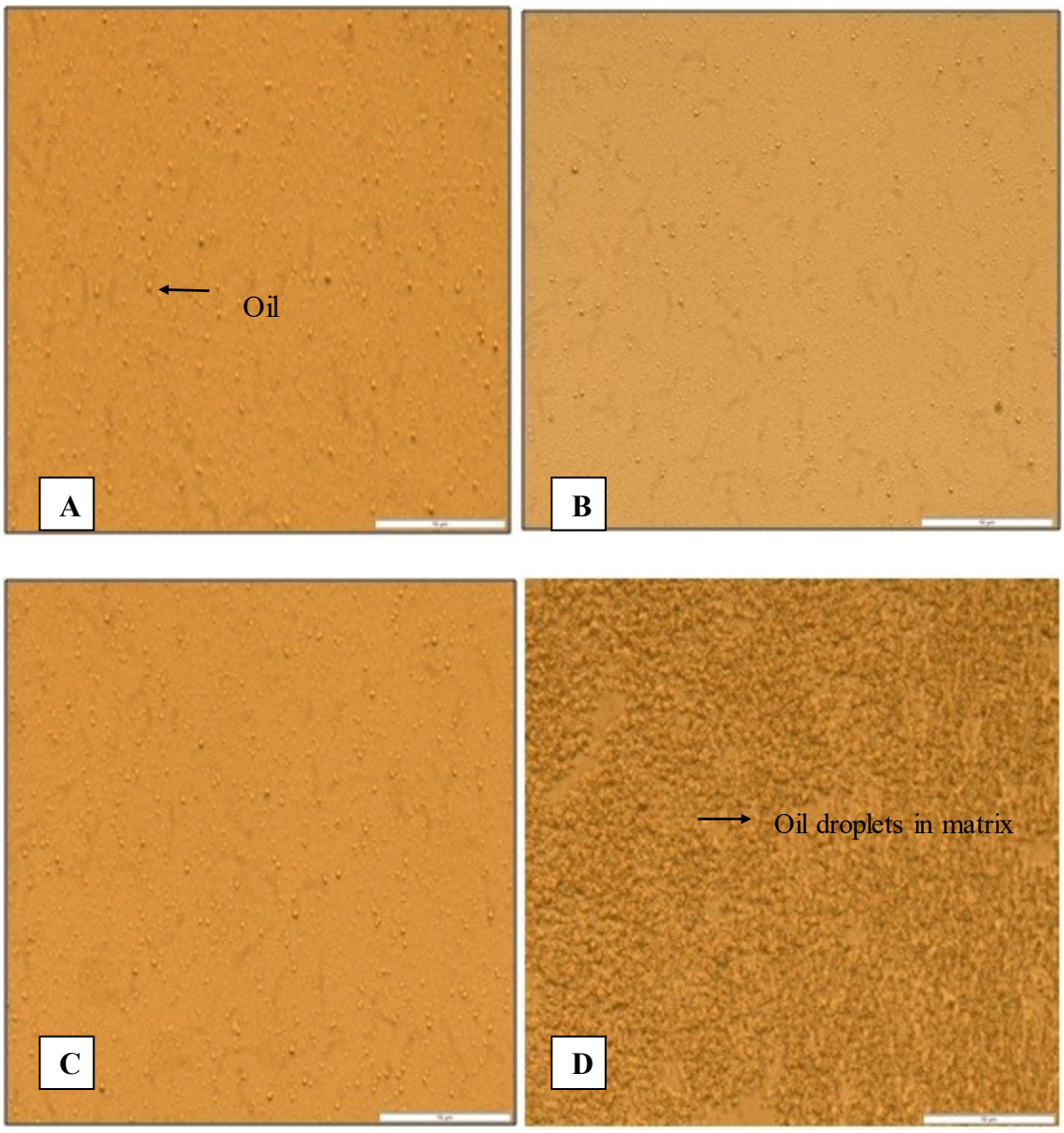
lower coacervate yields, possibly due to the formation of soluble rather than insoluble complexes occurring when either of the biopolymer is in excess. Soluble complexes are formed due to the charge imbalance and produce weaker electrostatic interaction, which results in lower coacervate yield (Ye, 2008). The biopolymer ratio and dispersion pH are known to alter the charge density of the complexes (Espinosa-Andrews et al., 2013). At pH 3.75 and WPI-to-GA ratio of 3:1, the electrostatic interaction between WPI and GA resulted in two phases specifically due to the formation of a soft dense coacervate phase rich in biopolymers and a dilute phase poor in biopolymer concentrations. Hence, this WPI-to-GA ratio of 3:1 was selected as the optimum ratio to produce complex coacervates.

3.3.2. Observation of microcapsule formation

Optical microscopy was used to study the formation of complex coacervates and their subsequent absorption onto oil droplets. As can be seen from Fig. 3.3A, 3.3B and 3.3C, no obvious complex coacervates were absorbed onto the oil droplets above pH 5.0. When the pH was further lowered to 4.5 (close to the IEP of WPI), the aggregation of oil droplets was observed (Fig. 3.3D). This may be due to weaker repulsion between negatively charged WPI and GA when the surface charge of WPI approaches neutral (Fig. 3.1A). Also, the steric repulsion between droplets was not enough to overcome the aggregation of the droplets. In this environment, the attractive interactions between the biopolymer molecules, such as van der Waals and hydrophobic, become dominant. Kulmyrzaev et al (2000) reported that excessive droplet aggregation occurs when the net charge on the droplet doesn't generate stronger electrostatic repulsive force than the strength of the attractive forces in the emulsion. It can be observed from Fig. 3.3E to 3.3H that when the pH of the dispersion was lowered below the isoelectric point of WPI (IEP = 4.4), complex

coacervation occurred in the surrounding continuous phase, and these coacervates migrated to the surface of the oil droplets, and formed a coacervates layer. It was observed that complex coacervation occurred and aggregation of the oil droplets started to take place below pH 4.4. This is because WPI became positively charged below its IEP and electrostatic attraction with negatively charged GA started to occur. Finally, a smooth layer of WPI-GA complex coacervates was formed uniformly around the oil droplets (Fig. 3.3G) at pH 3.75, which is consistent with the turbidity and coacervate yield data (Fig. 3.1B and Fig. 3.2A, 3.2B).

When the liquid microcapsules were cooled from ambient temperature to 5°C, the "free coacervates" which remained suspended in the continuous phase (at ambient temperature) began to absorb onto the surface of the aggregated oil droplets (Fig. 3.3H). This implies that cooling is an important step in stabilizing oil emulsions using complex coacervates. As can be seen from Fig. 3.3H, multicored microcapsules were formed due to the formation of WPI-GA complex coacervates. This may be partly due to homogenization occurring under high pressure when using a microfluidizer. Yeo et al (2005) reported that single core microcapsules were produced when a lower degree of homogenization was used while multi core microcapsules were produced under a higher degree of homogenization.


Oil
A
B
C
Oil droplets in matrix
D

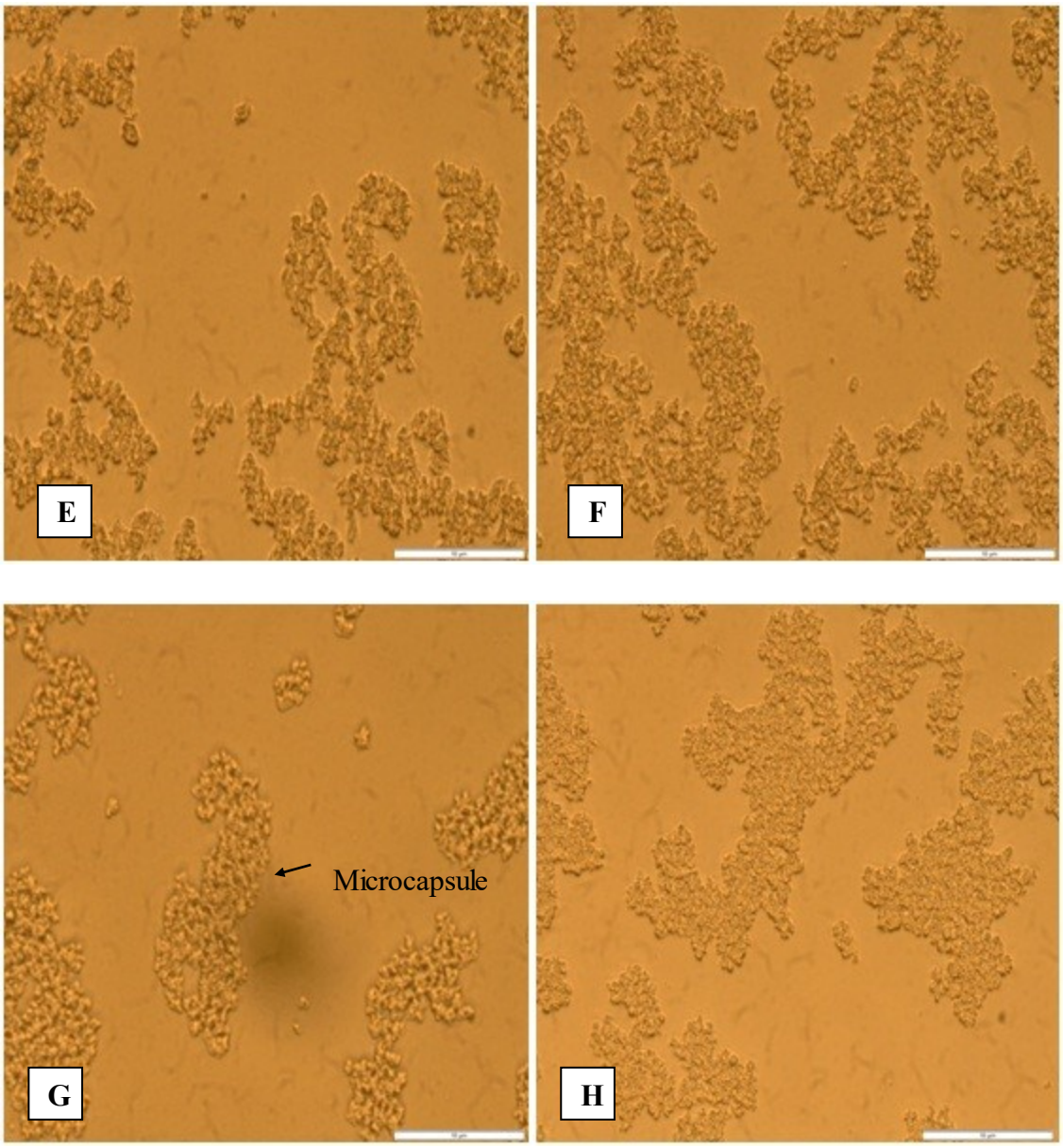
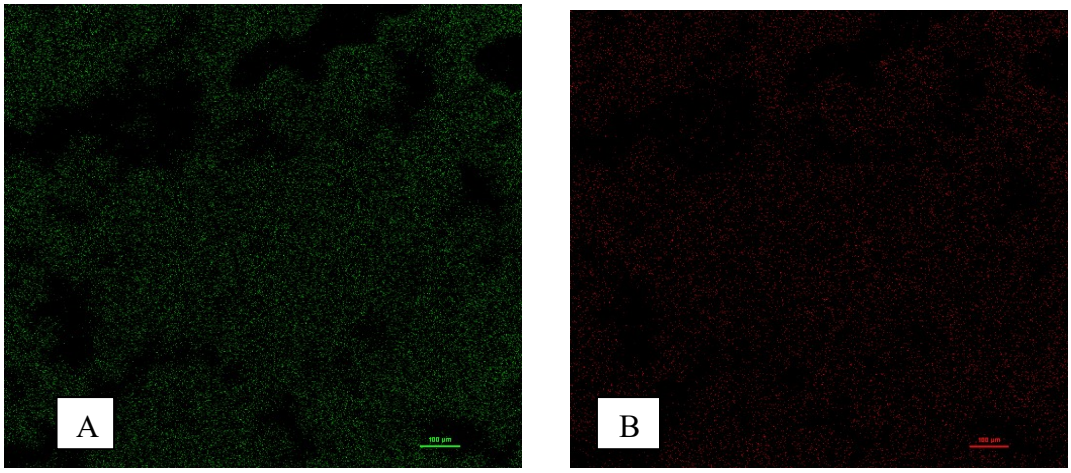


Fig 3.3: WPI-GA complex coacervates observed through light microscopy as a function of pH. (A) pH 6.0; (B) pH 5.5; (C) pH 5.0; (D) pH 4.5; (E) pH 4.0; (F) pH 3.8;(G) pH 3.75 and (H) pH 3.75 after cooling, scale bar = 10µm.

Confocal laser scanning microscopy was used to visualize complex coacervate formation between WPI and GA and to confirm the optimized processing conditions (pH 3.75 and WPI-to-GA ratio 3:1) and also to assess whether tuna oil droplets were microencapsulated

in the WPI-GA complex coacervates. WPI (labelled green) and GA (labelled red) in the dispersion before complex coacervation are shown in Fig. 3.4A and Fig. 3.4B. These figures are similar to the optical micrographs of dispersion before complex coacervation (Fig. 3.3A). WPI and GA are clearly visible in the complex coacervate as shown in Fig. 3.4C and Fig. 3.4D, respectively. The micrograph presented in Fig. 3.4C is WPI-GA complex coacervate (labelled WPI (green) only) formed under the optimized processing conditions. Similarly, Fig. 3.4D is the WPI-GA complex coacervate (labelled GA (red) only) formed under the same optimized conditions. Fig. 3.4E shows that WPI-GA complex coacervate (yellow in colour) was formed at pH 3.75 and at a WPI-to-GA ratio of 3:1, and neither WPI (green colour) nor GA (red colour) is dominant in Fig. 3.4E, indicating that complex coacervation has occurred between WPI and GA under the processing conditions used. These CLSM images are consistent with the optimum conditions for formation of complex coacervates between WPI and GA being pH 3.75 and a WPI-to-GA ratio of 3:1.



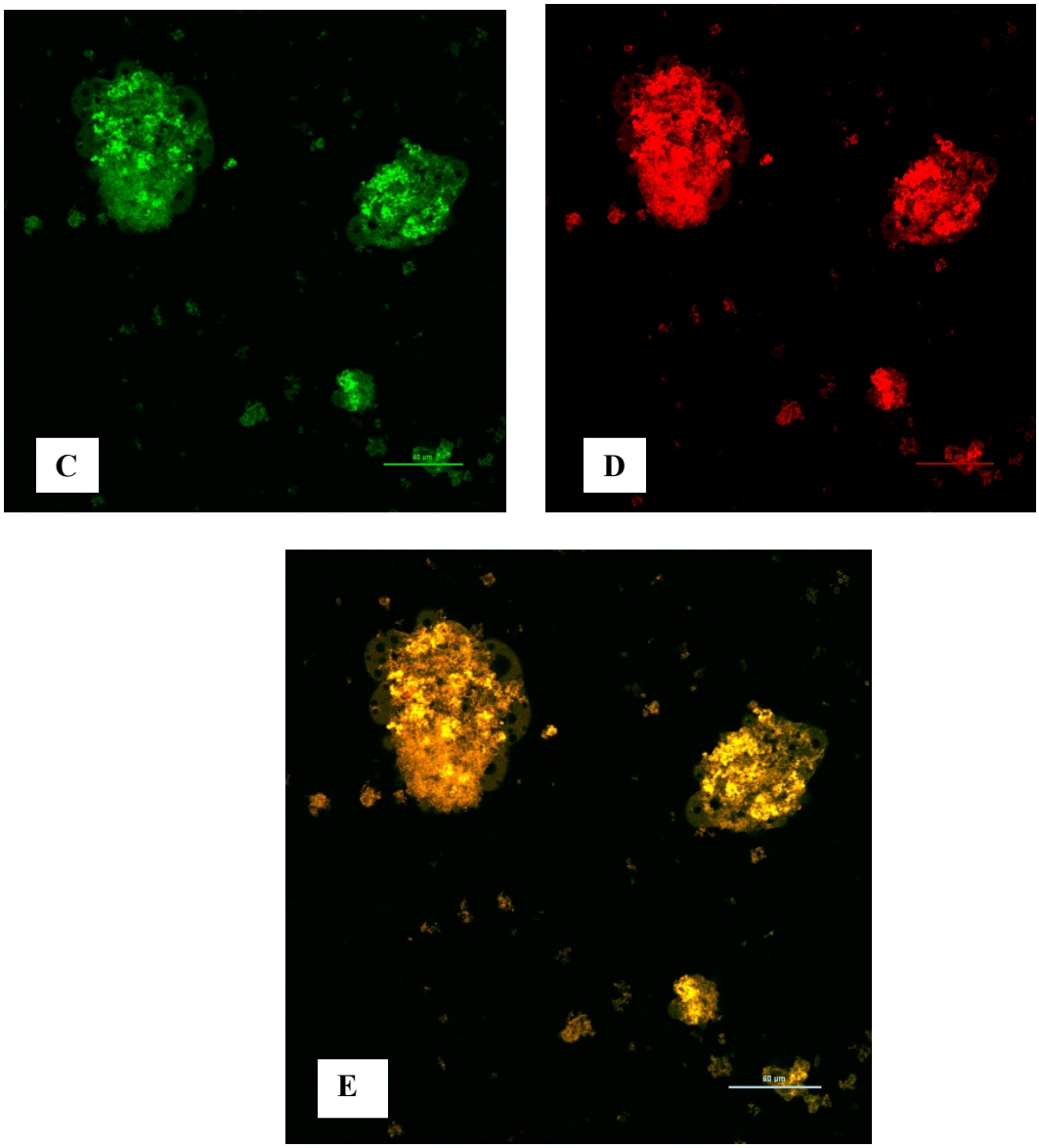


Fig 3.4: Confocal scanning laser micrographs of (A) WPI (green) in the dispersion before carrying out complex coacervation at pH 6.0, (B) GA (red) in the dispersion before carrying out complex coacervation at pH 6.0 (C) Complex coacervate with labelled WPI (green) at pH 3.75, (D) Complex coacervate with labelled GA (red) at pH 3.75 and (E) Complex coacervates of WPI and GA formed (yellow) at pH 3.75.

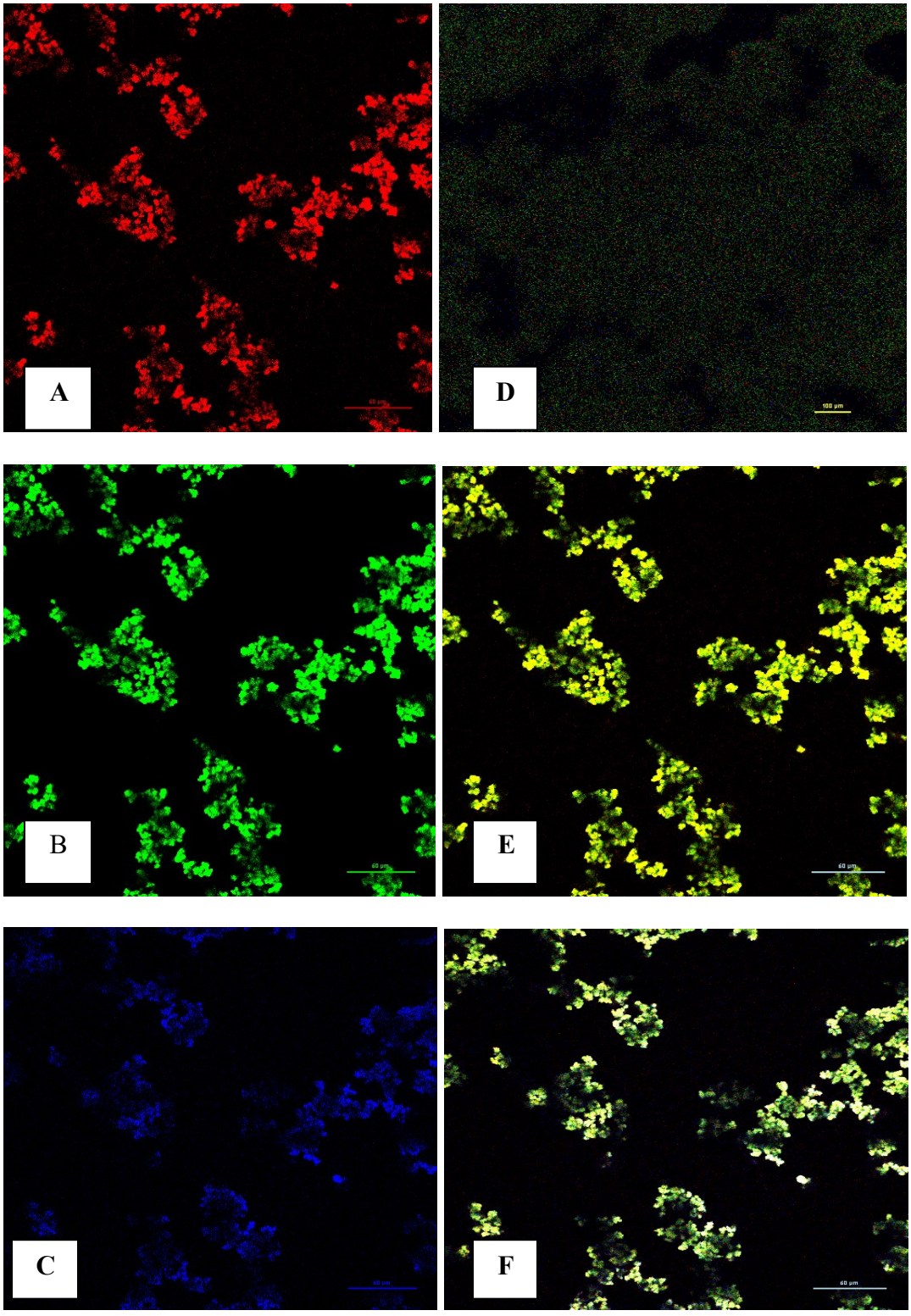


Fig. 3.5: Confocal scanning laser micrographs of (A) WPI in the coacervate microcapsules (green), (B) GA in the coacervate microcapsules (red), (C) Oil droplets in the coacervate microcapsules (blue), (D) Mixture of WPI (green), GA (red), and oil (blue) before carrying

out complex coacervation at pH 6.0, (E) Coacervate microcapsules not showing (blue) oil droplets at pH 3.75 and (F) Coacervate microcapsules showing (blue) oil droplets at pH 3.75.

Multiple labelling CLSM was used to visualize the distribution of tuna oil droplets (labelled blue) in the WPI-GA microcapsules (Fig. 3.5). Microcapsules without and with visualisation of oil droplets are shown in Fig. 3.5E and Fig. 3.5F, respectively, to assess whether complexation has occurred between WPI and GA at pH 3.75 and at the WPI-to-GA ratio of 3:1, and also to confirm the distribution of oil droplets in the microcapsules. The GA component in the microcapsule is shown in Fig. 3.5A (GA labelled red), the WPI component in Fig. 3.5B (WPI labelled green), and oil in Fig. 3.5C (oil droplets labelled blue). The mixture of oil, WPI and GA in the dispersion before carrying out complex coacervation process is presented in Fig. 3.5D. It can be clearly seen from Fig. 3.5D that no complex coacervates of WPI and GA was formed and oil droplets were just dispersed in the mixture at pH 6.0. An obvious formation of WPI-GA complex coacervate (yellow) and distribution of oil droplets in the coacervate can be clearly seen in Fig. 3.5E and Fig. 3.5F. The Z-average size of the oil droplets was 223.0 nm (polydispersity index (PdI) = 0.376) and these oil droplets are clearly microencapsulated in the WPI -GA matrix.

3.3.3. Effect of drying methods on the physiochemical characteristics of the microcapsules

Physiochemical properties (oxidative stability, surface oil, total oil, microencapsulation efficiency and morphological analysis through SEM) of spray and freeze dried tuna oil

microcapsules produced by microencapsulating with WPI-GA complex coacervates are discussed in this section.

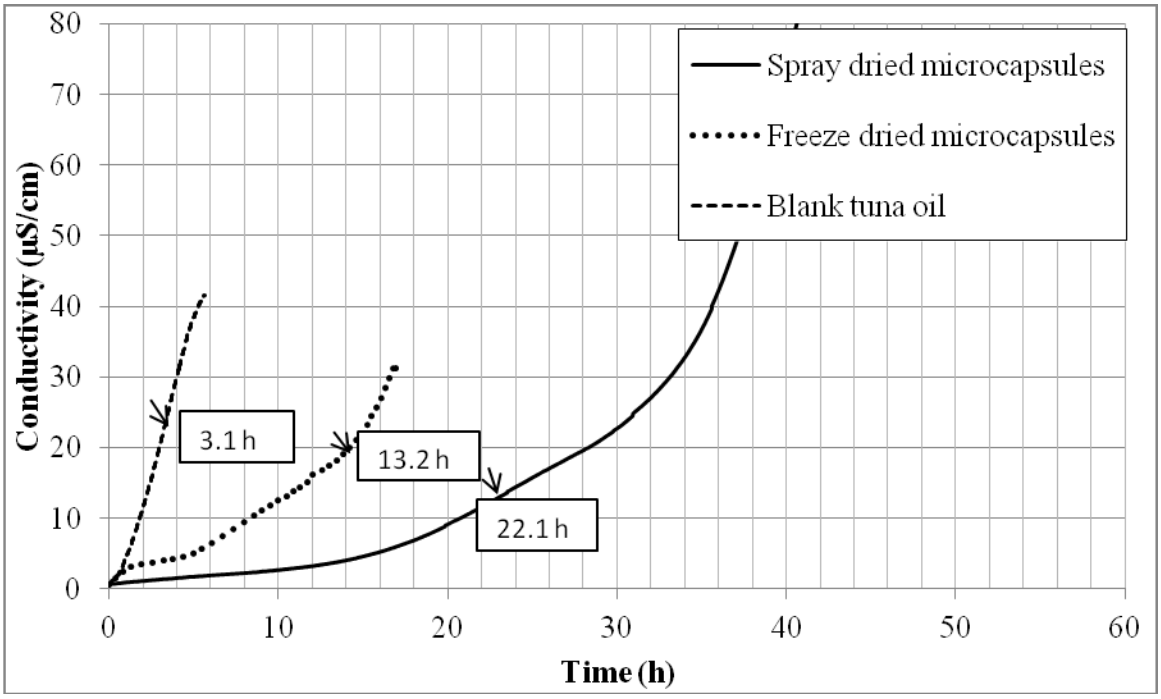


Fig. 3.6: Oxidative stability index of microcapsules measured by accelerated oxidation test using Rancimat™.

The *OSI* values of blank tuna oil (control), freeze dried and spray dried solid microcapsules are presented in Fig. 3.6. These *OSI* data suggest that both freeze and spray dried tuna oil microcapsules exhibited significantly ($p < 0.05$) better oxidative stability compared to the control (OSI = 3.1 h). The OSI value for freeze dried sample was 13.2 h compared to 22.1 h for spray dried sample implying that the freeze dried microcapsules were less stable against oxidation compared to the spray dried ones. This may be due to the highly porous structure of the freeze dried powder (Sablani et al., 2007). The porous structure makes it easier for oxygen to diffuse through the porous shell structure to access the encapsulated oil and weakens the oxidative stability (Rahman et al., 2002; Ezhilarasi et al., 2013). This relatively poor oxidative stability in freeze dried microcapsules can also be explained by

their morphology identified by SEM (Fig. 3.7B). Freeze dried microcapsules possess irregular shape, are flake-like and have a highly porous structure.

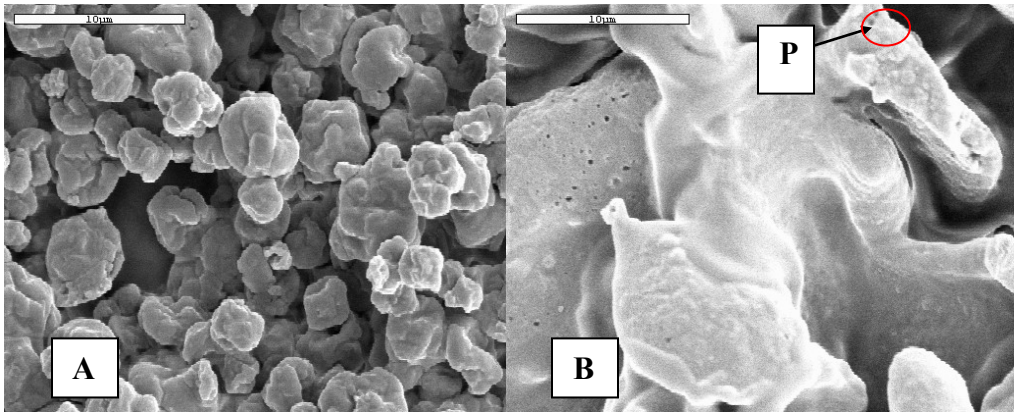


Fig. 3.7: SEM micrographs of spray dried (A) and freeze dried microcapsules (B). P indicates pores in the shell.

The spray dried microcapsules were found to be significantly more stable ($p < 0.05$) against oxidation, as compared to freeze dried microcapsules, even though much higher temperature was used in the spray drying process. This relatively high *OSI* of spray dried microcapsules can be attributed to the compact structure of the spray dried solid microcapsules (Fig. 3.7A). The outer topography of the spray dried particles indicates that there is no shell rupture and the shell is much less porous compared to freeze dried microcapsules. The absence of pores/ cracks on the particle surface is very important for preventing the inward diffusion of oxygen and hence for better protection of the encapsulated oil. The SEM micrograph shows that the spray dried microcapsules have uniform size distribution below 5 µm (based on 100 microcapsules) with wrinkled spherical shape, resulting from the protein in the wall material. The wrinkled surface is typical characteristics of spray dried powders with protein in the matrix (Soottitantawat et

al., 2005; Tonon et al., 2011; Xu et al., 2013). Moreover, this significantly enhanced oxidative stability for spray dried microcapsules compared to freeze dried ones is partly due to lower overall surface area and lower surface oil content.

The surface oil and total oil contents, and microencapsulation efficiency of the freeze and spray dried microcapsules, are shown in Fig. 3.8. The freeze dried solid microcapsules had higher surface oil content (11.41%) as shown in Fig. 3.8, and lower microencapsulation efficiency. Surface oil is the unencapsulated oil found on the surface of the microparticles, and can trigger lipid oxidation and is a result of lower oil encapsulation efficiency (Hardas et al., 2000). The microencapsulation efficiency of freeze dried microcapsules was 72.95 %, which is significantly ($p<0.05$) lower than that of spray dried microcapsules (Fig. 3.8). Similar result was also observed by Quispe-Condori et al (2011) when investigating the microencapsulation efficiency of freeze dried and spray dried flaxseed oil microcapsules.

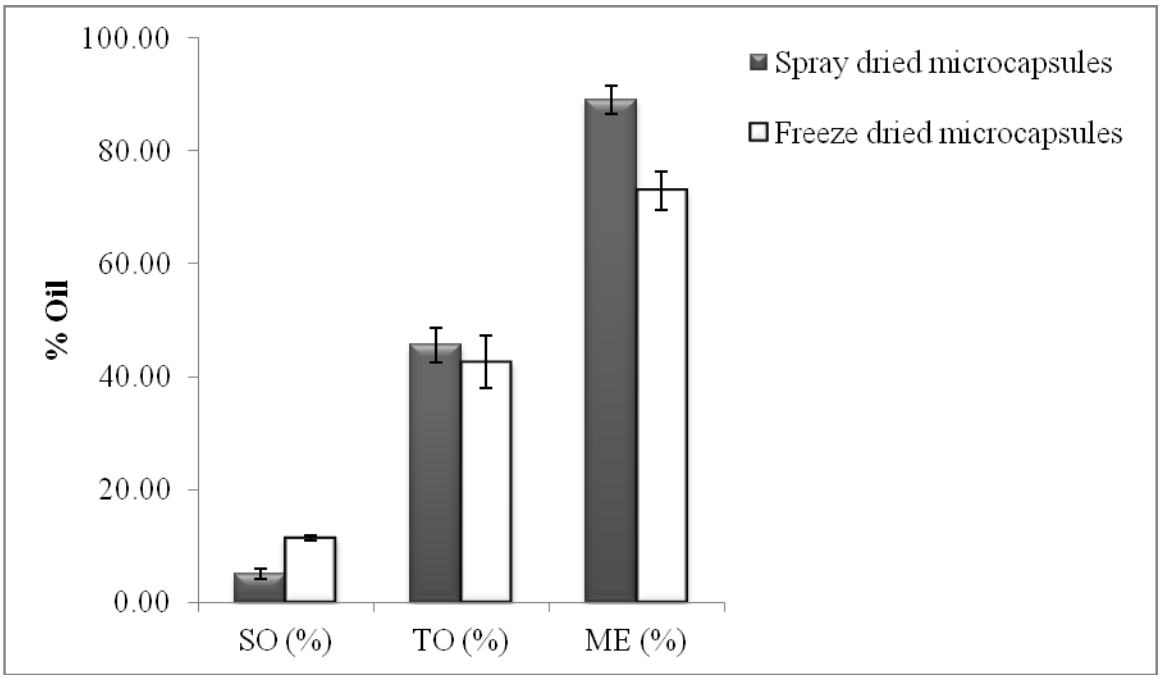


Fig. 3.8: Surface oil (SO), total oil (TO) and microencapsulation efficiency (ME) of spray dried and freeze dried complex coacervate microcapsules.

3.4. Conclusions

The complex coacervation process between WPI and GA was optimised in terms of pH and WPI-to-GA ratio. The WPI-GA complex coacervates were used to microencapsulate omega-3 rich tuna oil. Solid microcapsules of tuna oil were produced through spray drying and freeze drying. The optimal complexation pH and WPI-to-GA ratio were found to be 3.75 and 3:1, respectively. The spray dried microcapsules were found to be more stable against oxidation compared to those prepared by freeze drying. The spray dried microcapsules had the advantage of higher microencapsulation efficiency and lower surface oil content compared to the freeze dried samples. We conclude that WPI-GA complex coacervates can effectively microencapsulate omega-3 rich oils such as tuna oil and the solid microcapsules produced using spray drying will have high encapsulation efficiency and stability against oxidation.

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Chapter 4

Co-encapsulation and characterization of omega-3 fatty acids and probiotic bacteria in whey protein isolate-gum Arabic complex coacervates

Abstract

Omega-3 fatty acids and probiotic bacteria were co encapsulated in a single whey protein isolate (WPI)-gum Arabic (GA) complex coacervate microcapsule. Tuna oil (O) and L. casei 431 (P) were used as models of omega-3 and probiotic bacteria, respectively. The co microcapsules (WPI-P-O-GA) and L. casei containing microcapsules (WPI-P-GA) were converted into powder by using spray and freeze drying. The viability of L. casei was significantly higher in WPI-P O GA co microcapsules than in WPI-P-GA. The oxidative stability of tuna oil was significantly higher in spray dried co capsules than in freeze dried ones.

Key words: Omega-3 fatty acids, Probiotic bacteria, Microencapsulation, Whey protein isolate-gum Arabic complex coacervates, Spray drying, Freeze drying

4.1. Introduction

Probiotic bacteria are live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). Interest in the consumption of foods that contain probiotic bacteria is increasing steadily and probiotic products account for approximately 65 % of the world functional food market (Agrawal, 2005; Burgain et al., 2011). Maintaining the viability of probiotic bacteria in food during manufacture, storage, and delivery to the targeted site of gastrointestinal tract is still a major challenge (Ross et al., 2005; Mattila-Sandholm et al., 2002). The viability of probiotic bacteria depends on many factors such as strain of the bacteria, composition of food in which they are incorporated, and the processing conditions used (Saarela et al., 2000).

Lactobacillus and *Bifidobacterium* are the most commonly studied probiotics because of their proven health benefits and also their long history of use in foods (Parvez et al., 2006). *Lactobacillus casei* 431 is used as a model probiotic bacterium in this study. It has been reported that *Lactobacillus casei* is beneficial to people suffering from perennial allergic rhinitis (Peng et al., 2005). Supplementation of *L. casei* 431 is shown to improve the immune function by augmenting systemic and mucosal immune responses (Rizzaridini et al., 2012). *L. casei* has been successfully used in a number of cheese varieties and other fermented food products because it is acid tolerant (Mishra et al., 2005).

Omega-3 fatty acids are nutritionally essential for good health and are particularly recommended for individuals suffering from diseases such as cardiovascular disorders, Alzheimer's disease, immune response disorders, diabetes and cancer (Luchtman et al., 2013; Wen et al., 2014; McNamara et al., 2006; Rossmeisl et al., 2014). There is a high

commercial demand for omega-3 fatty acids; for example, the estimated global demand for omega-3 ingredients was worth USD 1595 million in 2010 and is estimated to exceed USD 4000 million in 2018 (Transparency Market Research, 2012). Fish oil usually contains a higher amount of omega-3 fatty acids than seed oils and microalgae (Rubio-Rodríguez et al., 2010). Oils containing a high amount of omega-3 fatty acids are highly susceptible to oxidation. The oxidation of omega-3 fatty acids produces peroxides that later convert to volatile compounds, some of which produce off-flavours and ultimately decrease the nutritional value of omega-3 fatty acids (Miyashita et al., 1993). Therefore, technologies to improve the stability and prolong the shelf-life of fish oil are necessary to enable the use of these oils in the food industry (Kamal-Eldin et al., 2002; Kaushik et al., 2014).

Microencapsulation is a technology used to protect bioactive unstable food materials. This technology retards or prevents rapid degradation and inactivation either by slowing down or by preventing degradation processes until the product is delivered to the desired site (Gouin, 2004; Barrow et al., 2009). The bioactive food compounds include not only polyunsaturated fatty acids, antioxidants, vitamins, peptides, phytosterols, lutein, lycopene but also living cells such as probiotic bacteria (Champagne et al., 2007; McClements et al., 2009; de Vos et al., 2010). A review of current literature suggests that the microencapsulation of omega-3 fatty acids and probiotic bacteria has so far been carried out separately (Pedroso et al., 2012; Chen et al., 2013; Tonon et al., 2011; Capela et al., 2006; Kim et al., 2008; Heinzelmann et al., 1999). Co-encapsulation of omega-3 fatty acids and probiotic bacteria in a single matrix has not been described. Halwani et al. (2008) reported that co-encapsulation of more than one core materials in an encapsulation system

that may enhance the bioactivity of individual components. Co-encapsulation concept has been widely used in pharmaceutical delivery systems, but the co-encapsulation of more than one bioactive component with different characteristics such as omega-3 oil (hydrophobic) and probiotic bacteria (hydrophilic) is challenging particularly using complex coacervation and has not been reported.

When probiotic bacteria and omega-3 fatty acids are co-encapsulated in a single product, there may be synergistic health benefits. A synergetic effect between omega-3 fatty acids and probiotic bacteria during digestion has been reported, where omega-3 lipids help probiotic bacteria attach to the intestinal wall (Das, 2002). There may also be stability benefits of co-microencapsulation. This study represents the first systematic attempt to develop a single microcapsule capable of delivering omega-3 fatty acids and probiotic bacteria together in one particle.

Microencapsulation by coacervation is carried out by phase separation of two or more biopolymers in a solution, followed by the subsequent deposition of the formed coacervate layer around the core material (Gouin, 2004). The liquid microcapsule obtained from the complex coacervation process can be dried using a variety of drying techniques. However, this step of the process is to remove the water and not to form the microcapsule. In complex coacervation, the size and type of particle formed is controlled during the wet process and not by the drying process, unlike spray dried emulsions. The microencapsulation of omega-3 rich tuna oil in whey protein isolate (WPI) – gum Arabic (GA) complex coacervates has been explored in detail (Eratte et al., 2014). The current work investigates on the microencapsulation of probiotic (P) bacteria *L. casei* 431 in WPI-

GA complex coacervate matrix forming WPI-P-GA microcapsules and the co-encapsulation of *L. casei* 431 and tuna oil (O) in WPI-GA matrix, forming WPI-P-O-GA microcapsules through complex coacervation. The novelty or significance of this study lies in the fact that it investigates whether probiotic bacteria and omega-3 fatty acids can be co-encapsulated in WPI-GA complex coacervate and if such co-encapsulation contributes synergistically to the survival of bacteria and the oxidative stability of the omega-3 fatty acids. This study compares the viability of probiotic bacteria in co-microcapsules (WPI-P-O-GA) with their viability in microcapsules containing only probiotic bacteria (WPI-P-GA) and substantiates the benefits of using WPI-GA complex coacervates for co-encapsulation of probiotic bacteria and omega-3 fatty acids.

A dry powder is desirable for extending the shelf-life and broadening the range of food applications and is normally formed using spray drying or freeze drying (Gouin, 2004). Freeze drying is suitable for drying sensitive biological materials as it exerts less thermal stress on the materials being dried; but is very expensive, has low throughput and a long drying time. Compared to freeze drying, the operational and capital costs of spray drying are reportedly 1/6 and 1/9, respectively, per unit mass of the product (Chavez and Ledeboer, 2007). However, the spray drying process can cause high viability and vitality loss as a result of simultaneous dehydration, thermal, and oxygen stresses imposed to bacteria during the drying process (Fang et al., 2012; Ghandi et al., 2013). To date, the drying behaviour of liquid capsules containing probiotic bacteria and omega-3 fatty acids has not been reported. Moreover, physicochemical characteristics of WPI-P-GA and WPI-P-O-GA microcapsules have not been studied in detail.

This study has the following key objectives: (1). To microencapsulate probiotic bacteria *L.casei* 431 in WPI-GA complex coacervates in order to produce probiotic bacteria containing microcapsules (WPI-P-GA). (2). To co-encapsulate probiotic bacteria *L.casei* 431 and omega-3 rich tuna oil in WPI-GA complex coacervates to produce WPI-P-O-GA microcapsules. (3). To produce solid microcapsules of WPI-P-GA and WPI-P-O-GA through spray and freeze drying. (4). To compare the viability (of *L.casei* 431) and oxidative stability (of omega-3 oil) in WPI-P-GA and WPI-P-O-GA microcapsules. (5). Finally to determine the moisture content, water activity, amorphous/crystalline nature and the surface elemental composition of the above mentioned solid microcapsules and the WPI-GA complex coacervates.

4.2. Materials and Methods

4.2.1. Materials

L. casei 431 was kindly donated by Chr.Hansen (Horsholm, Denmark). Tuna oil (HiDHA), containing was a gift from NuMega Ingredients Ltd. (Melbourne, Australia) and stored at 4 ^{o}C until use. According to data provided by the supplier, the major fatty acids in this tuna oil were as follows: DHA (docosahexaenoic acid) 29.4%, EPA (eicosapentaenoic acid) 6.0%, decosapentaenoic acid (DPA) 1.2%, arachidonic acid 2.0%, stearidonic acid 0.6%, linolenic acid 0.4%, linoleic acid 1.2%, oleic acid 12.8%, stearic acid 5.3%, palmitic acid 19.2%, myristic acid 2.4%. Whey protein isolate (WPI 895^{TM}) was donated by Fonterra Cooperative (Melbourne, Australia). Gum Arabic was purchased from Sigma-Aldrich Ltd. (Sydney, Australia). All other chemicals were purchased from Sigma–Aldrich Ltd. (Sydney, Australia) and were of analytical grade.

4.2.2. Preparation of *L.casei* 431 culture

The *L.casei* 431 was cultured for 18 h in sterile MRS broth (1%, w/v) at 37° C. This culture was further sub-cultured (37° C, 18 h) twice in the same broth to activate bacteria and to allow them to adapt. All the inoculation works were done under sterile biological hoods with laminar air flow. Finally, 1000 mL of media were used for bulk culturing and cells were harvested at stationary growth phase by centrifuging at 2200 × g for 15 min at 5° C. The supernatant was discarded and the precipitated cell mass was washed twice with sterile peptone water (0.2%, w/v). The final wet cell mass was weighed and divided into two equal portions. One portion was used to prepare WPI-P-GA microcapsules and the second half was used to prepare WPI-P-O-GA in order to make a good comparative study. For cell enumeration, spread plating method was carried out at 37° C for 48 h under anaerobic conditions. MRS agar medium was used to determine the number of colony forming units (cfu/mL).

4.2.3. Microencapsulation process

The complex coacervation and microencapsulation procedures were carried out as detailed below:

4.2.3.1. Preparation of WPI-P-GA liquid microcapsules

WPI (7.5g; 3%, w/v) solution was prepared in deionised water at ambient temperature. One portion of the probiotic cell mass prepared as detailed in Section 4.2.2 was added slowly into this WPI solution. Then GA solution (2.5 g; 1%, w/v) was added drop wise into this dispersion with continuous stirring at 400 rpm. The pH of the mixed dispersion was then

adjusted to 3.75 by adding 1% citric acid drop wise in order to induce electrostatic interaction between WPI and GA. The microencapsulation procedure was carried out at 25° C, followed by keeping the liquid microcapsules at 5° C for 48 h to ensure the complete formation of complex coacervates.

4.2.3.2. Preparation of WPI-P-O-GA liquid microcapsules

Firstly, WPI solution (7.5 g; 3%, w/v) was prepared at ambient temperature and 15 g of tuna oil was dispersed in this solution. The mixture was stirred using a mechanical stirrer (IKA® RW 20, Staufen, Germany) at 800 rpm for 10 min and was further homogenized using a microfluidizer (M110L, Microfluidics, Newton, Massachusetts, USA) at 45 MPa for 3 passes to produce an O/W emulsion. The second half portion of probiotic cell mass prepared as detailed in Section 4.2.2 was added into this O/W emulsion. Then GA solution (2.5g; 1%, w/w) was added drop wise into this dispersion with continuous stirring at 400 rpm. The pH of this emulsion was then adjusted to 3.75 by adding 1% citric acid drop wise in order to induce electrostatic interaction between WPI and GA. The microencapsulation procedure was carried out at 25° C, and the liquid microcapsules were kept at 5° C for 48 h to ensure complete formation of complex coacervates. A microscope (Vanox, Olympus Universal Research Microscope, Tokyo, Japan) was used to obtain optical images of the coacervates microcapsules.

4.2.4. Drying of coacervate microcapsules

A portion of suspension containing microcapsules produced as per Section 4.2.3 was spray dried (Mini spray dryer B-290, BÜCHI Labortechnik, Flawil, Switzerland) using inlet and outlet temperatures of 180 and 80 ° C, respectively. The spray dried solid microcapsules were collected and stored at 5 ° C for further characterization.

The second portion of the liquid microcapsules was frozen at -20° C overnight and was subsequently freeze dried (Christ Alpha 2-4LD, Osterode, Germany). The temperature of the ice condenser was set at -50° C and the vacuum pressure was set to 0.04 mbar. The frozen samples were dried for 30 h and the dried product was collected, pulverized and stored at 5° C for further tests.

4.2.5. Determination of physicochemical characteristics of WPI-P-GA and WPI-P-O-GA microcapsules

4.2.5.1. Oxidative stability

Accelerated oxidation tests were carried out on the oil and the solid WPI-P-O-GA microcapsules using a Rancimat (model 743, Metrohm, Herisau, Switzerland) (Mathäus, 1996). Four ml tuna oil or 1.5 g dried microcapsule powder was heated at 90° C under purified air (flow rate of 20L/h). The oxidative stability index (*OSI*) value of the samples is graphically determined by locating rapid change in slope of the conductivity versus time curve as described by Wang et al. (2014).

4.2.5.2. Microencapsulation efficiency

Microencapsulation efficiency was calculated by measuring the surface oil (solvent extractable) and total oil of the microcapsules. Surface oil was determined by the washing method described earlier by Liu et al. (2010) with slight modification as described in our previous paper (Eratte et al., 2014).

The total oil content in the dried microcapsules was determined by an acid digestion method using 4M HCl according to Eratte et al. (2014). The percent surface oil (SO), total oil (TO) and microencapsulation efficiency (ME) were calculated using equations (2), (3) and (4), respectively.

$$SO = \frac{w_s}{w_m} \times 100\% \quad (2)$$

$$TO = \frac{w_t}{w_m} \times 100\% \quad (3)$$

$$ME = \frac{w_t - w_s}{w_t} \times 100\% \quad (4)$$

where, w_t and w_s are the mass values (g) of total and surface oil of the microcapsules, respectively and w_m is the mass (g) of the microcapsules.

4.2.5.3. Surface morphology of the solid microcapsules

A scanning electron microscope (JEOL JSM6300 SEM, Tokyo, Japan) was used to acquire the morphology of dried microcapsules. Samples were lightly gold sputter coated (Sputter coater, Agar Aids, Stansted, UK) for 45 seconds and imaged under scanning electron microscope operated at 7kV and low beam current.

4.2.5.4. X-ray diffraction analysis

X-ray diffraction (XRD) traces were obtained from the powdered samples using a Siemens (D500, Siemens, Karlsruhe, Germany) diffractometer. Ni-filtered CuK_α radiation was used and the operating conditions were 40kV/30mA, 0.02θ/(2θ) step scan at 1° (2θ)/min, 1° divergence and receiving slits and a 0.15° scatter slit. A scan range of 5° to 50° (2θ) was used.

4.2.5.5. XPS analysis

The X-ray photoelectron spectroscopy was performed with an AXIS Ultra-DLD spectrometer (Kratos Analytical Ltd., Manchester, UK) equipped with a monochromated Al Kα X-ray source and a hemispherical analyser (fixed analyser transmission mode).

4.2.5.6. Moisture content and water activity

The moisture content of powdered microcapsules was determined by drying the sample in a hot air oven at 105° C for 12 h. The water activity of the powdered microcapsules was

determined using a water activity meter (Novasina, Switzerland). The temperature during the water activity tests was maintained at 24 ± 0.5 ° C.

4.2.6. Statistical Analysis

The Minitab 16 statistical software package (Minitab, Sydney, Australia) was used for the analysis of variance (ANOVA) to determine the significant differences. The confidence level of 95% ($p<0.05$) was used.

4.3 Results and discussions

4.3.1. Effect of WPI-GA complex coacervates, omega-3 oil and drying methods on the viability of *L. casei* 431

The viability of *L. casei* 431 in WPI-P-GA and WPI-P-O-GA liquid and solid microcapsules are presented in Fig. 4.1. As can be observed, there was about one log cycle loss of *L. casei* viable cells in liquid WPI-P-GA microcapsules (8.7 log cfu ml^{-1}) compared to the control (9.5 log cfu ml^{-1}) due to complex coacervation in the absence of omega-3 oil. This may be due to the stress on the *L. casei* 431 cells owing to the reduction of pH to 3.75 at which optimum complex coacervation between WPI and GA occurs (Eratte et al., 2014). Interestingly, there was no significant difference in viability ($p < 0.05$) of *L. casei* in liquid WPI-P-O-GA microcapsules (9.3 log cfu ml^{-1}) from the initial cell count.

The survival of *L. casei* in both liquid and solid microcapsules was much better in the presence of omega-3 oil (WPI-P-O-GA) than in its absence (WPI-P-GA). In order to verify whether or not the better survival of *L. casei* in WPI-P-O-GA is due to the effect of

omega-3 fatty acids, we repeated the entire microencapsulation process using coconut oil which does not readily undergo oxidation. The viability of *L. casei* cells in the presence of the coconut oil was as high as the viability in omega-3 rich oil (data not shown). Thus, it can be concluded that the viability of *L. casei* in currently used encapsulation process can be improved in the presence of oil whether or not it is omega-3 rich.

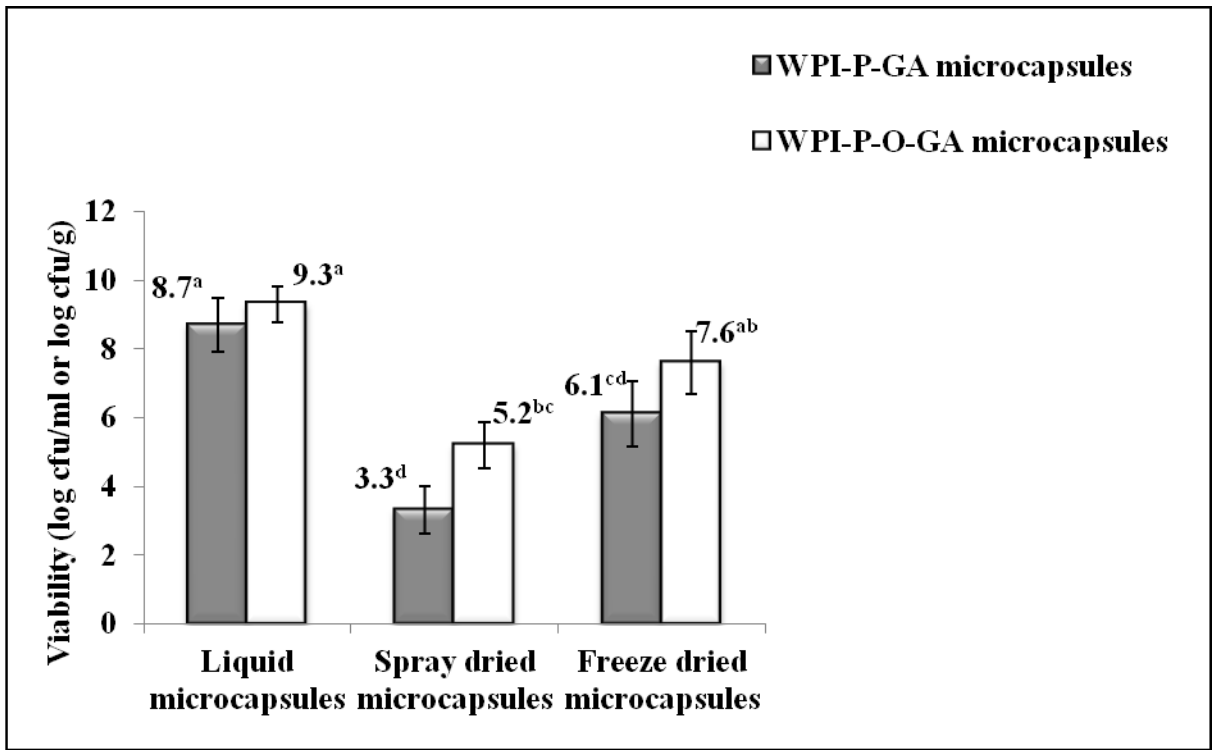


Fig. 4.1: Viability of *L. casei* 431 in WPI-P-GA and WPI-P-O-GA in liquid microcapsules and in dried powders.

[a,b,c,d]: Means followed by different letters are significantly different ($p < 0.05$).

The possible reason for the better survival of *L. casei* cells in WPI-P-O-GA microcapsules than in WPI-P-GA microcapsules can be explained with the aid of phase contrast micrographs (Fig. 4.2). As shown by the phase contrast micrographs, the composite microcapsules containing tuna oil and bacterial cells were formed at pH 3.75 in the case of WPI-P-O-GA microcapsules (Fig. 4.2F). The micrographs of WPI-P-GA (Fig 4.2A and

Fig. 4.2B) and WPI-P-O-GA microcapsules (Fig 4.2D and Fig. 4.2E) at pH 6.0 and pH 4.0 are presented to provide the process of formation of complex coacervates and the liquid microcapsules. It can be observed from Fig. 4.2C that the rod shaped bacterial cells are dispersed in the WPI-GA complex coacervate matrix at the optimum pH (3.75). In the case of WPI-P-O-GA microcapsules, aggregation of oil droplets occurred and each oil droplet was covered with the WPI-GA complex coacervates. This assembly better embedded the bacterial cells in the WPI-GA matrix. The embedding of the *L.casei* in the WPI-GA complex coacervates and the encapsulation of tuna oil in the *L. casei* embedded complex coacervate as capsule shell might have minimized the exposure of *L. casei* 431 cells to the acid stressor and this may have improved the cell viability. Lahtinen et al. (2007) reported a higher survival of *bifidobacteria* when immobilised in cocoa butter during storage. Pedroso et al. (2012) also reported a better survival of microorganisms encapsulated in lipid matrix than unencapsulated microorganisms in simulated gastric and intestinal fluids. Picot et al (2004) reported that diffusion of acid (H^+ ions) and oxygen in lipid based capsules are restricted and thus can provide further protection to the probiotic bacteria. The same authors reported that the whey protein based microcapsules can improve the survival of *bifidobacteria* in high acidic condition. Thus, we can conclude here that the presence of tuna oil in the microcapsules improved the survival of *L. casei* 431 in the liquid microcapsules.

The viability of *L. casei* cells in WPI-P-GA and WPI-P-O-GA solid microcapsules produced through spray and freeze drying is also presented in Fig. 4.1. The viability of *L. casei* cells was found to be higher in the presence of tuna oil in solid microcapsules as well. Therefore, we can conclude here that viability of *L. casei* cells could be improved by co-encapsulating or formulating dry WPI-P-O-GA microcapsules. The viability of *L. casei* in spray dried microcapsules was significantly lower ($p>0.05$) as compared to their viability in freeze dried microcapsules. This lower survival rate can be attributed to the higher inlet (180^{o} C) and outlet (80^{o} C) temperatures used and the associated thermal and dehydration induced stresses. These findings are similar to those reported by Rajam et al. (2012), Wang et al. (2004) and Chavez et al. (2007) who showed that the survival of bacterial cells was higher in freeze drying than in spray drying operations. However, Ying et al. (2010) reported that there was no difference in the loss of viability of *Lactobacillus rhamnosus GG* after spray and freeze drying whereas Perdana et al. (2013) reported that viability of probiotic bacteria in spray drying was higher than that in freeze drying.

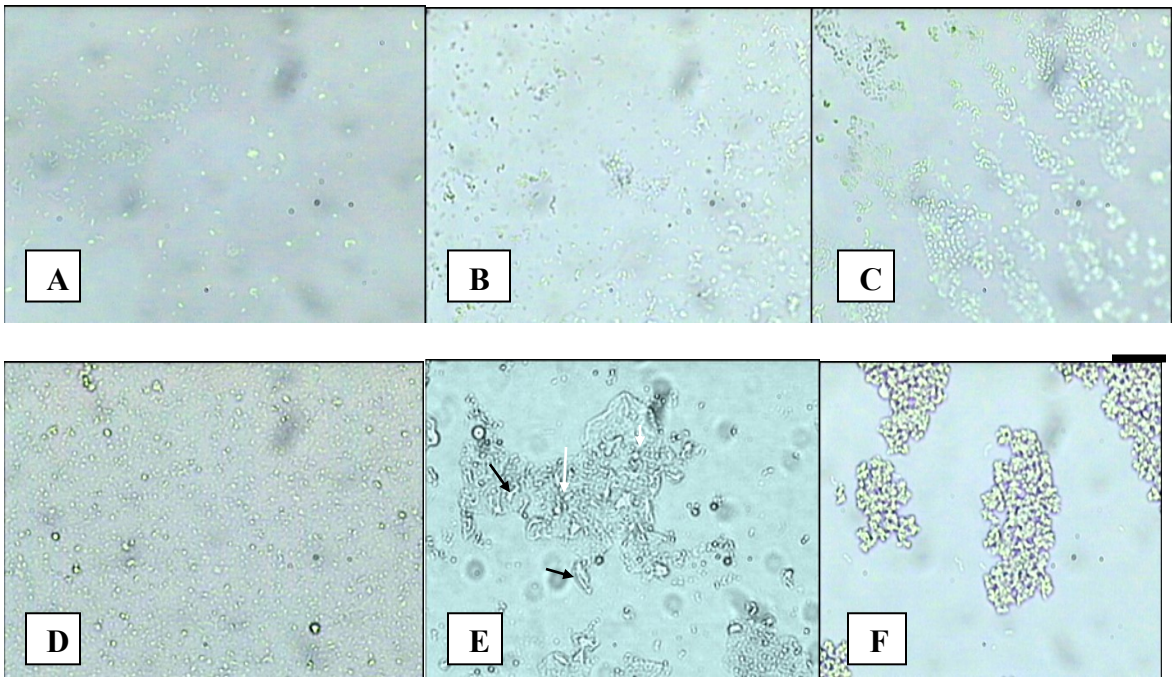


Fig. 4.2: Phase contrast micrographs of WPI-P-GA and WPI-P-O-GA liquid microcapsules as a function of pH. (A) WPI-P-GA microcapsules at pH 6.0; (B) WPI-P-GA microcapsules at pH 4.0; (C) WPI-P-O-GA microcapsules at pH 3.75; (D) WPI-P-O-GA microcapsules at pH 6.0; (E) WPI-P-O-GA microcapsules at pH 4.0; (F) WPI-P-O-GA microcapsules at pH 3.75. The black arrow represents rod shaped *L. casei* cells and white arrow represents round shaped oil droplets. Scale bar represents 10 μm.

4.3.2. Effect of drying methods on the physicochemical characteristics of the solid microcapsules

The physicochemical characteristics (moisture content, water activity, oxidative stability, surface oil, total oil, oil microencapsulation efficiency, survival of *L. casei* and morphological features) of spray and freeze dried WPI-P-GA and WPI-P-O-GA microcapsules are presented and discussed in this section.

Table 4.1: Physiochemical properties of spray dried and freeze dried WPI-P-GA and WPI-P-O-GA microcapsules. SD= spray dried, FD = Freeze dried, SO= Surface oil, TO = Total oil, ME = Microencapsulation efficiency

Microcapsules	Residual moisture content (%)	Water activity	SO (%)	TO (%)	Oil ME (%)	Cell survival (%)
SD WPI-P-GA	2.90± 0.2^{a}	0.23± 0.004^{d}	-	-	-	37.62^{d}
FD WPI-P-GA	3.94± 0.3^{a}	0.29± 0.003^{b}	-	-	-	70.23^{bc}
SD WPI-P-O-GA	3.19± 0.2^{b}	0.26± 0.014^{c}	3.3± 0.66^{a}	48.95± 2.2^{a}	93.35± 3.01^{a}	56.19^{cd}
FD WPI-P-O-GA	4.03± 0.3^{b}	0.32± 0.012^{a}	11.3± 2.5^{b}	47.57± 1.3^{a}	76.28± 5.1^{b}	84.95^{ab}

^{a,b,c,d}:Means followed by different letters within the same column are significantly different at $p < 0.05$.

The moisture content (Table 4.1) of both the spray and freeze dried microcapsules produced was lower than 4.03 % (w/w), and water activity was below 0.32. The moisture content and water activity values of freeze dried WPI-P-GA and WPI-P-O-GA microcapsules were slightly higher ($p<0.05$) than the corresponding values of spray dried microcapsules. On the other hand, WPI-P-O-GA microcapsules have higher moisture content ($p>0.05$) than that of WPI-P-GA microcapsules. It was reported that residual water contents of 4 % (w/w) or below improved the storability and stability of powders containing probiotic bacteria *L. salivarius* UCC 118 and *L. paracasei* NFBC 338 (Gardiner et al., 2000).

The OSI values of tuna oil (control), spray and freeze dried WPI-P-O-GA microcapsules are presented in supplementary data (Fig. 4.1S). As expected, the oxidative stability of WPI-P-O-GA microcapsules produced through both drying methods was higher than that of untreated tuna oil. However, there was a significant difference ($p>0.05$) in the oxidative stability between freeze and spray dried microcapsules. The spray dried WPI-P-O-GA microcapsules were more stable to oxidation (OSI= 14.2 h) than the freeze dried microcapsules (OSI= 7.5 h). Similar results were obtained in our previous work in which microencapsulation of omega-3 oil was carried out using WPI-GA complex coacervates in the absence of *L. casei*. The possible reason for this lower OSI in freeze dried WPI-P-O-GA microcapsule could be due to the relatively higher surface oil content (11.3%, w/w) compared to that (3.3%, w/w) in spray dried WPI-P-O-GA microcapsules (Table 4.1). The larger pore size, the presence of microscopic pores and rougher surface morphology of freeze dried microcapsules compared to spray dried ones (Fig. 4.3) may have resulted in lower oxidative stability. The oil encapsulation efficiency of freeze dried WPI-P-O-GA

microcapsules (76.28%) was significantly ($p>0.05$) lower than that of spray dried WPI-P-O-GA microcapsules (93.35%). However, the survival of *L. casei* in freeze dried microcapsules was higher than that in spray dried microcapsules. The survival of *L. casei* in WPI-P-O-GA microcapsules was higher than that in WPI-P-GA microcapsules, irrespective of the drying method (Table 4.1).

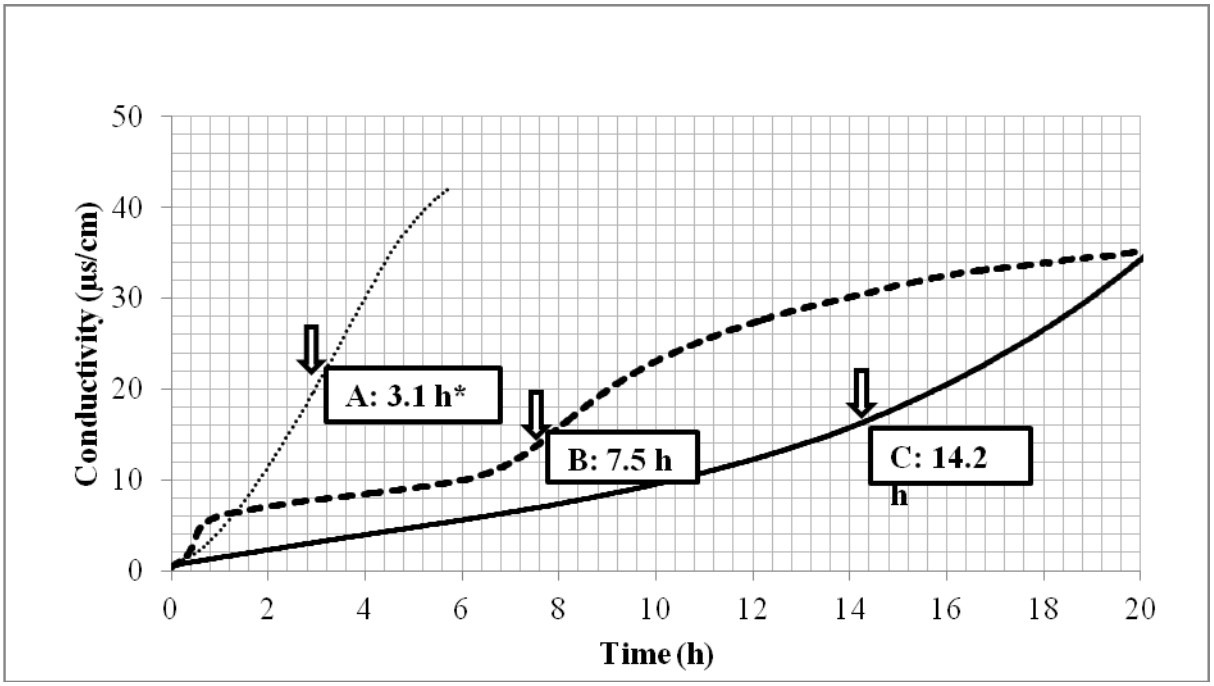


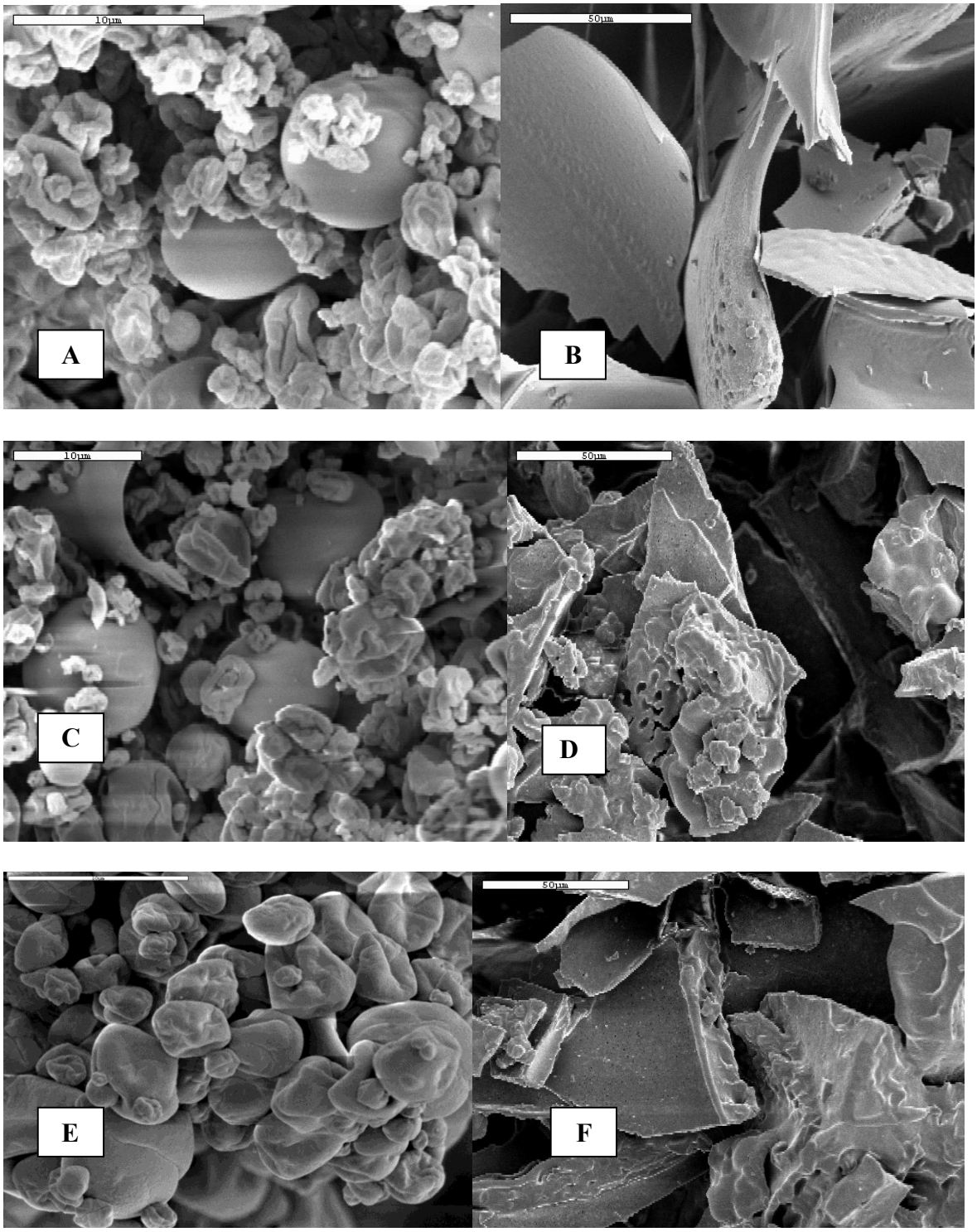
Fig. 4.1S: Oxidative stability index (OSI) of microcapsules measured by accelerated oxidation test using Rancimat^{TM}. A represents blank tuna oil (* taken from our previous work for comparison), B represents freeze dried WPI-P-O-GA and C represents spray dried WPI-P-O-GA with their corresponding OSI values in h.

The surface morphology of spray dried and freeze dried WPI-GA microcapsules, WPI-P-O-GA and WPI-P-GA microcapsules are presented in Fig. 4.3. The freeze dried microcapsules showed flake like porous morphology, while the spray dried microcapsules showed more or less spherical shape in various size with concavities typical of materials produced by spray drying. The spray dried WPI-P-O-GA microcapsules (Fig 4.3E) were

smoother with less concavities compared to the spray dried WPI-GA (control) complex coacervates, as well as the WPI-P-GA microcapsules (Fig. 4.3A and 4.3C). This smoother and lesser concavity in the case of spray dried WPI-P-O-GA microcapsules indicated that the presence of oil droplets in the microcapsules might have reduced the extent of moisture evaporation and also the extent of shrinkage of particles during drying. This might have been one of the reasons for retention of higher moisture content in WPI-P-O-GA microcapsules than in WPI-P-GA microcapsules (Table 4.1). The formation of concavities on the surface of the spray dried particles can be attributed to the shrinkage of the particles during the drying process due to rapid evaporation of water (Saenz et al., 2009). Therefore, the presence of oil in the liquid microcapsules is able to make the surface morphology of spray dried microcapsules smoother with less concavities.

The surface morphology of the freeze dried WPI-P-GA and WPI-P-O-GA microcapsules was quite similar (Fig. 4.3D and 4.3F). The SEM micrographs of freeze dried WPI-GA (control) microcapsules (Fig. 4.3B) showed much smoother surface compared to that in WPI-P-GA and WPI-P-O-GA microcapsules. The core materials were dispersed in the wall matrix which resulted into less smooth morphology in the case of freeze dried WPI-P-GA and WPI-P-O-GA microcapsules. The moisture content of freeze dried WPI-P-O-GA microcapsules was higher ($p > 0.05$) than that of freeze dried WPI-P-GA microcapsules. Therefore, the presence of oil might have acted as a vapour transport barrier during the sublimation process. The SEM micrographs (Fig. 4.3) revealed that there were no bacterial cells on the surface of both spray and freeze dried microcapsules indicating that *L. casei* cells were embedded within the wall matrix. The cross sectional SEM micrographs show that the composite matrix containing omega-3 oil and *L. casei* cells embedded in WPI-GA

coacervates was formed in the complex-coacervation followed by drying process (Fig. 4.3G and 4.3H); therefore WPI-P-O-GA composite microcapsules can be represented graphically as in Fig. 4.3I.



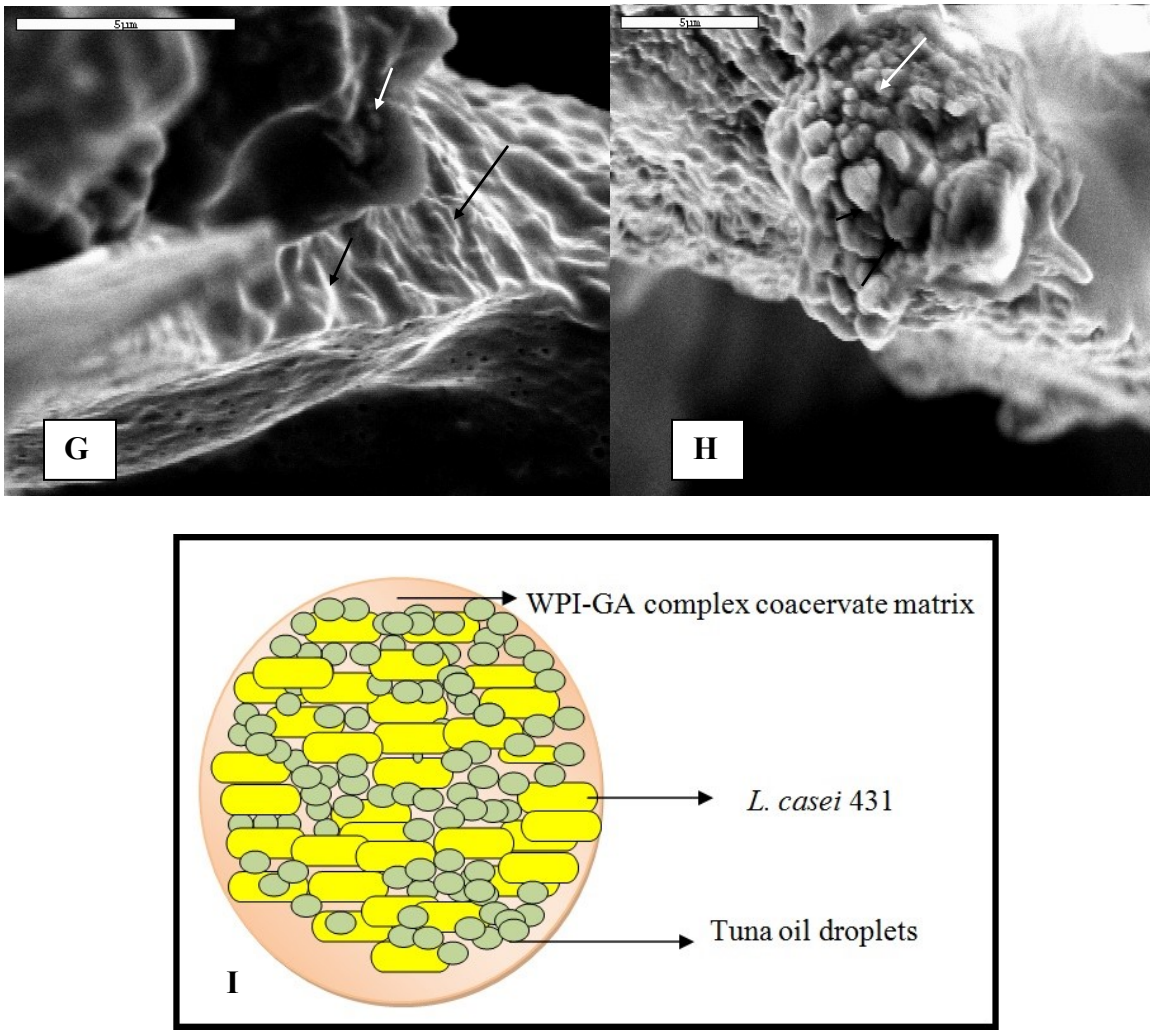


Fig. 4.3: SEM micrographs of solid microcapsules. (A) Spray dried WPI-GA microcapsules (control); (B) Freeze dried WPI-GA microcapsules (control); (C) Spray dried WPI-P-GA microcapsules; (D) Freeze dried WPI-P-GA microcapsules; (E) Spray dried WPI-P-O-GA microcapsules; (F) Freeze dried WPI-P-O-GA microcapsules; (G) Cross sectional images of freeze dried WPI-P-O-GA microcapsules; (H) Cross sectional image of spray dried WPI-P-O-GA microcapsules. The black arrow represents rod shaped L. casei cells and white arrow represents round shaped oil droplets. (I) Graphical representation of WPI-P-O-GA microcapsules inferred from SEM analysis. Scale bar 10

μm and 50 μm for spray dried and freeze dried microcapsules. Scale bar 5 μm for the cross sectional images.

4.3.3. Crystallinity/amorphous nature of the solid microcapsules

The crystalline/amorphous nature of the carrier materials (WPI and GA), spray dried and freeze dried microcapsules can be observed from supplementary data (Fig. 4.2S-A, Fig. 4.2S-B and Fig. 4.2S-C, respectively). It can be clearly seen from these figures that the carrier materials and microcapsules showed amorphous pattern irrespective of the drying methods used. Amorphous matrixes are known to be more soluble as well as more hygroscopic and they are able to release core material more readily (Botrel et al., 2014).

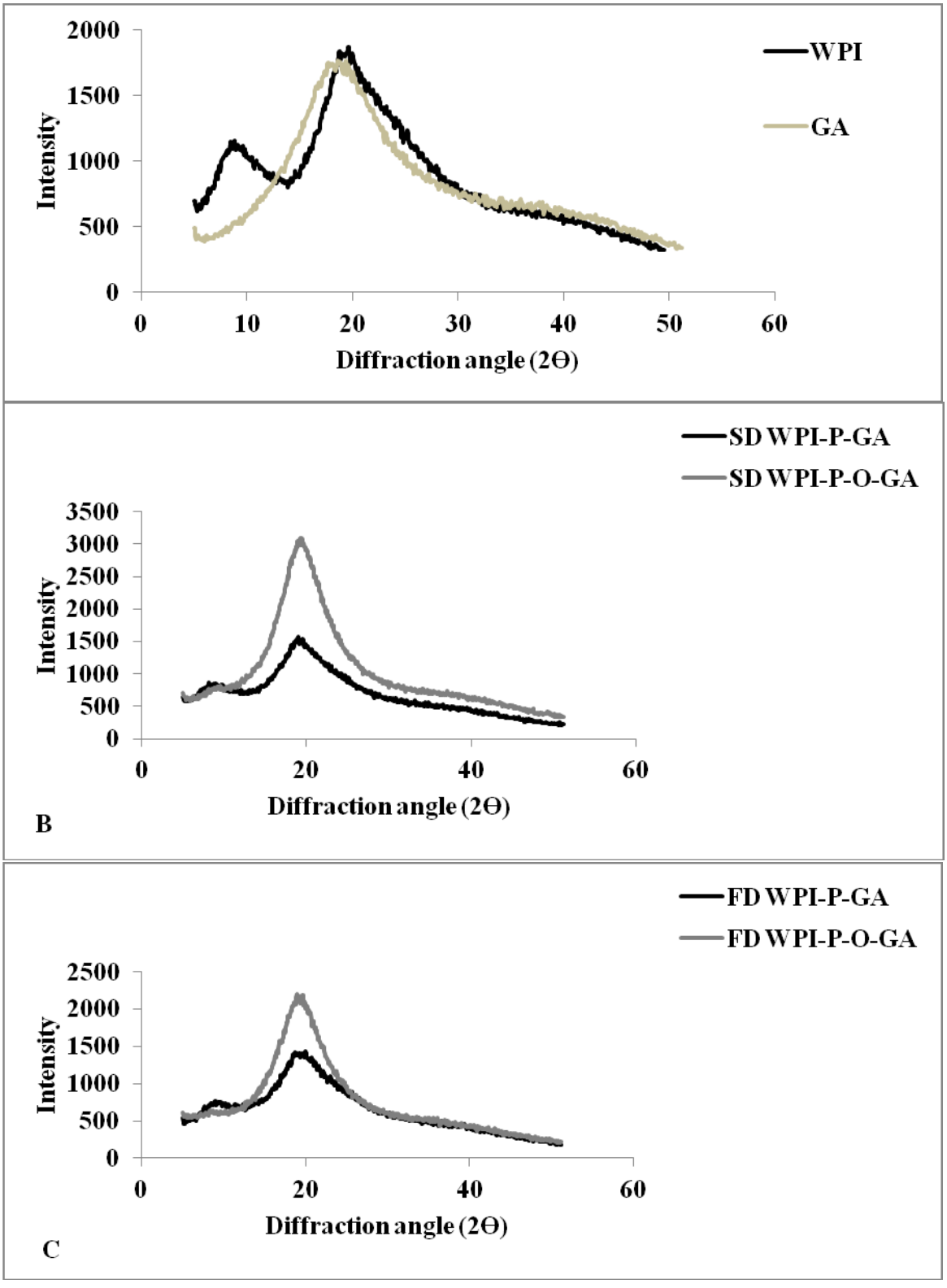


Fig. 4.2S: XRD patterns of: (A) Carrier materials (WPI and GA); (B) Spray dried microcapsules; (C) Freeze dried microcapsules (WPI-P-GA and WPI-P-O-GA).

4.3.4. Surface elemental analysis of microcapsules

XPS analysis was used to quantify the surface (20-100 Å) elemental and chemical composition of microcapsules, WPI, GA and freeze dried *L. casei* cells. Table 4.2B and 4.2C compares the oxygen and nitrogen concentrations as well as the concentrations of chemically different carbon species (all relative to the total concentration of carbon) of spray dried and freeze dried microcapsules (WPI-P-GA, WPI-P-O-GA) to those of WPI, GA and the *L. casei* cells (Table 4.2A). The concentration of different carbon species were determined by curve-fitting the high resolution C 1s spectra. The curve fit protocol is based on five components with the following assignments: C1+C2 (C-C, C-H), C3 (C-O, C-N), C4 (C=O, O-C-O, N-C=O), C5 (O-C=O). The relevant C 1s high resolution spectra are shown in Fig 4.4. The XPS analysis of oil was not carried out as it is in liquid form and therefore not compatible with ultra-high vacuum.

Table 4.2: Surface elemental composition (C, O, N) of (A) WPI, GA, L. casei, (B) spray-dried and (C) freeze-dried microcapsules. Presented are the mean (± std. dev.) of two measurements (atomic concentrations relative to total concentration of carbon).

Samples	WPI	GA	*L.casei*	FD cell pellets
C1+C2	0.741 ± *0.004*	0.168 ± *0.001*	0.158 ± *0.011*	0.411 ± *0.001*
C3	0.189 ± *0.006*	0.591 ± *0.001*	0.601 ± *0.015*	0.412 ± *0.006*
C4	0.041 ± *0.005*	0.212 ± *0.003*	0.210 ± *0.000*	0.137 ± *0.008*
C5	0.029 ± *0.003*	0.030 ± *0.001*	0.032 ± *0.004*	0.039 ± *0.002*
O	0.215 ± *0.003*	0.666 ± *0.000*	0.680 ± *0.016*	0.410 ± *0.001*
N	0.025 ± *0.000*	0.014 ± *0.000*	0.015 ± *0.001*	0.075 ± *0.001*

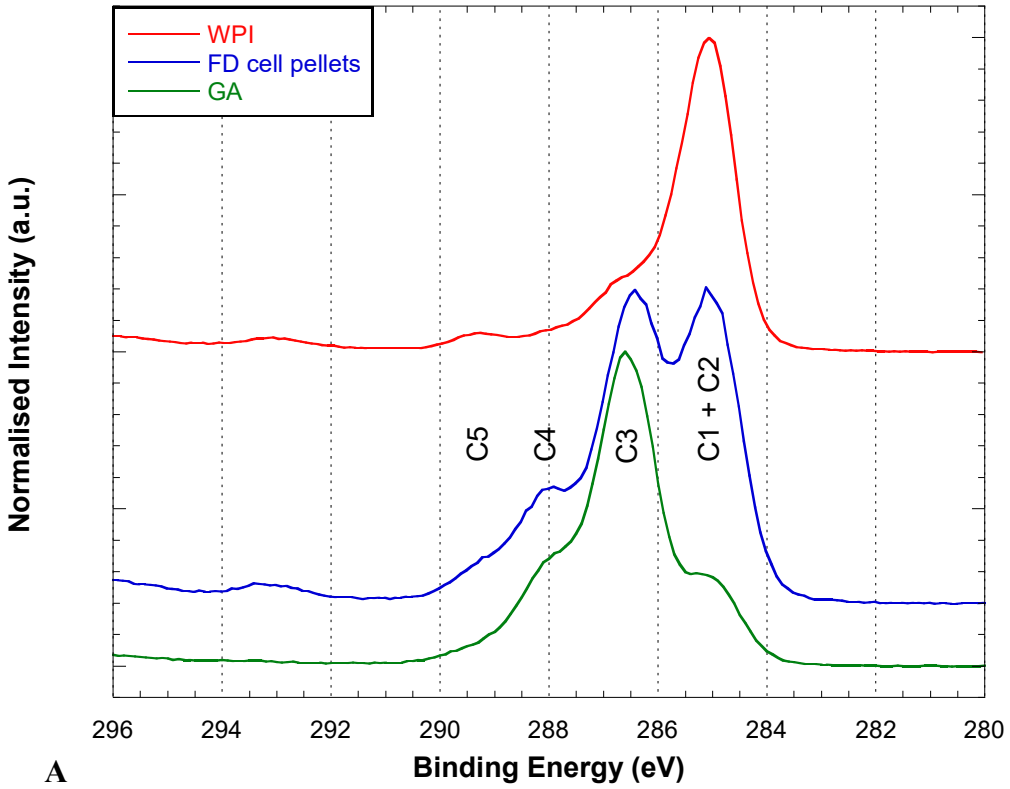
(A)

Samples	SD WPI-GA coacervates (control)	SD WPI-P-GA	SD WPI-P-O-GA
C1+C2	0.456 ± *0.002*	0.477 ± *0.002*	0.837 ± *0.001*
C3	0.275 ± *0.003*	0.273 ± *0.002*	0.098 ± *0.003*
C4	0.169 ± *0.002*	0.181 ± *0.005*	0.014 ± *0.002*
C5	0.100 ± *0.003*	0.068 ± *0.005*	0.051 ± *0.000*
O	0.428 ± *0.006*	0.377 ± *0.001*	0.119 ± *0.001*
N	0.167 ± *0.001*	0.175 ± *0.001*	0.014 ± *0.002*

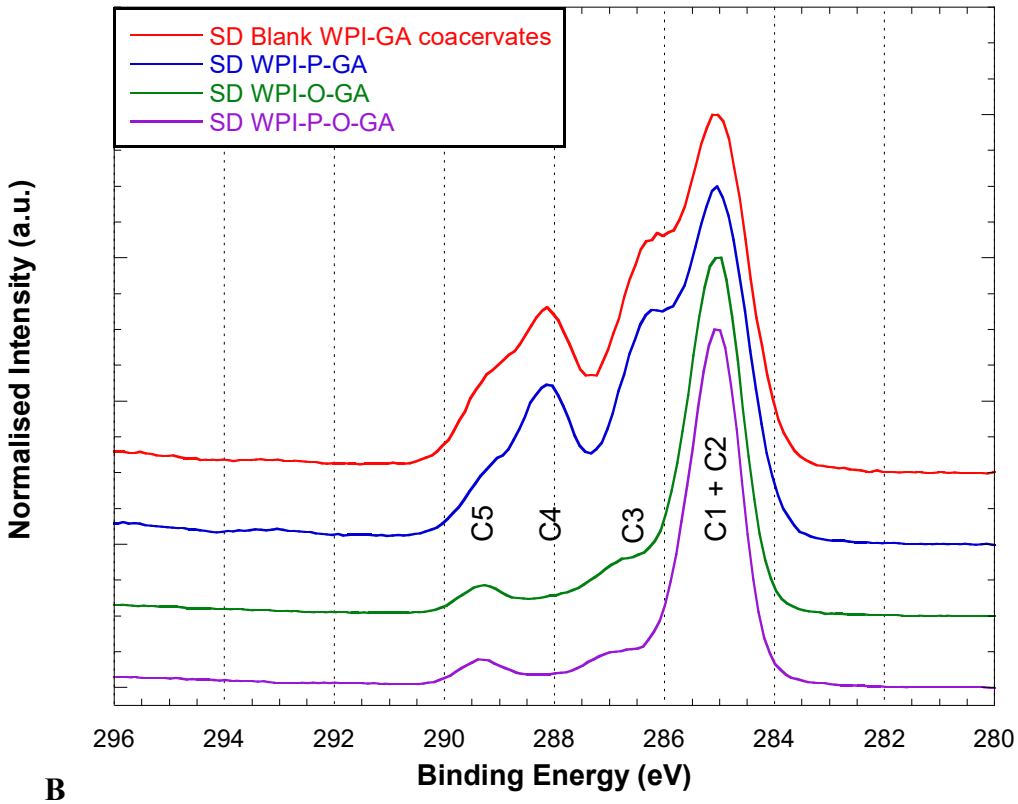
(B)

Samples	FD WPI-GA coacervates (control)	FD WPI-P-GA	FD WPI-P-O-GA
C1+C2	0.292 ± *0.006*	0.467 ± *0.015*	0.838 ± *0.001*
C3	0.460 ± *0.001*	0.339 ± *0.013*	0.096 ± *0.006*
C4	0.150 ± *0.004*	0.133 ± *0.002*	0.013 ± *0.004*
C5	0.097 ± *0.003*	0.060 ± *0.001*	0.052 ± *0.001*
O	0.591 ± *0.008*	0.407 ± *0.017*	0.129 ± *0.007*
N	0.020 ± *0.001*	0.035 ± *0.001*	0.006 ± *0.001*

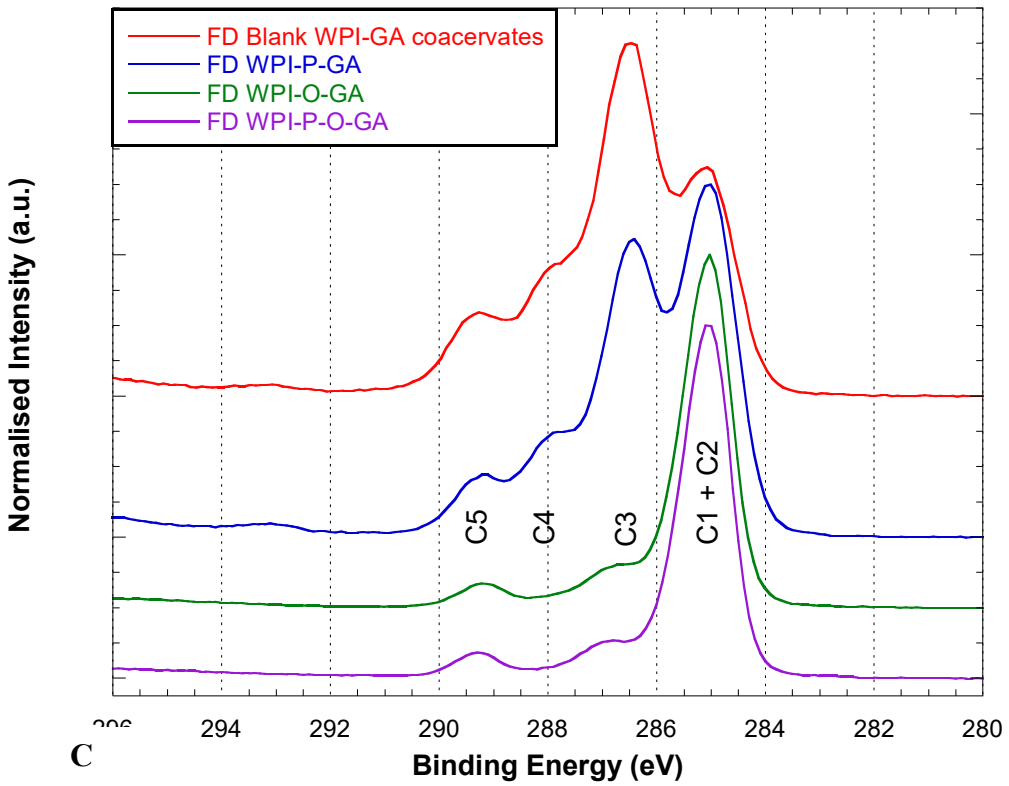
(C)


WPI
FD cell pellets
GA
Normalised Intensity (a.u.)
C5
C4
C3
C1 + C2
296
294
292
290
288
286
284
282
280
Binding Energy (eV)

A


SD Blank WPI-GA coacervates
SD WPI-P-GA
SD WPI-O-GA
SD WPI-P-O-GA
Normalised Intensity (a.u.)
C5
C4
C3
C1 + C2
296
294
292
290
288
286
284
282
280
Binding Energy (eV)

B



C

Fig. 4.4: C1s spectra of (A) shell material (WPI and GA) and freeze dried *L. casei* cells; (B) Spray dried microcapsules; (C) Freeze dried microcapsules. See text for details.

The C 1s spectrum of WPI is characterised by a particularly strong CH_x (C1+C2) contribution. This is not characteristic of proteins in general and indicates the presence of (most likely) lipids on the surface. This is confirmed by the very low level of nitrogen (uncharacteristic of proteins) as presented in Table. 4.2A. The C 1s of GA showed a typical polysaccharide spectrum, dominated by the C-O (C3) peak and a corresponding strong oxygen signal but almost no nitrogen (Fig. 4.4A). The data of freeze dried *L. casei* cells appear to combine characteristics of polysaccharides (prominent C-O peak together with high oxygen level) and proteins (presence of nitrogen) (Fig. 4.4A).

The application of spray and freeze drying to the same formulation results into different surface composition in the dried microcapsules. The surface composition of WPI-GA (control) coacervates produced through spray and freeze drying showed characteristics of both WPI and GA (Fig. 4.4A and 4.4B). However, the O-C=O (C5) peak, indicative of acid/ester groups, is significantly more intense compared to the those of WPI and GA suggesting to some interaction between WPI and GA occurring during the complex coacervation process. Spray-dried microcapsules had higher protein concentration at the surface (high level of nitrogen) whereas the freeze dried microcapsules had higher concentration of polysaccharides (high levels of oxygen and C-O) on the surface. The former may be due to greater exposure of the air-droplet interface (very high surface to volume ratio) and also due to the inability of proteins to counter diffuse when the water is rapidly evaporated. Moreover, whey protein instantly forms a skin when it comes in contact with hot air (Adhikari et al., 2007). This difference between spray and freeze drying (spray-dried microcapsules consistently had much higher concentration of protein on the surface) was observed for all the microcapsules (WPI-O-GA, WPI-P-GA and WPI-P-O-GA).

The surface composition of WPI-P-GA microcapsules was similar to that of WPI-GA (control) produced by both drying methods. It indicates that the bacterial cells were embedded within the complex coacervates and were not exposed to the surface. In the case of oil containing microcapsules, the XPS data clearly shows that the surface of these microcapsules was coated with the oil. The C 1s spectra of all the oil containing microcapsules are almost identical showing shapes and peaks typical of lipids: a very

intense CH_x peak (C1+C2), accompanied by two weaker peaks characteristic of ester groups (C-O and O-C=O).

4.4. Conclusions

Probiotic bacteria *L. casei* 431 was successfully encapsulated in WPI-GA complex coacervate matrix. *L casei* and omega-3 rich tuna oil were also successfully co-encapsulated in WPI-GA complex coacervate matrix. The liquid capsules containing bacterial cells and also bacterial cells together with tuna oil were converted into powder form using spray and freeze drying. The viability of the *L. casei* bacteria was significantly ($p>0.05$) higher when co-encapsulated with tuna oil in WPI-GA complex coacervates rather than being encapsulated on its own in the same shell matrix. The oxidative stability of tuna oil was greatly improved in the WPI-GA complex coacervates in the presence and absence of the bacterial cells. The surface elemental composition showed that in the spray dried microcapsules had high surface protein while freeze dried microcapsules had high surface carbohydrate when oil was not incorporated in the formulation. In oil-containing microcapsules, the surface of both spray and freeze dried microcapsules was consistently found to be enriched in oil. All the microcapsules produced were amorphous in nature. Composite microcapsules containing omega-3 fatty acids and probiotic bacteria will be useful for the stabilised delivery of these two important functional ingredients together into functional food and nutraceutical applications.

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Chapter 5

Survival, oxidative stability and surface characteristics of spray dried co-microcapsules containing omega-3 fatty acids and probiotic bacteria

Abstract

The objective of the study was to determine optimum inlet and outlet air temperatures of spray process for producing co-microcapsules containing omega-3 rich tuna oil and probiotic bacteria *L. casei*. These co-microcapsules were produced using whey protein isolate and gum Arabic complex coacervates as shell materials. Improved bacterial viability and oxidative stability of omega-3 oil were used as two main criteria of this study. Three sets of inlet (130° C, 150° C and 170° C) and outlet (55° C, 65° C and 75° C) air temperatures were used in nine combinations to produce powdered co-microcapsule. The viability of *L. casei*, oxidative stability of omega-3 oil, surface oil, oil microencapsulation efficiency, moisture content, surface elemental composition and morphology of the powdered samples were measured. There is no statistical difference in oxidative stability at two lower inlet air temperatures (130° C and 150° C). However, there was a significant decrease in oxidative stability when higher inlet temperature (170° C) was used. The viability of *L. casei* decreased with the increase in the inlet and outlet air temperatures. There was no difference in the surface elemental compositions and surface morphology of powdered co-microcapsules produced under these nine inlet/outlet temperature combinations. Of the range of conditions tested the co-microcapsules produced at inlet-outlet temperature 130-65° C showed the highest bacterial viability and oxidative stability of omega-3 and having the moisture content of 4.93 ± 0.05% (w/w). This research shows that powdered co-microcapsules of probiotic bacteria and omega-3 fatty acids with high

survival of the former and high stability against oxidation can be produced through spray drying.

Key words

Co-encapsulation, omega-3 fatty acids, probiotic bacteria, spray drying, surface composition and morphology

5.1. Introduction

The development of functional foods and nutraceuticals by incorporating bioactive ingredients, particularly omega-3 fatty acids, probiotic bacteria, and polyphenols is a significant focus of functional and nutritional food industries (Champagne et al., 2007; Olive Li et al., 2014). New nutraceutical and functional food products are being designed and manufactured to fulfil the growing demand for healthier foods (Earle, 1997; Annunziata et al., 2013). Two of the most widely utilised bioactive ingredients used in functional foods are probiotic bacteria (Nazzaro et al., 2012; Coman et al., 2012) and omega-3 fatty acids (Serfaty et al., 2014; Price Judge et al., 2014). The functional foods developed using probiotic bacteria and omega-3 oils have clinically proven health benefits (Kim et al., 2008; Nazzaro et al., 2012). Probiotic bacteria are a group of bacteria which are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit to the host' (FAO/WHO, 2001). *Lactobacillus* and *Bifidobacterium* are the most commonly studied probiotic bacteria because of their proven health benefits (Parvez et al., 2006; Gomes et al., 1999). *Lactobacillus (L.) casei* 431 is used as a model probiotic (P) bacterium in this study. Omega-3 fatty acids also have clinically proven health benefits in humans (Dinnetz et al., 2013). Tuna oil (O) is used as the source of omega-3 fatty acids

in this study. Whey proteins isolate (WPI) possesses very high nutritional value and good emulsifying properties (Sousa et al., 2012; Adhikari et al., 2007). Gum Arabic (GA) is a complex polysaccharide and it is commonly used as thickener and emulsifier in food formulations (Williams et al., 2009). For these reasons, these two are used as encapsulating wall materials.

Several functional foods are manufactured using microencapsulation technology which entraps potent and unstable core within relatively inert shell to enhance shelf-life and to achieve controlled release and targeted delivery (Desai et al., 2005; Sobel et al., 2014). Specifically, a number of microencapsulation techniques such as spray drying, fluidized bed drying, complex coacervation, extrusion, and emulsion have been used for the delivery of bioactive ingredients into food (Olive Li et al., 2014). Complex coacervation is generally regarded as a useful microencapsulation technology because it confers controlled and sustained release possibilities (Gouin, 2004). Complex coacervation based encapsulation technologies are utilized to protect bioactive ingredients such as omega-3 oil (Eratte et al., 2014), ascorbic acid (Comunian et al., 2013), sweet orange oil (Jun-Xia et al., 2011) and probiotic bacteria (Oliveira et al., 2007). The benefits of co-encapsulation of omega-3 oil and probiotic bacteria in WPI-GA complex coacervate matrix were reported in our previous work in terms of survival and oxidative stability (Eratte et al., 2015).

The liquid microcapsules produced through complex coacervation processes have to be converted into powder in order to increase shelf life, and to improve packaging, handling and storage properties (Anwar et al., 2011). Spray drying is one of the most hygienic and economical technologies used for microencapsulation and producing powders in food

industry (Re, 1998; Keshani et al., 2015). It is a thermally mild amongst the convective air drying methods because of its very short product residence time (Mazza et al., 2003). The spray drying is 4-7 times less expensive than that of freeze drying (Chavez et al., 2007). However, maintaining high viability of heat sensitive probiotic bacteria through spray drying is still a major challenge (Ghandi et al., 2012; Ghandi et al., 2013). Since spray drying is much more cost effective and it can process larger volumes and operate at higher energy efficiency compared to freeze drying, many studies endeavoured to optimize spray drying process parameters and product formulations towards highest probiotic viability and minimal activity losses (Knorr, 1998; Tripathi et al., 2014).

In our previous study, we investigated the co-microencapsulation of omega-3 oil and *L. casei* produced through complex coacervation followed by freeze and spray drying. It was found that spray dried co-microcapsule powders had advantages in terms of oxidative stability of omega-3 oil; however, the freeze dried co-microcapsules had higher viability of *L. casei.*[24] This work endeavours to optimise the spray drying process parameters so that co-microcapsules with higher bacterial viability as well as higher oxidative stability are produced. In addition, this study investigates the effect of inlet/outlet air temperatures on the physico-chemical and surface characteristics of spray dried co-microcapsules for the first time. These findings will benefit both the industry and the general public because of the synergistic health benefits of these powdered co-microcapsules. These co-microcapsules are robust enough to be incorporated in commonly used food items such as yoghurt and beverages.

This study had the following objectives: (1) To determine the optimum inlet/outlet air temperature combination to produce co-microcapsules (WPI-P-O-GA) with improved viability of probiotic bacteria and better oxidative stability of omega-3 oil. (2) To evaluate the effect of spray drying inlet/outlet air temperatures on the physico-chemical properties (moisture content, surface oil, total oil, oil microencapsulation efficiency) and morphological features and surface composition of co-microcapsules.

5.2. Materials and Methods

5.2.1. Materials

L. casei 431® was donated by Chr.Hansen (Horsholm, Denmark). Tuna oil (HiDHA), was obtained by courtesy of NuMega Ingredients (Melbourne, Australia) and stored at 4° C until used. According to data provided by the supplier, the major fatty acids in this tuna oil were as follows: DHA (docosahexaenoic acid) 29.4%, palmitic acid 19.2%, oleic acid 12.8%, EPA (eicosapentaenoic acid) 6.0%, stearic acid 5.3%, myristic acid 2.4%, arachidonic acid 2.0%, decosapentaenoic acid (DPA) 1.2%, linoleic acid 1.2%, stearidonic acid 0.6%, and linolenic acid 0.4%. Whey protein isolate (WPI 895™) was donated by Fonterra Cooperative (Melbourne, Australia). Gum Arabic was purchased from Sigma-Aldrich (Sydney, Australia). All other chemicals were purchased from Sigma–Aldrich Australia and were of analytical grade and used without further purification.

5.2.2. Preparation of probiotic cell pellets and co-microcapsules

The bacterial cell pellets and co-microcapsules were prepared as described in our earlier work (Eratte et al., 2015). Briefly, the *L. casei* was cultured for 18 h in sterile MRS broth

(1%, w/v) at 37° C. This culture was further sub-cultured (37° C, 18 h) twice in the same broth to activate bacteria and to allow them to adapt. All the inoculation works were carried out under sterile biological hoods with laminar air flow (Auramini, Laftech, Australia). Finally, 1000 mL of media was used for bulk culturing and cells were harvested at stationary growth phase by centrifuging at 2200×g for 15 min at 5° C. The supernatant was discarded and the precipitated cell mass was washed twice with sterile peptone water (0.2%, w/v) and these cell pellets were used for co-encapsulation process.

The co-encapsulation process was carried out as follows: Firstly, WPI solution (7.5 g; 3%, w/v) was prepared at ambient temperature and 15 g of tuna oil was dispersed in this solution. The mixture was stirred using a mechanical stirrer (IKA® RW 20, Staufen, Germany) at 800 rpm for 10 min and was further homogenized using a microfluidizer (M110L, Microfluidics, Newton, Massachusetts, USA) at 45 MPa for 3 passes to produce an O/W emulsion. The pure probiotic cell pellets prepared (wet mass 5g) as described above were then added into this O/W emulsion. Then GA solution (2.5 g; 1%, w/v) was added drop wise into this dispersion with continuous stirring at 400 rpm. The pH of this emulsion was then adjusted to 3.75 by adding 1% citric acid drop wise in order to induce complexation between WPI and GA. The co-microencapsulation procedure was carried out at 25° C, and the liquid co-microcapsules were kept at 5° C for 48 h to ensure complete formation of complex coacervates. Finally, the co-microcapsules were dried to produce solid or powder co-microcapsules (WPI-P-O-GA).

5.2.3. Spray drying of co-microcapsules

The liquid co-microcapsule samples prepared as described in Section 5.2.2 were spray dried (Mini spray dryer B-290, BÜCHI Labortechnik, Flawil, Switzerland) using three different inlet (130° C, 150° C, 170° C) and outlet (55° C, 65° C, 75° C) temperatures in nine combinations. The flow rate of the drying air was 35m^3/h at 100% fan aspiration. The pumping rate was adjusted to maintain the outlet air temperature (the feed rate varies from 1.2 mL/min to 3.6 mL / min). The powdered co-microcapsules were collected and stored at 5° C for further characterization. The entire process for the production of WPI-P-O-GA co-microcapsules was performed in triplicates for each inlet/outlet temperature set and the samples taken from three batches were used to determine their physico-chemical characteristics.

The thermal efficiency ($\eta_{thermal}$) of the spray drying operation was approximated using equation (1) (Hui, 2008).

$$\eta_{thermal}(\%) = \frac{(T_{inlet} - T_{outlet})}{(T_{inlet} - T_{ambient})} \times 100 \qquad (1)$$

where T_{inlet}, T_{outlet}, and $T_{ambient}$ are inlet, outlet and ambient (25° C) air temperatures, respectively.

5.2.4. Enumeration of bacterial viability in powdered co-microcapsules

The viability of *L. casei* in the co-microcapsules was assessed in MRS agar (CM0361, Thermo scientific, Melbourne, Australia) using spread plating technique as described in our earlier work (Eratte et al., 2015). Briefly, 1g of powder was diluted in 9 mL of sterile peptone water (0.2%, w/v). The bacterial cells were released from the capsules to the

medium using a stomacher. The cell suspension was serially diluted and plated on MRS agar plate and incubated under anaerobic condition (Oxoid™ Anaerojar™, Thermoscientific, Australia) at 37° C for 48h. The plating and enumeration were performed in triplicate and expressed viability as log cfu/g.

5.2.5. Assessment of oxidative stability

Accelerated oxidation tests were carried out on the powdered WPI-P-O-GA co-microcapsules using Rancimat™ test (model 743, Metrohm, Herisau, Switzerland) (Mathaus, 1996). Two grams of dried powder was heated at 90° C under purified air (flow rate of 20L/h). Briefly, when the encapsulated oil is oxidized, the conductivity of Milli-Q water in the collection chamber increases due to the increased solubility of volatile products (formic acid). The oxidative stability index (OSI) of the samples is graphically determined by locating tangential intersection point on experimental data (Eratte et al., 2014; Wang et al., 2014). The induction time (at which the conductivity of sample increases sharply due to oxidation) of the test sample was recorded and used as the OSI.

5.2.6. Microencapsulation efficiency

Microencapsulation efficiency (ME) was calculated by measuring the solvent extractable surface oil (SO) and total oil (TO) of the microcapsules. Surface oil was determined by the washing method (Eratte et al., 2014; Liu et al., 2010).

The total oil content in the dried microcapsules was determined by an acid digestion method using 4M HCl according to Eratte et al., (2014). The SO, TO and ME were calculated using equations (2), (3) and (4), respectively.

$$SO\ (\%) = \frac{W_s}{W_m} \times 100 \qquad (2)$$

$$TO\ (\%) = \frac{W_t}{W_m} \times 100 \qquad (3)$$

$$ME\ (\%) = \frac{W_t - W_s}{W_t} \times 100 \qquad (4)$$

where W_t and W_s are the mass (g) values of total and surface oil of the microcapsules and W_m is the mass (g) of the microcapsules.

5.2.7. Acquiring surface morphology of co-microcapsules

Scanning electron microscope (SEM) (JEOL JSM6300 SEM, Tokyo, Japan) was used to acquire the morphology of liquid, solid and reconstituted solid co-microcapsules. Solid samples were lightly gold sputter coated (Sputter coater, Agar Aids, England) for 45 seconds and imaged at 7kV and low beam current. The liquid co-microcapsules were subjected to additional treatment before gold sputter coating. Briefly, 20 mL of liquid co-microcapsules was fixed with 2% (w/v) glutaraldehyde in phosphate buffer (0.1 M; pH 7.2) for 12 h. The slurry containing the glutaraldehyde cross-linked co-microcapsules was dehydrated in ethanol at different concentrations (30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%), each for 30 min. The sample was transferred to a solution containing hexamethyldisilizane (HMDS) and 100% ethanol in the ratio of 1:2 and left undisturbed for 20 min. HMDS is used as an alternative of critical point drying. Then, the sample was transferred to a fresh solution with HMDS-to-100% ethanol ratio of 2:1 for another 20 min. Finally, the sample was transferred into 100% HMDS solution and capped loosely in the fume hood overnight. The HMDS evaporated and the resulting solid sample was gold sputter coated and imaged under SEM.

5.2.8. X-ray photoelectron spectroscopic (XPS) analysis

The surface chemical analysis was performed using an AXIS Ultra-DLD spectrometer (Kratos Analytical Ltd., UK) equipped with a monochromatic Al Kα X-ray source and a hemispherical analyser (fixed analyser transmission mode). Elemental compositions were determined from low-resolution survey spectra. High resolution C1s spectra were acquired to obtain detailed information about chemical map on the surface of the co-microcapsules.

5.2.9. Moisture content

The moisture content of powdered co-microcapsules was determined by drying the sample in a hot air oven at 105° C for 12 h. The dried samples were allowed to cool to room temperature in a desiccator containing silica gel before measuring sample mass.

5.2.10. Statistical analysis

All measurements were performed at least in triplicates unless otherwise stated and the results are reported as the mean ± standard deviation. The Minitab 17 statistical software package (University Park, Pennsylvania, United States) was used for the analysis of variance (ANOVA). The significant difference between any two means values was determined using Turkey test. A 95% ($p<0.05$) confidence level was used in all the cases.

5.3. Results and discussion

5.3.1. Effect of inlet-outlet air temperatures on the physico-chemical properties of co-microcapsules

In order to determine the optimal spray drying parameters of producing powdered co-microcapsules, the highest possible survival of encapsulated *L. casei,* highest oxidative stability (highest OSI) of encapsulated tuna oil and residual moisture content of 5% (w/w) were set as three overarching criteria.

5.3.1.1. Viability of co-encapsulated probiotic bacteria

The viability of *L. casei* in these powdered co-microcapsules produced at various inlet/outlet air temperature combinations is presented in Fig. 5.1. For a given inlet temperature, it was observed that there was a decrease in the viability of *L. casei*, when the outlet temperature increased from 55° C to 75° C. Various studies have indicated the importance of outlet air temperature associated with the bacterial viability (Knorr., 1988; Koc et al., 2010). As the droplet and particles are exposed to the outlet temperature in most of their residence time; hence, low outlet air temperatures better protect cells from being over-heated and thus improve the viability (Fu et al., 2013). Fig. 5.1 shows that the survival of *L. casei* in the co-microcapsules is lower at higher inlet temperatures. Similar findings on the effect of inlet temperatures on bacterial viability have been reported by others (Ghandi et al., 2012; Atalar et al., 2015). Therefore, it can be concluded here that the viability of *L. casei* in these co-microcapsules decreased with increase in inlet as well as outlet air temperatures. The highest bacterial survival in these co-microcapsules was observed at inlet/outlet temperature combination of 130/55° C (7.3 log cfu/g) followed by at 130/65° C and 150/55° C (7.1 log cfu/g). The survival of bacteria at these temperature

combinations was not significantly different ($p>0.05$) (Fig. 5.1). The lowest viability of *L. casei* was obtained at inlet/outlet temperature combination of 170/75° C (5.5 log cfu/g). These observations indicate that both inlet and outlet temperatures of spray dryer affect the survival of *L. casei* in WPI-P-O-GA co-microcapsules and that higher inlet and outlet air temperatures are detrimental to the viability of *L. casei* in these co-microcapsules.

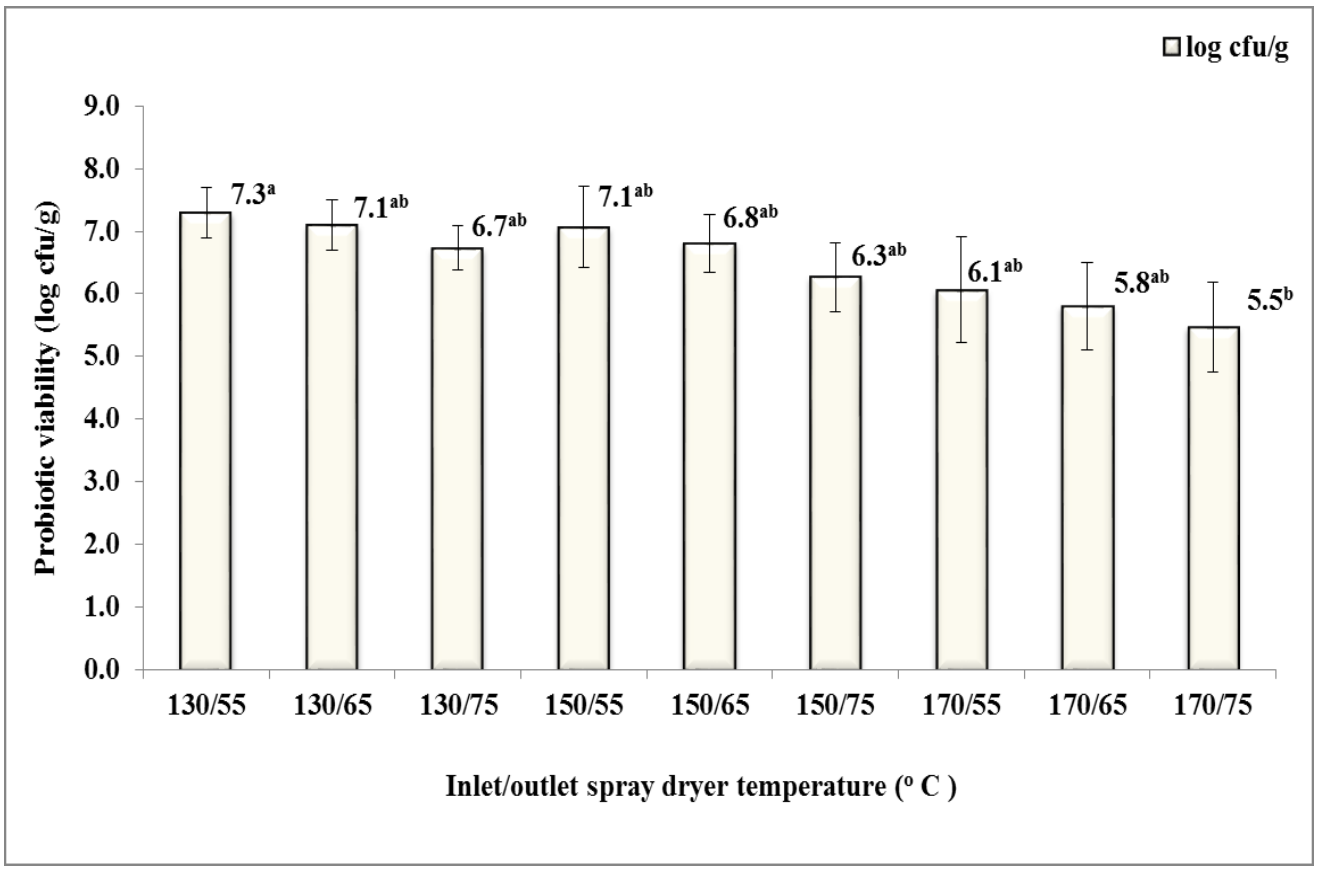

Fig. 5.1: Survival of *L. casei* in spray dried WPI-P-O-GA co-microcapsules at different inlet/outlet air temperature combinations. The labelled data corresponds to the viability in log (cfu/g). The bars with different letter in superscript indicate significant difference ($p < 0.05$).

5.3.1.2. Oxidative stability of co-encapsulated omega-3 oil

The OSI values of powders produced at different inlet/outlet temperature combinations are presented in Fig. 5.2. The OSI values of pure tuna oil (control) was measured as 3.1 h (Eratte et al., 2014). The oxidative stability of co-microcapsules produced at different inlet/outlet temperature combinations was significantly higher than that of untreated tuna oil. As can be observed, there is no statistical difference in OSI values at two lower inlet air temperatures (130° C and 150° C). However, there was a significant decrease in OSI value when higher inlet temperature (170° C) was used.

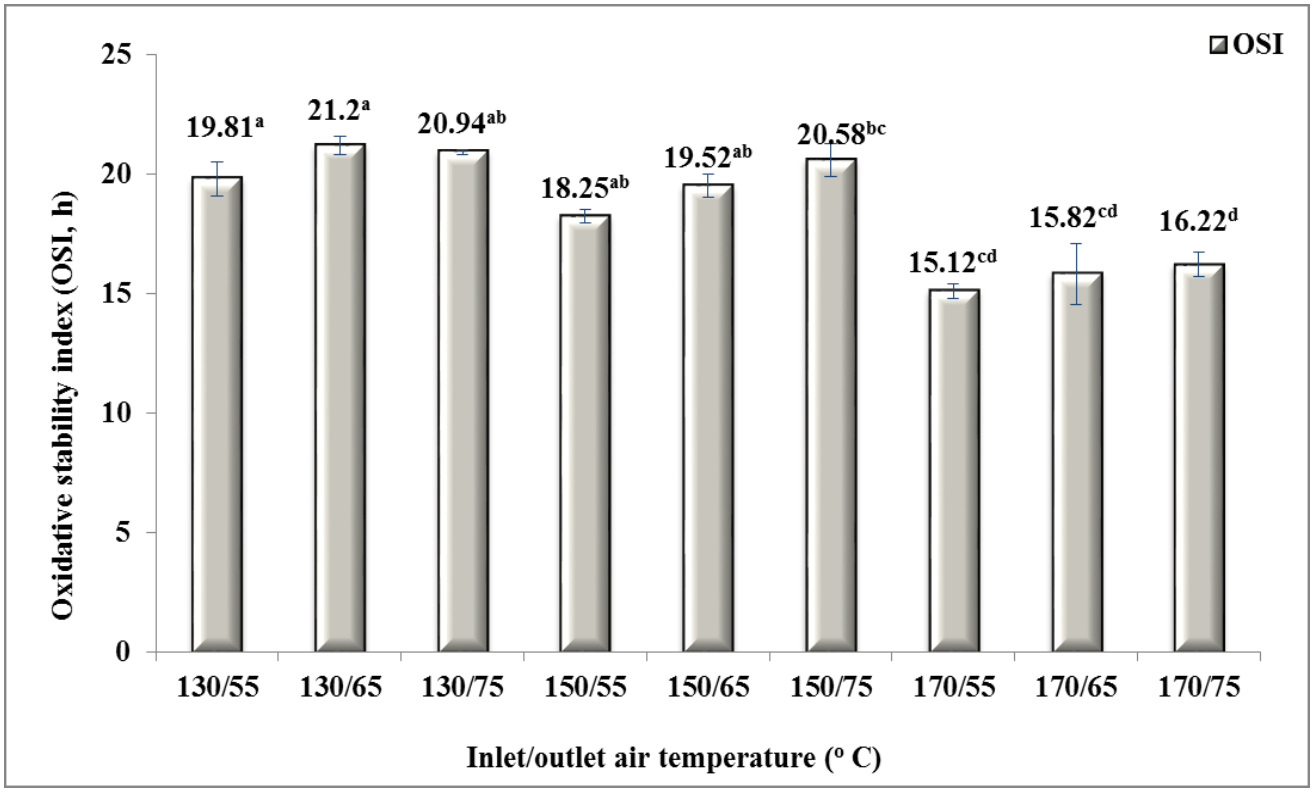

Fig. 5.2: Oxidative stability index (OSI) of encapsulated tuna oil in spray dried WPI-P-O-GA co-microcapsules at different inlet/outlet air temperature combinations. The labelled data indicates the oxidative stability index (OSI). Different lower case letters in superscript indicate significant difference ($p < 0.05$).

A large temperature gradient between the droplet/particle and the drying air can occur at high inlet temperature, resulting into faster heat, and moisture transfer rates. Faster outward diffusion of vapour when resisted by microcapsule shell of low moisture diffusivity can create higher number of cracks for unit surface area which can increase the inward diffusion or permeation of the oxygen. It was previously reported that high inlet air temperatures cause excessive bubble growth and surface imperfections (Jafari et al., 2008). This perhaps is the reason for lower OSI values in co-microcapsules dried at higher inlet temperature. Thus, so far it is technically and economically feasible, lower inlet air temperatures should be selected to produce oxidatively stable co-microcapsule powders. The highest and lowest OSI values of 21.2 h and 15.12 h were obtained at inlet/outlet combinations of 130/65° C and 170/55° C, respectively. The OSI values of the co-microcapsules increased with increase in the outlet air temperatures, for each inlet temperature tested except for 130° C (Fig. 5.2).

5.3.1.3. Physico-chemical characteristics of co-microcapsules

The residual moisture content, surface oil, total oil, and oil microencapsulation efficiency of spray dried co-microcapsules produced at different inlet/outlet air temperatures are shown in Table 5.1. As expected, the residual moisture content of powder decreased with increase in the inlet as well as outlet air temperatures in all the cases. This is due to the fact that the relative humidity decreases substantially at higher temperature, and it creates higher vapour gradient between the bulk air and the droplet/particle surface. The effect of higher dryer inlet and outlet temperatures showed similar trend in residual moisture content and bacterial survival. For example, the lowest residual moisture content and the lowest bacterial survival were observed at inlet/outlet temperature combination of 170/75° C.

Similarly the highest residual moisture content and the highest bacterial survival was observed at inlet/outlet temperature combination of 130/55° C. Given the residual moisture content of powders obtained from all these temperature combinations was close to 5% (w/w), due to highest bacterial survival and highest oxidative stability the inlet/outlet temperature combination of 130/65° C appeared to be optimal to produce WPI-P-O-GA co-microcapsule powder. The thermal efficiency calculated at the inlet/outlet temperature combination of 130/65° C was found to be 61.90% (Table 5.1).

There was no significant difference in surface oil (SO), total oil (TO) and oil microencapsulation efficiency (ME) in the spray dried WPI-P-O-GA co-microcapsules produced at different inlet/outlet temperature combinations (Table 5.1). This suggests that the complex coacervate matrix rather than the spray drying process determines the amount of surface oil on the WPI-P-O-GA co-microcapsules. This observation agrees with earlier findings that the surface oil in spray dried powders is not affected by outlet (Danviriyakul et al, 2002) and inlet (Klinkesorn et al., 2006) air temperatures. The ME depends on the amount of surface oil of the microcapsules in addition to the degree to which the wall matrix can hold or store the oil within (Hogan et al., 2001). Therefore, we can conclude here that surface oil and, hence, the microencapsulation efficiency of these co-microcapsules were mainly affected by the complex coacervate wall matrix rather than the inlet and outlet air temperatures used.

Table 5.1: Physico-chemical properties of spray dried WPI-P-O-GA co-microcapsules and thermal efficiency of spray drying process at different inlet/outlet air temperatures. SO = Surface oil, TO = Total oil, ME = Oil microencapsulation efficiency, $\eta_{thermal}$ = Thermal efficiency of spray drying process.

Inlet/outlet temperature (° C)	Residual moisture content (%)	SO (%)	TO (%)	Oil ME (%)	$\eta_{thermal}$ (%)
130/55	5.03 ± 0.07^{a}	2.85 ± 0.28^{a}	48.25 ± 1.11^{a}	94.08 ± 0.60^{a}	71.43
130/65	4.93 ± 0.05^{ab}	2.68 ± 0.70^{a}	47.57 ± 2.09^{a}	94.32 ± 1.68^{a}	61.90
130/75	4.72 ± 0.05^{abc}	2.80 ± 0.73^{a}	48.74 ± 1.40^{a}	94.24 ± 1.59^{a}	52.38
150/55	4.66 ± 0.33^{abcd}	2.83 ± 0.87^{a}	48.86 ± 0.85^{a}	94.23 ± 1.69^{a}	76.00
150/65	4.27 ± 0.28^{bcde}	2.96 ± 0.43^{a}	48.63 ± 1.50^{a}	93.91 ± 0.81^{a}	68.00
150/75	4.07 ± 0.51^{cdef}	2.61 ± 1.05^{a}	47.62 ± 1.50^{a}	94.49 ± 2.35^{a}	60.00
170/55	3.96 ± 0.30^{def}	3.14 ± 1.27^{a}	47.81 ± 1.27^{a}	93.39 ± 2.84^{a}	79.31
170/65	3.69 ± 0.23^{ef}	3.07 ± 1.07^{a}	48.66 ± 0.57^{a}	93.69 ± 2.18^{a}	72.41
170/75	3.32 ± 0.20^{f}	2.97 ± 0.79^{a}	47.87 ± 1.70^{a}	93.81 ± 1.46^{a}	65.52

Considering the bacterial viability, OSI and moisture content, it appears that the inlet temperatures >130° C and <170° C and outlet temperatures >55° C and < 75° C will produce high quality WPI-P-O-GA co-microcapsules in terms of viability of probiotic bacteria and oxidative stability of omega-3 fatty acids. The co-microcapsules produced at the inlet/outlet air temperatures of 130/65° C satisfied the three criteria of the highest

possible survival of the *L. casei* cells, improved oxidative stability of microencapsulated tuna oil and residual moisture content of <5% (w/w).

5.3.2. Morphology and surface characteristics of WPI-P-O-GA co-microcapsules

5.3.2.1. Morphology of co-microcapsules

Many important properties of microcapsules, such as protection of core materials, powder stability depend on their surface characteristics and surface morphology (Klinkesorn et al., 2006). The morphology of liquid co-microcapsules observed under phase contrast and SEM microscope (scale bar = 5 μm) is shown in Fig. 5.3A and Fig. 5.3B, respectively. The reconstituted spray dried co-microcapsules with additional biological treatment (Section 5.2.7) was observed under SEM to investigate the morphological change of rod shaped bacteria after drying, which is shown in Fig. 5.3C. The rod shaped bacteria (represented by white circles) and spherical oil droplets (represented by white arrows) are clearly distinguishable in the matrix (Fig. 5.3B and 5.3C). We observed that the morphology of the liquid co-microcapsules changed when observed under SEM (Fig. 5.3B) compared to the one observed under phase contrast (Fig. 5.3A), probably due the pre-treatment required before SEM imaging (Section 5.2.7). The sample pre-treatment was carried out in order to observe the structural change of bacterial cell before and after spray drying. There was no noticeable alteration in the bacterial cell structure (rod shape) in liquid co-microcapsules (Fig. 5.3B) and in reconstituted spray dried co-microcapsules (Fig. 5.3C). This can be attributed to the mild drying condition (inlet/outlet combination of 130/65° C), strong evaporative cooling effect, short residence time and resultant lower extent of bacterial denaturation (Arpagaus et al., 2012; Perdana et al., 2013; Eratte et al., 2016). Preservation

of structural integrity of bacteria during drying is important for preservation of their viability and vitality.

The SEM micrographs of spray dried WPI-P-O-GA solid co-microcapsules produced at different inlet/outlet temperature combinations are shown in Fig. 5.4 (scale bar = 20 μm). The solid microcapsules obtained are more or less spherical in shape and no bacterial cell was observed on the surface, indicating that *L. casei* cells were fully encapsulated within the shell matrix. This absence of bacterial cells on the surface of these co-microcapsules is also supported by the surface elemental composition data which will be presented shortly. In addition, there was no observable sign of fissures or cracks on the surface of these WPI-P-O-GA co-microcapsules. This observation further confirms good structural integrity of the co-microcapsule surface which corroborates to the low permeability of oxygen and better microencapsulation efficiency as observed in Table 5.1. The SEM micrograph of reconstituted WPI-P-O-GA co-microcapsules (Fig. 5.3C) substantiates the presence of *L. casei* cells and oil droplets inside the co-microcapsules after drying. There was no noticeable difference on the surface morphology in WPI-P-O-GA co-microcapsules produced at different inlet/outlet temperature combinations (Fig. 5.4).

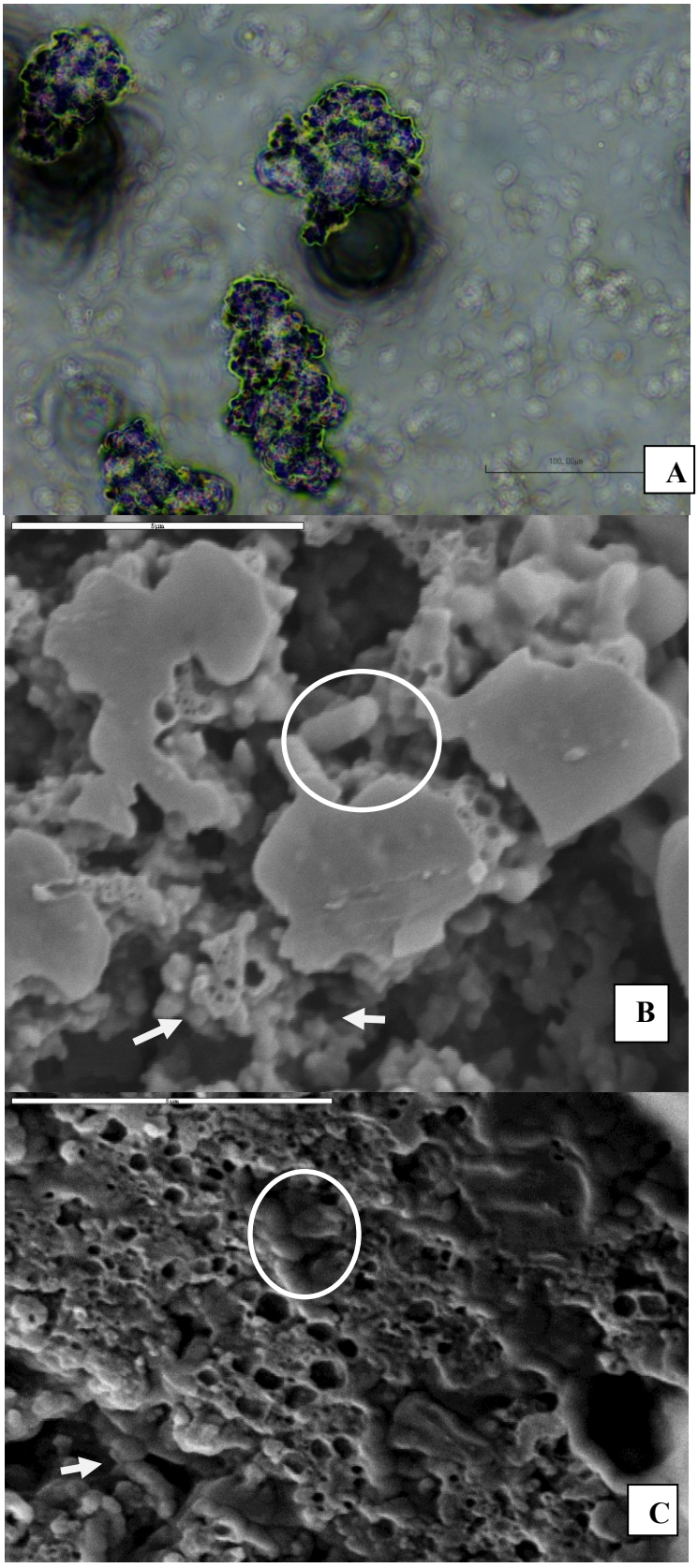
A
B
C

Fig. 5.3: Morphology of WPI-P-O-GA co-microcapsules: (A) Liquid co-microcapsules observed under phase contrast microscope; (B) Liquid co-microcapsules observed under SEM; (C) Reconstituted spray dried co-microcapsules obtained under SEM, scale bar = 5µm.

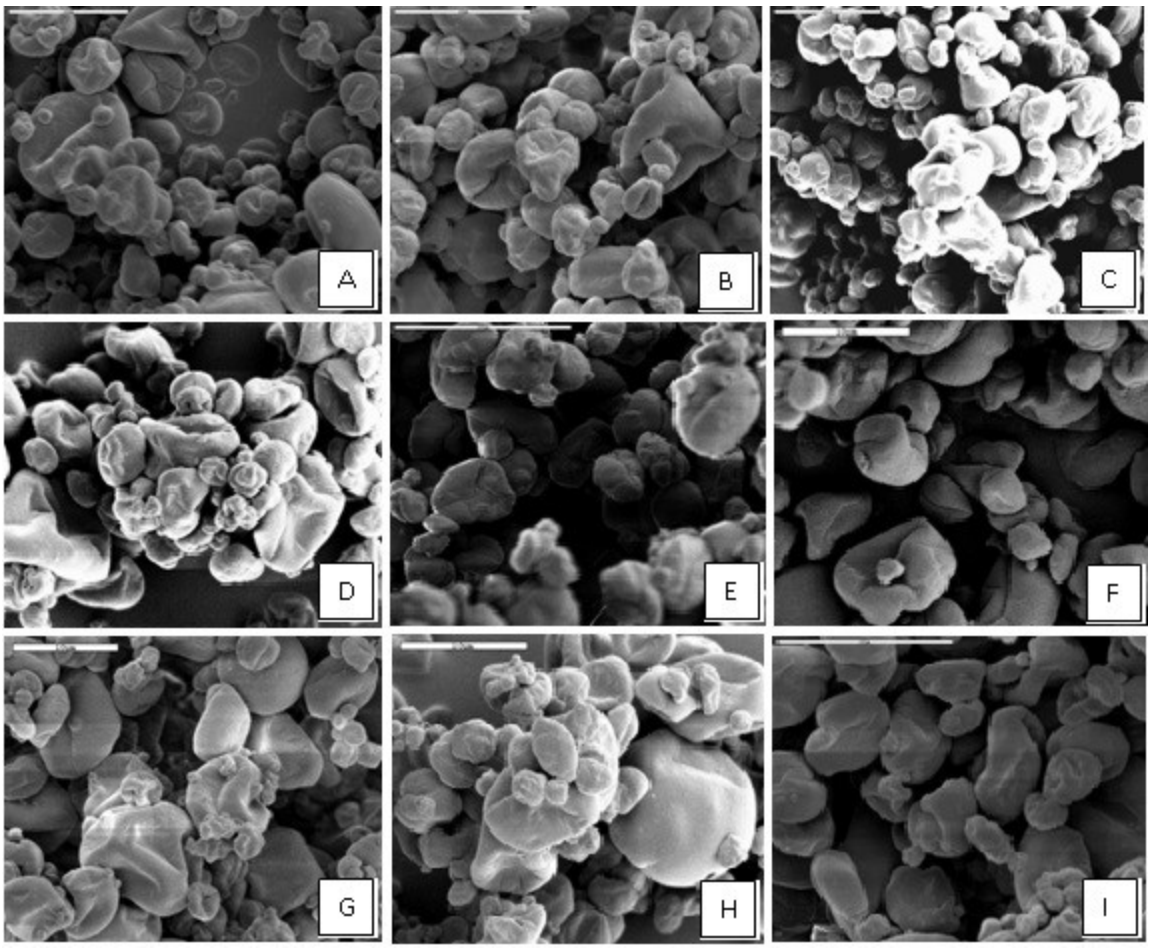

Fig. 5.4: SEM micrographs of WPI-P-O-GA co-microcapsules produced at different inlet/outlet temperature combinations (° C): (A) 130/55; (B) 130/65; (C) 130/75; (D) 150/55; (E) 150/65; (F) 150/75; (G) 170/55; (H) 170/65; (I) 170/75; scale bar = 20µm.

5.3.2.2. Surface characteristics of co-microcapsules

XPS analysis was used to quantify the surface (20-100 Å) elemental composition of WPI-P-O-GA co-microcapsules. The C1s spectra of these co-microcapsules at various inlet/outlet temperature combinations are shown in Fig. 5.5. The corresponding surface elemental composition in terms of carbon (C), oxygen (O), nitrogen (N) of these co-microcapsules are presented in Table 5.2.

Table 5.2: Surface elemental compositions [Carbon (C), Oxygen (O), and Nitrogen (N)] of spray-dried WPI-P-O-GA co-microcapsules produced at varying inlet/outlet air temperatures.

Inlet/outlet temperature (° C)	C1+C2	C3	C4	C5	O	N
130/55	0.716 ± .002	0.156 ± .000	0.064 ± .000	0.064 ± .002	0.215 ± .003	0.072 ± .001
130/65	0.736 ± .007	0.145 ± .002	0.058 ± .002	0.061 ± .003	0.196 ± .010	0.062 ± .003
130/75	0.714 ± .004	0.159 ± .005	0.067 ± .000	0.060 ± .001	0.209 ± .003	0.073 ± .001
150/55	0.733± .004	0.145 ± .002	0.058 ± .002	0.064 ± .000	0.202 ± .006	0.061 ± .003
150/65	0.720 ± .004	0.154 ± .003	0.067 ± .000	0.060 ± .001	0.209 ± .003	0.071 ± .002
150/75	0.715 ± .003	0.156 ± .004	0.068 ± .003	0.061 ± .002	0.214 ± .004	0.072 ± .001
170/55	0.726 ± .000	0.148 ± .003	0.064 ± .005	0.062 ± .001	0.208 ± .003	0.065 ± .000
170/65	0.719 ± .001	0.149± .001	0.070± .002	0.062 ± .000	0.220 ± .002	0.071 ± .001
170/75	0.716 ± .002	0.152 ± .001	0.071 ± .001	0.061 ± .001	0.218 ± .006	0.074 ± .005

The concentration of carbon atoms bonded differently or bonded with different elements was determined by curve-fitting the high resolution C1s spectra. The curve fitting protocol was based on five components with the following notations: C1+C2 (C-C, C-H), C3 (C-O, C-N), C4 (C=O, O-C-O, N-C=O), C5 (O-C=O). As can be seen from Fig. 5, the C1s spectra of all the co-microcapsules are overlapping with each other. This means that the spray dried WPI-P-O-GA co-microcapsules produced at different inlet/outlet temperature combinations had identical surface elemental composition and that the drying parameters made no difference on the surface composition. This observation also corroborates with the surface oil data (Table 5.1) which showed that there was no significant difference ($p>0.05$) in the surface oil content in WPI-P-O-GA co-microcapsules produced at different inlet/outlet temperature combinations. Furthermore, there was no significant difference in oxygen and nitrogen content on the surface of co-microcapsules produced at different inlet/outlet air temperature combinations (Table 5.2). However, the least oxygen content (0.196) was found on the surface of co-microcapsules produced at inlet/outlet temperature combination of 130/65^{o} C. This observation also corroborates with the oxidative stability index data (Fig. 5.2) which showed that co-microcapsules produced at inlet/outlet temperature combination of 130/65^{o} C exhibited highest oxidative stability (21.2 h) than those produced at other inlet/outlet temperature combinations.

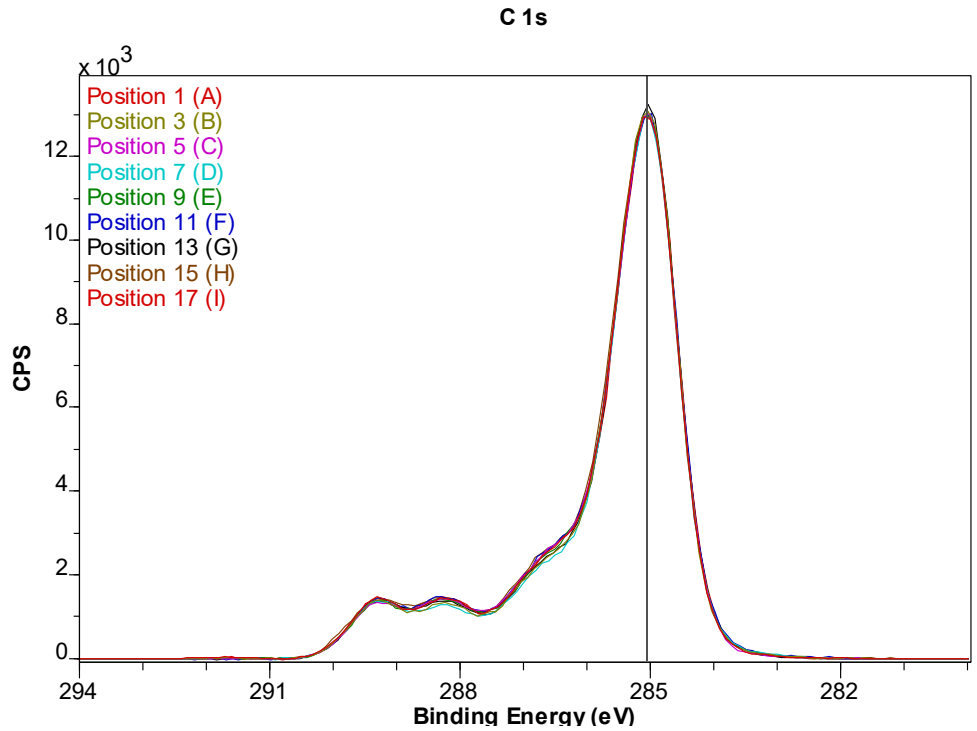

Fig. 5.5: C1s spectra of spray dried WPI-P-O-GA co-microcapsules produced at different inlet/outlet air temperature combinations (° C): (A) 130/55; (B) 130/65; (C) 130/75; (D) 150/55; (E) 150/65; (F) 150/75; (G) 170/55; (H) 170/65; (I) 170/75.

5.4. Conclusions

The probiotic bacteria *L. casei* and omega-3 rich tuna oil were co-encapsulated using WPI-GA complex coacervate as shell material and subsequently converted into powder using spray drying. Nine sets of spray dryer inlet/outlet temperature combinations were used to determine the effects of these temperature combinations on properties such as bacterial survival, stability of oil against oxidation, moisture content, surface oil, total oil, oil microencapsulation efficiency as well as surface morphology and surface elemental composition of these co-microcapsules. High inlet and outlet air temperatures decreased the survival of *L. casei*. High inlet air temperature also decreased oxidative stability of microencapsulated tuna oil. Surface oil content and hence the oil microencapsulation

efficiency of WPI-P-O-GA co-microcapsules was not affected by the inlet and outlet temperatures. The shape and the morphology of *L. casei* cells were not affected by the chosen spray drying temperatures. The surface morphology of co-microcapsules produced at different inlet/outlet temperature combinations was not significantly different. The inlet air temperatures from 130° C to 170° C and outlet temperatures between 55° C and 75° C different did not alter the surface elemental composition of these spray dried co-microcapsules. This study identified that the inlet/outlet temperature combination of 130/65° C produced WPI-P-O-GA co microcapsules with highest bacterial survival and highest oxidative stability under moisture content less than 5%. These findings of this study will benefit many potential applications in functional food, nutraceutical and pharmaceutical industries.

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Chapter 6

Survival and fermentation activity of probiotic bacteria and oxidative stability of omega-3 oil in co-microcapsules during storage

Abstract

Tuna oil (O) and probiotic bacteria *L. casei* (P) were co-microencapsulated in whey protein isolate (WPI)-gum Arabic (GA) complex coacervate. The co-microcapsules (WPI-P-O-GA), *L. casei* microcapsules (WPI-P-GA) and tuna oil microcapsules (WPI-O-GA) were converted into powder using spray and freeze drying. The interaction between probiotic bacteria and omega-3 oil in co-microcapsules, particularly in terms of oxidative stability of omega-3 oil and vitality/viability of probiotic bacteria and any synergistic outcome were studied. The effect of storage temperature (5 and 25° C) and time (90 days) on the survival and fermentation activity of *L. casei* and oxidative stability of tuna oil in the microcapsules/co-microcapsules were determined. A synergism between oxidative stability of omega-3 oil and vitality of probiotic bacteria was observed, when they were co-microencapsulated and spray dried. These co-microcapsules will likely have utility in functional food formulations due to simple and cost effective stabilisation and delivery of two important functional ingredients.

Key words: Probiotic bacteria, Omega-3 fatty acids, Co-microencapsulation, Viability, Vitality, Oxidative stability

6.1. Introduction

There is growing interest in producing functional foods containing encapsulated probiotic bacteria that provide health benefits to consumers (Mattila-Sandholm et al., 2002; Burgain et al., 2011). Probiotic bacteria are live microorganisms which when administered in adequate amounts confer health benefits to the host (FAO/WHO, 2001). These health benefits include the enhancement of gastrointestinal and immune functions (Macfarlane et al., 1999). Microencapsulation of probiotic bacteria has been investigated comprehensively including the strains, methods and matrix of microencapsulation, and the effect of drying and storage conditions in order to maintain their viability and vitality during manufacture, storage, and delivery to the targeted site of gastrointestinal tract (Tripathi et al., 2014).

The polyunsaturated fatty acid requirement of the human brain is substantial during the neonatal period and remains high throughout life, in order to preserve the integrity of cell membranes and their function. However, these very long chain fatty acids are poorly synthesised in our bodies from shorter precursors obtained from food (Bourre, 2004). Maintaining adequate levels of omega-3 content in brain cell membranes is crucial for memory-processing which may diminish with age and due to certain chronic brain disorders (Stough et al., 2012). Omega-3 fatty acids are considered as good fatty acids (Shahidi, 2015) and hence it is essential to take omega-3 fatty acids through food (Kris-Etherton et al., 2002). The broader consumption of omega-3 rich foods has been hindered because of their poor compatibility in water-based products and susceptibility to oxidative degradation, which can lead to off-flavours, off-odours, and loss in bioactivity (Barrow et al., 2007). Therefore, stabilisation of omega-3 rich oils using suitable microencapsulation technology is essential for successful incorporation of omega-3 rich oils in a broad

spectrum of food products and beverages (Sanguansri et al., 2006; Augustin et al., 2015). A remarkable emergent interest to enrich food products with omega-3 fatty acids has been observed in the last few decades (Lemahieu et al., 2015; de Camargo Talon et al., 2015).

We reported in an earlier work that the survival of probiotic bacteria is improved when they are co-microencapsulated with omega-3 in a single matrix (Eratte et al., 2015). Co-microencapsulation of omega-3 fatty acids and probiotic bacteria in whey protein isolate (WPI) - gum Arabic (GA) complex coacervate matrix significantly improved the survival of probiotic bacteria and stability of omega-3 rich tuna oil (Eratte et al., 2014; Eratte et al., 2015). In this study, we investigate the viability/vitality of probiotic bacteria and oxidative stability of omega-3 in co-microcapsules during specific storage conditions. For this purpose the viability/vitality of probiotic bacteria (P) and oxidative stability of tuna oil (O) in co-microcapsules (WPI-P-O-GA) and individual microcapsules containing either probiotic bacteria (WPI-P-GA) or tuna oil (WPI-O-GA) were studied during storage. Changes in viability/vitality and oxidative stability over time have not been previously reported for microcapsules containing both probiotic bacteria and omega-3 fatty acids.

Probiotic bacteria are dried and converted into powder for use in food and pharmaceutical applications, as the powder is easy to handle, transport and store (Ghandi et al., 2013a). Probiotic bacteria, particularly lactic acid bacteria (LAB), are added in fermented milk products in order to impart the desired physico-chemical, sensory and microbiological attributes in such products (Casarotti et al., 2014). Consumption of fermented probiotic milk provides health benefits such as alleviation of undesirable gastric and abdominal symptoms (Gomi et al., 2015), improved immune response (Bogsan et al., 2014), reduction

of total cholesterol (Ejtahed et al., 2011), and enhancement of intestinal microbiota (Wang et al., 2012).

For these reasons, we studied the vitality (fermentation ability) of microencapsulated and co-microencapsulated model probiotic bacteria (*L. casei*) in reconstituted skim milk powder. The fermentation activity of the probiotic bacteria was also determined in co-microcapsules as a function of storage temperature and time using microcapsules containing only the probiotic bacteria as a control. To date, no published study has addressed the viability and vitality of any probiotic bacteria in co-microcapsules containing omega-3 fatty acids as a function of storage temperature and time. Similarly, no study has been reported on the oxidative stability of omega-3 oil in co-microcapsules containing probiotic bacteria as a function of storage temperature and storage time by measuring and comparing different oxidation parameters, such as oxidative stability index (OSI) by Rancimat, peroxide value (PV), *p*-Anisidine value (AV) and total oxidation value (TOTOX). The novelty of this study lies in the facts that it is a further improvement of the concept of co-microencapsulation of omega-3 fatty acids and probiotic bacteria in one single matrix through a detailed examination of the viability/vitality of microencapsulated bacteria and the oxidative stability of the microencapsulated omega-3 at different storage temperatures and times.

Co-microcapsules provide the benefits of both omega-3 fatty acids and probiotic bacteria in one serving. This has cost and convenience advantages over individual microencapsulation of each ingredient. The cost of spray drying is six times lower per kilogram of water removed than the cost of freeze-drying (Knorr, 1998) and result in a

relatively stable powder that can be transported and stored in a stable form. Therefore, co-microcapsules can be preferred in functional foods where both quality and low cost are key parameters for commercial success.

This study had the following key objectives: (1) To compare the survival and fermentation activity of *L. casei* as a model probiotic bacteria in freeze and spray dried co-microcapsules (WPI-P-O-GA) as a function of storage temperature and time, in comparison with a control, of freeze and spray dried microcapsules containing only *L. casei* (WPI-P-GA). (2) To compare the oxidative stability of omega-3 rich tuna oil in the co-microcapsules (WPI-P-O-GA) as a function of storage temperature and time, in comparison with a control of freeze and spray dried microcapsules containing only tuna oil (WPI-O-GA).

6.2. Materials and Methods

6.2.1. Materials

L. casei 431 was used as the model of probiotic bacteria and was kindly donated by Chr. Hansen (Horsholm, Denmark). Tuna oil (HiDHA) (NuMega Ingredients Ltd, Melbourne, Australia) is rich in omega-3 fatty acids and was used as an omega-3 rich oil for this study. Tuna oil was stored at 4^{o} C until used. According to data provided by the supplier, the major fatty acids in this tuna oil were as follows: DHA (docosahexaenoic acid) 29.4%, palmitic acid 19.2%, oleic acid 12.8%, EPA (eicosapentaenoic acid) 6.0%, stearic acid 5.3%, myristic acid 2.4%, arachidonic acid 2.0%, decosapentaenoic acid (DPA) 1.2%, linoleic acid 1.2%, stearidonic acid 0.6%, and linolenic acid 0.4%. Whey protein isolate (WPI 895^{TM}) was donated by Fonterra Cooperative (Melbourne, Australia). Gum Arabic

was purchased from Sigma-Aldrich Ltd. (Sydney, Australia). All other chemicals were purchased from Sigma–Aldrich Ltd. (Sydney, Australia) and were of analytical grade. All of the above ingredients and chemicals were used as received without further purification or alteration.

6.2.2. Preparation of *L. casei* culture

The *L. casei* was cultured for 18 h in sterile MRS broth (1%, w/v) at 37° C. This culture was sub-cultured (37° C, 18 h) twice in the same broth to activate bacteria and to allow them to adapt. All the inoculation works were carried out under sterile biological hoods with laminar air flow (Auramini, Laftech, Melbourne, Australia). Finally, 1000 mL of media volume was used for bulk culturing and cells were harvested at stationary growth phase by centrifuging at 2200×g for 15 min at 5° C. The supernatant was discarded and the precipitated cell mass was washed twice with sterile peptone water (0.2%, w/v). The final wet cell mass was weighed and divided into two equal portions. One portion (wet mass 5g) was used to prepare WPI-P-GA microcapsules and the second half (wet mass 5g) was used to prepare WPI-P-O-GA co-microcapsules in order to facilitate a good comparative study (Eratte et al., 2015).

6.2.3. Microencapsulation process

The complex coacervation and microencapsulation procedures were performed as below, using a previously published methods with some modifications (Eratte et al., 2014; Eratte et al., 2015).

6.2.3.1. Microencapsulation of tuna oil

A solution of 15 g tuna oil and 250 mL WPI solution (12%, w/v) were stirred (IKA® RW 20 digital overhead stirrer, Staufen, Germany) at 800 rpm for 10 min and then homogenized using a microfluidizer at 45 MPa for 3 passes (M110L, Microfluidics, Newton, MA, USA) to produce an O/W emulsion. Then a 250 mL GA solution (4%, w/v) was added drop wise into this O/W emulsion with continuous stirring at 400 rpm and the pH adjusted to 3.75 by adding 1% citric acid drop wise in order to induce complexation between WPI and GA.

6.2.3.2. Microencapsulation of *L. casei*

Similar to above, the previously prepared probiotic cell mass was added slowly into a WPI solution, and a GA solution was then added drop wise with continuous stirring, after which the pH was adjusted to 3.75 as above.

6.2.3.3. Co-microencapsulation of omega-3 oil and *L. casei*

As above, tuna oil (15 g) was dispersed into a WPI solution and stirred for 10 min, followed by homogenizing using a microfluidizer to produce an O/W emulsion. Previously prepared probiotic cell mass was then added into this O/W emulsion and GA solution was added with continuous stirring at 400 rpm, and the pH adjusted to 3.75 as above.

The microencapsulation procedure in all the three cases were carried out at 25° C, and the liquid microcapsules/co-microcapsules were kept at 5° C for 48 h to ensure complete formation of complex coacervates. Finally, the microcapsules/co-microcapsules were dried to produce solid or powder co-microcapsules (WPI-O-GA, WPI-P-GA and WPI-P-O-GA).

6.2.4. Drying of coacervate liquid microcapsules

A portion of liquid microcapsules produced as per Section 6.2.3 was spray dried (B-290, BÜCHI Labortechnik, Flawil, Switzerland) using an inlet temperature of 130° C and outlet

temperature 65° C (Eratte et al., 2016). The second portion of the liquid microcapsules was frozen at -20° C overnight and was subsequently freeze dried (Christ Alpha 2-4LD, Osterode, Germany). The temperature of the ice condenser was set at -50° C and the vacuum pressure was set to 0.04 mbar. The frozen samples were dried for 48 h and the dried product was collected, pulverized and stored. This procedure is also adapted from our earlier work (Eratte et al., 2015).

6.2.5. Storage studies

The freeze dried and spray dried microcapsule powder containing tuna oil only (WPI-O-GA) and bacteria only (WPI-P-GA) and co-microcapsule powder (WPI-P-O-GA) were first flushed with nitrogen and stored for 90 days at two different temperatures (5 and 25° C) in dark airtight vials. They were further placed in aseptic plastic bags, vacuum sealed and kept in air-tight desiccator containing silica gel beads. Bacterial survival was measured at an interval of 15 days during 90 days of storage. Fermentation activity and oxidative stability by Rancimat^{TM} analysis were evaluated at an interval of 30 days (Läubli et al., 1986). The primary and secondary oxidation products such as peroxide value (PV) and *p*-Anisidine value (AV) were measured at intervals of 15 days during the storage.

6.2.6. Determination of bacterial survival during storage

The viability of *L. casei* in the microcapsules/co-microcapsules was assessed in MRS agar (CM0361, Thermoscientific, Melbourne, Australia) using spread plating. Briefly, 1g of powder was diluted in 9 mL of sterile peptone water (0.2%, w/v). The bacterial cells were completely released from the microcapsules to the medium within 15 min using a

stomacher. Maximum cell release was confirmed by testing cell viability over time (up to 20 min) in the preliminary test. The cell suspension was serially diluted and plated on MRS agar plate. The plates were then incubated under anaerobic condition (Oxoid^{TM} Anaerojar^{TM}, Thermoscientific, Melbourne, Australia) at 37^{o} C for 48h. The plating was performed in triplicate. Results are expressed as log colony forming units per gram of the powder used (log cfu/g powder).

The viability of the bacteria (*L. casei*) during storage was expressed as a logarithmic value (to the base 10) of the ratio of cell count at a particular storage time (N_t) to the cell count at the beginning of storage (N_0). Equation (1) was fitted to the experimental log (N_t/N_0) against storage time (t) in days for each temperature in order to determine the inactivation rate constant k (day^{-1}) during storage (Foerst et al., 2012).

$$\log\frac{N_t}{N_0} = -kt \qquad (1)$$

The survival percentage (S) of bacterial cells was calculated using equation (2) given below.

$$S(\%) = \frac{N_t}{N_0} \times 100 \qquad (2)$$

6.2.7. Fermentation process

The reconstituted skim milk (RSM) was prepared according to Casarotti et al., (2014) with slight modification. The skim milk powder- Regular High Heat (protein 32.9g/100g) (Fonterra, Melbourne, Australia) was reconstituted to 10% (w/v) in double distilled water. The RSM was thermally treated in sterile bottles at 90^{o} C for 5 min in a water bath, cooled in ice bath and transferred to a sterile glass flask sealed with a screw-cap sealed lid, all

inside a laminar flow chamber. The amounts of WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules powders added into 100 mL of sterile RSM are given in Table 6.1. These formulations provide the same initial cell count of 8 log cfu/g of microcapsules in 100 mL RSM for a good comparative study. These fermentation experiments were carried out using a shaking (100 rpm) incubator under sterile condition at 37° C for 72 h. The incubator was disinfected with 70% ethanol and all the glassware used were autoclaved before fermentation. These fermentation tests were carried out in triplicate.

6.2.8. Measurement of fermentation activity

The fermentation activity was determined in terms of change in pH and lactic acid production. The change of pH during fermentation was monitored intermittently over a period of 72 h using a pH meter (TPS WP-80M, Melbourne, Australia). The electrode was disinfected with 70% (v/v) ethanol, rinsed with sterile distilled water and placed in the fermentation flask under aseptic conditions in each measurement.

The following parameters were used to characterize the fermentation kinetics: (a) maximum acidification rate (V_{max}) which was calculated by differentiating the pH–time curve with respect to time (dpH/dt) and expressed in absolute values (10^{-3} pH units /min) (De Souza Oliveira et al., 2009); (b) the time at which pH 4.6 was reached ($t_{pH\ 4.6}$, h); (c) lactic acid productivity (P_{max}, g/L/h) was calculated by dividing the final lactic acid concentration by the total fermentation time (Dembczynski et al., 2002).

The fermented RSM was sampled at different time intervals (0h, 24h, 48h, 72h) to determine the amount of lactic acid produced. Briefly, the collected samples were centrifuged at 13,000 x g for 10 min. The supernatant was collected and further filtered by passing through 0.45 μm nylon syringe filter. The lactic acid concentration was then

determined by using ultra high performance liquid chromatography (LCMS-8030, Shimazdu, Columbia, Maryland, USA) with photodiode Array detector at λ=210 nm. An organic acid column (AllTech OA-1000 cat. No 9046; 300 × 7.8 mm) was used to determine the lactic acid content. The mobile phase (0.005M H_2SO_4) was vacuum filtered and fed using an isocratic elution with a flow rate of 0.6 mL/min and the oven temperature was kept at 65° C (Nazzaro et al., 2009).

6.2.9. Oxidative stability

6.2.9.1. Determination of oxidative stability index

Accelerated oxidation tests were carried on the solid WPI-P-O-GA co-microcapsules and WPI-O-GA microcapsules using a Rancimat (model 743, Metrohm, Herisau, Switzerland) (Mathäus, 1996). Dried microcapsule powder (1.5 g) was heated at 90° C under purified air flow rate of 20L/h. The oxidative stability index (OSI) value of the samples was graphically determined by locating the rapid change of slope of the conductivity versus time curve. These tests were carried out in triplicate.

6.2.9.2. Primary and secondary oil oxidation products

Primary oxidation products in the extracted oil were measured by peroxide value (PV) according to the methodology described by Karaca et al. (2013). Secondary oxidation products, specifically the aldehydes produced from the breakdown of oil peroxides, were measured by *p*-Anisidine value (AV) according to Lee et al. (2007). The total oxidation (TOTOX) value was calculated using equation (3) given below (Ifeduba et al., 2015).

$$\text{TOTOX} = 2\text{PV} + \text{AV} \qquad (3)$$

6.2.10. Statistical Analysis

All the experiments were carried out either in duplicate or triplicate, as stated. The Minitab 16^{TM} statistical software package (University Park, PA, USA) was used for the analysis of variance (ANOVA) to determine the significant differences. The confidence level of 95% ($p<0.05$) was used to determine significant difference between mean values.

6.3. Results and discussion

In this section, the viability and vitality (fermentation activity) of the microencapsulated probiotic bacteria and the oxidative stability of microencapsulated omega-3 oil during storage are discussed based on four parameters: Microencapsulation process; drying method used (spray and freeze drying); storage time (up to 90 days); and storage temperature (5 and 25^{o} C).

6.3.1. Survival of *L. casei* in WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules

The initial cell count, final survival percentage, and inactivation rate constant of *L. casei* in freeze dried and spray dried WPI-P-GA microcapsules (control) and WPI-P-O-GA co-microcapsules are presented in Table 6.1. We had reported earlier that the count of viable cells (viability) of *L. casei* in WPI-P-O-GA co-microcapsules was greater than that of WPI-P-GA microcapsule powders obtained from both spray and freeze drying (Eratte et al., 2015). The initial viability data obtained in this study (Table 6.1) confirmed those findings. The initial cell count was found to be greater in FD WPI-P-O-GA (8.47 log cfu/g) than all the other solid microcapsules. However, after storage for 90 days, the highest

bacterial survival (45.72 %) was measured in spray dried (WPI-P-O-GA) co-microcapsules stored at 5° C. Some denaturation and aggregation of whey protein occurred during spray drying, enhancing adsorption at the interface and resulting in the formation of a thin gel-like protective layer (Adhikari et al., 2007). This has resulted in enhanced protection of the bacterial cells during storage for the spray dried co-microcapsules compared to the freeze dried co-microcapsules. Lian et al, (2002) reported that GA can act as a thermo-protectant to bacterial cells during spray drying and help to preserve cell viability on storage. Moreover, the presence of more oxidation-sensitive lipids in the co-microcapsules appears to further protect bacterial cells during spray drying and storage, resulting in greater viability of *L. casei* in spray dried WPI-P-O-GA during storage. The less porous nature of the outer surface (skin) and lower surface oil content of spray dried microcapsules compared to freeze dried microcapsules has resulted in greater storage stability for spray dried microcapsules (Eratte et al., 2014).

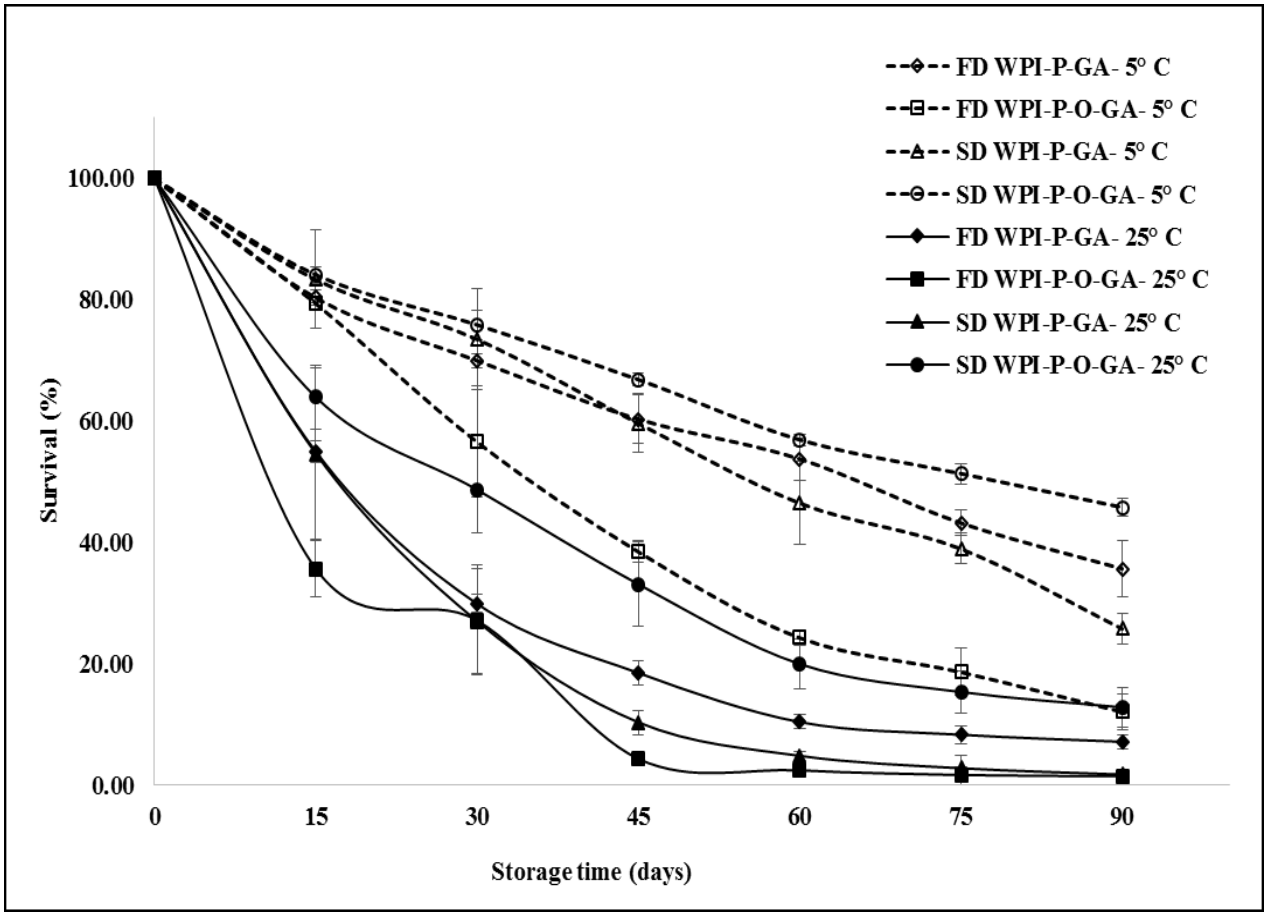


Fig. 6.1: The survival of *L.casei* in freeze (FD) and spray (SD) dried WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules as a function of storage temperature (5° C and 25° C) and storage time. Data represent survival percentage in mean ± SD of three replicate.

It can be seen from Table 6.1 that the survival of *L. casei* decreased with storage time in all the cases. Bacterial death was more rapid when the powders were stored at 25° C than at 5° C. The highest survival percentage of *L. casei* was found in spray dried WPI-P-O-GA at both temperatures when stored for 90 days. The lowest survival percentage was found in freeze dried WPI-P-O-GA (1.44 %) stored at 25° C. Thus, it can be concluded that the upstream drying methods significantly ($p<0.05$) affect the survival of microencapsulated

bacteria during storage. The bacterial cells in spray dried co-microcapsules had higher survival percentage whereas the bacterial cells in the freeze dried co-microcapsules had the lowest survival percentage compared to survival in their respective controls (microcapsules containing only bacteria) during storage. The presence of omega-3 rich oil in the liquid microcapsules resulted in improved survival of bacterial cells in spray dried WPI-P-O-GA compared to when the oil was absent (i.e. in spray dried WPI-P-GA). To the contrary, the presence of omega-3 rich oil in freeze dried WPI-P-O-GA hindered the survival of bacterial cells during storage when compared to its control (freeze dried WPI-P-GA). The mechanism behind this observation is assumed to be related to the oxidative stability of co-microencapsulated omega-3 oil, which will be discussed in detail in the Section 6.3.4.

The bacterial survival data over storage time is presented in Fig. 6.1. The survival of *L. casei* in the microcapsules was significantly ($p<0.05$) lower in the samples stored at room temperature. The viability of dried cells in WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules during storage is clearly reflected on the inactivation rate constant (k) (Table 6.1). This constant also enables the comparison of viability of *L. casei* cells at different storage temperature. It is observed that k values increased with increase in the storage temperature, indicating poorer survival at higher temperatures. As indicated by these k values, the type of microcapsules, drying methods used, and the choice of storage temperature all affected the viability of encapsulated bacteria. The drying methods impacted the viability of bacterial cells in WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules in different ways. The spray dried WPI-P-O-GA co-microcapsules had lower inactivation rate constants than that of spray dried WPI-P-GA microcapsules,

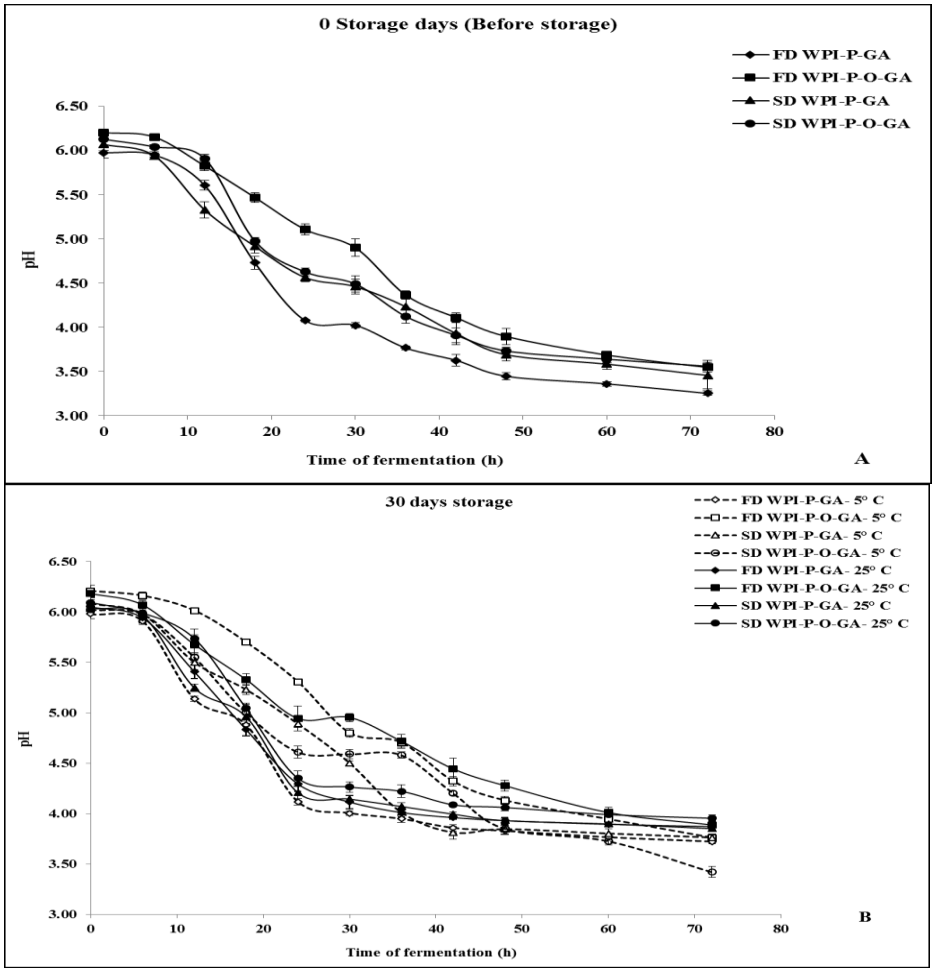
whereas the freeze dried WPI-P-GA microcapsules have lower inactivation rate constant than that of freeze dried WPI-P-O-GA co-microcapsules on storage.

It was observed clearly that spray dried WPI-P-O-GA co-microcapsules had the lowest k both at 5° C and 25° C, indicating that the co-microencapsulation of bacterial cells with omega-3 rich oil can have higher survival during storage than when they are encapsulated without it. Interestingly, the freeze dried WPI-P-O-GA co-microcapsules had a higher k value than that of the freeze dried WPI-P-GA microcapsules. Therefore, it is concluded that although the initial viability of bacterial cells in freeze dried WPI-P-O-GA co-microcapsules was the highest, they had the poorest viability during storage at both temperatures (5 and 25° C). The porous structure of freeze dried microcapsules might have enhanced the moisture and oxygen diffusion inward and this might have reduced the viability of probiotic cells upon storage.

6.3.2. Fermentation activity of *L. casei* in WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules

The panels A, B, C, and D in Fig. 6.2 show the acidification profile of *L.casei* in WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules. These microcapsules were stored at 5° C and 25° C for a period of 0, 30, 60 and 90 days before these fermentation tests were carried out. The fermentation was carried out for 72 h at 37° C. The change in the pH over the fermentation time due to the production of lactic acid was measured as an indicator of fermentation activity (Ghandi et al., 2013a). It was observed that (Fig. 6.2) the fermentation activity (decrease in pH value) was affected by type of microcapsules, drying methods used, time and temperature of storage of microcapsules. The different

acidification profile of *L. casei* in different microcapsules, when the initial bacterial count was kept identical, may be due to the different extent of injury or shock caused by different upstream drying methods in the case of microcapsules which were subjected to fermentation tests immediately after they were produced (zero days of storage). Although significant differences were noticed in pH versus time profiles, the acidification profile of all of the microcapsules exhibited three distinct and noticeable phases. Firstly, there was an initial lag phase in which the pH did not decrease or decreased only insignificantly ($p>0.05$). The lag phase was followed by a second period at which pH values decreased rapidly, and finally the pH decreased only slightly or stopped, indicating completion or termination of the fermentation process. This phenomenon is due to the chemical and biochemical reactions that take place in bacterial cytoplasm during the lactic acid production process (Kashket, 1987).


0 Storage days (Before storage)
FD WPI-P-GA
FD WPI-P-O-GA
SD WPI-P-GA
SD WPI-P-O-GA
pH
Time of fermentation (h)
A
30 days storage
FD WPI-P-GA- 5° C
FD WPI-P-O-GA- 5° C
SD WPI-P-GA- 5° C
SD WPI-P-O-GA- 5° C
FD WPI-P-GA- 25° C
FD WPI-P-O-GA- 25° C
SD WPI-P-GA- 25° C
SD WPI-P-O-GA- 25° C
pH
Time of fermentation (h)
B

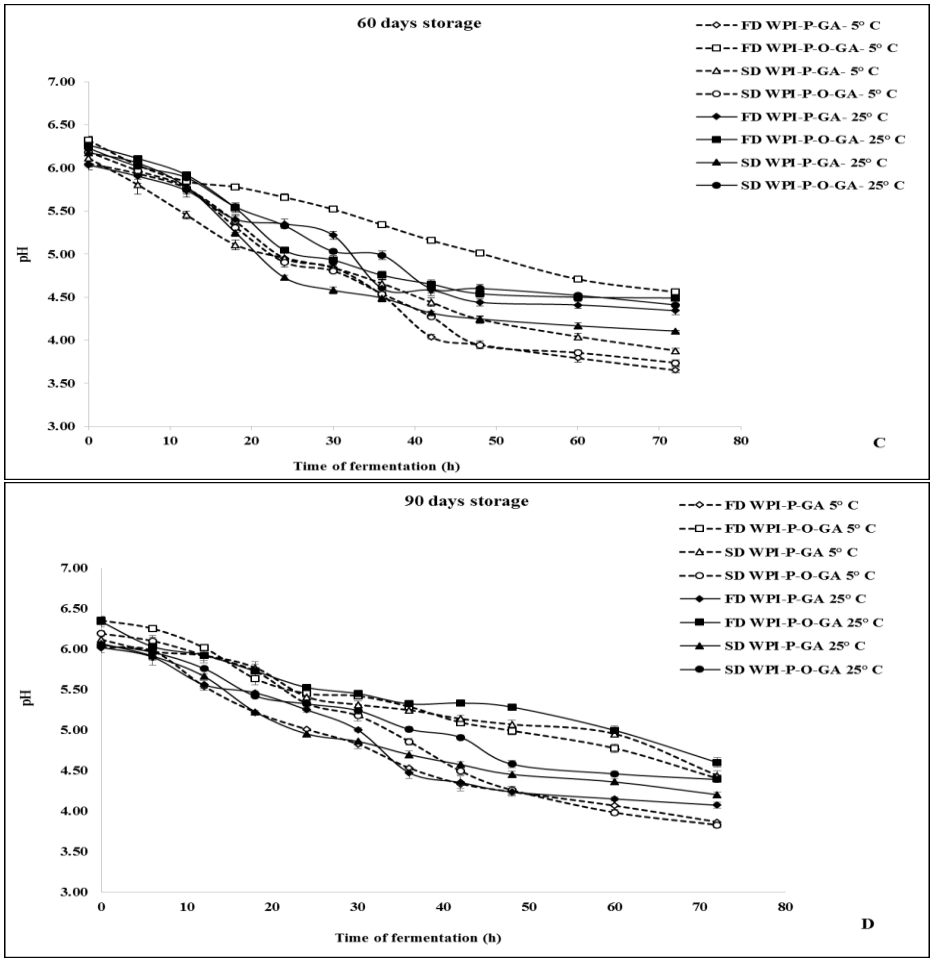


Fig. 6.2: The fermentation activity (pH versus time) profile of freeze (FD) and spray (SD) dried WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules in RSM. (A) Microcapsules immediately after drying; (B) Microcapsules stored for 30 days; (C) Microcapsules stored for 60 days; (D) Microcapsules stored for 90 days. Data represent pH in mean ± SD of three replicate.

The acidification kinetics of bacterial cells contained within different microcapsules was further evaluated in terms of the maximum acidification rate (V_{max}), the time taken to reach pH 4.6 ($T_{pH\ 4.6}$), which represents the isoelectric point of bovine casein, and the maximum rate of production of lactic acid (P_{max}). These results are shown in Table 6.1. In general, V_{max} of all the microcapsules decreased with increase in storage time and temperature. This

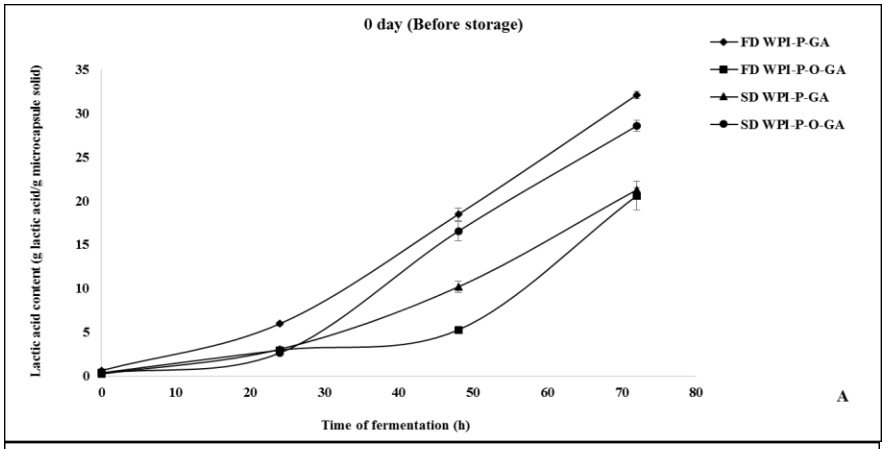
may be due to the decrease in the reproductive rate or vitality of the bacterial cells in the fermentation medium during storage. An opposite trend was noted in the case of time taken to reach pH 4.6 (Table 6.1), which increased with the storage time and temperature. Curdling of medium was observed at and below pH 4.6 in all cases due to coagulation of casein. The highest V_{max} after 90 days storage was observed in the encapsulated cells in spray dried WPI-P-O-GA and the lowest value was obtained in the case of freeze dried WPI-P-O-GA indicating that the vitality of *L. casei* in the freeze dried co-microcapsules was the lowest in this study. These findings are consistent with the viability data (Fig. 6.1).

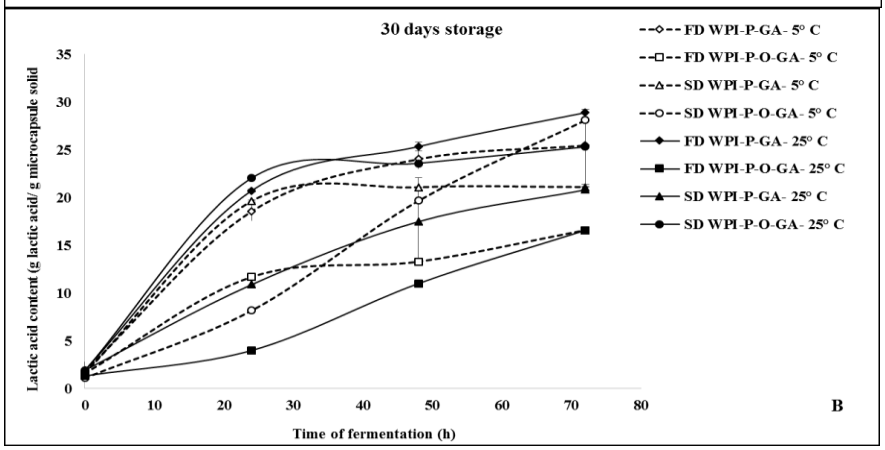
Table 6.1: Survival and fermentation kinetics of L. casei in freeze (FD) and spray (SD) dried WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules

Table 1: Survival and fermentation kinetics of *L. casei* in freeze (FD) and spray (SD) dried WPI-P-GA microcapsules and WPI-P-O-GA co-microca								
Microcapsules/ co-microcapsules	Storage Temperature (^{o} C)	Storage days	Initial cell count (cfu/g)	Final survival (%)	Inactivation rate constant (k, day^{-1})	Samples taken/ 100 ml of RSM (g)	V_{max} (x10^{-3} pH units/min)	$t_{pH\ (4.6)}$ (h)
		0					2.49 ± 0.07^{a}	18.50 ± 0.71^{o}
		30					2.14 ± 0.02^{b}	20.00 ± 0.00^{no}
		60			0.0003		1.49 ± 0.01^{efgh}	35.00 ± 0.00^{hi}
	5	90	7.10 ± 0.01^{c}	35.63 ± 4.62^{b}	(0.9916)	1.13	1.32 ± 0.01^{ghi}	36.50 ± 0.71^{hi}
		30					1.61 ± 0.03^{de}	20.38 ± 0.53^{no}
		60			0.001		1.37 ± 0.04^{ghi}	36.50 ± 0.71^{hi}
FD WPI-P-GA	25	90	7.10 ± 0.01^{c}	7.13 ± 1.15^{cd}	(0.9634)	1.13	1.22 ± 0.01^{ijk}	36.25 ± 0.35^{hi}
		0					1.51 ± 0.03^{defg}	32.50 ± 0.71^{k}
		30					1.10 ± 0.01^{jkl}	37.75 ± 0.35^{gh}
		60			0.0005		1.08 ± 0.02^{jkl}	65.50 ± 0.71^{c}
	5	90	8.47 ± 0.07^{a}	12.06 ± 2.93^{c}	(0.9866)	0.94	1.05 ± 0.03^{kl}	68.75 ± 1.06^{b}
		30					1.09 ± 0.03^{jkl}	32.00 ± 0.00^{k}
		60			0.0015		1.09 ± 0.04^{jkl}	36.25 ± 0.35^{hi}
FD WPI-P-O-GA	25	90	8.47 ± 0.07^{a}	1.44 ± 0.59^{d}	(0.8779)	0.94	1.02 ± 0.01^{l}	72.00 ± 0.56^{a}
		0					1.70 ± 0.01^{d}	23.15 ± 0.21^{m}
		30					1.57 ± 0.03^{def}	28.25 ± 0.35^{l}
		60			0.0004		1.40 ± 0.02^{fghi}	38.20 ± 0.28^{fgh}
	5	90	6.55 ± 0.07^{d}	25.77 ± 2.51^{b}	(0.9611)	1.22	0.99 ± 0.04^{l}	68.35 ± 0.49^{c}
		30					1.35 ± 0.03^{ghi}	22.05 ± 0.07^{mn}
		60			0.0017		1.27 ± 0.04^{ij}	28.25 ± 0.35^{l}
SD WPI-P-GA	25	90	6.55 ± 0.07^{d}	1.72 ± 0.08d	(0.9920)	1.22	0.98 ± 0.02^{l}	40.25 ± 0.35^{ef}
		0					2.60 ± 0.01^{a}	23.50 ± 0.71^{m}
		30					2.06 ± 0.02^{bc}	24.00 ± 0.00^{m}
		60			0.0002		1.63 ± 0.06^{de}	33.50 ± 0.71^{jk}
	5	90	7.98 ± 0.04^{b}	45.72 ± 1.48^{a}	(0.9949)	1.00	1.61 ± 0.21^{de}	39.45 ± 0.78^{fg}
		30					1.92 ± 0.02^{c}	24.00 ± 1.41^{m}
		60			0.0006		1.32 ± 0.01^{ghi}	42.50 ± 0.71^{e}
SD WPI-P-O-GA	25	90	7.98 ± 0.04^{b}	12.80 ± 3.30^{c}	(0.9885)	1.00	1.31 ± 0.03^{hi}	48.00 ± 0.00^{d}

The decrease in pH in the RSM medium is due to the production of lactic acid as a consequence of the metabolic activity of *L. casei* (fermentation of lactose). The amount of lactic acid produced by the microencapsulated bacteria in freshly freeze dried and spray dried WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules in RSM as a function of time is plotted in Fig. 6.3A. The lactic acid content-time profiles of microcapsules

stored at 5 and 25° C for a period of 30, 60, and 90 days are shown in Fig. 6.3B, 6.3C, 6.3D, respectively. The lactic acid production was found to vary across the different microcapsule types. The quantity of lactic acid produced by *L. casei* was higher in spray dried WPI-P-O-GA compared to its control (spray dried WPI-P-GA) at 90 days of storage. This can be attributed to the fact that highest survival and preservation of metabolic activity of *L. casei* was observed in spray dried WPI-P-O-GA on storage (Table 6.1). The amount of lactic acid production was lower in freeze dried WPI-P-O-GA compared to its control (freeze dried WPI-P-GA). This can also be attributed to the lower percentage of survival of *L. casei* in freeze dried WPI-P-O-GA upon storage (Table 6.1). Injury or damage of cell wall and cell membrane due to membrane lipid oxidation would decrease the vitality of the bacterial cells and may play a role in this result (Edward et al., 2011; Ghandi et al., 2013b). To confirm that storage stability is adequate for commercial use more extensive accelerated storage studies showing up to 2 years shelf-life of the ingredient, and storage studies specific to each food application, will be required.


0 day (Before storage)
FD WPI-P-GA
FD WPI-P-O-GA
SD WPI-P-GA
SD WPI-P-O-GA
Lactic acid content (g lactic acid/g microcapsule solid)
Time of fermentation (h)
A


30 days storage
FD WPI-P-GA- 5° C
FD WPI-P-O-GA- 5° C
SD WPI-P-GA- 5° C
SD WPI-P-O-GA- 5° C
FD WPI-P-GA- 25° C
FD WPI-P-O-GA- 25° C
SD WPI-P-GA- 25° C
SD WPI-P-O-GA- 25° C
Lactic acid content (g lactic acid/ g microcapsule solid
Time of fermentation (h)
B

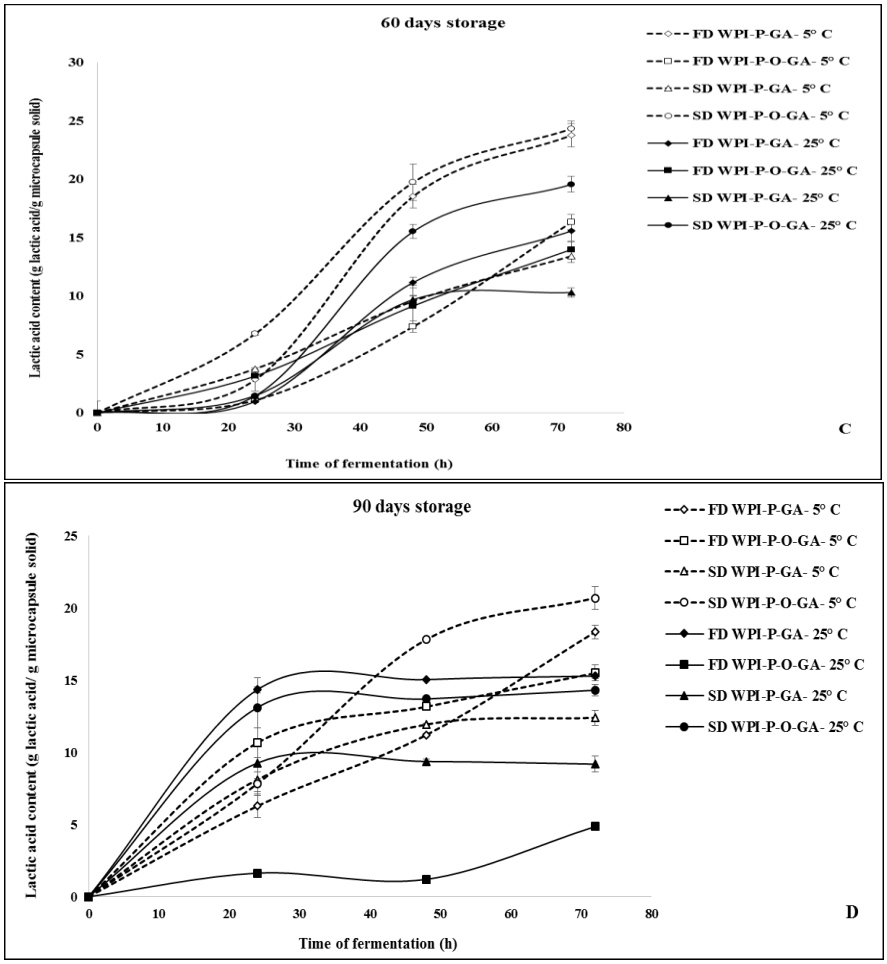


Fig. 6.3: Fermentation activity [Lactic acid content (g lactic acid/g microcapsule solid) versus time] of freeze (FD) and spray (SD) dried WPI-P-GA microcapsules and WPI-P-O-GA co-microcapsules in RSM: (A) Microcapsules immediately after drying; (B) Microcapsules stored for 30 days; (C) Microcapsules stored for 60 days; (D) Microcapsules stored for 90 days. Data represent lactic acid in mean ± SD of two replicate.

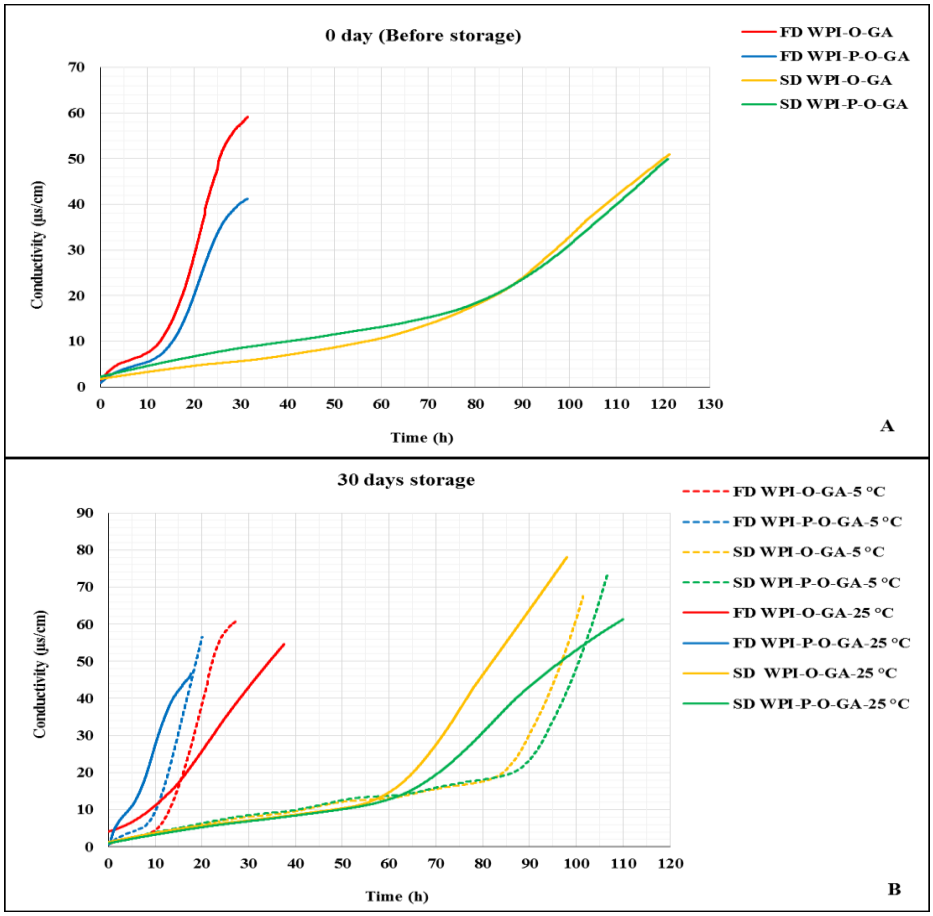
The lactic acid production by encapsulated *L. casei* decreased with increase in the storage time and storage temperature. The highest lactic acid production was observed in the spray dried WPI-P-O-GA stored at 5° C. Therefore, it can be concluded that co-microencapsulation of omega-3 rich oil and *L. casei* in WPI-GA complex coacervates followed by spray drying of the resultant co-microcapsules results in improved viability

and vitality. As expected, storing these microcapsules at 5° C better preserves the viability and vitality of *L. casei* than storing at 25° C up to the maximum 90 days tested. The freeze drying of WPI-P-O-GA co-microcapsules resulted in lower viability and fermentation activity during storage, even though it had the highest initial cell count. The mechanism behind this phenomenon is also related to the oxidative stability of co-microencapsulated omega-3 oil and this will be discussed in detail in the Section 6.3.4.

6.3.3. Oxidative stability of omega-3 oil in WPI-O-GA microcapsules and WPI-P-O-GA co-microcapsules

6.3.3.1. Oxidation stability index (OSI) on storage

The OSI graphs of freeze dried and spray dried solid microcapsules and co-microcapsules stored at 5° C and 25° C for 90 days are presented in Fig. 6.4. The OSI value for freeze dried samples was significantly lower than that of spray dried samples, implying that the freeze dried microcapsules and co-microcapsules were less stable against oxidation when compared to the spray dried ones. This relatively poor oxidative stability in freeze dried microcapsules is attributed to the highly porous structure of freeze dried sample that allow easier diffusion of oxygen through to the encapsulated oil and also increases the amount of surface oil (Eratte et al., 2014). The relatively high OSI value of spray dried microcapsules/co-microcapsules can be attributed to the compact structure of the spray dried solid microcapsules, less pores on the shell and lower surface oil content (Eratte et al., 2014).


0 day (Before storage)
FD WPI-O-GA
FD WPI-P-O-GA
SD WPI-O-GA
SD WPI-P-O-GA
Conductivity (µs/cm)
Time (h)
A
30 days storage
FD WPI-O-GA-5 °C
FD WPI-P-O-GA-5 °C
SD WPI-O-GA-5 °C
SD WPI-P-O-GA-5 °C
FD WPI-O-GA-25 °C
FD WPI-P-O-GA-25 °C
SD WPI-O-GA-25 °C
SD WPI-P-O-GA-25 °C
Conductivity (µs/cm)
Time (h)
B

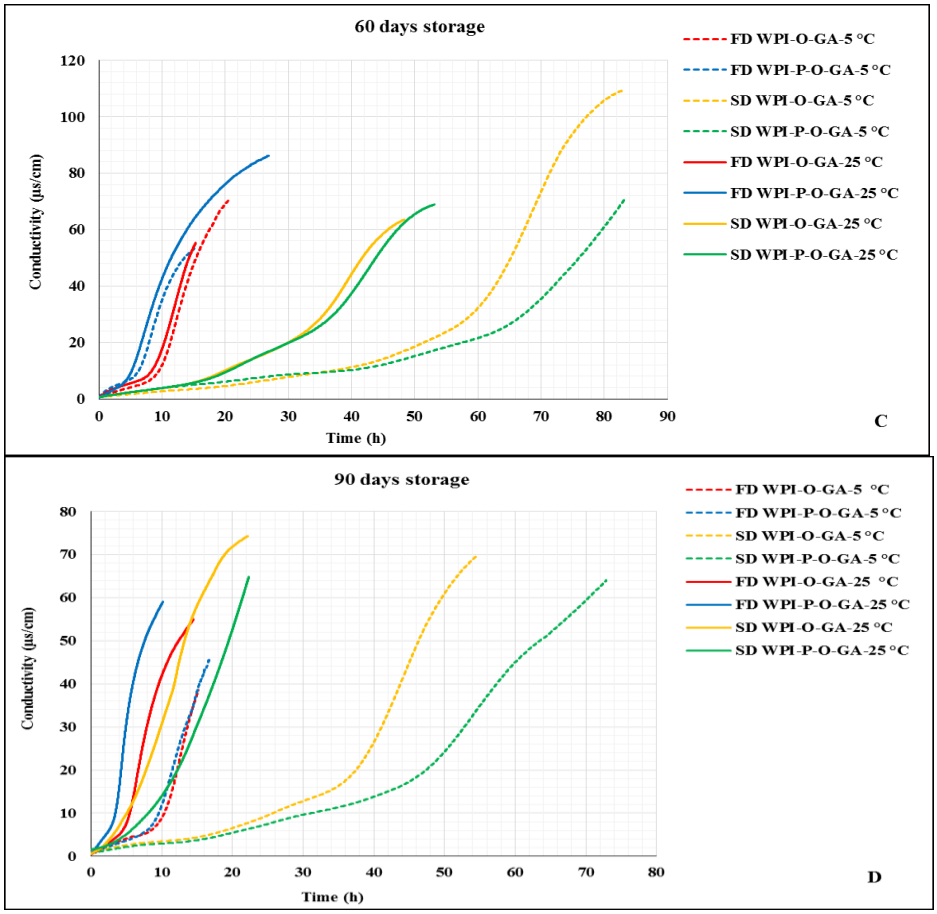


Fig. 6.4: Oxidative stability index (OSI) of freeze (FD) and spray (SD) dried microcapsules/ co-microcapsules on storage measured by accelerated oxidation test using Rancimat^{TM}. (A) Microcapsules immediately after drying; (B) Microcapsules stored for 30 days; (C) Microcapsules stored for 60 days; (D) Microcapsules stored for 90 days. Data represents OSI in mean ± SD of three replicate.

The OSI value was found to decrease with increasing storage temperature and time and was found to be higher in co-microcapsules (containing both *L. casei* and tuna oil) than in microcapsule (containing only tuna oil) obtained from spray drying. There was no statistically significant ($p>0.05$) difference between OSI values of freeze dried WPI-O-GA and freeze dried WPI-P-O-GA. It has been reported that lactic acid bacteria do not have pro-oxidant properties but do provide some antioxidant benefits to unsaturated free fatty

acids (Talon et al., 2000). In general, the spray dried co-microcapsule containing *L. casei* and tuna oil had better oxidative stability during storage compared to that of spray dried microcapsules containing only tuna oil. This could be due to the presence of probiotic bacterial cells on the encapsulation matrix and subsequent denser packaging of co-microcapsule particles compared to that of microcapsules, thereby minimizing the diffusion of oxygen and moisture and enhancing oxidative stability.

6.3.3.2. Primary and secondary oxidation products on storage

Both the primary and secondary oxidation products were analysed as indicators of lipid oxidation of the microencapsulated tuna oil. Primary oxidation of microencapsulated tuna oil was determined by measuring peroxide value (PV). Changes in the PVs of encapsulated omega-3 in co-microcapsules and microcapsules at 5 and 25° C for the entire storage period are plotted in Fig. 6.5A. These PV results reveal that the oxidation gradually increased with increasing storage time. AV indicates the amount of secondary oxidation products such as α and β-alkenals and related compounds that react with *p*-Anisidine. Fig. 6.5B shows AV values for microencapsulated omega-3 during storage. Storage temperature also exerted notable effect on the lipid oxidation as the microcapsules were more oxidative stable at 5 than at 25° C. A comparison of Fig. 6.5A (PV values) with Fig. 6.5B (AV values) shows that both the primary and secondary oxidation products are produced simultaneously. This is because oxidation is triggered by the presence of free radicals that abstract a hydrogen atom from the carbon atoms adjacent to the double bond (Vandamme et al., 2015). Reactive oxygen species then react with these lipid radicals and produces lipid hydroperoxide, which eventually gets degraded into secondary lipid oxidation products (Frankel, 1991).

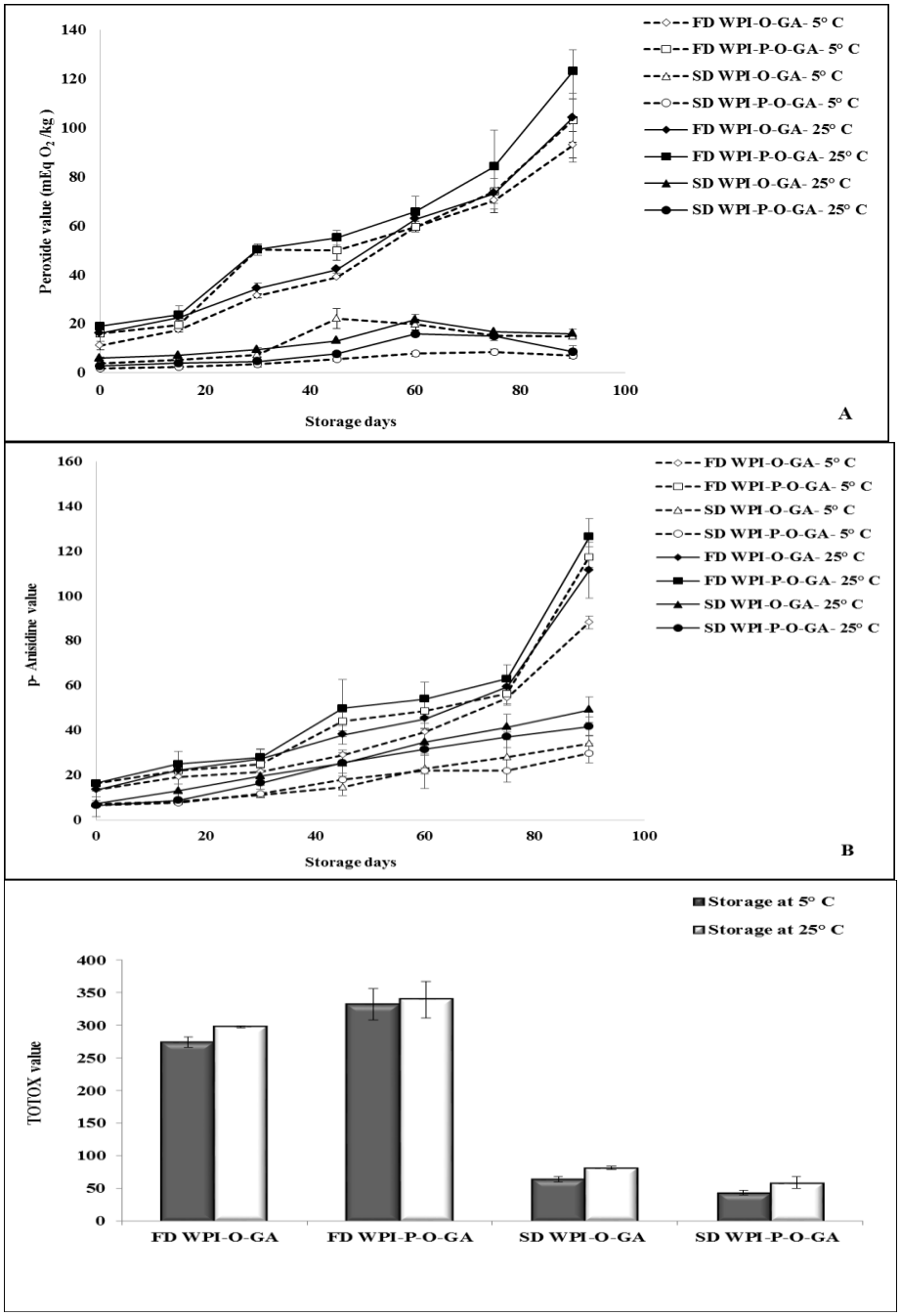


Fig. 6.5: Oxidation profile of freeze (FD) and spray (SD) dried microcapsules of omega-3 rich tuna oil when stored for 90 days at 5° C and 25° C: (A) Peroxide value (PV); (B) p-Anisidine value (AV); (C) Total oxidation value (TOTOX) after 90 days of storage at 5° C and 25° C. Data represent oxidation profile in mean ± SD of three replicate.

In general, the freeze dried WPI-O-GA and WPI-P-O-GA powders had significantly ($p<0.05$) higher PV and AV values than that of corresponding spray dried ones. These results are consistent with the OSI data (Fig. 6.4). The highly porous structure, larger surface area and comparatively greater surface oil have likely caused the decrease in the oxidative stability of freeze dried microcapsules (Eratte et al., 2014).

Total oxidation (TOTOX) values of microencapsulated omega-3 at 90 days of storage at both storage temperatures are shown in Fig. 6.5C. TOTOX provides information on the production of both primary and secondary oxidation products (Abramovic et al., 2005). TOTOX values were found to be significantly lower in spray dried WPI-P-O-GA co-microcapsules stored at both temperatures compared to those of other microcapsules. Limits on the amount of primary and secondary oxidation products are based on the palatability rather than health impacts (Albert et al., 2015). We set limits for the spray dried WPI-P-O-GA stored at 5° C for 30 days at PV, AV and TOTOX values of < 5 meq/kg, < 20 and < 26, respectively.

6.3.4. Correlation between the viability of *L. casei* and oxidative stability of omega-3 oil in WPI-P-O-GA co-microcapsules during storage

Synergism was observed between the oxidative stability of omega-3 oil and vitality/viability of *L. casei* in the freshly prepared WPI-P-O-GA co-microcapsules produced by both spray and freeze drying. However, these drying techniques affected the storage stability in different ways. Spray dried WPI-P-O-GA was found to have the highest oxidative stability at the tested storage temperature (5 and 25° C) and the storage time (90 days). Similarly, the vitality and viability of *L. casei* were highest in spray dried WPI-P-O-

GA. To the contrary, the oxidative stability of omega-3 oil was significantly ($p<0.05$) lower in freeze dried WPI-P-O-GA co-microcapsules. The storage stability and vitality of *L. casei* were poor in freeze dried WPI-P-O-GA. The viability and vitality of *L. casei* in freeze dried WPI-P-GA was higher than that of freeze dried WPI-P-O-GA, indicating that the presence of omega-3 rich oil in the freeze dried co-microcapsules negatively affects the viability and vitality of bacteria due to greater access of oxygen through the porous structure. The spray dried co-microcapsules (WPI-P-O-GA) had higher oxidative stability compared to that of spray dried capsules containing only the omega-3 rich oil (WPI-O-GA) or the control, which indicates that the presence of bacterial cells in microcapsule shell provides protection against oil oxidation. The vitality of *L. casei* in spray dried WPI-P-O-GA was higher than that in the spray dried WPI-P-GA (control) due to the protective effect of omega-3 oil on the bacterial cells. Therefore, co-encapsulation of omega-3 oil and *L. casei* in WPI-GA matrix followed by spray drying provides a synergistic effect in terms of oxidative stability of omega-3 oil and survival and vitality of *L. casei* on storage. The co-microencapsulation of omega-3 oil and *L. casei* in WPI-GA matrix followed by freeze drying exhibited synergism in the freshly prepared co-microcapsules; however, this synergism was lost during storage.

6.4. Conclusions

The effects of storage temperature and time on the survival and fermentation activity of *L. casei* and oxidative stability of omega-3 rich oil when they were microencapsulated together in whey protein isolate (WPI)-gum Arabic (GA) complex coacervate solid co-microcapsules (WPI-P-O-GA) were determined, and compared with results from microencapsulation of omega-3 rich oil and the probiotic alone. Synergistically enhanced

oxidative stability of omega-3 rich tuna oil and viability and vitality of *L. casei* was observed in these co-microcapsules during storage in those produced through spray drying. Thus, the co-encapsulation of omega-3 oil and *L. casei* in WPI-GA complex coacervate followed by spray drying produces co-microcapsules with higher fermentability of bacteria and higher oxidative stability of omega-3 oil. The mechanism for improved stability of the oil in co-microcapsules is unclear, but may involve shielding of oil by the probiotic rich WPI-GA complexed shell matrix and resultant denser packing providing an improved oxygen barrier. Further work is required to determine the mechanisms involved. Whatever the mechanism, it appears that a synergetic interaction occurs between omega-3 oil and probiotic bacteria in co-microcapsules that enhances the stability of the omega-3 oil and the viability and vitality of the probiotic bacteria. These co-microcapsules provide the potential benefit of both omega-3 fatty acids and probiotic bacteria in a single functional food ingredient, providing compatible shell matrix for these two quite different materials functional ingredients for delivery to be incorporated in functional foods.

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Chapter 7

In-vitro digestion of probiotic bacteria and omega-3 oil co-microencapsulated in whey protein isolate-gum Arabic complex coacervates

Abstract

Solid co-microcapsules of omega-3 rich tuna oil (O) and probiotic bacteria *L. casei* (P) were produced using whey protein isolate (WPI)–gum Arabic (GA) complex coacervate as wall material. The in-vitro digestibility of the co-microcapsules (WPI-P-O-GA) and microcapsules (WPI-P-GA and WPI-O-GA) was studied in terms of survival of *L. casei* and release of oil in sequential exposure to simulated salivary (SSF), gastric (SGF) and intestinal (SIF) fluids. Co-microencapsulation significantly increased the survival and surface hydrophobicity and the ability of *L. casei* to adhere to the intestinal wall. No significant difference in the assimilative reduction of cholesterol was observed between the microencapsulated and co-microencapsulated *L. casei*. The pattern of release of oil from the microcapsules and co-microcapsules was similar. However, the content of total chemically intact omega-3 fatty acids was higher in the oil released from co-microcapsules than the oil released from microcapsules. The co-microencapsulation can deliver bacterial cells and omega-3 fatty acids to human intestinal system with less impact on their functional properties.

Key words:

Co-microencapsulation, Omega-3 oil, Probiotic bacteria, In-vitro digestion, Survival, Simulated digestive fluids

7.1. Introduction

There is a significant commercial interest for functional foods containing long chain omega-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The estimated global demand for omega-3 ingredients was 1.6 billion USD in 2010 and is expected to be greater than 4.0 billion USD in 2018 (Transparency Market Research, 2013). Long chain PUFAs play an important role in human health (Shahidi, 2015). The dietary intake of these fatty acids has been associated with improving cardiovascular health (Bonafini et al., 2015), combating neural disorders (Blondeau, 2016), helping in infant brain and vision development (Wu et al., 2015), and combating some forms of cancer (Rose et al., 1999). However, omega-3 fatty acids are highly susceptible to oxidative degradation due to high degree of unsaturation (Jacobsen, 2010). Lipid oxidation impairs flavour and nutritional value, and shortens the shelf-life of products containing omega-3 fatty acids (Kaushik et al., 2015). Microencapsulation is effective in stabilizing omega-3 fatty acids and for masking the inherent fishy odour of fish oils (Nickerson et al., 2014). Microencapsulated omega-3 fatty acids have been used to fortify a range of food products such as breads, milk, fruit juices, tortillas, chocolate, and yogurt drinks (Kaushik et al., 2015; Kadam et al., 2010).

Alternatively, probiotic bacteria are live microorganism which provide numerous health benefits in human (Salminen et al., 2016). These benefits include control of serum cholesterol levels and intestinal infection, positively influencing the immune system (de Roos et al., 2000), improving lactose utilization (Post, 2013) and providing anticarcinogenic activities (Maleki et al., 2016). Many studies report on various aspects of incorporation of probiotic bacteria into a range of food products (Farnworth et al., 2016;

Shori, 2016). One of the major challenges regarding the application of probiotic bacterial cultures in functional foods is the maintenance of their viability during processing and passage through the host's gastrointestinal system (De Prisco et al., 2016). Microencapsulation is a promising technology for introducing viable probiotic bacteria in various foods as the encapsulating shell material provides protection to the encapsulated bacterial cells against environmental stressors such as temperature, pH and mechanical shear (Mattila-Sandholm et al., 2002). The microcapsules containing bacterial cells, if properly designed, are able to maintain their integrity during passage through the gastrointestinal tract until they reach their target destination (colon), where they should break down and release the probiotic bacteria (Del Piano et al., 2011).

In our previous work, we reported the co-microencapsulation of omega-3 rich tuna oil and probiotic bacteria *L. casei* in whey protein isolate-gum Arabic complex coacervate matrix and detailed the synergistic effect of this co-encapsulation to bacterial survival and stability against oxidation during storage (Eratte et al., 2015; Eratte et al., 2016b). In this study, address the in-vitro digestion of co-microcapsules containing omega-3 oil and probiotic bacteria when they are sequentially exposed to simulated salivary (SSF), gastric (SGF) and intestinal (SIF) fluids. In-vitro digestion behaviour of individual microcapsules containing probiotic bacteria (WPI-P-GA) or tuna oil (WPI-O-GA) distinctly were also studied as controls. To date, there is no study which reports the digestion behaviour of co-microcapsules containing two distinct bioactive ingredients such as omega-3 oil and probiotic bacteria.

The site-specific delivery of bioactive ingredients to a targeted site in the gastrointestinal tract is of great interest (Hirsch et al., 1999; Kosaraju et al., 2009). Hence, in order to ensure the delivery of co-microencapsulated omega-3 oil and probiotic bacteria to targeted sites of the human digestive system, it is important to test the stability and release of both omega-3 and probiotic bacterial cells either in-vivo or in-vitro using simulated digestive fluids. In the human digestive system, food is first exposed to salivary enzymes at pH ~ 7.0 and then digested by gastric pepsin at low pH of ~ 3.0, and eventually reaches the intestine having pH ~ 7.0 (Minekus et al., 2014). To understand the possible release of omega-3 oil and survival of probiotic bacteria during their passage through the human gastrointestinal digestion system, the co-microcapsules were subjected to SSF, SGF and SIF and the survival of *L. casei* and release of omega-3 oil were evaluated. The novelty of this study lies in that it advances the concept of co-microencapsulation of probiotic bacteria and omega-3 fatty acids by quantifying viability of the former and release of the latter in-vitro using SSF, SGF and SIF.

In this context, this study addresses two major research objectives. Firstly, to quantify the survival of *L. casei* in co-microcapsules (WPI-P-O-GA) when sequentially exposed to SSF, SGF and SIF, in comparison to microcapsules containing only *L. casei* (WPI-P-GA). Secondly, to quantify and compare the release properties of major omega-3 fatty acids from co-encapsulated tuna oil (WPI-P-O-GA) when sequentially exposed to SSF, SGF and SIF with that of microcapsules containing only tuna oil (WPI-O-GA).

7.2. Materials and Methods

7.2.1. Materials

L. casei 431 was used as a model probiotic bacteria and was kindly donated by Chr.Hansen (Horsholm, Denmark). Tuna oil (HiDHA) was donated by NuMega Ingredients Ltd. (Melbourne, Australia). Tuna oil is rich in omega-3 fatty acids particularly DHA. The oil samples were stored at 4±0.5° C until use. Whey protein isolate (WPI 895^{TM}) was donated by Fonterra Cooperative (Melbourne, Australia). Gum Arabic was purchased from Sigma-Aldrich Ltd. (Sydney, Australia). Enzymes including α-amylase from human saliva (Type 1X-A, 1000-3000 units/mg protein), pepsin from porcine gastric mucosa (3200–4500 units/mg protein), pancreatin from porcine pancreas, bile salt (B8756) were obtained from Sigma–Aldrich Ltd. (Sydney, Australia). All other chemicals were purchased from Sigma–Aldrich and were of analytical grade. All of the above ingredients and chemicals were used as received without further purification or alteration.

7.2.2. Preparation of *L. casei* culture

L. casei was cultured for 18 h in sterile MRS broth (1%, w/v) at 37±0.5° C. This culture was further sub-cultured (37±0.5° C, 18 h) twice in the same broth to activate bacterial cells and to allow them to adapt. All inoculation works were carried out under a sterile biological hoods with laminar air flow (Auramini, Laftech, Australia). Finally, 1000 mL of media was used for bulk culturing and cells were harvested at the stationary growth phase by centrifuging at 2200 × g for 15 min at 5° C. The supernatant was discarded and the precipitated cell mass was washed twice with sterile peptone water (0.2%, w/v). The final wet cell mass was weighed and divided into two equal portions. One portion (wet mass ~5

g) was used to prepare WPI-P-GA microcapsules and the second equal half was used to prepare WPI-P-O-GA co-microcapsules (Eratte et al., 2015).

7.2.3. Microencapsulation process

The complex coacervation and microencapsulation procedures were carried out according to the following procedures described in our previous works (Eratte et al., 2014; Eratte et al., 2015) with slight modification. These processes are briefly presented in subsections 7.2.3.1, 7.2.3.2 and 7.2.3.3.

7.2.3.1. Microencapsulation of tuna oil

A solution of 15 g tuna oil and 250 mL WPI solution (12%, w/v) were stirred (IKA® RW 20 digital overhead stirrer, Staufen, Germany) at 800 rpm for 10 min and then homogenized using a microfluidizer at 45 MPa for 3 passes (M110L, Microfluidics, Newton, MA, USA) to produce an O/W emulsion. Then a 250 mL GA solution (4%, w/v) was added drop wise into this O/W emulsion with continuous stirring at 400 rpm and the pH adjusted to 3.75 by adding 1% citric acid drop wise in order to induce complexation between WPI and GA.

7.2.3.2. Microencapsulation of *L. casei*

Similar to above, the previously prepared probiotic cell mass (Section 7.2.2) was added slowly into a WPI solution, and a GA solution was then added drop wise with continuous stirring, after which the pH was adjusted to 3.75.

7.2.3.3. Co-microencapsulation of omega-3 oil and *L. casei*

As above, tuna oil (15 g) was dispersed into a WPI solution and stirred for 10 min, followed by homogenizing using a microfluidizer to produce an O/W emulsion. Previously

prepared probiotic cell mass was then added into this O/W emulsion and GA solution was added with continuous stirring at 400 rpm, and the pH adjusted to 3.75 as above.

The microencapsulation procedure in all the three cases were carried out at 25° C, followed by keeping the liquid microcapsules at 5° C for 24 h to ensure the complete formation of complex coacervates. After maintaining the sample at 5° C for 24 h, 100 mL of 4% (w/w) transglutaminase dispersion was added to induce the crosslinking. The slurry was heated to 25° C to activate the enzyme and was kept at 25° C for 2 h to allow complete crosslinking. Finally, the microcapsules/co-microcapsules were dried to produce solid or powder co-microcapsules (WPI-O-GA, WPI-P-GA and WPI-P-O-GA).

7.2.4. Drying of coacervate liquid microcapsules

The liquid microcapsules produced as detailed in Section 7.2.3 were spray dried (B-290, BÜCHI Labortechnik, Flawil, Switzerland) using inlet and outlet temperatures of 130° C and 65° C, respectively. The flow rate of the drying air was 35 m^3/h at 100% fan aspiration. The powdered microcapsules/co-microcapsules were collected and stored at 5° C for further characterizations.

7.2.5. Preparation of simulated digestive fluids and in-vitro digestion of microcapsules

Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the method proposed by Minekus et al., (2014) with slight modification as presented in Table 7.1 and includes the details of the stock solutions used. The pH of the stock solution was adjusted using 1.0 M NaOH and 1.0 M HCl. Calcium chloride ($CaCl_2$) stock solution was prepared by dissolving 44.1g/L. $CaCl_2(H_2O)_2$

was not added to the electrolyte stock solutions to avoid precipitation. Instead, it was added to the final mixture of simulated digestive fluid and microcapsule/ co-microcapsule powder (Table 7.1).

Table 7.1: Detailed composition of stock solutions used to prepare simulated digestive fluids. The final volume was adjusted to 500 mL with distilled water for each simulated fluid. SSF = Simulated Salivary Fluid, SGF = Simulated Gastric Fluid, SIF = Simulated Intestinal Fluid

Components	Stock concentration (g/L)	SSF (pH 7.0)	SGF (pH 3.0)	SIF (pH 7.0)
		Volume of stock (mL)	Volume of stock (mL)	Volume of stock (mL)
KCl	37.3	20.1	6.9	6.8
KH_2PO_4	68	8.7	0.9	0.8
$NaHCO_3$	84	11.8	12.5	42.5
NaCl	117	-	11.8	9.6
$MgCl_2(H_2O)_6$	30.5	1.0	0.4	1.1
$(NH_4)_2CO_3$	48	0.12	0.5	-

7.2.5.1. In-vitro digestion in the oral phase

Five grams of microcapsule/co-microcapsule powder was mixed with 7 mL of SSF electrolyte stock solution. Then, 0.5 mL salivary α-amylase solution (1500 units/mL) prepared with SSF electrolyte stock solution was added. This was followed by addition of

50 µL 0.3 M $CaCl_2$ and 2 mL water and was thoroughly mixed. The pH was adjusted to 7.0 and the oral bolus thus prepared was kept at 37° C for 2 min.

7.2.5.2. In-vitro digestion in the gastric phase

The oral bolus obtained was mixed with 7.5 mL of SGF electrolyte stock solution. Then, 1.6 mL porcine pepsin stock solution (25000 units/ mL) prepared with SGF electrolyte stock solution was mixed thoroughly followed by addition of 5 µL 0.3 M $CaCl_2$, and 0.695 µL water. Then, the pH was adjusted to 3.0 with HCl. The gastric chime thus prepared was kept at shaking incubator at 100 rpm at 37° C for 2h.

7.2.5.3. In-vitro digestion in the intestinal phase

The gastric chime obtained was mixed with 11 mL of SIF electrolyte stock solution, 5.0 mL of a pancreatin solution (800 units/ mL) prepared with SIF electrolyte stock solution, 2.5 mL fresh bile (160 mM in fresh bile), 40 µL of 0.3 M $CaCl_2$, and 1.5 mL of water. The pH of the mixture was adjusted to 7.0 using NaOH. The intestinal phase thus prepared was kept at shaking incubator at 100 rpm at 37° C for 3h.

7.2.6. Enumeration of bacterial viability

The viability of *L. casei* was assessed in MRS agar (CM0361, Thermo scientific, Melbourne, Australia) using the spread plating technique as described in our earlier work (Eratte et al., 2015). Samples were taken from SSF every 30 seconds and every 30 min from SGF and SIF. Briefly, the sample (1 mL) was diluted in 9 mL of sterile peptone water (0.2%, w/v) and placed in a stomacher for 30 sec. The cell suspension was then serially diluted and plated on MRS agar plate and incubated under anaerobic condition (Oxoid™ Anaerojar™, Thermoscientific, Australia) at 37° C for 48 h. The plating and enumeration were performed in triplicate.

7.2.7. Assimilation of cholesterol by microencapsulated and co-microencapsulated *L. casei*

7.2.7.1. Preparation of cholesterol micelles

The cholesterol micelles were prepared according to the methodology given by Kim et al., (2008). Briefly, cholesterol (9.1 mg) and α-lecithin (0.2 mL) were inoculated in 1.0 mL chloroform. A stream of nitrogen gas was used to facilitate the evaporation of solvent and then 10.0 mL of 0.4 mole/L sucrose solution was added. The samples were ultra-sonicated (M220 Focused-ultrasonicator™, Massachusetts, USA) for 15 min and cooled for 5 min. Once the ultra-sonication was repeated for five times, the samples were centrifuged at 24,471 × g at 20° C for 30 min. The supernatant was sterile-filtered through a filter (0.45 mm, Minisart® high flow syringe filters) and was then used for cholesterol assimilation study.

7.2.7.2. Determination of cholesterol assimilation ability

The cholesterol assimilation ability of microencapsulated and co-microencapsulated *L. casei* was carried out according to the method described by Kim et al., (2008). Firstly, MRS broth supplemented with 0.2 g/100 mL of thioglycolate and 0.3 g/100 mL of oxgall was prepared and sterilized at 121° C for 20 min. Each of the microencapsulated and co-microencapsulated probiotic bacteria preparations (~ 10^7 cfu/g) were inoculated into a mixture (9:1) of MRS broth and cholesterol–phosphatidylcholine micelles and incubated at 37° C for 18 h under anaerobic condition. After incubation, probiotic bacteria were removed by centrifugation for 10 min at 2000 × g at 4° C. The supernatant (1 mL) was placed into two clean sterile test tubes. Three mL of 95% ethanol and 2 mL of 50%

potassium hydroxide were added to each tube and were thoroughly mixed. Both test tubes were heated at 60° C for 10 min in a water bath and then cooled down to room temperature (25° C). Five millilitres of hexane was dispensed into each tube and mixed with aliquots for 30 s. The test tubes were allowed to stand for 15 min at room temperature to permit phase separation. Then 2.5 mL of the hexane layer was transferred into a clean test tube. The hexane was evaporated by the flow of nitrogen gas and subsequently by heating at 60° C. Four millilitres of o-phthalaldehyde reagent containing 0.55 mg of o-phthalaldehyde per millilitre of glacial acetic acid was added to each tube and allowed stand for 10 min at ambient temperature. Two millilitres of sulfuric acid was pipetted slowly down the inside of each tube. Each tube was immediately mixed and was allowed to stand at ambient temperature for 10 min. The absorbance was measured at 550 nm using a UV spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Triplicate runs were carried out for each sample. The cholesterol assimilation rate was calculated using equation (1):

$$C\ (\%) = 100 - \left(\frac{A}{B} \times 100 \right) \qquad (1)$$

whereas C indicates the percentage (%) of cholesterol reduction, A indicates optical density of microencapsulated or co-microencapsulated probiotic bacteria after incubation for 18 h at 37° C and B indicates optical density of control in MRS broth supplemented with cholesterol–phosphatidylcholine micelles.

7.2.8. Surface hydrophobicity

The measurement of surface hydrophobicity was carried out according to the method given by Vinderola et al., (2003). Bacterial cells were released from microcapsules/co-microcapsules using 0.4 M sodium phosphate buffer. The released bacterial cells were

centrifuged at 14000 × *g* for 5 min at 4° C, washed twice with 50 mM K_2HPO_4 (pH 7.0), and then resuspended in 50 mM K_2HPO_4 to realise an absorbance value of 0.5 at 560 nm. Five millilitre of the suspension was mixed with 1 mL of *n*-hexadecane by vortexing for 120 s and was incubated for 1 h at room temperature. Changes in the absorbance (A) of probiotic bacterial suspension were recorded using a UV–Vis spectrophotometer. The surface hydrophobicity was determined using the equation (2), given below.

$$SH\ (\%) = \frac{(A_0 - A)}{A_0} \times 100 \qquad (2)$$

where SH, A_0 and A are the surface hydrophobicity (%), absorbance before and after extraction with *n*-hexadecane, respectively.

7.2.9. Estimation of released oil and omega-3 fatty acids during in-vitro digestion

The release of omega-3 oil before and after subjecting the microcapsules/co-microcapsules to the digestion fluids was estimated using the method reported (Kosaraju et al., 2009) with slight modification. Briefly, petroleum ether (75 mL) was added to the release medium containing microencapsulated/co-microencapsulated omega-3 oil and the mixture was shaken thoroughly on an orbital shaker (Stuart SSL-1, Carl Roth, Karlsruhe, Germany). The solvent phase was then filtered using filter papers (Whatman, 5 µm) and the solid particles caught on the filter were washed three times with 20 mL of petroleum in each wash. The filtrate was dried under nitrogen followed by drying at 100° C for 1 h in an oven to remove the residual solvent. The amount of oil released from the sample was expressed as % of total oil present in the microcapsule sample.

The omega-3 fatty acid profile of the released oil was determined using gas chromatography. Before undertaking the chromatographic experiments, the oil samples were transesterified to their respective fatty acids methyl esters (FAMEs) according to Kolanowski (2010). The resulting FAMEs were analysed using a GC column BPX70, (60 m × 0.22 mm ×0.25µm) installed in a gas chromatograph system (Agilent 7890A GC, California, USA) equipped with a flame ionisation detector. The initial oven temperature was 180° C, which was maintained for 5 min before increasing to 210° C at a rate of 1° C/min and then maintained at 210° C for 60 minutes. Helium was used as the carrier gas and the column head pressure was 76 kPa. The split ratio used was 20:1. Peaks were identified using retention times obtained for standard FAME mixture. The major omega-3 fatty acids such as hexadecatrienoic acid (HTA, 16:3w3), α-linolenic acid (ALA, 18:3w3), stearidonic acid (SDA, 18:4w3), eicosatetraenoic acid (ETA, 20:4w3), eicosapentaenoic acid (EPA, 20:5w3), docosapentaenoic acid (DPA, 22:5w3), and docosahexaenoic acid (DHA, 22:6w3) in the released oil were expressed as mg/ g of oil.

7.2.10. Statistical Analysis

All the experiments were carried out in duplicate or triplicate, as stated. The Minitab 16™ statistical software package (University Park, Pennsylvania, United States) was used for the analysis of variance (ANOVA) to determine the significant differences. The confidence level of 95% ($p < 0.05$) was used to determine significant difference between any two mean values.

7.3. Results and discussion

7.3.1. Survival of unencapsulated, microencapsulated and co-microencapsulated *L. casei* in simulated digestive fluids

The survival or viability of the unencapsulated (P), microencapsulated (WPI-P-GA) and co-microencapsulated (WPI-P-O-GA) *L. casei* in simulated digestive fluids (SSF, SGF, and SIF) is shown in Fig. 7.1. This strain showed a steady loss in viability when exposed to simulated digestive fluids; however, its susceptibility to SSF, SGF and SIF was quite different. No significant ($p>0.05$) decrease in probiotic cell counts was detected after exposure to simulated mouth conditions (SSF) for both microencapsulated and co-microencapsulated *L. casei* (Fig. 7.1: inset). There was a significant loss of viability (~ 0.5 log cfu/mL) of unencapsulated free bacterial cells when exposed to SSF. This indicates that microencapsulation of *L. casei* in complex coacervate shell material helps to improve its survival in SSF.

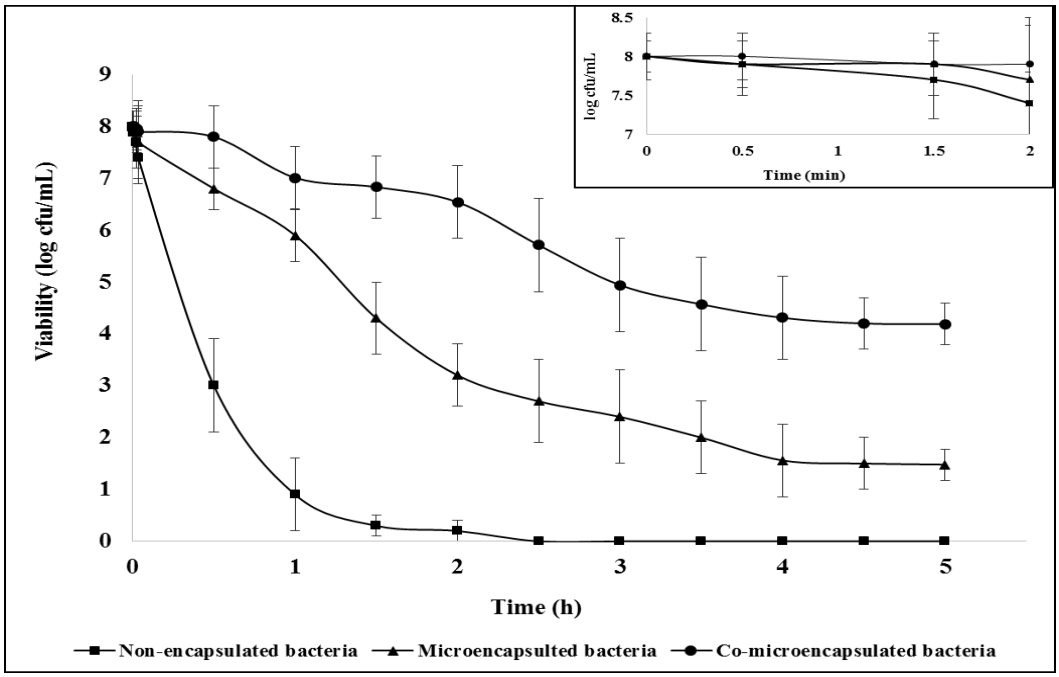

Fig. 7.1: Survival of unencapsulated, microencapsulated and co-microencapsulated *L. casei* upon sequential exposure to simulated salivary fluid (SSF) for 2 min, simulated gastric fluid (SGF) for 2 h and simulated intestinal fluid (SIF) for 3h. The inset represents the survival of cells when exposed in SSF for 2 min.

When the samples were exposed to the gastric condition, the number of viable cells significantly ($p < 0.05$) decreased in all three cases. The unencapsulated or free bacterial cells lost all viability within 1.5 h in SGF. There was a significantly ($p<0.05$) greater decrease in the viability of microencapsulated cells (4.8 log cfu/mL), when compared to the co-microencapsulated cells (1.5 log cfu/mL) in the SGF condition. This result indicate that the presence of omega-3 oil together with the bacterial cells provides better protection to the latter during the gastric simulation.

No viable cells of *L. casei* managed to reach the SGF when they were not protected. The co-microencapsulated bacterial cells survived better in SIF than the microencapsulated bacterial cells. This indicates that the presence of omega-3 oil in general greatly improves the survival of bacterial cells in SIF. It has been shown recently that the dietary PUFAs, particularly eicosapentaenoic acid (EPA), promoted the secretion of immune signalling protein TGF-β1 (Transforming Growth Factor β1) by gut bacteria and thus improved their viability (Bentley-Hewitt et al., 2015). Our results, as articulated above, strongly support that the co-microencapsulation of omega-3 oil and probiotic bacteria improves the survival of probiotic bacteria in the human digestive system.

7.3.2. Assimilation of cholesterol by microencapsulated and co-microencapsulated *L. casei*

The cholesterol assimilation study was undertaken to access the degree to which co-microencapsulation preserves the health promoting properties of probiotic bacteria in the human digestive system. The percentage of cholesterol reduction by microencapsulated and co-microencapsulated *L. casei* is shown in Fig. 7.2. No significant difference ($p > 0.05$) in the extent of cholesterol assimilation was observed between the microencapsulated and co-microencapsulated *L. casei*. These results show that co-microencapsulation of omega-3 oil with *L. casei* improved the survival but did not improve the degree of cholesterol assimilation. It has been reported that non-viable cells of probiotic bacteria can also act as cholesterol reducing agents (Miremadi et al., 2014). This could explain the similar cholesterol reducing effects of microencapsulated and co-microencapsulated *L. casei*.

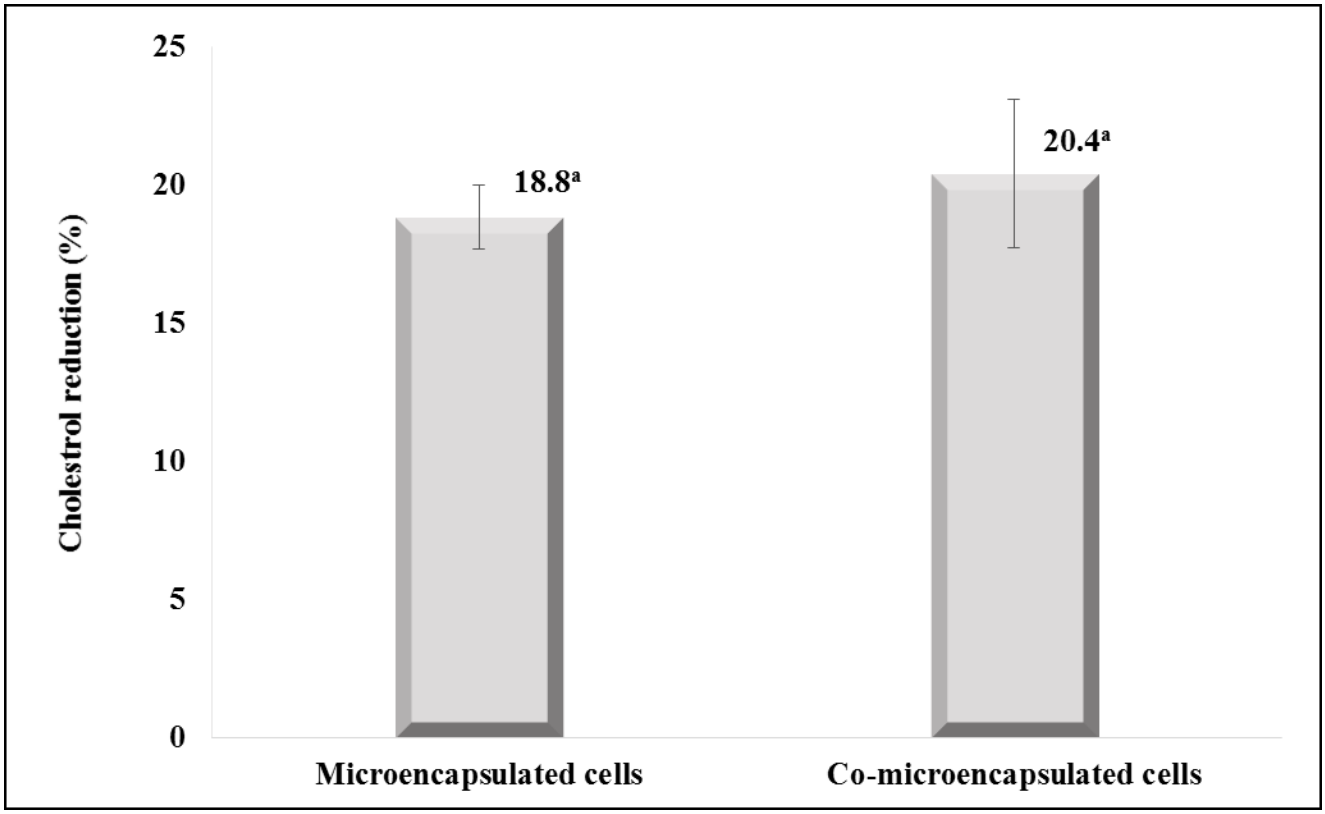

Fig. 7.2: The percentage assimilation of cholesterol by microencapsulated and co-microencapsulated *L. casei.*

7.3.3. Surface hydrophobicity of microencapsulated and co-microencapsulated *L. casei*

The surface hydrophobicity of bacterial cells is an indirect measure of the ability of these cells to adhere to the intestinal lining. The surface hydrophobicity of microencapsulated and co-microencapsulated *L. casei* is shown in Fig. 7.3. It was reported that surface hydrophobicity correlated with the ability of bacterial cells to adhere to the intestinal wall (Botes et al., 2008). The surface hydrophobicity of co-microencapsulated *L. casei* cells (43.7%) was significantly ($p<0.05$) greater than that of microencapsulated cells (31.3%). It has also been reported that the higher the surface hydrophobicity of probiotic bacteria, the

greater is its ability to adhere to the epithelial cells of intestines (Duary et al., 2011). Higher surface hydrophobicity in co-microencapsulated *L. casei* cells also indicated that the structural integrity of cell membrane (phospholipids and glycolipids) was better preserved in co-microcapsules than in microcapsules. Thus, the better preserved hydrophobic components of the membrane, such as fatty acids or S-protein, could better promote the adherence of cells to hydrophobic components of intestinal lining.

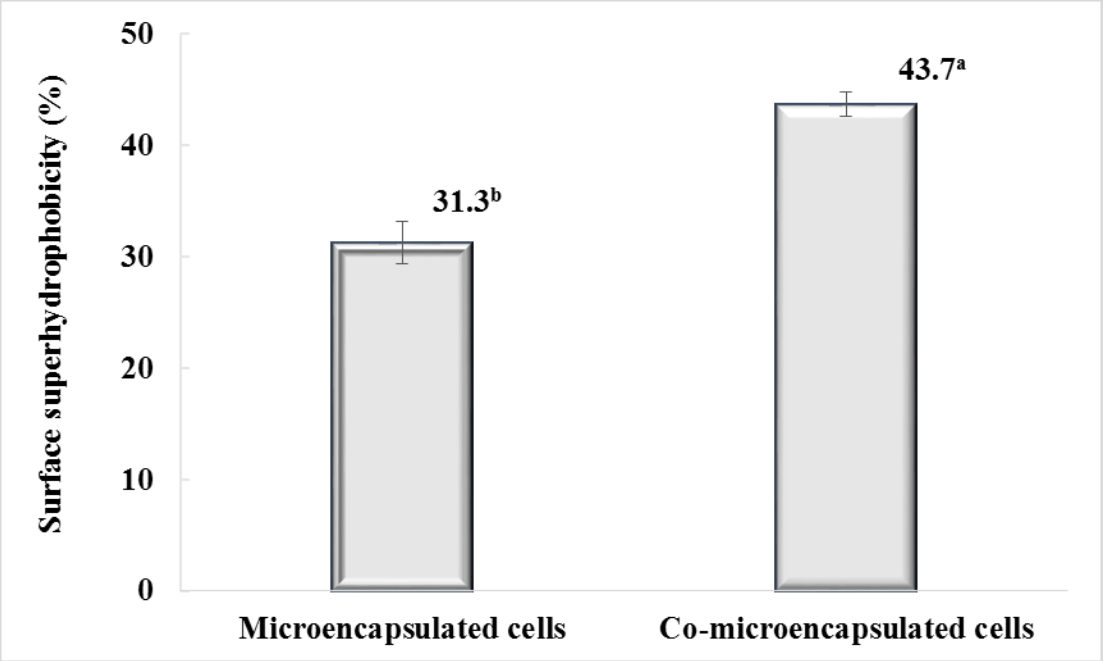

Fig. 7.3: Surface hydrophobicity as a measure of intestinal lining ability of microencapsulated and co-microencapsulated *L. casei*. Different lower case letters in superscript indicate significant difference ($p < 0.05$).

7.3.4. In-vitro release of omega-3 from microcapsules and co-microcapsules

It is essential to evaluate the release behaviour of omega-3 oil from microcapsules and co-microcapsules during gastrointestinal transit in order to estimate the effectiveness of these microcapsules and co-microcapsules to deliver it to the targeted site of the gastrointestinal

tract. The percentage of oil released upon sequential exposure to SSF, SGF and SIF is shown in Fig. 7.4. As shown, there was no significant difference ($p > 0.05$) in the percentage of oil released from WPI-O-GA microcapsules and WPI-P-O-GA co-microcapsules in all the simulated digestive fluids. However, there was a significant difference in the percentage of oil released among SSF, SGF, and SIF. These differences can be attributed to the different pH and the presence of different enzymes in these different digestive fluids.

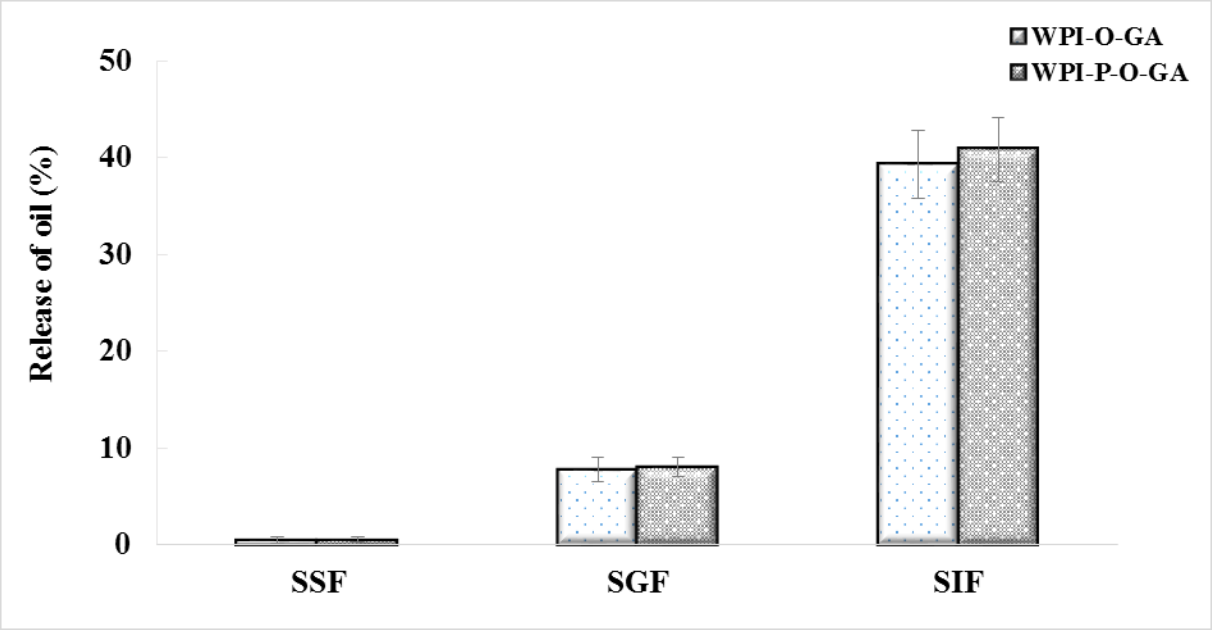

Fig. 7.4: Release of omega-3 oil from WPI-O-GA microcapsules and WPI-P-O-GA co-microcapsules upon sequential exposure to simulated salivary fluid (SSF) for 2 min, simulated gastric fluid (SGF) for 2 h and simulated intestinal fluid (SIF) for 3h.

It is expected that the presence of different digestive enzymes and different prevailing pH degrade WPI-GA complex coacervate wall matrix. This different degree of degradation of encapsulating shell releases significantly different amounts of encapsulated oil. As expected, the lowest amount of oil was released in SSF and the highest amount of oil was

released in SIF. The much lower amount of oil released in SSF and SGF compared to SIF may be attributed to the much lower extent of degradation of WPI-GA complex coacervate shell material by the salivary and gastric enzymes. It has been reported that chemical crosslinking of protein in complex coacervates slowed down the proteolytic activity of gastric enzyme and resulted in slower release of oil in simulated gastric conditions (Kosaraju et al., 2009). Interestingly, the low pH (pH=3.0) does not seem to erode the WPI-GA matrix significantly, and we postulate this may be due to stability of these crosslinked complex coacervates at this pH. The larger amount of oil release in SIF (pH 7.0) compared to SGF (pH 3.0) can be attributed to the breaking down of both protein and polysaccharide by pancreatin present in SIF. The breaking down of WPI-GA complex coacervate matrix in SIF facilitated the extraction of oil in solvent.

7.3.5. Evaluation of major omega-3 fatty acids in the released oil

The amount of major omega-3 fatty acids such as hexadecatrienoic acid (HTA, 16:3w3), α-linolenic acid (ALA, 18:3w3), stearidonic acid (SDA, 18:4w3), eicosatetraenoic acid (ETA, 20:4w3), eicosapentaenoic acid (EPA, 20:5w3), eicosapentaenoic acid (DPA, 22:5w3), and docosahexaenoic acid (DHA, 22:6w3) in the unencapsulated or control tuna oil, and the oil extracted from solid WPI-O-GA microcapsules and released from WPI-O-GA microcapsules upon sequential exposure to SSF, SGF and SIF, are presented in Table 7.2A. The major omega-3 fatty acids in the oil extracted from WPI-P-O-GA co-microcapsules and in the released oil obtained from sequential exposure of WPI-P-O-GA co-microcapsules to SSF, SGF and SIF are given in Table 7.2B.

Table 7.1A: The major omega-3 fatty acids in the blank tuna oil, in the oil extracted from WPI-O-GA microcapsules and in the released oil from WPI-O-GA microcapsules during in-vitro digestion; SSF = Simulated Salivary Fluid, SGF = Simulated Gastric Fluid, SIF = Simulated Intestinal Fluid. Different lower case letters in superscript indicate significant difference ($p < 0.05$).

Omega-3 fatty acids	Blank tuna oil (mg/g of oil)	WPI-O-GA (mg/g of oil)	SSF (mg/g of oil)	SGF (mg/g of oil)	SIF (mg/g of oil)
16:3w3	9.70 ± 0.0^{no}	9.7 ± 0.1^{no}	8 ± 0.7^{no}	7.1 ± 0.1^{op}	6.9 ± 0.1^{pq}
18:3w3 ALA	4.10 ± 0.1^{rst}	3.35 ± 0.2^{t}	3.5 ± 0.7^{t}	3.25 ± 0.3^{t}	3.05 ± 0.1^{t}
18:4w3	6.05 ± 0.1^{pqr}	5.9 ± 0.1^{qrs}	4.75 ± 0.4^{rst}	4.65 ± 0.5^{rst}	4.45 ± 0.1^{rst}
20:4w3	4.60 ± 0.0^{rst}	3.95 ± 0.1^{st}	3.65 ± 0.2^{t}	3.4 ± 0.1^{t}	3.05 ± 0.1^{t}
20:5w3 EPA	59.81 ± 0.0^{j}	53.3 ± 0.4^{k}	48.88 ± 0.2^{l}	48.65 ± 0.2^{l}	47.85 ± 0.4^{l}
22:5w3	12.05 ± 0.1^{m}	11 ± 0.1^{mn}	10 ± 0.3^{n}	10 ± 0.0^{n}	9.85 ± 0.2^{no}
22:6w3 DHA	294.21 ± 0.0^{f}	280.5 ± 0.7^{g}	269 ± 1.4^{h}	266.8 ± 0.3^{i}	265.75 ± 0.4^{i}
Sum Omega-3 - PUFAS	390.52 ± 0.3^{a}	367.7 ± 0.7^{b}	347.78 ± 1.2^{c}	343.85 ± 1.3^{d}	340.9 ± 0.4^{e}

Table 7.2B: The major omega-3 fatty acids in the oil extracted from WPI-P-O-GA co microcapsules and in the released oil from WPI-P-O-GA co-microcapsules during in-vitro digestion; SSF = Simulated Salivary Fluid, SGF = Simulated Gastric Fluid, SIF = Simulated Intestinal Fluid. Different lower case letters in superscript indicate significant difference ($p < 0.05$).

Omega-3 fatty acids	WPI-P-O-GA (mg/g of oil)	SSF (mg/ g of oil)	SGF (mg/g of oil)	SIF (mg/g of oil)
16:3w3	9.65 ± 0.1^{jk}	9.65 ± 0.1^{jk}	7.04 ± 0.1^{kl}	7.04 ± 0.1^{kl}
18:3w3 ALA	3.95 ± 0.1^{lm}	3.91 ± 0.0^{lm}	3.67 ± 0.2^{lm}	3.04 ± 0.1^{m}
18:4w3	5.98 ± 0.0^{lm}	5.93 ± 0.1^{lm}	5.87 ± 0.1^{lm}	5.86 ± 0.0^{lm}
20:4w3	4.60 ± 0.0^{lm}	4.57 ± 0.1^{lm}	3.06 ± 0.1^{m}	3.02 ± 0.0^{m}
20:5w3 EPA	55.65 ± 0.8^{g}	55.2 ± 0.1^{gh}	52.10 ± 1.4^{hi}	51.74 ± 1.1^{i}
22:5w3	11.95 ± 0.1^{j}	11.95 ± 0.0^{j}	10.00 ± 0.3^{jk}	10.00 ± 0.3^{jk}
22:6w3 DHA	290.00 ± 1.4^{d}	289.5 ± 0.7^{d}	277.30 ± 2.4^{e}	273.25 ± 0.4^{f}
Sum Omega-3 - PUFAS	381.78 ± 2.1^{a}	380.68 ± 0.7^{a}	359.04 ± 1.3^{b}	353.94 ± 1.8^{c}

In general, there is a decrease in the omega-3 fatty acid content in the oil extracted from WPI-O-GA microcapsules and WPI-P-O-GA co-microcapsules when compared to that amount in unencapsulated or control tuna oil. There was also a significant decrease in EPA and DHA contents in the oil extracted from microcapsules and co-microcapsules from their content in the original tuna oil. The complex coacervation and spray drying processes might have caused this drop, possibly due to oxidative degradation. The total identified omega-3 fatty acids content also decreased upon microencapsulation (367.7 mg/g of oil)

and co-microencapsulation (381.78 mg/g of oil) compared to that in unencapsulated sample (390.52 mg/g of oil). This suggests that the PUFAs present in the encapsulated oil underwent some oxidative damage, which is not unexpected (Czerniak et al., 2015). However, a significant ($p < 0.05$) retention of total omega-3 fatty acids were observed from the oil released from co-microcapsules compared to that from microcapsules. This is probably due to the greater oxidative stability of co-microcapsules as compared to that of microcapsules (Eratte et al., 2016a).

There was no significant drop ($p > 0.05$) in total omega-3 fatty acids in the released oil from SSF digesta of WPI-P-O-GA microcapsules (380.68±0.7 mg/g of oil) when compared to that in oil released from undigested WPI-P-O-GA co-microcapsules (381.7± 2.1 mg/g of oil). However, there was a significant ($p<0.05$) drop in total omega-3 fatty acids content in the SSF digesta of WPI-O-GA microcapsules (347.78 ± 1.2 mg/g of oil). Salts contained in the SSF might not have interacted with co-microencapsulated PUFAs to the same extent as with microencapsulated PUFAs. There was a decrease in the total omega-3 fatty acids in the oil released from the digesta of SGF and SIF in both microcapsules and co-microcapsules. However, the decrease was comparatively higher for WPI-O-GA microcapsules. A significant decrease in DHA in the released oil was observed from microcapsules and co-microcapsules in all digestive fluids, compared to that of other omega-3 fatty acids tested. Retention of total omega-3 fatty acids in the oil extracted from WPI-P-O-GA co-microcapsules (90.63%) was significantly ($p<0.05$) higher than that in WPI-O-GA microcapsules (87.29%). The above observations show that although there was no significant effect of co-microencapsulation on the release properties of oil from microcapsules/co-microcapsules, there was a significantly ($p<0.05$) higher retention of

omega-3 fatty acids in co-microcapsules compared to that in microcapsules during oral, gastric and intestinal digestion phases. This may be due to the co-microencapsulation of omega-3 oil with probiotic bacteria providing better oxidative stability to omega-3 oil (Eratte et al., 2016b).

7.4. Conclusions

The in-vitro release and oxidative stability of omega-3 oil in co-microcapsules containing *L. casei* were determined. The co-microcapsules were prepared using WPI-GA complex coacervates. The co-microcapsules (WPI-P-O-GA) were sequentially subjected to simulated digestive fluids (SSF, SGF, and SIF) *in vitro*. The in-vitro digestion behaviour of microcapsules containing only *L. casei* (WPI-P-GA) and omega-3 rich tuna oil (WPI-O-GA) was also determined. The viability, cholesterol assimilation ability and cell surface hydrophobicity of *L. casei* in the microcapsules and co-microcapsules were determined. Co-microencapsulation was found to significantly improve the viability of probiotic bacteria during digestion; however, the cholesterol assimilation ability of *L. casei* did not improve. The cell surface hydrophobicity, which measures the ability of bacteria to adhere to the intestinal wall, was found to significantly improve when co-microencapsulated. No significant difference in the release properties of encapsulated omega-3 oil was observed between the microcapsules and co-microcapsules during the *in-vitro* digestion. However, the retention of total omega-3 fatty acids in the released oil was significantly higher in co-microencapsulated samples. This study shows that the co-microencapsulation of omega-3 rich oil with probiotic bacteria improved the survival and intestinal adhering ability and also helped to retain a higher proportion of omega-3 fatty acids in the human digestive

system. In-vivo clinical studies are required to further evaluate the survival/oxidative stability/ bioavailability of co-encapsulated probiotic bacteria and omega-3 fatty acids.

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Chapter 8

General Discussion, Conclusions and Recommendations

8.1. Introduction

The overarching goal of this research was to successfully co-encapsulate omega-3 rich tuna oil (O) and probiotic bacteria *L. casei* (P) in whey protein isolate (WPI) - gum Arabic (GA) complex coacervate matrix. Further studies on the interaction (synergistic/ competitive) between encapsulated omega-3 oil and encapsulated bacterial cells in terms of oxidative stability of the former and cell viability of the latter until it reached the human gastro-intestinal tracts were carried out. This thesis describes the detailed investigations on the underpinning science involved in the design, production and characterisation of physico-chemical properties of composite microcapsule comprising omega-3 fatty acids and probiotic bacteria in a single microcapsule. The outcomes contribute to both fundamental science and technological aspects in the body of knowledge of functional foods.

The co-encapsulation concept has been widely used in pharmaceutical delivery systems; however, co-encapsulation of more than one bioactive components with different characteristics such as omega-3 oil (hydrophobic) and probiotic bacteria (hydrophilic) is challenging and illustrates the novelty of this research. Powdered co-microcapsules containing omega-3 oil (tuna oil) and probiotic bacteria (*L. casei*) were produced through spray and freeze drying and their physico-chemical properties were studied during storage. Simulated digestion studies were also carried in these solid co-microcapsules to investigate the applicability of these co-microcapsules to the food industry. The outcomes of the

research contribute to the body of knowledge related to complex coacervation and co-encapsulation in food science. The importance of key findings obtained from the above mentioned investigations is articulated in the ensuing sections. The contributions made by this study to the body of knowledge are also presented. Finally, recommendations for future studies are outlined towards the end of this chapter.

8.2. Research Aspects and Key Findings

The research was designed in a systematic way by first developing and optimising the microencapsulation of omega-3 fatty acids and probiotic bacteria separately. The knowledge gained from these individual encapsulation experiments was used to optimise the combined encapsulation of these two components in single matrix. The key findings obtained from the research study and their significances are articulated in the ensuing sections:

8.2.1. Complex coacervation of WPI and GA

The process of complex coacervate formation between cationic whey protein isolate and anionic gum Arabic was explored in detail and key aspects are presented in Chapter 3. WPI is prepared from whey which is a by-product of cheese making and it has been widely used in the food processing industry due to its nutritional qualities and unique physio-chemical properties, such as emulsion stabilization and gel formation. WPI is effective in lowering interfacial tension at the oil-water interface, promptly migrates to the interface from bulk solution, and possesses good skin forming ability when it is dried. GA is one of the most commonly used stabilizers in the food and pharmaceutical industries. The complex

coacervation process between WPI and GA was optimised in terms of pH, and WPI-to-GA ratio, using zeta potential and turbidity measurements, and acquiring morphology of the microcapsules. The optimum pH and WPI-to-GA ratio for complex coacervation was found to be 3.75 and 3:1, respectively. Confocal laser scanning microscopy (CLSM) and optical microscopy were used to visualize process of complex coacervate formation between WPI and GA and to confirm the optimized processing conditions.

8.2.2. Co-encapsulation of omega-3 oil and probiotic bacteria in WPI-GA complex coacervates

Microencapsulation of omega-3 rich tuna oil (Chapter 3) and probiotic bacteria *L. casei* (Chapter 4) in WPI-GA complex coacervate were carried out separately followed by co-encapsulation of both (Chapter 4).

Omega-3 rich tuna oil and probiotic bacteria *L. casei* 431 were successfully encapsulated individually in WPI-GA complex coacervate matrix. *L casei* and omega-3 rich tuna oil were also successfully co-encapsulated in WPI-GA complex coacervate matrix. The liquid capsules containing bacterial cells, tuna oil and bacterial cells together with tuna oil were converted into powder form using spray and freeze drying. The viability of bacterial cells and oxidative stability of tuna oil were studied in detail in microcapsules and co-capsules. Furthermore, the morphology and surface elemental composition of microcapsules were compared. The viability of the *L. casei* bacteria was significantly higher when co-encapsulated with tuna oil in WPI-GA complex coacervates rather than being encapsulated on its own in the same shell matrix both in liquid and dried microcapsules. Freeze dried WPI-P-O-GA microcapsules exhibited greater survival compared to that in spray dried microcapsules. The higher inlet and outlet temperatures (180/80^{o} C) resulted into lower

viability of bacterial cells. The oxidative stability of tuna oil was greatly improved in the WPI-GA complex coacervates in the presence of the bacterial cells. However, spray dried WPI-P-O-GA microcapsules exhibited greater oxidative stability than that of freeze dried microcapsules. The surface elemental composition showed that the spray dried microcapsules had high surface protein content while freeze dried microcapsules had high surface carbohydrate when oil was not incorporated in the formulation. The freeze dried microcapsules showed flake like porous morphology, while the spray dried microcapsules showed more or less spherical shapes in various size with concavities typical of polymeric materials produced by spray drying. All the microcapsules produced were amorphous in nature. In conclusion, we found that spray dried co-microcapsule powders had advantages in terms of oxidative stability of omega-3 oil; however, the freeze dried co-microcapsules had higher viability of *L. casei.* Since spray drying is one of the most hygienic and economical technologies used for microencapsulation and production of powders in food industry and is also less expensive than that of freeze drying, the spray drying process parameters were optimised so that co-microcapsules with higher bacterial viability as well as higher oxidative stability could be produced (Chapter 5). The effect of three sets of inlet (130^{o} C, 150^{o} C and 170^{o} C) and outlet (55^{o} C, 65^{o} C and 75^{o} C) air temperatures on the physico-chemical and surface characteristics of spray dried co-microcapsules were determined. The viability of *L. casei* decreased with increase in the inlet and outlet air temperatures. There was no difference in the surface elemental compositions and surface morphology of powdered co-microcapsules produced under these nine inlet/outlet temperature combinations. The co-microcapsules produced at inlet-outlet air temperature 130-65^{o} C had better bacterial viability and oxidative stability of omega-3 at moisture content 4.93 % (w/w). This research shows that powdered co-microcapsules of probiotic

bacteria and omega-3 fatty acids with high survival of the former and high stability against oxidation can be produced through spray drying, which confirms the commercial suitability of production of these composite microcapsules.

8.2.3. Is the interaction synergistic or competitive in co-capsules?

The complex coacervation process, co-encapsulation and drying parameters to produce co-capsules with highest possible bacterial cell viability and better oxidative stability for tuna oil have been optimized. Further, the storage stability of co-capsules were tested and key results are presented in Chapter 6. Whether or not the interaction between probiotic bacteria and omega-3 oil in co-microcapsules, particularly in terms of oxidative stability of omega-3 oil and vitality/viability of probiotic bacteria was synergistic outcome was tested. The effect of storage temperature (5 and 25° C) and time (90 days) on the survival and fermentation activity of *L. casei* and oxidative stability of tuna oil in the microcapsules/co-microcapsules were determined.

Synergism was observed between the oxidative stability of omega-3 oil and vitality/viability of *L. casei* in the freshly prepared WPI-P-O-GA co-microcapsules produced by both spray and freeze drying. However, these drying techniques affected the storage stability in different ways. Spray dried WPI-P-O-GA was found to have the highest oxidative stability at the tested storage temperature (5 and 25° C) and the storage time (90 days). Similarly, the vitality and viability of *L. casei* were highest in spray dried WPI-P-O-GA. The oxidative stability of omega-3 oil was significantly lower in freeze dried WPI-P-O-GA co-microcapsules. The storage stability and vitality of *L. casei* were poor in the freeze dried WPI-P-O-GA. The viability and vitality of *L. casei* in freeze dried WPI-P-GA

was higher than that of freeze dried WPI-P-O-GA, indicating that the presence of omega-3 rich oil in the freeze dried co-microcapsules negatively affected the viability and vitality of bacteria due to greater access of oxygen through the porous structure. The spray dried co-microcapsules (WPI-P-O-GA) had higher oxidative stability compared to that of spray dried capsules containing only the omega-3 rich oil (WPI-O-GA) or the control, which indicated that the presence of bacterial cells in microcapsule shell provided protection against oil oxidation. The vitality of *L. casei* in spray dried WPI-P-O-GA was higher than that in the spray dried WPI-P-GA (control) due to the protective effect of omega-3 oil on the bacterial cells. Therefore, co-encapsulation of omega-3 oil and *L. casei* in WPI-GA matrix followed by spray drying provided a synergistic effect in terms of oxidative stability of omega-3 oil and survival and vitality of *L. casei* on storage. The co-microencapsulation of omega-3 oil and *L. casei* in WPI-GA matrix followed by freeze drying exhibited synergism in the freshly prepared co-microcapsules; however, this positive effect was lost during storage.

8.2.5. In vitro digestion of microcapsules and co-microcapsules (*in vitro)*

The in-vitro digestibility of the co-microcapsules (WPI-P-O-GA) and microcapsules (WPI-P-GA and WPI-O-GA) was studied in terms of survival of *L. casei* and release of oil in sequential exposure to simulated salivary, gastric and intestinal fluids. Co-microencapsulation significantly increased the survival and surface hydrophobicity and the ability of *L. casei* to adhere to the intestinal wall. No significant difference in the assimilative reduction of cholesterol was observed between the microencapsulated and co-microencapsulated *L. casei*. The pattern of release of oil from the microcapsules and co-microcapsules was similar. However, the content of total chemically intact omega-3 fatty acids was higher in the oil released from co-microcapsules than the oil released from

microcapsules. The co-microencapsulation was able to deliver bacterial cells and omega-3 fatty acids to human intestinal system with less negative impact on their functional properties.

8.3. Contributions made by this Thesis

This thesis has made significant contributions to the body of knowledge as follows:

It demonstrated that it is possible to co-encapsulate omega-3 oil and probiotic bacteria in a single capsule.

It showed that synergism between viability/vitality and oxidative stability occurs in in spray dried co-microcapsules.

Co-encapsulation of omega-3 promoted the survival of probiotic cells in simulated gut system.

The amount of total omega-3 fatty acids in the released oil was higher in co-microcapsules.

A total of six peer reviewed journal papers were generated from this work out of which five are published. The review chapter (Chapter 2) is currently under peer-review.

In summary, the findings of this study paves the way for co-encapsulation of omega-3 and probiotic bacteria into one matrix, and this technology may have a wide range of applications in functional powder formulations. Consumers can get a two-fold health benefits in one serving, which has commercial potential. Furthermore, the delivery of stable multiple bioactive ingredients through a single product can facilitate the incorporation of these ingredients into functional foods.

8.4. Recommendations for further research

Food industry is increasingly interested in developing microencapsulated bioactive products to incorporate into processed foods. In this study, WPI-GA complex coacervate matrix was used as wall material to co-encapsulate omega-3 oil and probiotic bacteria *L. casei* 431. Further investigations on different wall materials and probiotic bacterial strains could broaden the scope of this technology.

In this study, WPI and GA were successfully complexed and crosslinked to produce a wall material to protect tuna oil and probiotic bacteria. However, the cross linker used in this study is transglutaminase. There is an increasing interest in application of plant polyphenols as cross linker due to their unique health benefits. Hence, further work using polyphenols as crosslinking agent can add value to the research done in this study.

The mechanism for improved stability of the oil in co-microcapsules is not yet clearly understood. This may involve shielding of oil by the probiotic rich WPI-GA complex coacervate shell matrix and resultant denser packing providing an improved oxygen barrier. Further work is required to determine the mechanisms involved. Similarly, the trend on the fermentation ability of encapsulated probiotic bacteria and the mechanism of lactic acid generation during much prolonged storage period and higher temperature need to be explored further.

It is of industrially important to investigate the incorporation aspect this co-microcapsules in many commercial food products. These co-microcapsules can be used in a variety of food products such as bakery products, beverages, dairy products, baby foods, nutrition bars, soups and salad dressings. The performance of the encapsulated oil and encapsulated

probiotic bacteria in these food products can be investigated by employing simulated digestive conditions and subsequently testing its bioavailability. In-vivo and clinical human studies will further confirm the bioavailability and health benefits of these co-microcapsules.