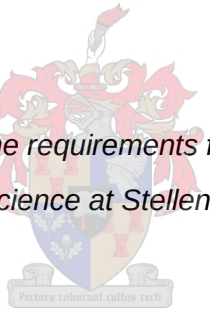
The application of astaxanthin producing bacteria in poultry feed

by

Tersia Andrea Conradie



Thesis presented in fulfilment of the requirements for the degree Master of Science in the Faculty of Science at Stellenbosch University

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“The key to everything is patience.

You get the chicken by hatching the egg, not by smashing it.”

- Arnold H. Glasow

Declaration

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Summary

In the food industry, the colour of the product is important to the consumer as it gives an indication of the freshness and quality of the product. Hens are not able to produce pigments and absorb pigments through their diet. This has led to a rapidly emerging trend in poultry farming to enhance egg yolk colour as the yolk colour is influenced by the diet of the hen. Over the years, natural or synthetic carotenoids have been added to poultry feed. Several studies have focused on using astaxanthin producing microorganisms, such as the microalga, *Haematococcus pluvialis*, and yeast, *Xanthophyllomyces dendrorhous*. However, the production costs are expensive and the thick cell walls of the microalga and yeast limits its whole cell application as a feed additive. Some bacterial species are also able to produce astaxanthin, including the bacterium *Paracoccus marcusii*, and have previously not been used as a feed additive to enhance yolk colour. The purpose of this study was, therefore, to determine the whole cell application of *P. marcusii* as a feed additive to enhance egg yolk colour, without the need to homogenise the cells or extract the pigment.

In the first experimental chapter (Chapter 2), the growth conditions and astaxanthin production of *P. marcusii* was optimised. Furthermore, the stability of the astaxanthin molecule under different storage conditions, namely lyophilisation and microencapsulation, was determined. The optimum growth conditions for *P. marcusii* and for astaxanthin production was at 26 °C in a specialised medium containing yeast extract (5 g/L), bacteriological peptone (10 g/L) and NaCl (3%) at a pH between 6 – 7. Astaxanthin is a valuable compound with several health promoting benefits for humans and animals. However, the molecule is unstable when exposed to oxygen, light and temperature. After lyophilisation in sucrose (10% m/v), there was an 85% loss in astaxanthin concentration after 3 weeks. However, the loss in cell viability was low. When *P. marcusii* was microencapsulated in calcium alginate beads, cell viability significantly decreased when stored at 20 °C compared to 4 °C. However, only 30% of the total astaxanthin concentration was lost after 3 weeks at both storage temperatures. The microencapsulation significantly improved the stability of astaxanthin under storage. The highest concentration of astaxanthin obtained was 24.25 µg/g dry cell weight.

Chapter 3 examined the pigmentation effect of *P. marcusii* when fed to laying hens to enhance egg yolk colour. *Paracoccus marcusii* was fed to hens daily either in a sucrose solution (10% m/v) or microencapsulated in calcium alginate beads. After the pilot study, it was clear that a diet free of all pigments was needed to effectively determine the pigmentation effect of *P. marcusii*. In all the experimental trials there was a significant increase in yolk colour and no negative effect on egg quality, laying rate or hen weight was observed. There was also an increase in whole egg and yolk

weight when compared to the control groups. Furthermore, the microbial communities of the duodenum and caeca were investigated after a prolonged feeding of *P. marcusii* to detect any changes that might have occurred (Chapter 4). The microbial community of the hen's gastro-intestinal tract (GIT) starts out as a simple community in the small intestines which becomes increasingly diverse and complex further down the intestinal tract. The findings in this study revealed a similar pattern when considering the results obtained from the Shannon diversity index and total number of operational taxonomic units (OTUs). Starting in the duodenum, the index ranged between 2.14 – 2.59 and increased in the caeca to between 2.45 – 3.03. OTUs increased from 21.44 – 28.60 in the duodenum to 28.30 – 38.00 in the caeca. A significant difference was only observed for the OTUs of the experimental group compared to the control groups in both the duodenum and caeca. There was no significant difference observed in the microbial community structure of the duodenum. However, distinct patterns and clusters formed in the caeca between the experimental diet group compared to the control diet groups. Since no mortalities were recorded during the trial and all hens appeared in excellent health, it is safe to assume that the change in microbial community structure of the caeca was not negative. Therefore, *P. marcusii* is safe to use as a feed additive for laying hens.

Finally, Chapter 5 evaluated the costs associated with the small-scale production of *P. marcusii* microencapsulated in calcium alginate beads and its feasibility in the poultry industry. Based on the economic assessment, the total cost for one month's production of 210 g calcium alginate beads is estimated at R2912.88. This is too expensive and not practical to be used by poultry farmers. Possible solutions can include the use of inexpensive peptones, production on a larger scale and also increasing the concentration of bacterium encapsulated in the bead.

Opsomming

In die voedselbedryf is die kleur van ’n produk baie belangrik vir die verbruiker, aangesien dit ‘n indruk skep van ‘n vars produk van goeie gehalte. Hoenders is nie daartoe instaat om self pigmente te produseer nie en is afhanklik van hul dieet om dit in te neem. Dit het gelei tot ‘n toenemende neiging onder pluimveeboere om die kleur van die eiergeel te manipuleer deur natuurlike of sintetiese kleurmiddels, byvoorbeeld karotenoïede, by die voer te meng. Karotenoïede word oor ‘n lang tydperk reeds by pluimveevoer gevoeg. Meeste van vandag se navorsing fokus op die gebruik van ‘n astazantien produserende mikroalge, *Haematococcus pluvialis*, en gis, *Xanthophyllomyces dendrorhous*. Die gebruik van hierdie mikroörganismes as ‘n bymiddel word egter beperk aangesien die produksie kostes baie hoog is. Hierdie mikroörganismes het ook ‘n baie dik selwand wat die vrystelling van astazantien bemoeilik. Sommige bakterieë, byvoorbeeld die bakterium *Paracoccus marcusii,* is ook daartoe instaat om astazantien te produseer, maar is voorheen nog nie gebruik as a bymiddel nie. Die doel van hierdie studie was dus om te bepaal of *P. marcusii* gebruik kan word as ‘n bymiddel om die kleur van eiergeel te manipuleer sonder om die selle te homogeniseer of die pigmente uit te haal.

In die eerste eksperimentele hoofstuk (Hoofstuk 2), is die optimale groeitoestande van *P. marcusii* vir ‘n hoë produksie astazantien bepaal. Verder is die stabiliteit van die astazantien molekule onder verskillende berging metodes ook bepaal, naamlik vriesdroging en mikroënkapsulering. Die optimale groeitoestande van *P. marcusii* was by 26 °C in ‘n gespesialiseerde medium met ‘n pH van tussen 6 – 7, wat gis ekstrak (5 g/L), bakteriologiese peptone (10 g/L) en NaCl (3%) bevat. Astazantien is ‘n baie waardevolle pigment met verskeie gesondheids voordele vir beide mense en diere. Hierdie molekuul is egter onstabiel wanneer dit blootgestel word aan suurstof, lig en hoë temperature. Drie weke na vriesdroging in sukrose (10% m/v) was daar ‘n 85% verlies in astazantien konsentrasie, maar die lewensvatbaarheid van die selle was nog hoog. Die mikroënkapsulering van *P. marcusii* in kalsiumalginaat-balletjies het ‘n laer sellewensvatbaarheid gehad by 20 °C in vergelyking met 4 °C en slegs 30% van die astazantien konsentrasie het verlore gegaan na drie weke by albei temperature. Die mikroënkapsulering het dus die stabiliteit van die astazantien molekule aansienlik verbeter. Die hoogste konsentrasie astazantien wat verkry is, was 24.25 µg/g droë sel gewig.

Hoofstuk 3 het gekyk na die effek wat *P. marcusii* op die kleur van die eiergeel uitoefen wanneer dit vir lê-henne gevoer word. Die henne het daagliks ‘n dosis van die bakterium gekry in óf ‘n sukrose oplossing (10% m/v) óf gemikroënkapsuleer in kalsiumalginaat-balletjies. Na die loodsstudie was dit

duidelik dat ‘n voer sonder enige pigmente nodig is om die kleuringseffek van die bakterium te kan bepaal. Daar was ‘n beduidende toename in die kleur van die eiergeel by al die eksperimentele proewe en geen newe-effekte is waargeneem in terme van eierkwaliteit, hoeveelheid eiers wat gelê is of die gewig van die hoender nie. In vergelyking met die kontrolegroepe was daar ‘n effense toename in die gewig van die eier en die eiergeel. Verder is daar ook gekyk na die effek van die bakterium op die mikrobiese gemeenskap van die duodenum en seka (Hoofstuk 4). Die mikrobiese gemeenskap van die spysverteringskanaal (SVK) van die hoender begin as ‘n eenvoudinge samestelling van bakterieë in die dunderm wat meer kompleks en divers raak verder af in die SVK tot by die seka. Die bevindinge in hierdie studie het gedui op soortgelyke patrone deur die resultate van die Shannon indeks en totale aantal operasionele taksonomiese eenhede (OTE) te bestudeer. Die indeks van die duodenum was tussen 2.14 – 2.59 en het toegeneem tot 2.45 – 3.03 in die seka. Die OTE het ook toegeneem van 21.44 – 28.60 tot 28.30 – 38.00. ‘n Beduidende verskil was slegs waargeneem tussen die OTE waar die eksperimentele groep ‘n laer OTE gehad het as die kontrole groepe. Daar was geen verskil tussen die mikrobiese samestellings van die duodenum nie, maar duidelike patrone en groepe was waargeneem tussen die eksperimentele groep en kontroles van die seka. Aangesien daar geen mortaliteite was nie en alle hoenders gesondheid was, kan afgelei word dat die verandering in die mikrobiese samestelling van die seka nie negatief was nie. Daarom is dit veilig om *P. marcusii* te gebruik as ‘n bymiddel vir lê-henne.

In die finale hoofstuk (Hoofstuk 5) is die kostes verbonde aan die produksie van mikrogeënkapsuleerde *P. marcusii* in kalsiumalginaat-balletjies, en die ekonomiese haalbaarheid daarvan in die pluimveebedryf, bespreek. Die maandelike koste van 210 g kalsiumalginaat-balletjies beloop tans R2912.88. Die kostes is egter baie duur en nie haalbaar vir ‘n pluimveeboer nie. Moontlike oplossings kan die gebruik van ‘n goedkoper bron van peptone insluit, asook grootskaalse produksie en ‘n verhoogde konsentrasie van die bakterium in die kalsiumalginaat-balletjies.

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Chapter 1

Literature review

Literature review

There is a trend among farmers to use natural ingredients free of synthetic pigments, antibiotics and other chemicals in feed (Fanatico *et al*., 2009 and USDA, 2016). This is partially due to the demand from the consumer for a more natural and organic product and because of legislative actions that eliminate the use of chemical additives (Cherian *et al*., 2002; Sean, 2002 and USDA, 2016). Chicken eggs are one example of such a food group as colourants are added to the feed to enhance egg yolk colour.

Poultry eggs

Nutritional value and health aspects of chicken eggs

Chicken eggs are considered to be a wholesome food source that contains minerals, proteins, vitamins and high quality and quantity lipids (Fredriksson *et al*., 2006). The total nutrient content of one large raw egg compared to the Recommended Dietary Allowance (RDA) is set out in Table 1 (USDA, 2005).

Table 1 – Nutritional value of one large egg (± 50 g) compared to the Recommended Dietary Allowance
(USDA, 2005)

Nutrient		Whole egg	RDA	Total of RDA (%)
Macronutrients and energy	Protein	6.3 g	0.8 g/kg body weight	N/A
	Carbohydrate	0.4 g	130 g	0.31
	Total fat	5.0 g	65 g	7.7
	Cholesterol	212 mg	< 300 mg	71
Vitamins	Vitamin A	244 IU	3000 IU	8.1
	Vitamin B_6	0.07 mg	1.3 mg	5.4
	Vitamin B_{12}	0.65 µg	2.4 µg	27
	Vitamin D	18 IU	600 IU	3
	Vitamin E	0.48 mg	1000 mg	0.05
	Choline	126 mg	**	**
	Folate	24 µg	400 µg	6
	Riboflavin	0.24 mg	1.3 mg	18.5
	Thiamine	0.04 mg	1.2 mg	3.3
Minerals	Calcium	26 mg	1000 mg	2.6
	Iron	0.92 mg	8 mg	11.5
	Phosphorus	96 mg	700 mg	13.7
	Potassium	67 mg	4700 mg	1.4
	Magnesium	6 mg	400 – 420 mg	1.4 – 1.5
	Selenium	15.8 µg	70 µg	22.6
	Sodium	70 mg	2300 mg	3.0
	Zinc	0.56 mg	11 mg	5.1
Carotenoids	Lutein and Zeaxanthin	166 µg	**	**

RDA – Recommended Dietary Allowance
N/A – Not applicable
** Not established

These nutrients not only protect against age related health risks, but the antioxidants (zeaxanthin and lutein) in the yolk have numerous health benefits. These include the maintenance of normal eye health, improved skin health and immune system function and a reduced risk of cancer and heart disease (Boon *et al.*, 2010 and Schonfeldt *et al*., 2013). In addition, the relatively high content of vitamin D is noteworthy as only a few foods are recognised sources for vitamin D, such as fatty fish (tuna and salmon), liver and cheese (Ruxton *et al*., 2010). According to the South African Food-Based Dietary Guidelines (FBDG), eggs can be consumed daily, but the recommended number of eggs are three to four eggs per week (Schonfeldt *et al*., 2013 and Vorster *et al*., 2013). Each year there is more evidence emerging that suggests that consuming eggs are associated with a good quality diet and also weight management (Van der Wal *et al*., 2005; 2008; Ratliff *et al*., 2010 and Vorster *et al*., 2013).

South African egg industry

Whole chicken eggs are perishable and cannot be transported over long distances. For this reason, eggs are produced in every province of South Africa. According to a census taken in 2014 of layer hen distributions across South Africa, Gauteng had the highest percentage of layer hens (26.1%) followed by the Western Cape (17.2%), Free State (15.4%), KwaZulu Natal (14.6%), Limpopo (7.4%), Mpumalanga (4.6%) and Eastern Cape (4%). The Northern Cape had the lowest percentage of layer hens with only 0.2% (Fig. 1) (South African Poultry Association, 2014).

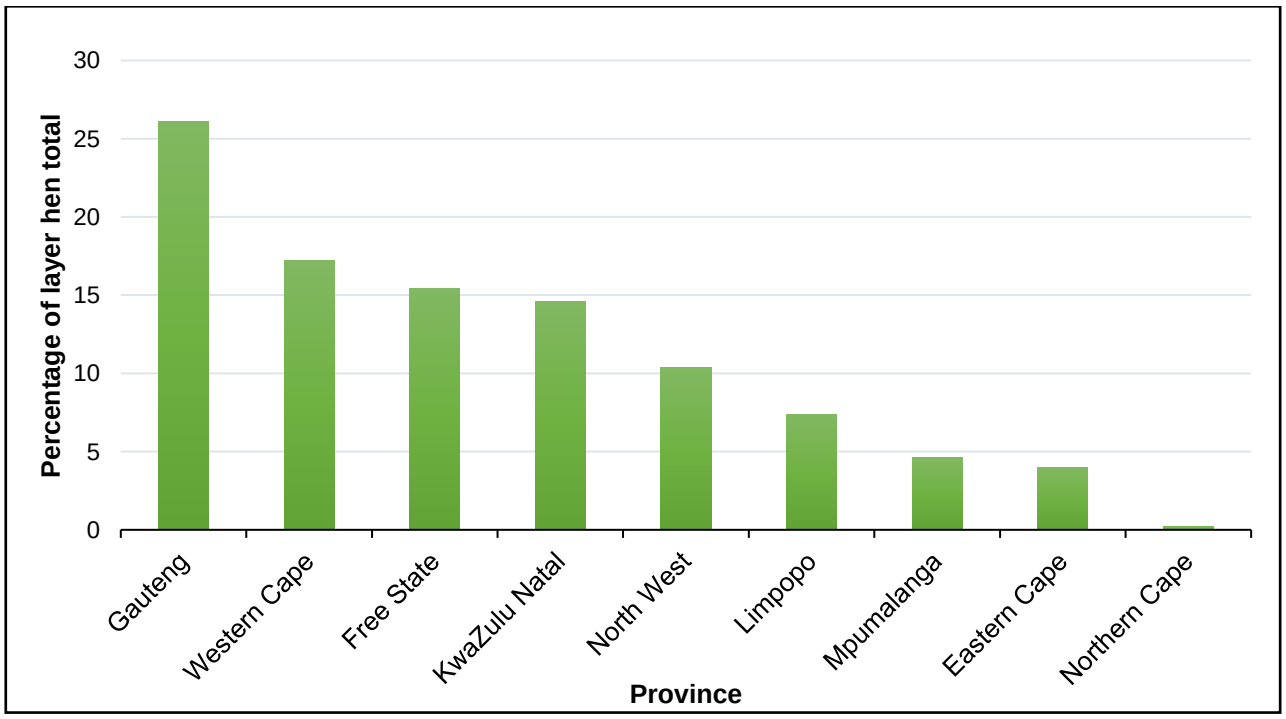

Figure 1 – Percentage distribution of layer hens across South Africa (adopted from South African Poultry Association, 2014).

Other egg products are also exported to other countries. These egg exports include fertilised eggs (chickens and ostriches) for hatcheries and products including dried and liquid yolks, albumen and shell products. During 2016, the main countries for export of South African eggs and egg products were Mozambique (69%), Zimbabwe (9.9%), Swaziland (7.9%), Lesotho (4.1%), Namibia (3.1%), Angola (2.1%), Cote d'Ivoire (1.9%) and Botswana (1.6%). A small percentage of eggs and egg products (mostly dried eggs) are imported from other countries, including India, Italy, France, Germany, United States and Lesotho (South African Poultry Association, 2016).

Quality of eggs

The quality of any food product was first defined by Kramer (1951) as the properties of the food that have an influence on the rejection or acceptance by the consumer (Giusti *et al*., 2007). Egg quality was first defined by Stadelman (1977) as the characteristics most important to the consumer. These included yolk colour (light or dark), texture and firmness together with albumen appearance and consistency (Gerber, 2006). Several factors can influence the internal quality of the egg. These include environmental conditions, such as humidity and temperature, but the most influential is the hen's age and storage time (Roberts *et al*., 2013 and Chung and Lee, 2014). Young hens lay eggs with longer pores and thicker shells than older hens (Britton, 1977 and Akyurek and Okur, 2009). The function of the shell and membrane is to retain the moisture content and to prevent bacterial infections (Burley and Vadehra, 1989; Davies and Breslin, 2003 and Svobodová and Tůmová, 2014). As the egg ages during storage, moisture and carbon dioxide is lost through the pores, causing an increase in albumen pH and a larger air pocket inside the egg (Akyurek and Okur, 2009). Although the internal quality starts to decline with a longer storage period, the nutritional composition remains the same with only a change in moisture content (Al-Obaidi *et al*., 2011). The average fresh egg consists out of 10% shell, 32% yolk and 58% albumen. The albumen contains mainly water (88%) and protein (9%) and the egg yolk contains water (51%), protein (16%) and fat (31%) (Roberts, 2004 and Coutts and Wilson, 2007).

A good quality yolk should be firm and free of all blemishes such as pigment spots, blood spots and meat spots (Gerber, 2006 and Coutts and Wilson, 2007). Egg yolks lose their firmness when the perivitelline membrane surrounding the yolk becomes weakened during storage, causing the yolk to break easily (Kirunda and McKee, 2000). For many years, the colour of the yolk has been manipulated by adding synthetic or natural carotenoids to the diet of the hen. Some of the most important natural sources of carotenoids include grass meals, corn and maize gluten (Gerber, 2006). Some synthetic products available on the market include canthaxanthin for orange yolks and beta-apo-8-carotene ethyl ester and beta-apo-8'-carotenal for yellow yolks (Bunnell *et al*., 1962; Sunde, 1962; Norman *et al*., 1973 and Fletcher *et al*., 1978). Yolk colour preference varies between

countries and corn and maize alone does not add enough colour to the yolk that is satisfactory to the consumer (Roberts, 2004). By adding these synthetic or other natural supplements to the feed, different market demands can be met. For example, on the yolk colour fan ranging from 1 to 15 (Fig. 2), France prefers a yolk colour of 14, England a yolk colour of 12 and in Australia a yolk colour of 11 is preferred (Beardsworth and Hernandez, 2004 and Roberts, 2004).

Figure 2 – DSM yolk colour fan.

To measure albumen quality, the following formula is used to calculate the Haugh Unit:

$$HU = 100 \times \log(h - 1.7w^{0.37} + 7.6)$$

where *h* is the thick albumen height and *w* is the weight of the whole egg (Haugh, 1937). The average *HU* value of the egg that leaves the farm after processing ranges between 75 – 85 and by the time it reaches the consumer it is about 60. A higher *HU* value corresponds to a better-quality egg (Adamiec *et al*., 2002). The albumen of a fresh egg should be a slight yellow to green colour and transparent. Any discolouration to pink or a darker yellow or green may be the result of an excess of cottonseed meal, riboflavin and specific weed seeds in the diet (Gerber, 2006 and Coutts and Wilson, 2007).

Egg formation

The egg starts as a single ovum, also known as the yolk, which takes about 24 hours to completely pass through different sections of the oviduct (Coutts and Wilson, 2007). In the first step, the yolk is released from the ovary into the funnel where chalaza covers the yolk. From here the yolk passes into the magnum where the albumen is layered around the yolk. The next part is called the isthmus and this is where the outer membranes are created to hold the liquid portion of the egg together.

From the isthmus, the egg moves to the uterus where it spends the majority of time as the calcium carbonate shell is built before it is passed through the cloaca (Coutts and Wilson, 2007). Figure 3 illustrates the flow of egg formation and the time spent in each section of the oviduct.

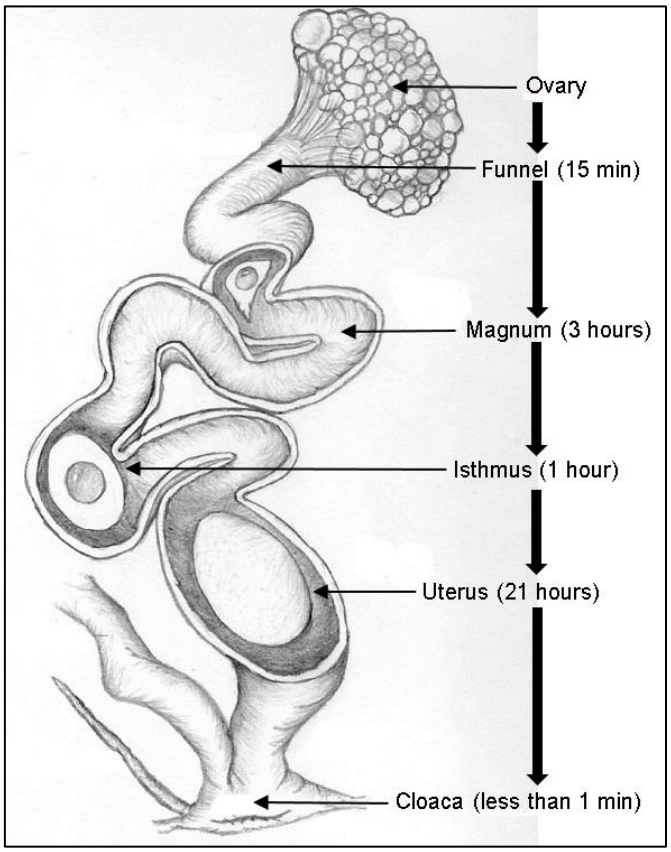

Figure 3 – Oviduct of the hen and the time spent in each section (adapted from Curbstone Valley Farm, 2010).

Microbial contamination of eggs

Microorganisms can contaminate eggs either horizontally after the egg is laid (Barrow and Lowell, 1991), or vertically, also known as the trans-ovarian route, in the reproductive organs of the hen (Keller *et al*., 1995 and Miyamoto *et al*., 1997). Figure 4 illustrates these possible routes of egg contamination when contaminated with *Salmonella.* Most eggs are sterile when they are laid and only get contaminated when it leaves the oviduct (Fig. 4A). The temperature of the egg once it is laid is around 42 °C and as it cools down a negative pressure is created inside the egg and has a potential to pull material into the pores of the shell. The egg can, therefore, potentially get contaminated with any surface area it comes into contact with after it is laid. This can include faeces, soil, cage material, hands of workers, water, nesting material and insects (Davies and Breslin, 2003 and Svobodová and Tůmová, 2014). Vertical trans-ovarian contamination mainly occurs because of infected ovaries or from contaminated cloaca into the lower regions of the oviduct, also known as ascending infections (Keller *et al*., 1995 and Miyamoto *et al*., 1997). Infected oviduct tissue or

ovaries directly contaminate the egg content even before the shell is formed (Fig. 4B) (Messens *et al*., 2005).

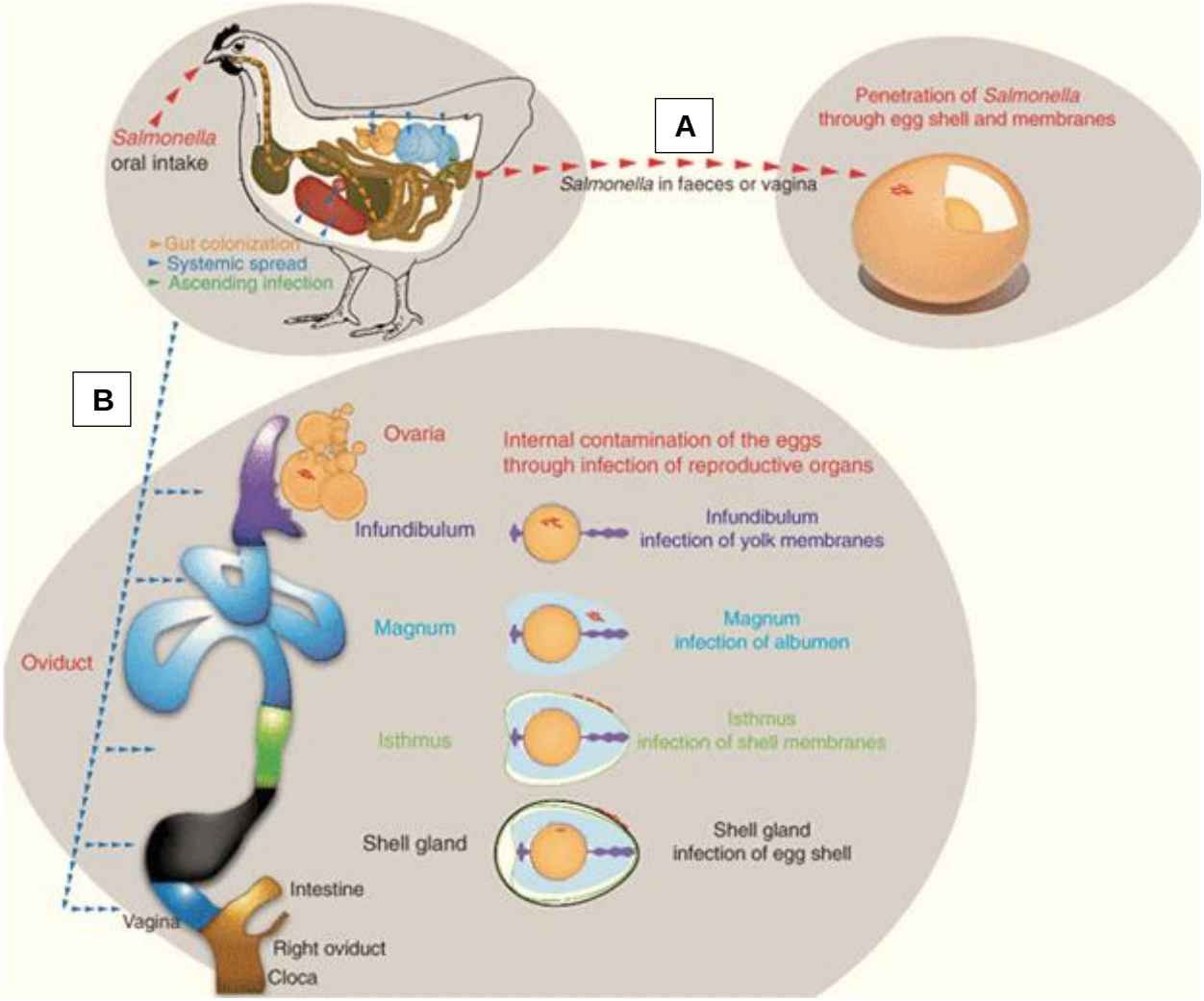

Figure 4 – Pathogenesis by e.g. *Salmonella* of contaminated eggs. *Salmonella* is taken up orally by the hen and spreads through gut colonisation, systemic spread or ascending infection. **A.** Horizontal contamination though faeces or vagina. **B.** Vertical trans-ovarian contamination through different sections of the oviduct (adapted from Gantois *et al*., 2009).

On the egg shell surface, the microbial population is dominated by Gram-positive bacteria. In contrast, the internal egg is mostly contaminated by Gram-negative bacteria and some Gram-positive bacteria (Stadelman and Cotterill, 1995 and De Rue *et al*., 2006). Some of the most common bacterial contaminants include the genera *Athrobacter, Alcaligenes, Bacillus, Escherichia, Flavobacterium, Pseudomonas, Micrococcus* and *Staphylococcus* (Stadelman and Cotterill, 1995 and Svobodová and Tůmová, 2014).

Poultry health

The microbiome of any living organism plays an important role in maintaining overall health and function of the host. These organisms have a role in nutrient absorption, immune system function and feed digestion (Gong *et al*., 2002a; 2002b; 2007 and Stanley *et al*., 2012). The microbiome is important in animal health and production. It positively influences the host's gastrointestinal development, physiology, immunology, biochemistry and nonspecific resistance to infections (Torok *et al*., 2008).

The gastrointestinal tract (GIT) of poultry differs from mammals in that the type of microorganisms that will colonise are primarily influenced by the surrounding environment (Oakley *et al*., 2013). Several other factors also influence the GIT of poultry and include feed additives, treatments with antibiotics, age of the hen, hygiene level, type of hen, diet, climate and geography (Shaufi *et al*., 2015). Newly hatched chicks from farming industries do not come into contact with adult hens, therefore, the environmental microbial communities serve as a first 'inoculum' that will start the development of the GIT (Oakley *et al*., 2013). The microorganisms that colonise the GIT in newly hatched chicks form a synergistic relationship with the host. These organisms are important for absorption and utilisation of energy and nutrients and for the response of poultry to feed additives and enzymes (Torok *et al*., 2008). As the hen ages, the microbiome becomes more diverse until it reaches a stable state (Pan and Yu, 2014).

The gastrointestinal tract of the hen

The GIT of the hen has several compartments each with different characteristics. After the oral cavity, three segments follow; the crop (Fig. 5A), proventriculus (or stomach) (Fig. 5B) and gizzard (Fig. 5C). The crop is a fermentation and food storage organ. Food can reside in the crop for minutes to several hours before the food is acidified in the proventriculus and ground in the gizzard (Savory, 1999 and Sekelja *et al*., 2012). The lower gut consists out of the small intestines (Fig. 5D), colon (Fig. 5E) and caeca (Fig. 5F). The caeca are two fermentation chambers and can store contents for longer than the small intestines (Clench and Mathias, 1992). The different compartments differ in pH, nutrient availability, atmospheric pressure, water and salt levels. These variations in the different compartments select for different microorganisms along the GIT (Pedroso and Lee, 2015).

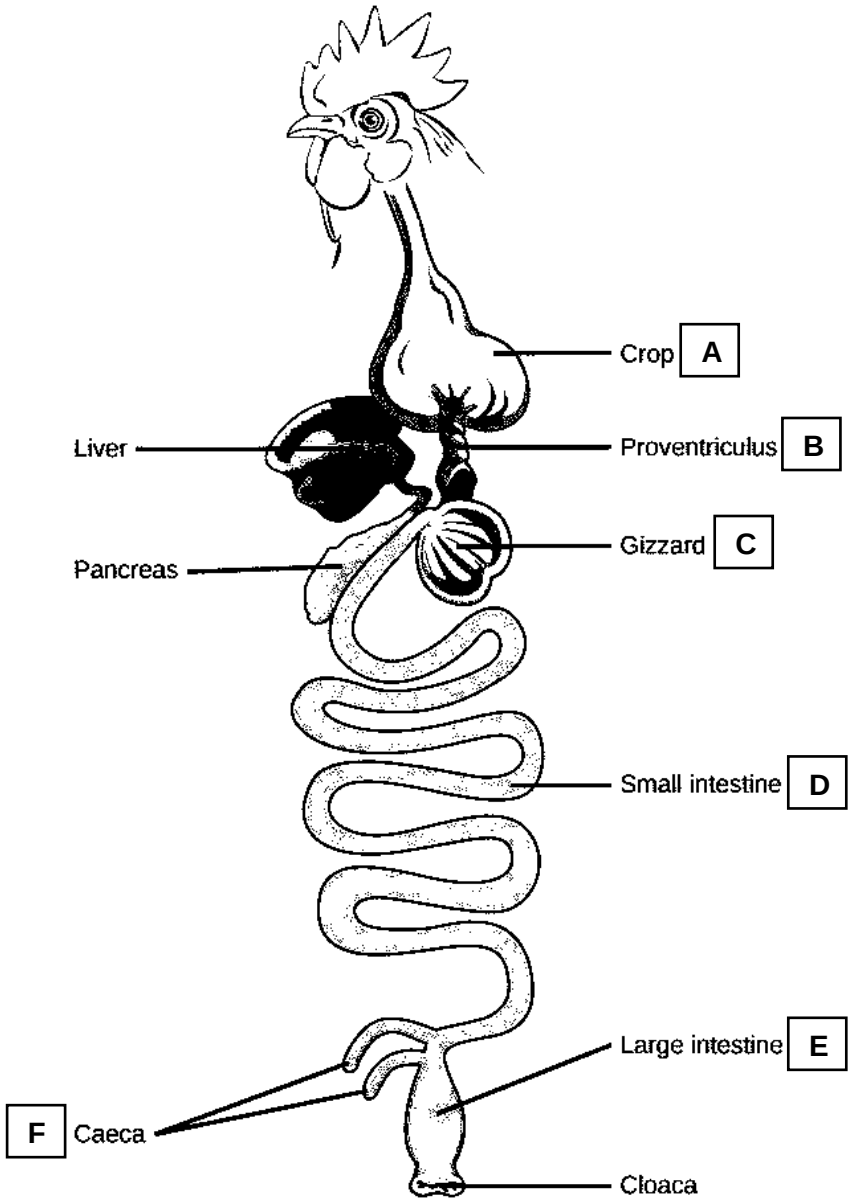

Figure 5 – Hen digestive system (adapted from Boundless, 2016).

Microbial composition of the gastrointestinal tract

Microorganisms are found across the entire length of the GIT. Each location shows spatial variation in microbial community composition (Gong *et al*., 2007). Table 2 is a summary of all major taxa found along the hens GIT taken from different studies (Gong *et al*., 2002a; Saengkerdsub *et al*., 2007a; 2007b; Qu *et al*., 2008 and Yeoman *et al.,* 2012). Specialised microbial communities throughout the GIT have an important role in digesting feed that passes through the GIT. In the crop, the community is dominated by Firmicutes (mostly *Lactobacillus*) for starch hydrolysis and lactate fermentation. The cell densities can reach up to 10^9 CFU/g (van der Wielen *et al*., 2000). In the proventriculus and gizzard, the cell numbers are limited to below 10^8 CFU/g, because of the low pH of the gastric juices that contains pepsin and hydrochloric acid (Yeoman *et al*., 2012). The small

intestines can harbour large populations of bacteria dominated by the Firmicutes and Proteobacteria. The density and diversity of the microbial community is greatest in the caeca pouches, 10^{10} – 10^{11} CFU/g, where digestion is longest (12 to 20 hours) (Qu *et al*., 2008). This allows for a more substantial microbial fermentation (Rehman *et al*., 2007). The caeca are important for the recycling of urea, carbohydrate fermentation and water retention (Sergeant *et al*., 2014).

Table 2 – Spatial distribution of major taxa at phyla and genus level

(Gong *et al*., 2002a; Saengkerdsub *et al*., 2007a; 2007b; Qu *et al*., 2008 and Yeoman *et al*., 2012)

GIT location	Cell density (CFU/g)	Phyla	Genera
Crop	10^8 – 10^9	Firmicutes	*Lactobacillus*
		Actinobacteria	*Bifidobacterium*
		Proteobacteria	*Enterobacter*
Gizzard	10^7 – 10^8	Firmicutes	*Lactobacillus* *Enterococcus*
Small intestines	10^8 – 10^9	Firmicutes	*Lactobacillus* *Candidatis* *Arthromitus* *Ruminococcus*
		Proteobacteria	*Escherichia* *Enterococcus*
Large intestines	**	Firmicutes	*Lactobacillus*
		Proteobacteria	*Escherichia*
Caeca	10^{10} – 10^{11}	Firmicutes	*Ruminococcus* *Faecalibacterium* *Pseudobutyrivibrio* *Subdoligranulum* *Acetanaerobacterium* *Clostridium* *Lactobacillus* *Megamonas* *Sporobacter* *Peptococcus*
		Bacteroidetes	*Bacteroides*
		Proteobacteria	*Escherichia* *Bilophila*
		Archaea	*Methanobacterium* *Methanothermus* *Methanopyrus* *Methanococcus*
		Fungi	*Candida*

**Cell density not known

Poultry microbiome interactions

There are several interactions that the gut microbiome has with the host, the diet and among the individual microorganisms (Fig. 6) (Pan and Yu, 2014).

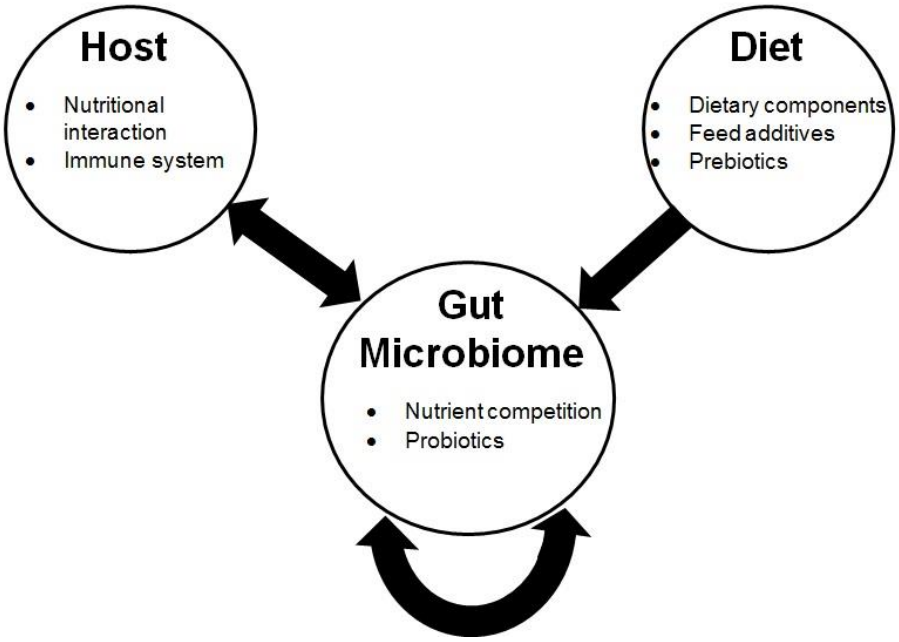

Figure 6 – Model for the interactions between the gut microbiome, diet and host (adapted from Pan and Yu, 2014).

There is a two-way interaction between the bacteria in the gut and the hen in terms of nutritional interaction. Both the hen and the microbiome benefit from each other by providing nutrients during the digestion time. Digestible carbohydrates are easily absorbed in the gut by the hen, but indigestible and residual carbohydrates are broken down by the bacteria residing in the gut (Hooper *et al*., 2002). Indigestible carbohydrates such as oligosaccharides, polysaccharides and disaccharides can be hydrolysed by bacteria to form sugars. Bacteria then ferment the sugars to yield acetate, butyrate and propionate (short chain fatty acids: SCFAs). These SCFAs are then utilised by the hen as a carbon and energy source (van der Wielen *et al*., 2000 and Hooper *et al*., 2002). In comparison, while the human gut microbiome prefers proteins and polysaccharides for a balanced growth, the hen's microbiome requires only simple sugars and peptides (Lei *et al.,* 2012).

It is well known that an advantageous microbiome helps in maintaining normal physiological homeostasis, influencing organ development and metabolism and modulating the host's immune system (Sommer and Backhed, 2013; Belkaid and Hand, 2014; Rodríguez *et al*., 2015 and Thaiss *et al*., 2016). The interaction between the host immune system and microbiome takes place in the inner layer of the gut. The gut is lined with a thick layer of mucus formed from mucin glycoproteins (Forder *et al*., 2012). The mucin layer consists of an outer loose layer and an inner compact layer (Hansson and Johansson, 2010). The outer loose layer is where intestinal microorganisms colonise and the inner compact layer prevents most bacteria from penetrating the intestinal epithelium. This serves as a first line of defence against infections (Brisbin *et al*., 2008). Another form of immune defence in the gut is the presence of antimicrobial peptides (AMPs) in the intestinal epithelial surface

(Brisbin *et al*., 2008). The most important and well-studied AMPs in poultry, are β-defensins. These peptides are small, cationic peptides produced by epithelial cells, heterophils and macrophages. These peptides can kill a variety of pathogens by disrupting the permeability of the cell membrane, leading to cell death (Jenssen *et al*., 2006).

The diet of the hen serves as a source of substrates for the growth of intestinal bacteria and has the greatest potential to have an impact on the microbial composition. A small change in the type of cereal grain used in the feed can affect the bacterial composition at strain level (Engberg *et al*., 2002 and Hammons *et al.,* 2010). Hammons *et al*. (2010) showed that a standard ration of corn to soybean favoured the growth of *Lactobacillus agilis* R5. By changing the diet to a ration high in wheat, the growth of *Lactobacillus agilis* R1 was favoured. The results of this study are important in understanding the implications of feed composition on the application of probiotics and other microbial feed supplements. *Lactobacillus* strains are often used as a probiotic and its effectiveness is reliant on the successful colonisation of the bacterium in the GIT (Schrezenmeir and de Vrese, 2001; Borriello *et al*., 2003 and Barrons and Tassone, 2008). However, if composition of the feed selects for which strain becomes more dominant, successful establishment of the probiotic in the GIT will then also depend on the type of feed. It was, therefore, suggested that a diet be chosen that is compatible with the probiotic or other microbial feed supplements to ensure efficiency (Hammons *et al.,* 2010).

Feed additives and prebiotics are commonly used in poultry diets to reduce the growth of pathogens and promote the growth of beneficial bacteria present in the intestines (Van Immerseel *et al*., 2004 and 2009). Prebiotics are indigestible ingredients in a diet that serves as a nutrient source for one or more beneficial bacteria that are present in the intestines. Two well-known prebiotics are galactooligosaccharides (GOS) and fructooligosaccharides (FOS). The inclusion of GOS promoted the growth of bifidobacteria in the gut of broiler hens (Jung *et al*., 2008) and the inclusion of FOS decreased *Salmonella* counts in laying hens (Donalson *et al*., 2008).

In the microbiome of all living organisms, the different organisms present in the gut will have different interactions with each other. All these microorganisms will be competing for nutrients and an attachment site (Soler *et al*., 2010). In healthy hens, competitive exclusion is achieved with a layer of dense and complex communities on the surface of the mucus layer. These complex communities prevent the attachment of possible pathogenic organisms by occupying the attachment sites, competing for resources in a chemical or physical niche or by attacking a potential colonist either chemically or physically (Oakley *et al.*, 2014). The use of probiotics in poultry has been extensively

studied. Several lactobacilli and bacilli strains isolated from the gut of hens have shown to decrease the growth of *Salmonella* (Pascual *et al*., 1999), *Campylobacter* (Nakphaichit *et al*., 2011) and *Escherichia coli* (Molnár *et al*., 2011). Not only were these potential probiotics able to decrease the number of pathogens, but an increase in feed efficiency and body weight was also observed (Oakley *et al.,* 2014). These probiotics can potentially be used to improve the safety of food by reducing the number of human pathogens present in the hen.

Carotenoids

For many years carotenoids have been used to manipulate the colour of egg yolk to obtain a desired colour (Adams, 1985). Carotenoids can be health promoting in hens by stimulating a secondary antibody response for virus infections and also has an anti-inflammatory effect (Bedecarrats and Leeson, 2006; Rajput *et al*., 2013 and Moraes *et al*., 2016). After the hen has ingested the feed, the carotenoids are released with enzymes and absorbed by the small intestines. The free carotenoids are then emulsified to form oil droplets (or portomicrons) and delivered to the liver. These molecules are incorporated into very low density lipoproteins (VLDL) by the liver and are delivered to the yolk (Surai *et al.,* 2001 and Bortolotti *et al*., 2003).

Carotenoids are a group of natural pigments that are utilised as colourants, nutraceuticals, cosmetics, feed supplements and for other biotechnological uses (Martin *et al*., 2008). Carotenoids are yellow to intensely red coloured pigments that are lipid soluble, water-insoluble molecules. These pigments can be found in nature and are responsible for pigmentation, ranging from the flesh of fish to the feathers of birds and consist of more than 750 different compounds (Britton, 1995 and Kirti *et al*., 2014). These molecules also have an added health benefit for plants and animals (Boon *et al.*, 2010). Some microorganisms produce carotenoids and include bacteria (*Chryseobacterium indologenes*) (Bhosale and Bernstein, 2004), microalga (*Haematococcus pluvialis*) (Jeon *et al*., 2006), yeasts (*Xanthophyllomyces dendrorhous*) (An *et al*., 2001) and some fungal species (*Phycomyces blakesleeanus*) (Kuzina and Cerda-Olmedo, 2006).

Some examples of carotenoids include zeaxanthin, lycopene, β-carotene and lutein, where β-carotene is a major contributor of carotenoids found in the diet of animals and humans (Johnson, 2002). Carotenoids can be degraded by multiple mechanisms (Fig. 7) and because of its liposoluble characteristic, it can easily be incorporated into food products through emulsification with surfactants or with proteins. These molecules are then oxidatively and physically stable in the emulsified form (Boon *et al.*, 2010).

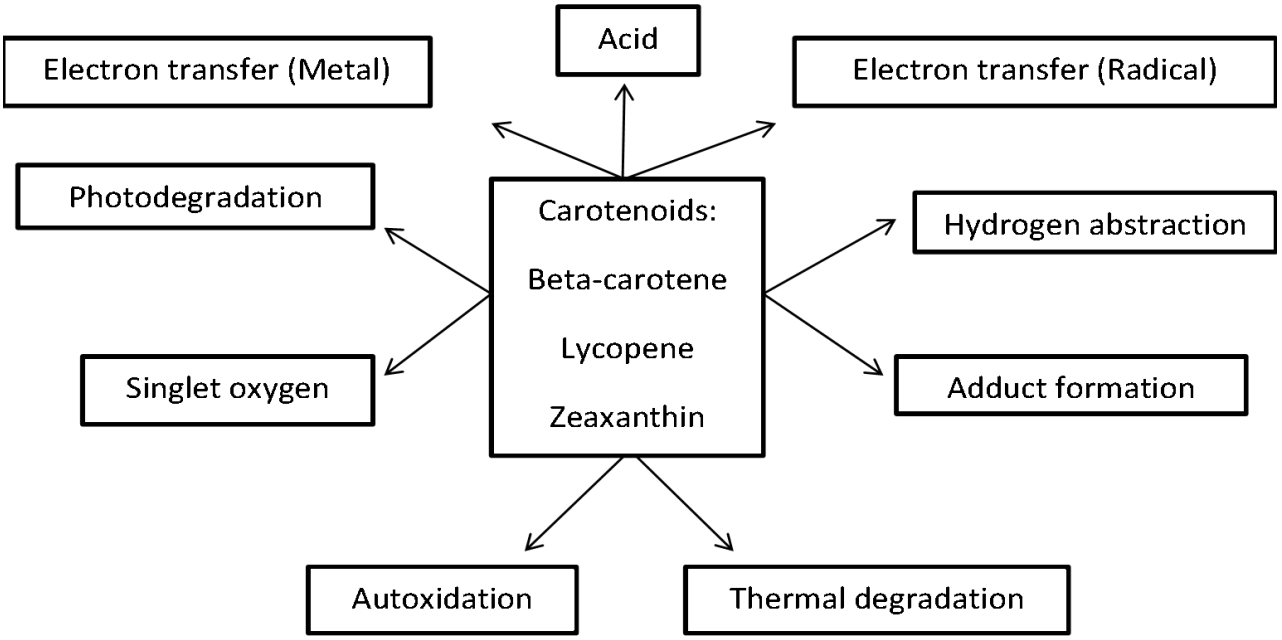

Figure 7 - Degradation mechanisms of some carotenoids, including β-carotene, lycopene and zeaxanthin (adapted from Boon *et al.,* 2010).

Astaxanthin

Astaxanthin (3,3'-dihydroxy-β, β'carotene-4,4'-dione), a xanthophyll carotenoid, is in the same family as lutein, β-carotene and lycopene (Higuera-Ciapara *et al*., 2006 and Ambati *et al*., 2014). Astaxanthin is used in a wide range of applications that include the production of food for humans and feed for animals, cosmetics, nutraceutical and pharmaceutical industries (Higuera-Ciapara *et al*., 2006; Villalobos-Castillejos *et al.*, 2013; Ambati *et al*., 2014 and Shah *et al*., 2016). Astaxanthin has been described as the king of all antioxidants because it is 50 times stronger than β-carotene, a 100 times more active than α-tocopherol, 65 times stronger than vitamin C and 10 times more potent than canthaxanthin, zeaxanthin and lutein (Miki, 1991, Perez-Galvez and Mınguez-Mosquera, 2005; Pérez-López *et al*., 2014 and Shah *et al*., 2016). Astaxanthin ($C_{40}H_{52}O_4$) has a molar mass of 596.84 g/mol and consists of two chiral centres joined by a polyene chain (conjugated double bond structure), asymmetric carbons at positions 3 and 3' and a hydroxyl group (-OH) at each end (Fig. 8) (Ambati *et al*., 2014). Natural astaxanthin is either in *cis* or *trans* form, but *trans* isomers are more common as they are thermodynamically more stable than the *cis* isomers (Higuera-Ciapara *et al*., 2006 and Chen *et al*., 2007a). The two isomers commonly found in nature are (3R, 3'R) and (3S, 3'S) (Rao *et al*., 2014).

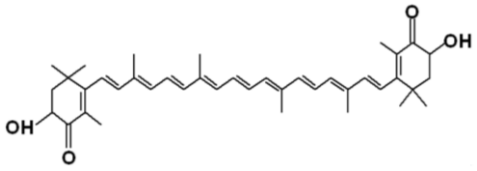

Figure 8 – Astaxanthin molecular structure (adopted from Ambati *et al*., 2014).

In its free form outside of its biological structure, astaxanthin is highly unstable in the presence of light, oxygen, and temperature with temperature being the most influential environmental factor (Villalobos-Castillejos *et al.*, 2013). There have been several attempts to improve the stability of astaxanthin. Some of these strategies included nanoencapsulation (Tachaprutinun *et al*., 2009), inclusion matrixes with hydroxypropyl-β-cyclodextrin or calcium ions (Chen *et al*., 2007 and Yuan *et al*., 2008), calcium alginate or chitosan microencapsulation (Higuera-Ciapara *et al*., 2004 and Lin *et al*., 2016) and incorporation into suspensions, emulsions and liposomes (Matsushita, 2000 and Ribeiro *et al*., 2005).

Health benefits of astaxanthin

Astaxanthin has a unique action in the cell membrane and has numerous clinical benefits. Astaxanthin has a superior position in the cell membrane of humans and microorganisms, because the polar head and tail and lipid backbone of the molecule allows it to span the entire width of the cell membrane (Fig. 9) (Kidd, 2011). This provides antioxidant protection on both sides of the membrane and throughout the lipid bilayer by intercepting reactive molecular species (McNulty *et al*., 2007). Other antioxidants such as β-carotene and vitamin C can only offer protection at a specific location of the membrane (Capelli and Cycewski, 2012).

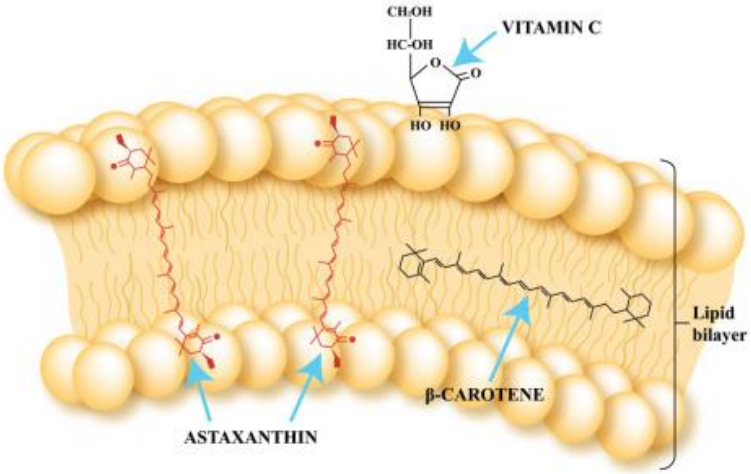

Figure 9 – Molecule orientation of vitamin C, β-carotene and astaxanthin in the cell membrane (taken from Capelli and Cycewski, 2012).

Astaxanthin has several biological functions. It is a precursor for vitamin A, enhances the immune response and acts as a scavenger of free radicals (Matsuno, 1985; Jyonouchi *et al.,* 1991; Miki, 1991; Jyonouchi *et al.,* 1993 and Miki *et al*., 1994). Over the years, the focus has been on the application of astaxanthin as a nutraceutical and medical ingredient for the treatment and prevention of several conditions including macular degeneration (Santocono *et al*., 2007), infections by *Helicobacter pylori* (Wang *et al*., 2001), inflammation (Ohgami *et al*., 2003) and cancer (Nishino *et al*., 2005) to name a few. Figure 10 shows the main health promoting benefits of astaxanthin. In addition, natural astaxanthin extracted from *Haematococcus pluvialis* has been granted "GRAS" status (Generally Recognized As Safe) by the US FDA (Food and Drug Administration) (Capelli and Cycewski, 2012).

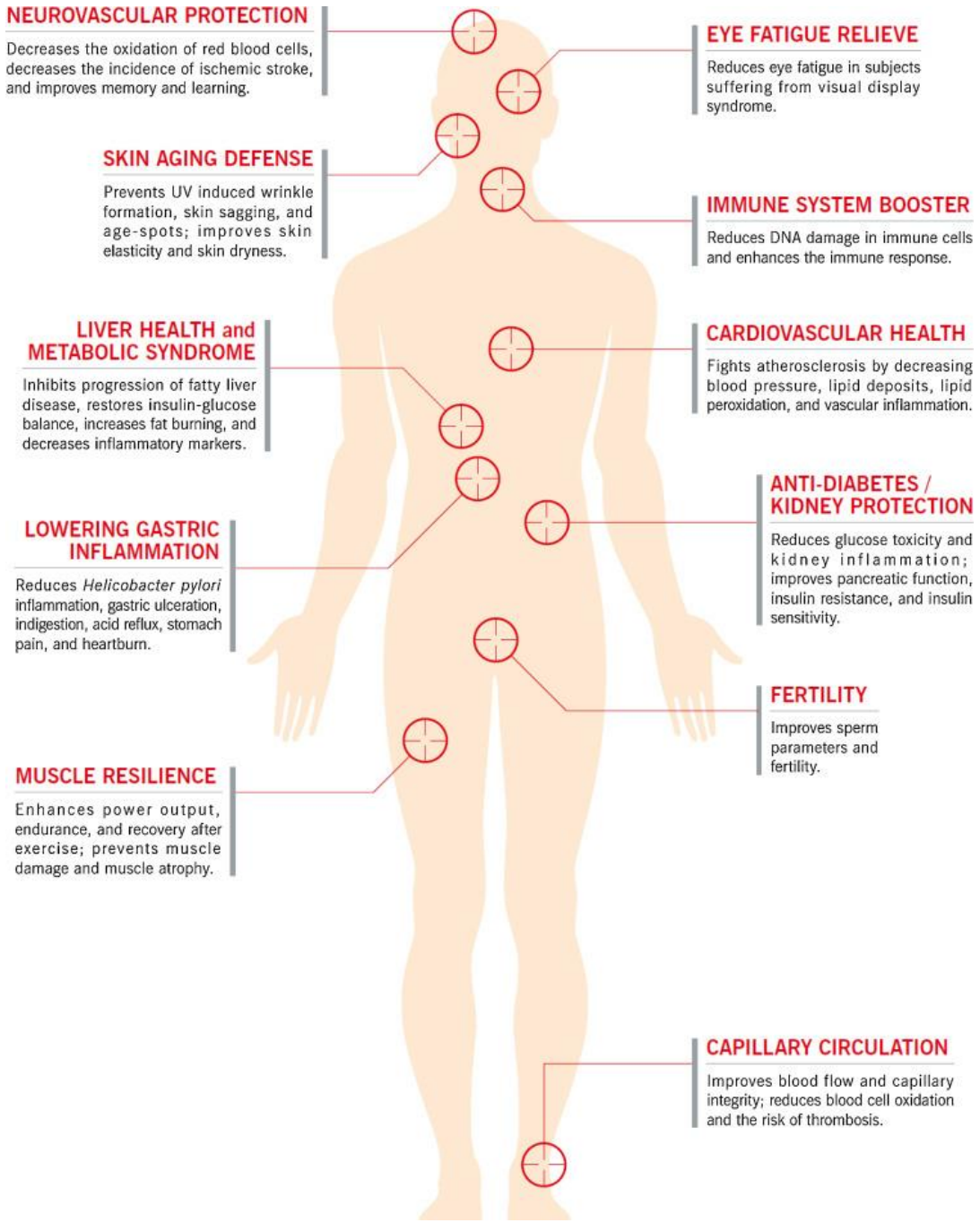

Figure 10 – Main health benefits of astaxanthin for humans (adopted from Yamashita, 2015).

Biosynthesis of astaxanthin in microorganisms

There are five genes involved in the biosynthesis of carotenoids, *crtB, crtI, crtY, crtW* and *crtZ* (Fig. 11). In 1995, Misawa *et al*. determined the function of all five of the biosynthesis genes through chromatographic and spectroscopic analysis. The results are summarised in Table 3.

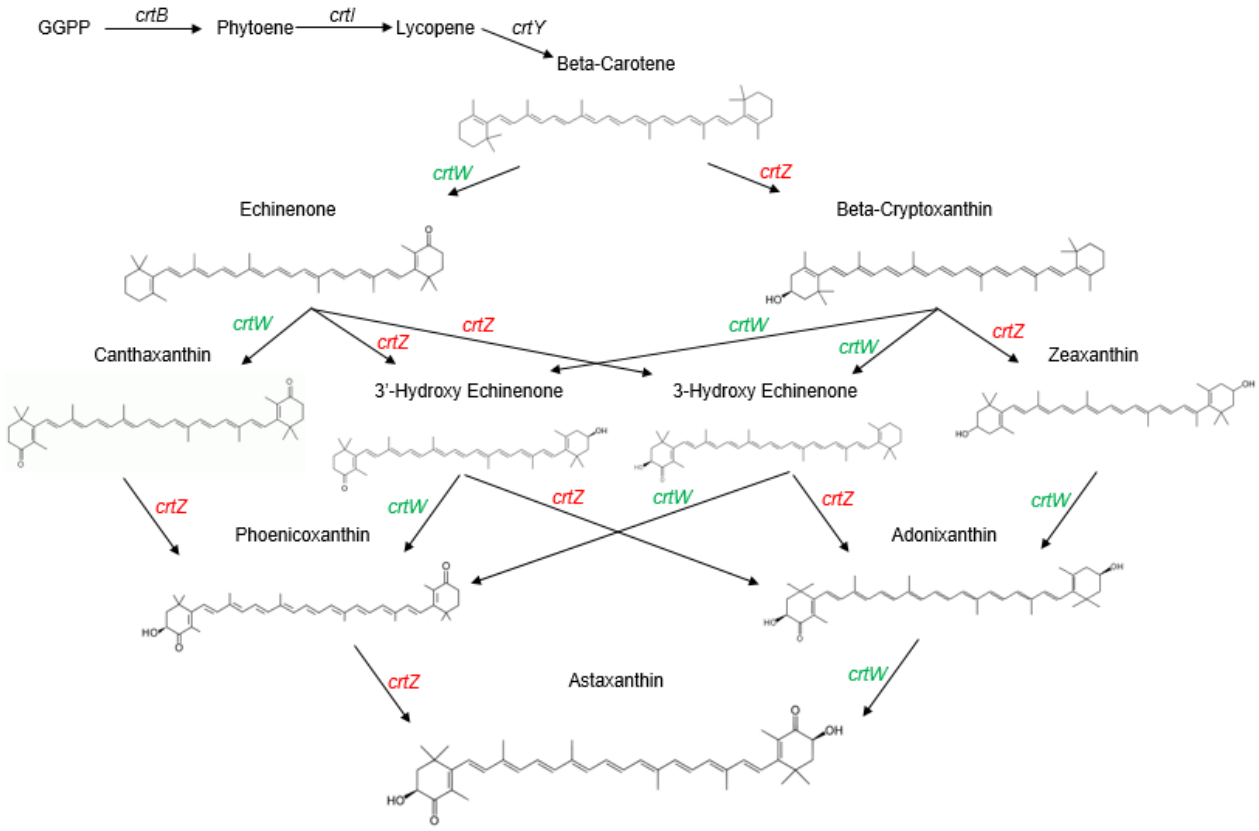

Figure 11 – Proposed biosynthetic pathway of astaxanthin production at gene level in the marine bacterium, *Paracoccus* sp. (adapted from Misawa *et al.,* 1995).

Table 3 – Genes involved in the biosynthesis of carotenoids and their function
(Misawa *et al*., 1995)

Gene	Enzyme	Function
crtB	Phytoene synthase	Catalyses the condensation reaction of two GGPP molecules to produce 15,15'-*cis*-phytoene
crtI	Phytoene desaturase	Conversion of 15,15'-*cis*-phytoene to all-*trans*-lycopene
crtY	Lycopene cyclase	Catalyses the terminal cyclisation reaction from all-*trans*-lycopene to all-*trans*-β-carotene
crtW	β-carotene ketolase	Catalyses the conversion of methylene to keto groups to synthesise canthaxanthin from β-carotene via echinenone
crtZ	β-carotene hydroxylase	Catalyses the hydroxylation reaction from β-carotene to (3*R*,2'*R*)-zeaxanthin via β-cryptoxanthin

GGPP: Geranylgeranyl pyrophosphate

Only two genes are involved in the synthesis of astaxanthin from β-carotene (Fig. 11), i.e., *crtW* and *crtZ* (Misawa *et al*., 1995). These gene products are believed to be bifunctional, because specific

enzyme assays indicated that the CrtZ enzymes formed astaxanthin from canthaxanthin, as well as zeaxanthin from β-carotene. The CrtW enzymes also formed astaxanthin from zeaxanthin, as well as canthaxanthin from β-carotene (Misawa *et al*., 1995 and Fraser *et al*., 1997).

In previous studies, the addition of cofactors significantly increased the activity of CrtW and Z, leading to an increase in astaxanthin production (Fraser *et al*., 1997 and Chougle and Singhal, 2012). A dioxygenase mixture consisting out of Fe^{2+}, ascorbic acid, 2-oxoglutarate and catalase stimulated the activity of CrtZ six-fold more and CrtW four-fold more than the normal activity (Fraser *et al.,* 1997). Further experimentation indicated that Fe^{2+} was the most powerful effector and is involved in the catalysis performed by CrtW and Z. The presence of Fe^{2+} enhances 4,4'-oxygenation by CrtW or 3,3'-hydroxylation by CrtZ (Fraser *et al.,* 1997). The other cofactors (ascorbic acid, 2-oxoglutarate and catalase) had no independent stimulation and their role is hypothesised to be an oxidisable cosubstrate to generate reactive Fe^{2+} in the process (Dawson *et al*., 1993).

Astaxanthin in industry

Astaxanthin produced by microorganisms have been commercialised and applied in the colouration of cosmetics, beverages, dairy products, and meats (Del Campo *et al*., 2000; Guerin *et al*., 2003; Liang *et al*., 2004; Pulz and Gross, 2004 and Chandi and Gill, 2011). There is an increase in demand for naturally derived astaxanthin from microorganisms instead of synthetic astaxanthin, since natural astaxanthin has a higher antioxidant activity when compared to synthetic astaxanthin (Capelli *et al.,* 2013). The yeast, *Xanthophyllomyces dendrorhous*, and microalga, *Haematococcus pluvialis*, are currently used for the large-scale cultivation of astaxanthin. Many studies have used these microorganisms in developing biotechnological processes to produce astaxanthin in large quantities (Lorenz and Cysewski, 2000; Dufosse *et al*., 2005; Schmidt *et al*., 2011; Rodríguez-Sáiz *et al*., 2010 and Mata-Gómez *et al*., 2014).

The microalga, *H. pluvialis*, can accumulate up to 2% of astaxanthin (Dufosse *et al*., 2005). During cultivation for astaxanthin production, the cells undergo a two-step batch process in which the physical properties and nutrient requirements of the microalga changes (Mata-Gómez *et al*., 2014). The first stage is known as the growth phase where nutrients are abundant, pH and temperature are controlled and there are low levels of irradiation (Fig. 12A). In the second stage, also known as the "reddening" phase, the cells are introduced to different stress conditions, such as nutrient deprivation, high levels of irradiation and high concentrations of NaCl, forcing the production of astaxanthin (Fig. 12B). The microalga cells are harvested through centrifugation, dried and milled

for astaxanthin extraction (Lorenz and Cysewski, 2000; Schmidt *et al*., 2011 and Mata-Gómez *et al*., 2014).

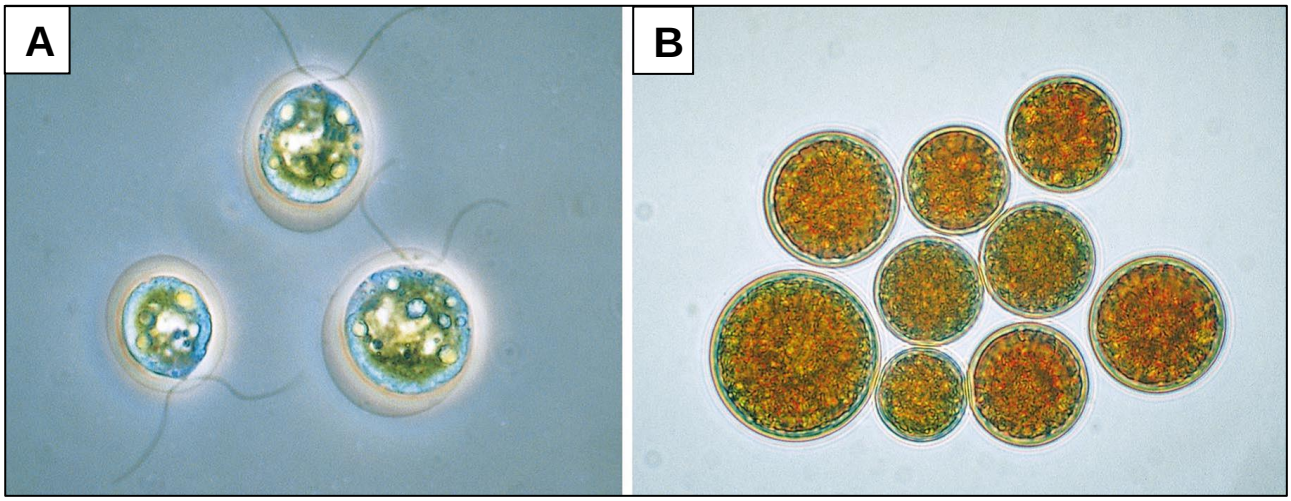

Figure 12 – Different phases of the cell during the two-stage batch process. **A.** *Haematococcus pluvialis* growth phase. **B.** "Reddening" phase of *Haematococcus pluvialis* aplanospores (adopted from Lorenz and Cysewski, 2000).

There are some drawbacks in using this microalga to produce astaxanthin. Firstly, the microalga requires a completely closed photobioreactor with artificial light to ensure that there is no contamination from other microorganisms (Lorenz and Cysewski, 2000). Secondly, during the growth phases the microalga cells physically change from a motile cell (Fig. 12B) to non-motile aplanospores (Fig. 12B) with thick walls containing the astaxanthin. Consequently, the walls of the aplanospores need to be cracked to extract the astaxanthin (Dufosse *et al*., 2005 and Lorenz and Cysewski, 2000). These growth requirements and downstream processing to extract the astaxanthin can be expensive. Without rupturing the thick walls of the microalga, the astaxanthin is not released from the cell and cannot be incorporated into egg yolks or the flesh of animals, limiting the application of a whole cell microalga (Lorenz and Cysewski, 2000).

The yeast, *X. dendrorhous,* is commonly used as a pigmentation source in crustaceans, egg yolks and aquaculture (Mata-Gómez *et al*., 2014). The astaxanthin yield of *X. dendrorhous* ranges between 50 – 350 µg/g dry cell weight. This is lower than the microalga (Chandi and Gill, 2011). Factors that have been tested to increase astaxanthin production included temperature, pH, oxygen, nutrients, aeration and light (Rodríguez-Sáiz *et al*., 2010 and Chandi and Gill, 2011). Light positively affects the production of astaxanthin in microalga and yeast cells. Carotenogenesis is a photoprotective mechanism that prevents harmful wavelengths of light from damaging the cells (Chandi and Gill, 2011 and Mata-Gómez *et al*., 2014). Like the microalga, the yeast cells must be

milled or enzymatically digested before it can be used as a pigmentation source (Johnson *et al*., 1977; 1980). These methods for extraction are also expensive (An and Choi, 2003).

Currently, bacteria are not used as a natural source of astaxanthin for industrial production. Some bacterial species that are known to produce astaxanthin include *Agrobacterium aurantiacum, Paracoccus carotinifaciens, Paracoccus haeundaensis and Paracoccus marcusii* (Misawa *et al*., 1995; Harker *et al*., 1998; Tsubokura *et al.,* 1999; Lee *et al*., 2004 and Schmidt *et al*., 2011). Species of the genus *Paracoccus* are Gram-negative, oxidase and catalase positive bacteria that grow aerobically. Table 4 illustrates a species comparison of the genus *Paracoccus* taken from different studies (Harker *et al*., 1998; Tsubokura *et al.,* 1999 and Lee *et al*., 2004).

Table 4 – A comparison between known astaxanthin producing *Paracoccus* species

	P. marcusii (Harker *et al.,* 1998)	***P. carotinifaciens*** (Tsubokura *et al.,* 1999)	***P. haeundaensis*** (Lee *et al.,* 2004)
Motility	No	Yes	No
Nitrate reduction	No	No	Yes
Flagella	N/A*	Peritrichous flagella	N/A*
Cell shape	Cocci to short rods	Rods	Rods
DNA G+C content	66 mol %	67 mol %	66.9 mol %
Utilization of:			
Arabinose	Yes	No	Yes
Fructose	Yes	Yes	No
Galactose	Yes	Yes	Yes
Glucose	Yes	Yes	No
Mannose	Yes	Yes	No
Mannitol	Yes	Yes	No

* N/A – Not applicable

In previous studies, yeast and microalga have been used as a pigmentation source in the diet of trout (Choubert and Heinrich, 1992 and Storebakken *et al.*, 2004), salmon (Lorenz and Cysewski, 2000) and laying hens (Johnson *et al*., 2003). A significant difference was only observed after partial homogenisation, enzymatic digestion or by cracking the cells to increase the release of the available pigments (Choubert and Heinrich, 1992; Lorenz and Cysewski, 2000; Johnson *et al*., 2003 and Storebakken *et al.*, 2004). However, there was no need to enzymatically digest or homogenise the bacterium, *P. marcusii*, when fed to rainbow trout (*Oncorhynchus mykiss*) for pigmentation effect (De Bruyn, 2013). The results obtained by De Bruyn (2013) indicated that an astaxanthin-producing bacterium could be used as a pigmentation source, instead of yeast or microalga.

Aim of this study

The aim of this study was to optimise the growth and quantify astaxanthin production by *Paracoccus marcusii* and to prolong the stability during storage of the whole cell with astaxanthin. Furthermore, the aim was to evaluate the potential application of *P. marcusii* as a feed additive to poultry to enhance egg yolk colour and the effect it might have on the microbial diversity structure in the gastrointestinal tract of the laying hen. Additionally, an economical assessment was also done to determine the cost benefit of producing astaxanthin with *P. marcusii*.

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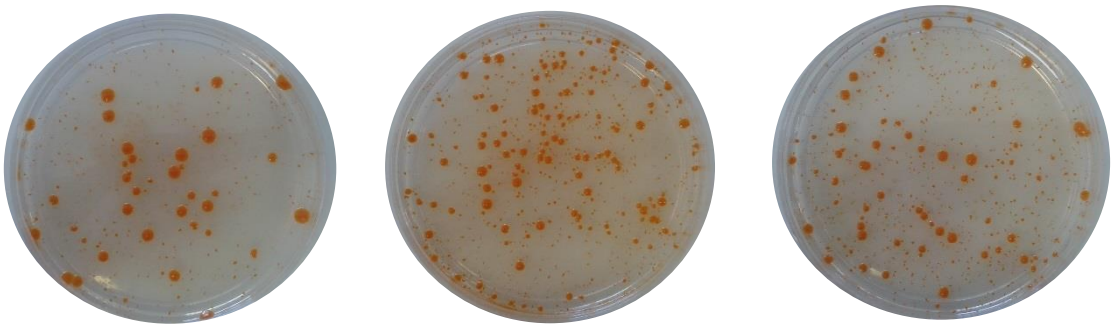
Chapter 2

Optimisation, quantification and storage stability of astaxanthin produced by the bacterium, *Paracoccus marcusii*



Abstract

Astaxanthin is a potent antioxidant that provides several health benefits to humans and animals. Some microorganisms, including the Gram-negative bacterium *Paracoccus marcusii*, are able to produce astaxanthin naturally. This study investigated the ability of *P. marcusii* to produce astaxanthin, as well as the stability of the molecule during storage together with the cell viability after whole cell lyophilisation or microencapsulation. The growth conditions for cell growth and astaxanthin production were optimised. The optimum growth conditions for *P. marcusii* and for astaxanthin production was at 26 °C in a specialised medium containing yeast extract (5 g/L), bacteriological peptone (10 g/L) and NaCl (3%) at a pH between 6 – 7. Harvested cells were either lyophilised in sucrose (10% m/v) or microencapsulated in calcium alginate beads and stored for 3 weeks at either 4 °C or room temperature (± 20°C). Astaxanthin was extracted every week with methanol in the dark at 26 °C on a rotary shaker (150 rpm). Lyophilisation ensured viability of the cells but there was a significant loss in astaxanthin concentration. Astaxanthin was more stable in the calcium alginate beads after 3 weeks even though viability of the cells significantly decreased at 20 °C. This study shows promising results in using calcium alginate beads as a possible storage method compared to lyophilisation.

Introduction

Over the years there have been an increase interest in carotenoids and the biological functions of the molecules. Carotenoids are widely distributed in plants as well as microorganisms (Ausich, 1997 and Bhosale and Bernstein, 2005). One carotenoid, astaxanthin, has captured the most attention, because of its potent activity as an antioxidant (Miki, 1991, Pérez-López *et al*., 2014 and Shah *et al*., 2016). Astaxanthin (3,3'-dihydroxy-β, β'carotene-4,4'-dione) is a byproduct of the glycolytic metabolic pathway in several microorganisms. The most well-known microbial sources of astaxanthin include the yeast, *Xanthophyllomyces dendrorhous* (An *et al*., 2001), and microalga, *Haematococcus pluvialis* (Jeon *et al*., 2006). Some bacteria are also able to produce astaxanthin, but are currently not used in the industry for large-scale production. These bacteria include species of the genus *Paracoccus* (Harker *et al.,* 1998; Tsubokura *et al.,* 1999 and Lee *et al.,* 2004) and *Agrobacterium aurantiacum* (Misawa *et al*., 1995).

Synthetic astaxanthin has been used as a colouring agent and as a feed additive in aquaculture and poultry (Guerin *et al*., 2003). Synthetic astaxanthin is, however, not available for human consumption. This is a result of concerns around the use of carcinogenic petrochemicals in the production of astaxanthin (Newsome, 1986). There are several methods of production of synthetic astaxanthin that include the reaction between two C_{15}-phosphonium salts with C_{10}-dialdehyde (Widmer *et al*., 1981), canthaxanthin hydroxylation (Bernhard *et al*., 1984) and lutein isomerisation to zeaxanthin from marigold which is then oxidised to astaxanthin (Schloemer and Davis, 2001). Synthetic astaxanthin is a mixture of three isomers (3S, 3'S), (3R, 3'R) and (3R, 3'S) in a ratio of 1:1:2 (Schiedt *et al*., 1988 and Higuera-Ciapara *et al*., 2006). Natural astaxanthin is either in *cis* or *trans* form, but *trans* isomers are more common as they are thermodynamically more stable than the *cis* isomers as can be seen in Figure 1 (Higuera-Ciapara *et al*., 2006 and Chen *et al*., 2007a). The two isomers commonly found in nature are (3R, 3'R) and (3S, 3'S) (Rao *et al*., 2014).

The use of natural astaxanthin in the food industry is restricted because of its unstable conjugated double bond structure (polyene chain) and the 4-keto and 3-hydroxy groups at both ends of the molecule (Mendes-Pinto *et al*., 2001). When astaxanthin is removed from the stable biological matrix, it becomes highly unstable and is vulnerable to degradation when exposed to light, oxygen or high temperatures (Chen *et al*., 2007a; Boon *et al*., 2010 and Villalobos-Castillejos *et al.*, 2013). Several studies have been done to improve the stability of astaxanthin and prevent loss of pigmentation. Some strategies included lyophilisation or storage at low temperatures, addition of antioxidants such as β-carotene, avoiding direct contact with light or oxygen and storage under nitrogen (Mendes-Pinto *et al*., 2001; Boon *et al*., 2010 and Villalobos-Castillejos *et al.*, 2013). Some

studies have also reported a greater astaxanthin stability when it is kept within the biomass structure compared to the extracted astaxanthin (Gouveia and Empis, 2003 and Delgado *et al*., 2016).

The aim of this study, therefore, was to determine the optimum growth conditions of *Paracoccus marcusii* and to quantify the astaxanthin produced. We also aimed to determine the viability of the cells and the stability of astaxanthin within the cell under different storage conditions, namely lyophilisation and microencapsulation.

Materials and Methods

Origin of *Paracoccus marcusii*

The bacterium used in all experimental work was previously isolated from the gastrointestinal tract of the South African abalone (*Haliotis midae*) (De Bruyn, 2013).

Optimum growth conditions of *Paracoccus marcusii*

Temperature

Twelve test tubes containing 9.9 ml Luria Bertani (LB) broth (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) (Biolab, South Africa) were prepared. Each test tube was inoculated with 0.1 ml of the starter culture (OD_{550nm} – 0.781). An absorbance value was measured and the test tubes were incubated in triplicate on a wheel at 20 °C, 26 °C, 30 °C and 37 °C, respectively. Every 12 hours for 120 hours an absorbance value was measured for all test tubes.

NaCl concentration

Twenty-one test tubes containing 9.9 ml bacteriological peptone (10 g/L) (Oxoid, United Kingdom) and yeast extract (5 g/L) (Biolab, South Africa) were prepared. Different concentrations of NaCl were added to obtain concentrations of 0%, 1.5%, 3%, 4.5%, 6%, 7.5% and 9%, respectively. Each test tube was inoculated with 0.1 ml of the starter culture (OD_{550nm} – 0.781). An absorbance value was measured and the test tubes were incubated on a wheel at 26 °C. Every 12 hours for 120 hours an absorbance value was measured for all test tubes. All experiments were done in triplicate.

pH range

Twenty-one test tubes containing 9.9 ml LB broth were prepared. The pH of each test tube was adjusted with 10 M NaOH pellets or 1 M HCl solution to pH 4, 5, 6, 7, 8, 9 and 10, respectively. Each test tube was inoculated with 0.1 ml of the starter culture (OD_{550nm} – 0.781). An absorbance value was measured and the test tubes were incubated on a wheel at 26 °C. Every 12 hours for 50 hours an absorbance value was measured for all test tubes. All experiments were done in triplicate.

Optimum growth conditions for astaxanthin production

Culturing

The following growth media were used to determine the ability of *P. marcusii* to produce astaxanthin: Nutrient broth (1 g/L meat extract, 2 g/L yeast extract, 5 g/L peptone and 8 g/L NaCl) (Biolab, South Africa), LB broth (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) (Biolab, South Africa) and a specialised medium (5 g/L yeast extract (Biolab, South Africa), 10 g/L bacteriological peptone (Oxoid, United Kingdom) and 3% NaCl (m/v)). The NaCl concentration of the Nutrient and LB broth was adjusted to 3% (m/v). The different media were incubated at 26 °C until an absorbance value of 1 at 550 nm was measured. Aliquots of cells were harvested through centrifugation at 10000 rpm for 5 minutes and washed once with sterile dH_2O.

Extraction of astaxanthin and Liquid Chromatography-Mass Spectrometry (LC-MS)

Carotenoids were extracted in the dark with 1 ml methanol at 26 °C for 90 minutes on a shaker (150 rpm) (Harker *et al*., 1998). The extracted carotenoids were then separated from the cell content through centrifugation at 10000 rpm for 10 minutes. The methanol (now containing the carotenoids) was transferred into an amber vial and sent to CAF (Central Analytical Facility), Stellenbosch University, for analysis. Liquid chromatography-mass spectrometry was performed as previously described with minor modifications, where a mobile phase of acetonitrile was used instead of acetonitrile/methanol/isopropyl alcohol (90:6:40, v/v/v) (Fraser *et al*., 1997). Figure 2 indicates the mobile phase gradient profile. Column: Waters UPLC HSS C18, 2.1 x 150 mm. Solvent A: 1% Formic Acid. Solvent B: Acetonitrile. Source: Electrospray positive. Capillary voltage 3 kV. Cone voltage 15 V. Mass spectra obtained by scanning from m/z 200 – 700. A standard (3S, 3′S) *trans*-astaxanthin solution (from *Haematococcus pluvialis,* SML0982, Sigma-Aldrich, United States) was prepared with concentrations 2, 4, 6, 8, 10 and 20 ppm (Fig. 3).

Final growth conditions and media used for further experimental work

The media selected for further culturing of *P. marcusii* contained 5 g/L yeast extract, 10 g/L bacteriological peptone and 3% NaCl at a pH between 6 – 7. The incubation temperature for further experimental work was 26 °C.

Cell viability of *Paracoccus marcusii* and stability of astaxanthin

In preparation for lyophilisation in sucrose and microencapsulation in calcium alginate beads, cells were cultured in 2 L Schott bottles containing 1 L of specialised medium and incubated at 26 °C for 4 – 7 days. The cells were harvested through centrifugation at 10000 rpm for 5 minutes and washed once with sterile dH_2O.

Paracoccus marcusii *lyophilised in sucrose*

Lyophilisation process

Cell pellets from every 1 L of harvested cells were resuspended in 200 ml sucrose (10% m/v) and transferred into sterile 500 ml Schott bottles. The solution was frozen overnight at -80 °C and lyophilised until dry (VirTis benchtop K, model 6KBTEL-85, SP Scientific, United States) (Heckly, 1961). Lyophilised cells were stored in a dark airtight container at 4 °C.

Cell viability

A dilution series of 10^{-2} – 10^{-8} was prepared by adding 0.1 g of lyophilised cells to 9.9 ml of sterile saline solution (0.9% NaCl). The dilutions were plated onto Nutrient Agar (1 g/L meat extract, 2 g/L yeast extract, 5 g/L peptone, 8 g/L NaCl and 12 g/L agar-agar) (Biolab, South Africa) and incubated for 4 – 5 days at 26 °C. Dilutions and plating were performed in triplicate. The total colony forming units (CFU) were counted for each plate and the average calculated. Viability of cells was determined directly after lyophilisation and then every week for 3 weeks.

Astaxanthin extraction and storage stability

One gram of lyophilised cells was resuspended in 10 ml of dH_2O to dissolve the sucrose before extraction. The cells were harvested through centrifugation at 10000 rpm for 5 minutes and the supernatant discarded. Carotenoids were extracted from the pellet in a 50 ml Falcon tube with 10 ml methanol at 26 °C on a rotary shaker at 150 rpm for 90 minutes in the dark. The samples

were centrifuged at 5000 rpm for 10 minutes and the methanol transferred to an amber vial. The samples were sent to CAF for analysis, as previously described. Carotenoids were extracted directly after lyophilisation and then every week for 3 weeks. Extractions were performed in triplicate.

Paracoccus marcusii *microencapsulated in calcium alginate beads*

Microencapsulation process

Every 3 L of harvested cells were resuspended in 200 ml of 2% sodium alginate solution. The extrusion method was used to encapsulate whole *P. marcusii* cells in calcium alginate beads (Lin *et al*., 2016). The sodium alginate solution containing *P. marcusii* was added drop-wise using a 21G x 1.5" hypodermic needle and syringe into 200 ml of 2% $CaCl_2$ solution under constant stirring of 150 rpm (Fig. 4A). The resulting beads (Fig. 4B) were separated from the $CaCl_2$ solution using a sieve and allowed to dry overnight in a laminar flow cabinet at room temperature (Fig. 5A and B). Equal volumes of control beads were also made and dried over-night (Fig. 6). This solution contained no *P. marcusii* cells. The dried control and *P. marcusii* beads were weighed (Fig. 7) and the total weight of *P. marcusii* in 1 g of beads was calculated. The size of the beads was measured with an electronic digital calliper and morphology of the beads were examined using a light microscope (Model: UB200i, Lasec, South Africa) under 40x and 100x magnification. The beads were stored in a dark container at 4 °C and room temperature (± 20 °C).

Cell viability

One gram of calcium alginate beads was first dissolved in 200 ml of 0.05 M Na_2CO_3/ 0.02 M citric acid buffer (Mater *et al*., 1995). This buffer solution allows for 100% cell recovery and viability. The buffer solution containing the beads were placed on a rotary shaker at 200 rpm until all the beads were dissolved (Fig. 8A). A dilution series of 10^{-1} – 10^{-8} were prepared by adding 1 ml of the dissolved solution to 9 ml of sterile saline solution (0.9% NaCl). The dilutions were plated onto Nutrient Agar (1 g/L meat extract, 2 g/L yeast extract, 5 g/L peptone, 8 g/L NaCl and 12 g/L agar-agar) (Biolab, South Africa) and incubated for 4 – 5 days at 26 °C. Dilutions and plating were performed in triplicate. The total colony forming units (CFU) were counted for each plate and the average calculated. Viability of cells was determined directly after microencapsulation and then every week for 3 weeks.

Astaxanthin extraction and storage stability

One gram of calcium alginate beads was first dissolved as described above. The cells were separated from the alginate solution through centrifugation at 14000 rpm for 20 minutes. The water and alginate content (Fig. 8B) was discarded and the pellet rinsed once with dH_2O. The carotenoids were extracted from the pellet in a 50 ml Falcon tube with 10 ml methanol at 26 °C on a rotary shaker (150 rpm) for 90 minutes in the dark. The samples were centrifuged at 5000 rpm for 10 minutes and the methanol supernatant (Fig. 8C) was transferred to an amber vial. The samples were sent to CAF for analysis, as described previously. Carotenoids were extracted directly after microencapsulation and then every week for 3 weeks. Extractions were performed in triplicate.

Results

Optimum growth conditions of *Paracoccus marcusii*

Temperature

Paracoccus marcusii was able to grow at all temperatures, with an optimum growth temperature observed at 26 °C (Fig. 9). A lag phase was only observed at 20 °C and 26 °C. After 24 hours, the absorbance values were similar for the temperatures 26 °C, 30 °C and 37 °C (OD_{550nm} ± 1.10). After 36 hours, the absorbance value started to decrease at 37 °C and after 60 hours at 30 °C, while absorbance still increased at 26 °C until it reached a plateau after 60 hours (OD_{550nm} ± 1.6). Pigmentation was observed to be less at the higher temperatures (30 °C and 37 °C; yellow to light orange) and higher at the lower temperatures (20 °C and 26 °C; bright to dark orange).

NaCl concentration

Paracoccus marcusii was unable to grow at all NaCl concentrations tested (Fig. 10). An optimum growth was observed between 1.5% and 3% (OD_{550nm} ± 1.45). Even though *P. marcusii* was able to grow at 0% NaCl, pigmentation was lower compared to 1.5% and 3%. There was an initial lag phase between 4.5% and 6%, but after 60 hours all absorbance values were similar (OD_{550nm} ± 1.43). No growth was observed at concentrations of 7.5% and 9%.

pH level

Optimum growth was observed at a pH between 6 (1.40 ± 0.02) and 7 (1.31 ± 0.05) (Fig. 11). With an increase in pH (8, 9 and 10) there was a decrease in absorbance value (lower than 1.11), although relatively good growth was still observed. There was no growth observed at pH of 4 and 5.

Optimum growth conditions for astaxanthin production

The mean concentration of astaxanthin produced by *P. marcusii* was the highest in the specialised medium with 3.35 ppm (± 0.21), followed by LB broth with 2.1 ppm (± 0.14) and Nutrient broth with 1.65 ppm (± 0.49) (Fig. 12). Figure 13 is one example of the typical results obtained of extracted carotenoids from *P. marcusii*. Five individual peaks can be seen on the extracted sample run (Fig. 13A) with the first peak aligning with the standard solution peak (Fig. 13B).

Cell viability of *Paracoccus marcusii* and stability of astaxanthin

Paracoccus marcusii *lyophilised in sucrose*

Cell viability and astaxanthin storage stability

The viability of *P. marcusii* in sucrose and the loss of astaxanthin concentration over time is illustrated in Figure 14. Directly after lyophilisation, the mean cell count was 16 x 10^6 CFU/ml (Fig. 14, dotted line). However, one week after lyophilisation the cell count decreased significantly (3.04 x 10^6 CFU/ml), but remained similar after 2 and 3 weeks with a cell count of 2.49 x 10^6 CFU/ml and 1.87 x 10^6 CFU/ml, respectively. After lyophilisation the mean astaxanthin concentration was 2.78 ppm (± 0.06) (Fig. 14, bars). In the first week, the concentration decreased to 2.39 ppm (± 0.05) and then 1.87 ppm (± 0.06) in the second week. There was a significant loss in astaxanthin concentration in the final week (week 3) with a concentration of 0.40 ppm (± 0.15) detected.

Paracoccus marcusii *microencapsulated in calcium alginate beads*

Microencapsulation of Paracoccus marcusii

The total weight of the dried control and *P. marcusii* beads were 4.5 g and 7.5 g, respectively. The total dry cell weight per gram of beads is estimated to be 400 mg. The shape of the beads was spherical and uniform in size (± 5 mm) after being dripped into the $CaCl_2$ solution. However, after drying the beads lost their spherical shape and were ellipsoidal with curvy edges and some flat surfaces (Fig. 15A and B). The diameters of the dried beads ranged between 0.9 mm and 1.10 mm.

Cell viability and astaxanthin storage stability

The viability of *P. marcusii* in the calcium alginate beads and the loss of astaxanthin concentration over time stored at 4 °C and room temperature (± 20 °C) is illustrated in Figure 16. The mean cell count after microencapsulation was 44.5 x 10^8 CFU/ml. The cell count decreased for both temperatures with a significant loss in viability at 20 °C after 3 weeks with a cell count of 5.98 x 10^8 CFU/ml, compared to 29.85 x 10^8 CFU/ml at 4 °C (Fig. 16, dotted lines). The concentration of astaxanthin decreased over time, but remained relatively stable (Fig. 16, bars). After microencapsulation, the mean astaxanthin concentration was 9.65 ppm (± 0.07) (24.25 µg/g dry cell weight) and after 3 weeks it was 6.55 ppm (± 0.13) and 6.65 ppm (± 0.05) at 4 °C and 20 °C, respectively. There was no significant difference between 4 °C and 20 °C in terms of astaxanthin concentration.

Discussion

Astaxanthin (3,3'-dihydroxy-β, β'carotene-4,4'-dione), a xanthophyll carotenoid, has been described as the king of all carotenoids because of its potent antioxidant activity and wide range of applications in the food and health industry (Perez-Galvez and Mınguez-Mosquera, 2005; Villalobos-Castillejos *et al.*, 2013 and Shah *et al*., 2016). Microorganisms that are well known to produce astaxanthin in large quantities include the microalga, *Haematococcus pluvialis*, and yeast, *Xanthophyllomyces dendrorhous* (Johnson *et al*., 1977; 1980 and Dufosse *et al*., 2005). Some bacteria are also able to produce astaxanthin and include the bacterium, *Paracoccus marcusii* (Harker *et al*., 1998 and Chougle and Singhal, 2012). This is a Gram-negative, catalase and oxidase positive bacterium that grow aerobically and was isolated from the gastrointestinal tract (GIT) of the South African abalone, *Haliotis midae* (Harker *et al*., 1998 and De Bruyn, 2013). The stability of astaxanthin is strongly influenced by temperature, oxygen and light, with high temperatures being the most influencial factor (Villalobos-Castillejos *et al.*, 2013 and Lin *et al*., 2016). In order to use *P. marcusii* as a possible pigmentation source for application in poultry feed, large quantities of the bacterium are needed. Therefore, in this study we aimed to determine the optimum growth conditions of *P. marcusii* and to quantify and enhance the production of astaxanthin. Furthermore, we also aimed to determine the viability of the cells and the stability of astaxanthin under two storage conditions, namely lyophilisation in a sucrose solution and microencapsulation in calcium alginate.

The optimum growth temperature was determined at 26 °C even though *P. marcusii* was also able to grow relatively well at 20 °C, followed by 30 °C and 37 °C (Fig. 9). The colour of the growth at 30 °C and 37 °C appeared yellow to light orange compared to the 20 °C and 26 °C which had a

bright to dark orange colour. It has been suggested by Sandmann *et al*. (1999) that lower temperatures promote a slower expression of the genes responsible for carotenogenesis which leads to an enhanced activity of enzymes and added phenyl pyrophosphates (carotenoid precursors). Several other reports have indicated the importance of incubation temperature on carotenoid production as it triggers a survival mechanism in microorganisms to accumulate carotenoids (Bhosale and Gadre, 2002; Bhosale, 2004 and Durmaz *et al*., 2009). The optimum NaCl concentration of between 1.5% - 3% (Fig. 10) and pH between 6 – 7 (Fig. 11) is similar to the conditions of sea water from which the bacterium was isolated (Mater *et al*., 1995 and De Bruyn, 2013). These optimum growth conditions correlate with a previous study conducted by De Bruyn (2013).

With the optimum conditions determined for biomass production, the use of different combinations of nitrogen and carbon sources to produce astaxanthin was also determined. The highest concentration of 3.35 ppm (± 0.21) of astaxanthin was produced in the specialised medium containing 5 g/L yeast extract, 10 g/L bacteriological peptone and 3% NaCl (3.35 ppm), followed by LB broth and Nutrient broth with 2.1 ppm (± 0.14) and 1.65 ppm (± 0.49), respectively (Fig. 12). The difference in astaxanthin production can be explained by the available carbon and nitrogen compounds in the media. Nitrogen and carbon are the energy source and building blocks of the cell and are also involved in the formation of biomolecules, such as astaxanthin (Chougle and Singhal, 2012). Chougle and Singhal (2012) compared the production of astaxanthin by *Paracoccus* MBIC 01143 with different nitrogen sources. They found that the highest concentration of astaxanthin was obtained with media containing bacteriological peptone, followed closely by yeast extract and the lowest with meat extract. However, a combination of yeast extract and bacteriological peptone obtained even higher concentrations (Chougle and Singhal, 2012). The results of this study are in agreement with Chougle and Singhal (2012), as the highest concentration of astaxanthin was obtained with a combination of yeast extract (5 g/L) and bacteriological peptone (10 g/L) compared to LB broth and Nutrient broth.

Lyophilisation is a method commonly used to preserve biological samples (Delgado *et al*., 2016). However, the process may affect the stability of astaxanthin as the lyophilised sample will have a porous surface, allowing for oxidation of the molecule (Rodriguez-Amaya, 2001 and Wessman *et al*., 2011). Another method includes the microencapsulation of compounds in calcium alginate beads for storage stability and a controlled release (Delgado *et al*., 2016). This method is simple, there is less stress on the cell and molecules and the matrix created provides protection from environmental conditions (Lin *et al*., 2016). Therefore, in this study, we determined the stability of astaxanthin in *P. marcusii* lyophilised in sucrose (10% m/v) and whole cell microencapsulation in calcium alginate

beads. Skimmed milk and sucrose are both common lyoprotectants used to obtain a higher survival rate of bacteria after lyophilisation (Leslie *et al*., 1995; Schoug *et al*., 2006 and Peiren *et al*., 2015). However, hens are lactose intolerant and for this reason sucrose was used as a lyoprotectant in this study. The results obtained from the first few weeks after lyophilisation in sucrose showed some promise, but after 3 weeks there is a significant loss in astaxanthin concentration (less than 0.5 ppm, Fig. 14). However, the cells remained viable at total cell counts ranging between 1.5 – 2.5 x 10^6 CFU/ml. Lyophilisation with sucrose may be suitable for preserving viable cells, but not for long term storage of astaxanthin.

The microencapsulation of whole *P. marcusii* cells showed promise to be used as a method for long term storage. The astaxanthin concentration over time was similar for both storage temperatures and loss of concentration was low compared to the lyophilisation. After 3 weeks, only 30% of the astaxanthin content was lost with the microencapsulated beads at both temperatures compared to the lyophilisation in sucrose with a total loss of 85%. However, the viability of the cells was lower at 20 °C (Fig. 16). Since the aim is to use *P. marcusii* as a feed additive to enhance egg yolk colour, the viability of the cells is not important but rather the storage stability of the astaxanthin molecule. These results indicate the ability of calcium alginate beads to improve the thermal stability of astaxanthin as both storage temperatures had similar concentrations every week even though the cell viability was the lowest at 20 °C. As cell viability decreases, astaxanthin is released from the cells, but are still stable within the bead matrix. Calcium ions are believed to have a bifunctional role in the calcium alginate beads matrix. First, calcium ions act as a crosslinker to link the chains of alginate to form the matrix layer, preventing exposure to oxygen. Second, calcium is able to form a complex with the free astaxanthin molecule, enhancing the stability of the molecule (Chen *et al*., 2007b and Lin *et al*., 2016).

Conclusion

In this study, we aimed to determine the optimum growth conditions for astaxanthin production by *Paracoccus marcusii* and a possible storage method for a longer stability of astaxanthin over time. The optimum growth conditions for *P. marcusii* and for astaxanthin production was at 26 °C in a specialised medium containing yeast extract (5 g/L), bacteriological peptone (10 g/L) and NaCl (3% m/v) at a pH between 6 – 7. The astaxanthin concentration of the lyophilised cells decreased significantly compared to the microencapsulated cells (4 °C and 20 °C), but viability was more stable for the lyophilised cells and microencapsulated cells stored at 4 °C. The stability of astaxanthin was similar for the two temperatures of calcium alginate beads, indicating a relatively thermostable product. The highest concentration of astaxanthin detected was 9.65 ppm (24.25 µg/g dry cell weight) in the microencapsulated beads directly after microencapsulation. Further studies need to be done to further optimise the production of astaxanthin and storage stability. Also, future studies can include a total quantification of other possible carotenoids and astaxanthin isomers produced by *P. marcusii.*

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Figures

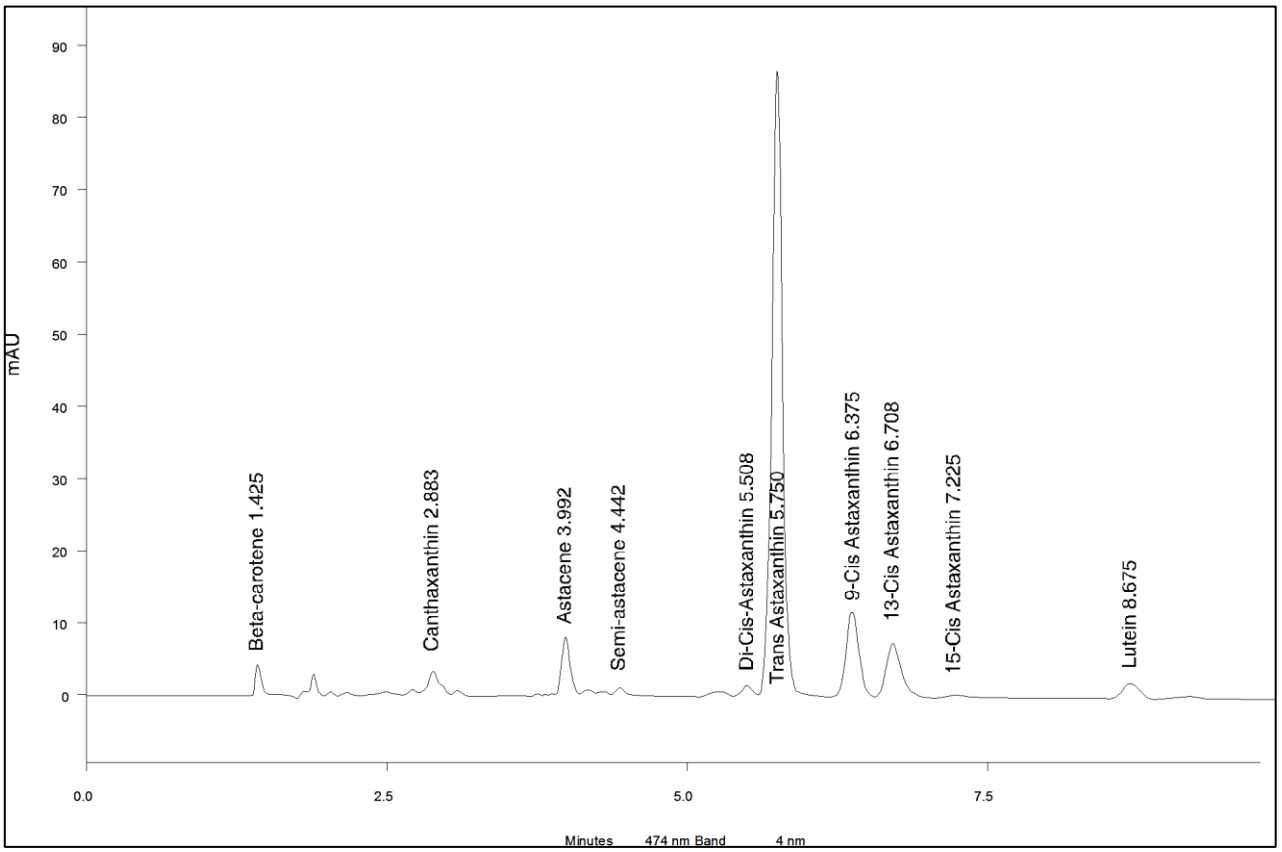

Figure 1 – Chromatogram example of astaxanthin isomers compared to other well-known carotenoids.

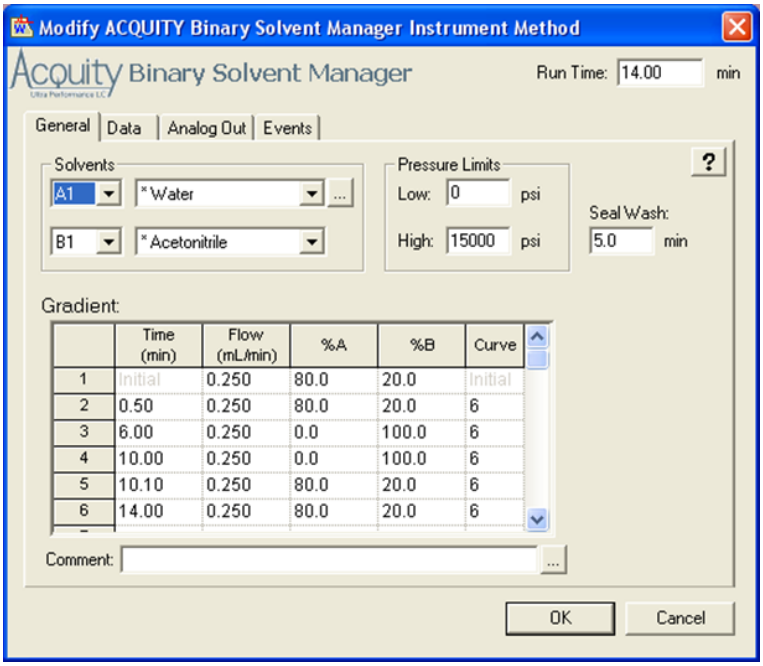

Figure 2 – Mobile phase gradient profile. Column: Waters UPLC HSS C18, 2.1 x 150 mm. Solvent A: 1% Formic Acid. Solvent B: Acetonitrile. Source: Electrospray positive. Capillary voltage 3 kV. Cone voltage 15 V. Mass spectra obtained by scanning from *m/z* 200 – 700.

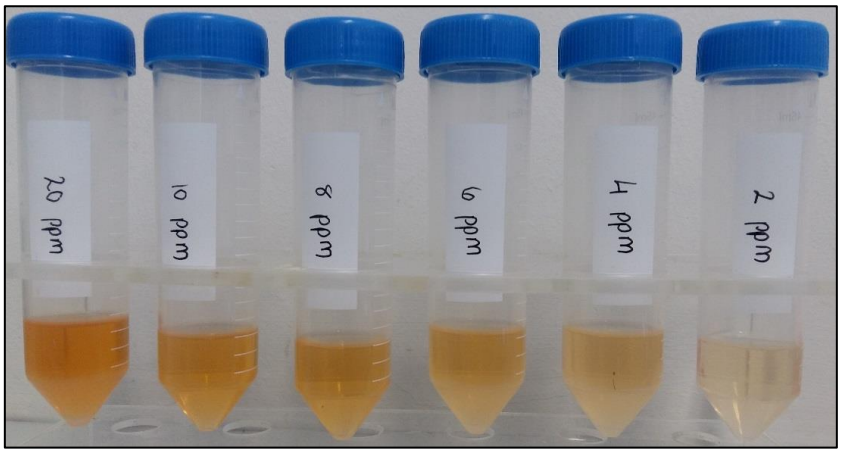

Figure 3 – Astaxanthin standards used for calibration curve.

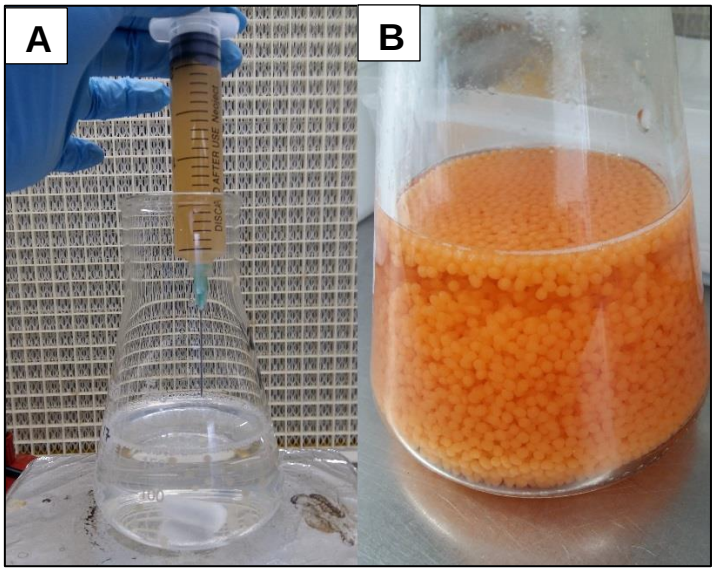

Figure 4 – Extrusion method. **A.** Needle and syringe containing sodium alginate and *P. marcusii* polymer solution. **B.** Calcium alginate beads with *P. marcusii* in $CaCl_2$ solution.

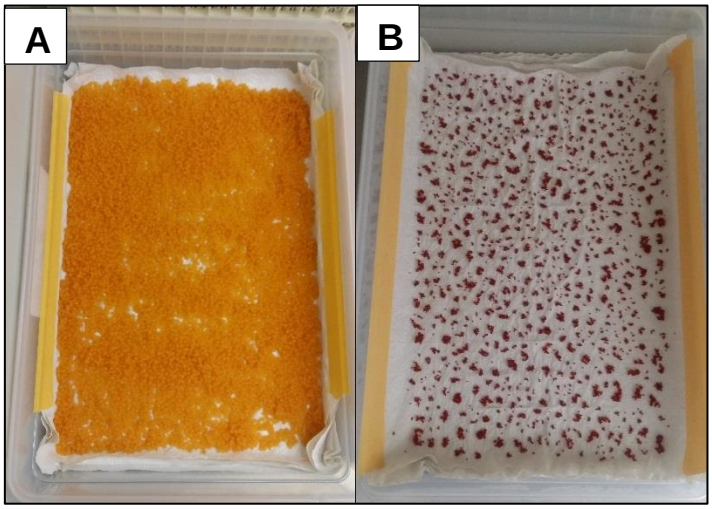

Figure 5 – Microencapsulated *P. marcusii* in calcium alginate beads. **A.** Wet beads. **B.** Desiccated beads after drying.

Figure 6 – Control calcium alginate beads before drying, containing no *P. marcusii*.

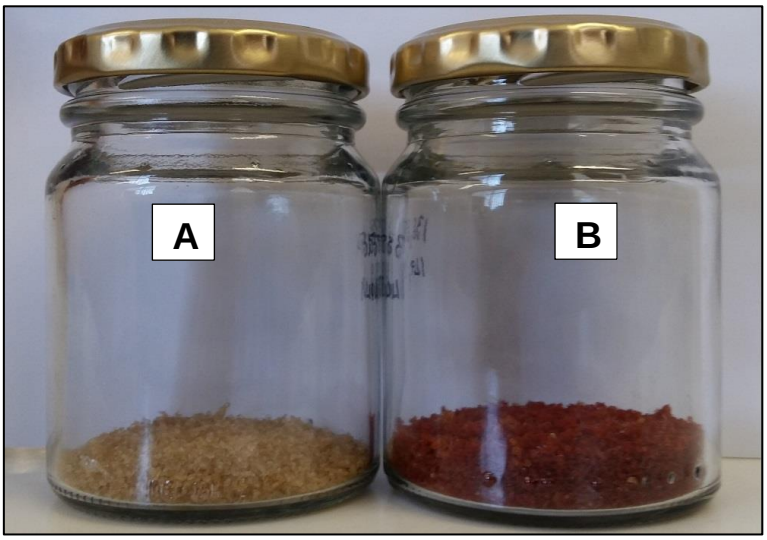

Figure 7 – Dried calcium alginate beads. **A.** Control beads. **B.** Beads with *P. marcusii*.

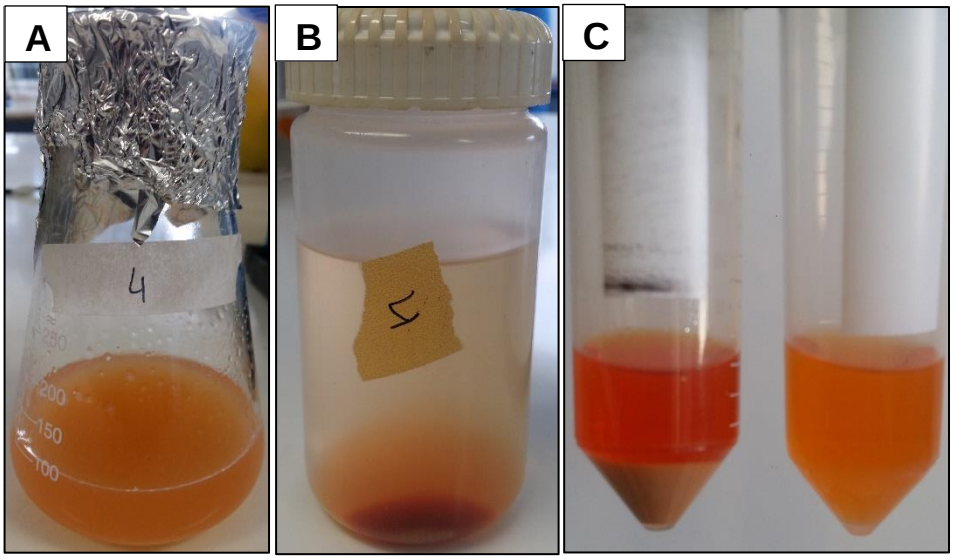

Figure 8 – Astaxanthin extraction process from calcium alginate beads containing *P. marcusii*. **A.** Calcium alginate beads dissolved in 0.05 M Na_2CO_3/ 0.02 M citric acid buffer solution. **B.** *Paracoccus marcusii* pellet after centrifugation. **C.** Total carotenoids extracted, left, compared to the astaxanthin standard, right.

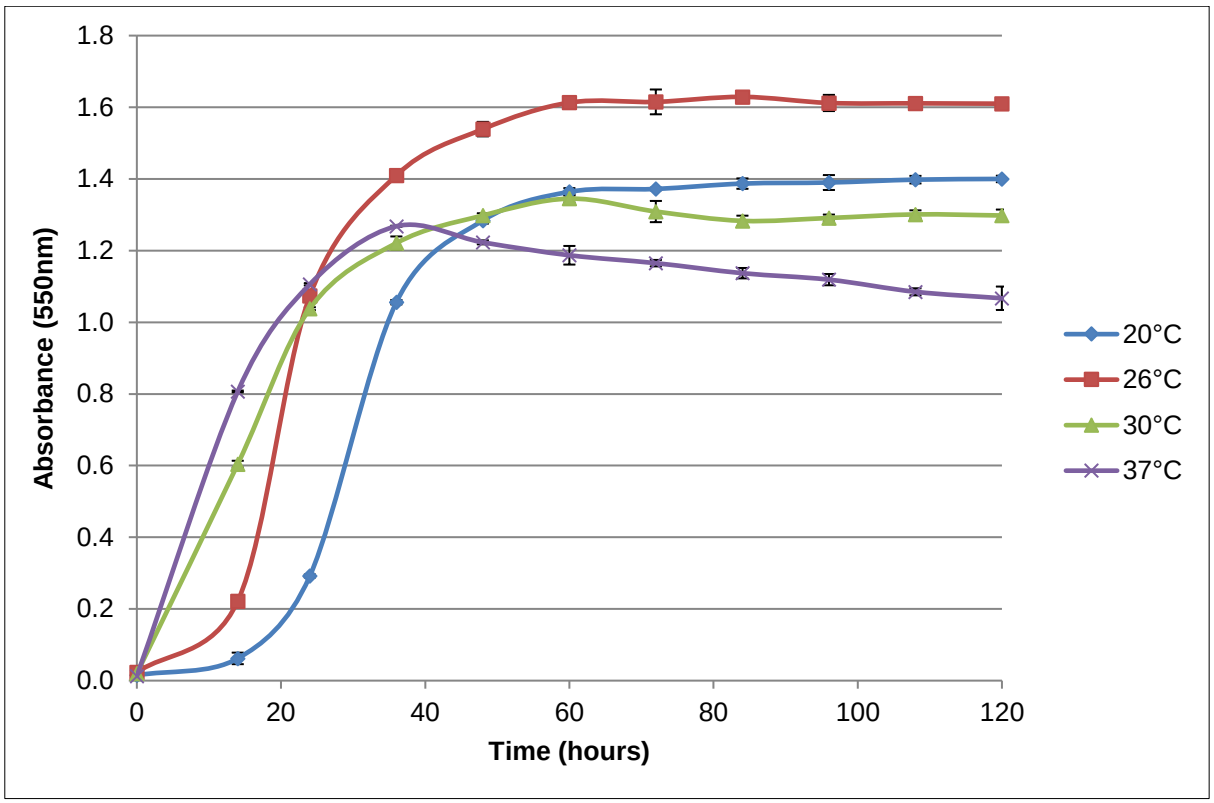

Figure 9 – Growth curve of *P. marcusii* at different temperatures.

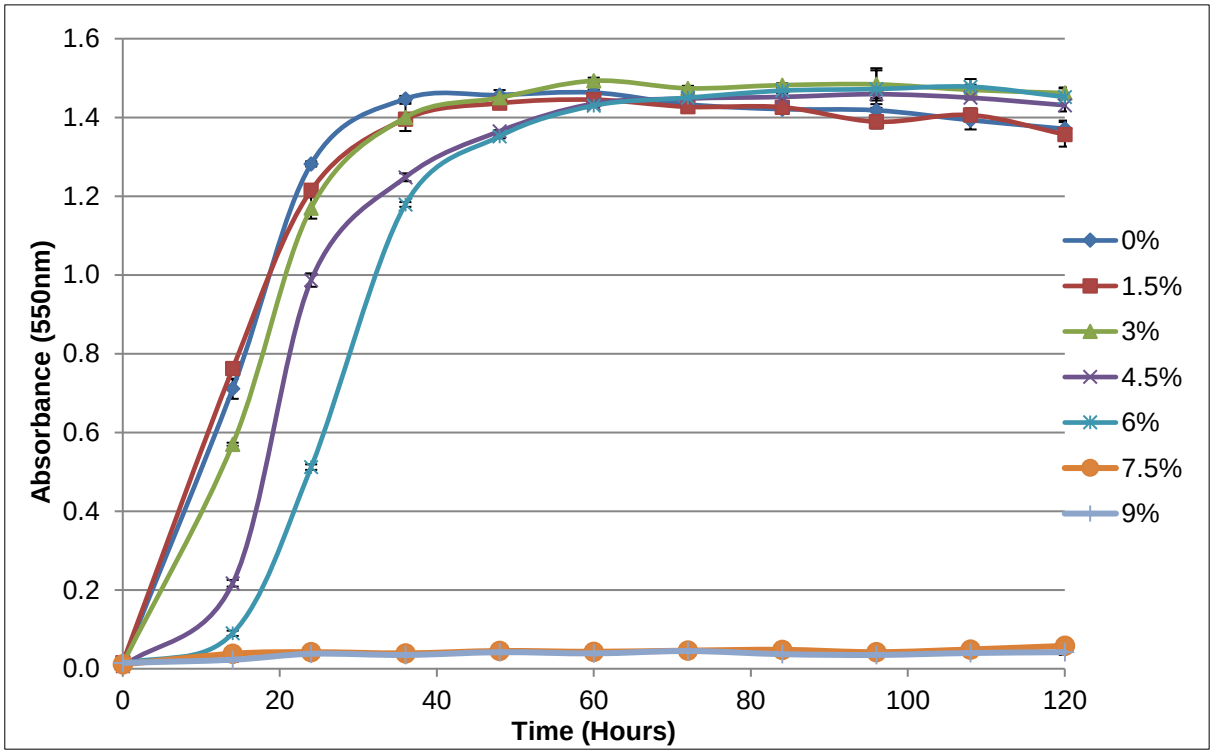

Figure 10 – Growth curve of *P. marcusii* at different NaCl concentrations.

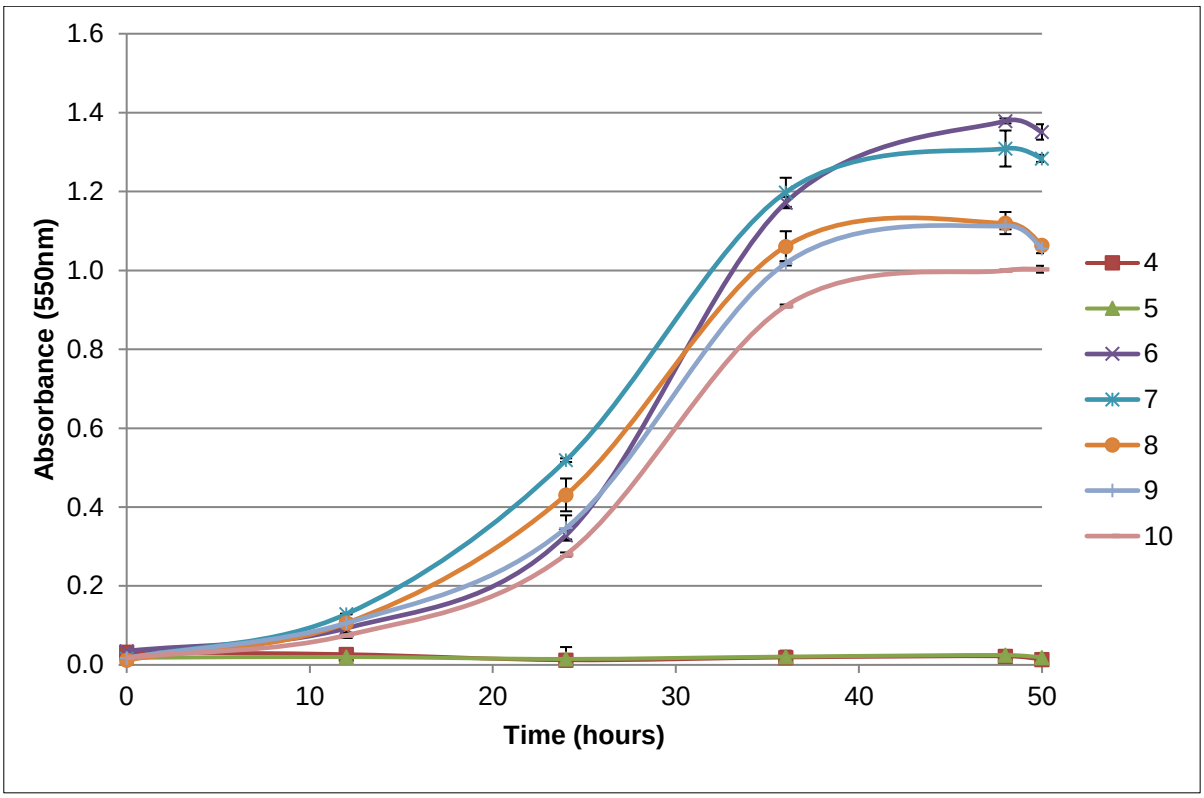

Figure 11 – Growth curve of *P. marcusii* at different pH ranges.

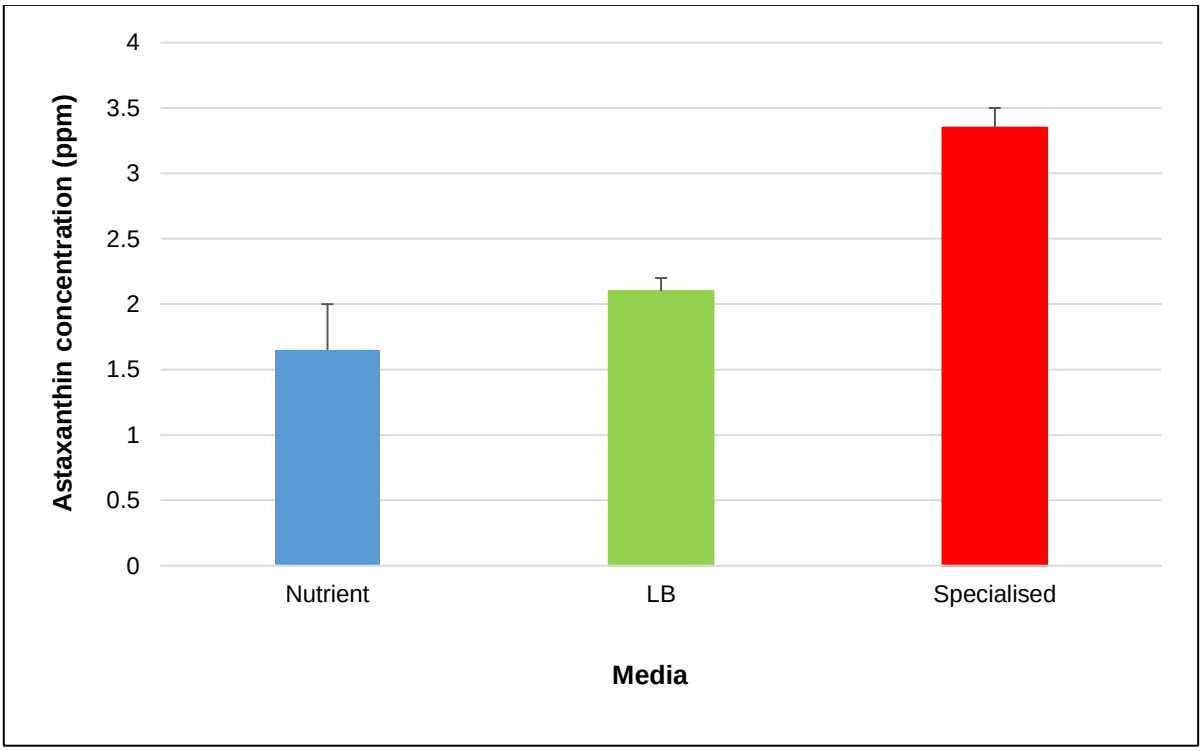

Figure 12 – Astaxanthin concentration (in parts per million) of *P. marcusii* grown in different growth media.

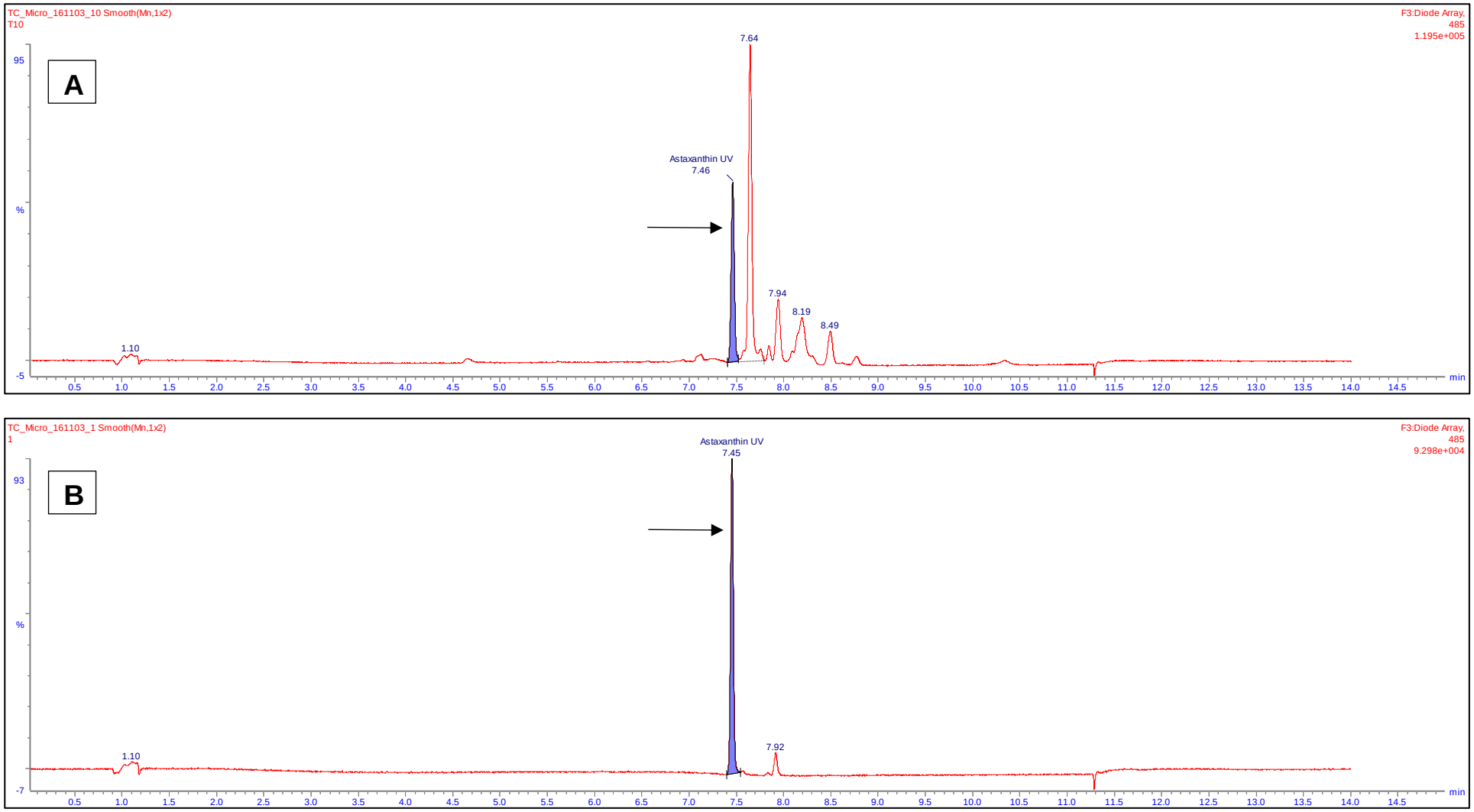

Figure 13 – Example of LC-MS chromatogram results of extracted astaxanthin compared to the standard. **A.** Extracted astaxanthin peak at 7.46 minutes followed by other possible carotenoids or isomers of astaxanthin. **B.** Astaxanthin standard peak at 7.45 minutes.

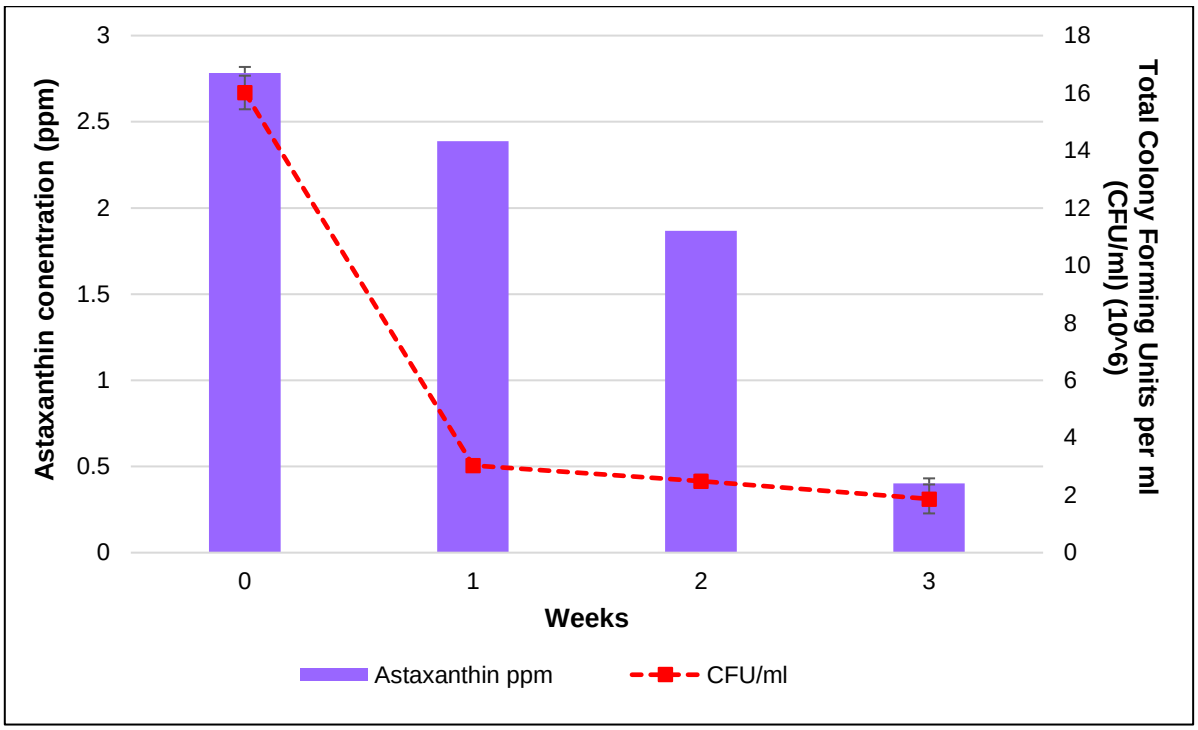

Figure 14 – Combined graph of loss of astaxanthin concentration (purple bars) and total colony forming units (dotted red line) of *P. marcusii* lyophilised in sucrose over a 3-week period stored at 4 °C.

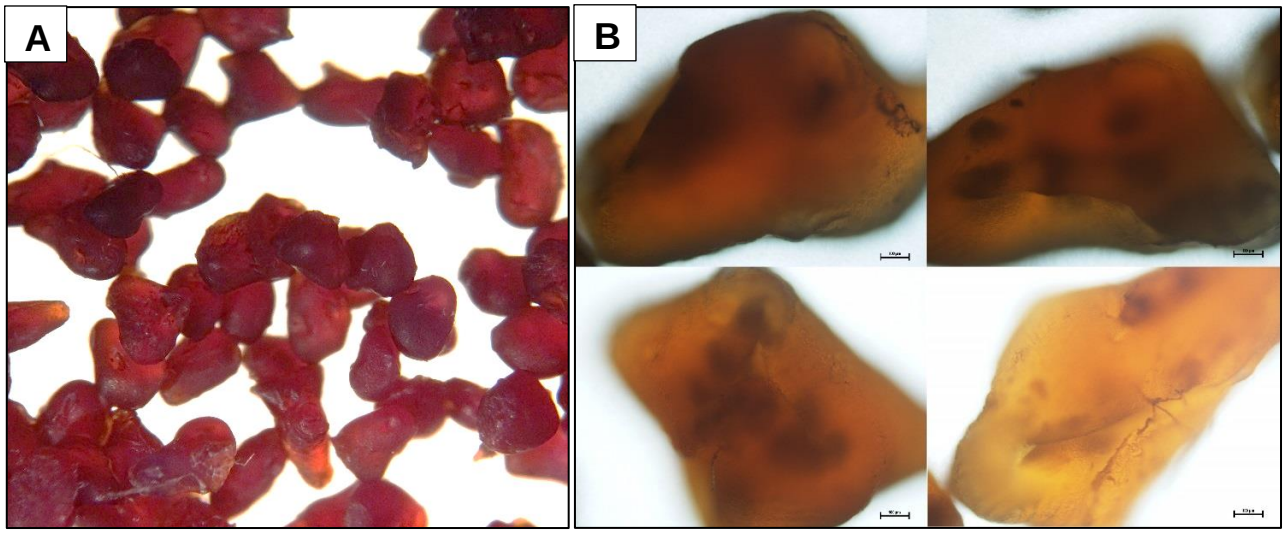

Figure 15 – Microscopic view of morphological features of *P. marcusii* calcium alginate beads at **A.** 40x and **B.** 100x magnification.

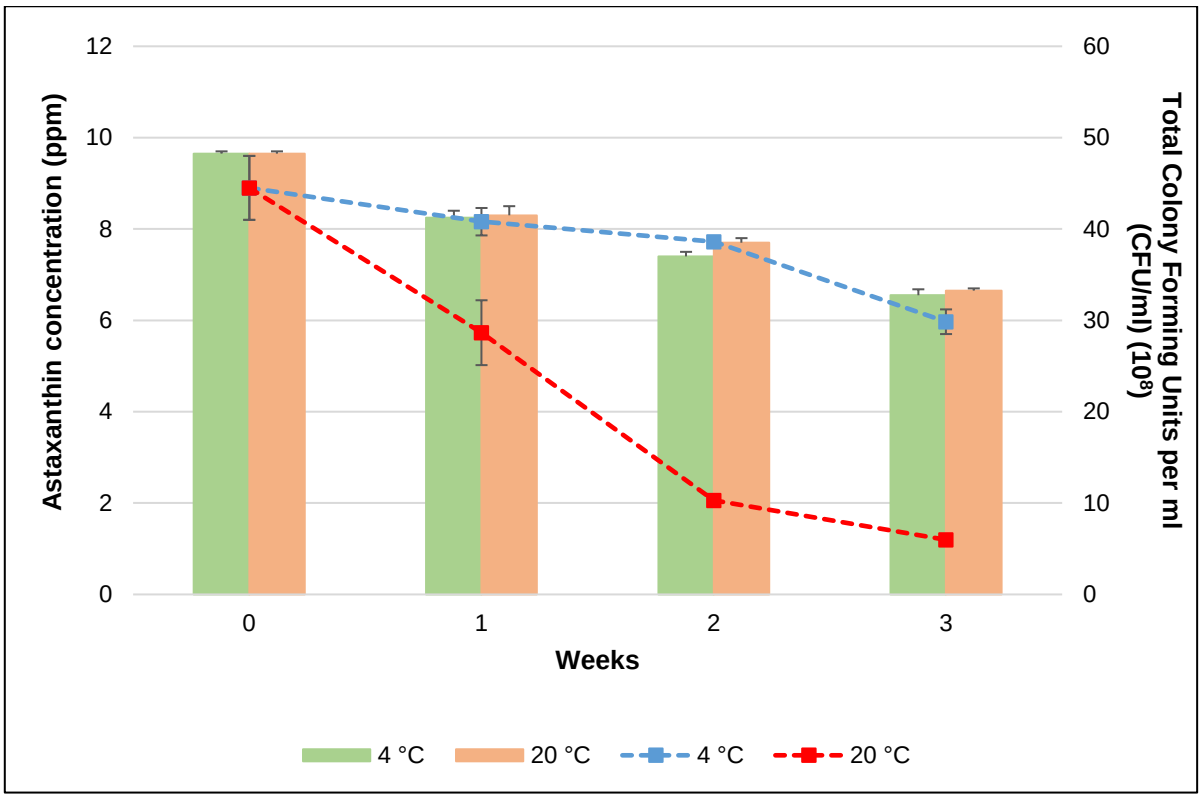

Figure 16 – Combined graph of loss of astaxanthin concentration (light green and orange bars) and total colony forming units (dotted red and blue lines) of microencapsulated *P. marcusii* in calcium alginate beads over a 3-week period stored at 4 °C and room temperature (± 20 °C).

Chapter 3

Application of *Paracoccus marcusii* as a potential feed additive for laying hens

Abstract

Carotenoids have been used for many years as an added pigment source to enhance egg yolk colour. One such carotenoid, astaxanthin, has a strong antioxidant activity and is produced by several microorganisms, including the bacterium *Paracoccus marcusii*, and has shown promise to be used as a feed additive. Therefore, this study investigated the use of *P. marcusii* as a possible source of pigmentation in layer hen feed to enhance egg yolk colour. *Paracoccus marcusii* was fed to hens either in a sucrose solution (10% m/v) or microencapsulated in calcium alginate beads. The hens were fed daily and eggs were collected for analysis. Dilutions of egg contents were plated out onto selective media to detect the presence of known food pathogens (*E. coli*, *Listeria* and *Salmonella*). In all the feeding trials there was no negative effect on the weight of the hen, the laying rate or the overall quality of the egg. All trials indicated a significant increase ($P \leq 0.05$) in yolk colour as well as an increase in whole egg and yolk weight. There were also no known food pathogens detected in any of the egg samples. This study has shown promising results in using this bacterium as an effective feed additive for laying hens.

Introduction

The chicken (*Gallus gallus domesticus*) is a domesticated subspecies of the red junglefowl (*Gallus gallus*). Chickens have become one of the most common domesticated animals and are mainly kept as a source of food for meat and eggs. In 2015, the South African layer flock size was 24.9 million hens and has since increased to 25.05 million hens in 2016 (South African Poultry Association, 2016). Chicken eggs remain one of the largest animal product sectors in the South African agriculture after beef, chicken meat and milk with a gross turnover of R9.83 billion in 2015 (Department of Agriculture, Forestry and Fisheries (DAFF), South Africa).

Egg quality is an important factor that contributes to the price of table eggs (Monira *et al*., 2003). Stadelman (1977) first defined egg quality as the characteristics important to the consumers. These characteristics affect the acceptability by the consumer and it is of great importance that the quality is maintained. Egg quality parameters include eggs size, whole egg weight, shell thickness, shell weight, yolk height, yolk colour, albumen height and Haugh Unit. Each of these parameters differs within species, breeds, lines, strains and families (Buss, 1982). In some studies they have shown that coloured feathered hens generally lay bigger eggs than white feathered hens (Halaj and Grofík, 1994 and Vits *et al*., 2005). Other external quality parameters include freshness and cleanliness. As soon as the egg is laid its internal quality starts to decline (Dudusola, 2010). The quality of the egg is heavily influenced by the age, nutrition and management of the hen and the storage and handling of the eggs before it reaches the consumer (Gerber, 2006).

For many years carotenoids have been used to manipulate the colour of egg yolk to obtain a desired colour (Adams, 1985). Poultry can easily absorb carotenoids from their diet (Hudon, 1994). After the hen has ingested the feed, the carotenoids are released by enzymes and absorbed in the small intestines. The free carotenoids are then emulsified to form oil droplets (or portomicrons) and delivered to the liver. These molecules are incorporated into very low density lipoproteins (VLDL) and delivered to the yolk (Surai *et al.,* 2001 and Bortolotti *et al*., 2003).

Astaxanthin produced by microorganisms have been commercialised and applied in the colouration of cosmetics, beverages, dairy products, and meats (Del Campo *et al*., 2000; Guerin *et al*., 2003; Liang *et al*., 2004; Pulz and Gross, 2004 and Chandi and Gill, 2011). There is an increase in demand for naturally derived astaxanthin from microorganisms instead of synthetic astaxanthin, since natural astaxanthin has a higher antioxidant activity when compared to synthetic astaxanthin (Capelli *et al.,* 2013). The yeast, *Xanthophyllomyces dendrorhous,* and microalga, *Haematococcus pluvialis*, are

currently used for the large scale cultivation for astaxanthin production. Many studies have used these microorganisms in developing biotechnological processes to produce astaxanthin in large quantities (Lorenz and Cysewski, 2000; Dufosse *et al*., 2005; Schmidt *et al*., 2011 and Mata-Gómez *et al*., 2014).

In previous studies, the yeast and microalga have been used as a pigmentation source in the diet of trout (Choubert and Heinrich, 1992 and Storebakken *et al.*, 2004), salmon (Lorenz and Cysewski, 2000) and laying hens (Johnson *et al*., 2003). A significant difference was only observed after partial homogenisation, enzymatic digestion or by cracking the cells to increase the release of the available pigments (Choubert and Heinrich, 1992; Lorenz and Cysewski, 2000; Johnson *et al*., 2003 and Storebakken *et al.*, 2004). However, there was no need to enzymatically digest or homogenise the bacterium, *P. marcusii*, when fed to rainbow trout (*Oncorhynchus mykiss*) for pigmentation effect (De Bruyn, 2013). The results obtained by De Bruyn (2013) showed the promising application of using an astaxanthin producing bacterium as a pigmentation source, instead of the yeast or microalga.

The aim of this study was, therefore, to determine if whole *Paracoccus marcusii* cells can be used as a possible pigmentation source to enhance egg yolk colour and possibly improve egg quality without the need to extract the pigment from the cell.

Materials and Methods

Experimental layout, hens and feed

All three feeding trials took place at the poultry unit of Mariendahl Experimental Farm, University of Stellenbosch. The facility consisted of a hen house equipped with a two-tier A-shape battery system. Each row contained 12 cages with sliding doors, slightly slanted floors which allowed the eggs to roll out of the cage for easy collection and a feeding tray in front of the cage (Fig. 1). Water was provided *ad libitum* by two nipple-type drinkers per cage (Ethical clearance protocol number: SU-ACUM14-0034 (Pilot study) and SU-ACUD15-00088 (Experimental trials)).

Pilot study

A total of 50 hens of 36 weeks of age were selected. Ten hens were randomly assigned into a group. The hens were allowed to adjust to their new surroundings for four days before starting with the

experimental diets. After five weeks of the experimental treatments the hens were fed a basal diet for two weeks to determine if the change in pigmentation was because of the bacterium. The basal feed consisted of commercially available layer hen feed.

Experimental trials

Bacterium in sucrose solution

A total of 120 hens of 16 weeks of age were selected. Twenty hens were randomly assigned into a group. For the first four weeks, all hens were fed prelay feed containing white maize before starting with the experimental diets for eight weeks on peaking feed containing either yellow maize (Fig. 2A) or white maize (Fig. 2B) (Table 1).

Bacterium microencapsulated in calcium alginate beads

After the bacterium in sucrose trial, the beads trial followed. A total of 60 hens of 28 weeks of age were selected. Fifteen hens were randomly assigned into a group. The hens were allowed to adjust to their new surroundings for four days before starting with the experimental diets for three weeks. The basal feed consisted of peaking feed containing either yellow maize or white maize (Table 1).

Experimental treatments

All dosages given every day at each trial is indicated in Table 2. Dosages were given by hand every day either in liquid or in bead form to ensure that the hens were fed equal amounts of the control or the bacterium. The next day all of the feeding trays were checked to observe if any of the previous days' bacterium was still present.

Pilot study

Five different diets were prepared and randomly assigned to each group of 10 hens. Treatment 1 and 2 served as negative controls which consisted of a basal feed with no additives (Control) and 1 ml sucrose solution (10% m/v) (Sucrose Control). Treatments 3 – 5 consisted of a basal feed and a dosage of *Paracoccus marcusii*, either freeze-dried (PM-Freeze-dried) and resuspended in sterile dH_2O or live cells (PM-1 and PM-5), which were dripped onto the feed.

Experimental trials

To effectively determine the pigmentation effect of the bacterium to enhance egg yolk colour, all experimental diet groups (PM-Feed and PM-Beads) were fed a diet containing white maize, except for the positive control diet groups which were fed a diet containing yellow maize.

Bacterium in sucrose solution

Six different diets were prepared and randomly assigned to each group of 20 hens. Treatments 1 – 4 served as the control groups. For treatments 5 and 6, a sucrose solution containing the bacterium, *P. marcusii*, was dripped onto the feed (Fig. 3A) (PM-Feed) or added to the water system (PM-Water) every day.

Bacterium microencapsulated in calcium alginate beads

Four different diets were prepared and randomly assigned to each group of 15 hens. Treatments 1 – 3 served as the control groups which consisted of a basal feed containing yellow maize as the positive control (Yellow Maize Control) and two negative controls which consisted of a White Maize Control with no calcium alginate beads and a White Maize Beads Control where calcium alginate beads containing no bacterium was sprinkled over the feed every day. For treatment 4 (PM-Beads), calcium alginate beads containing the bacterium was sprinkled over the feed every day (Fig. 3B).

Preparation of *Paracoccus marcusii* for feeding trials

In preparation for the feeding trials, *P. marcusii* was cultured in 2 L Schott bottles containing 1 L of specialised medium (10 g/L bacteriological peptone (Oxoid, United Kingdom), 5 g/L yeast extract (Biolab, South Africa), 3% NaCl and pH 7-8). Schott bottles were incubated at 26 °C for 4 – 7 days (see Chapter 2). When an absorbance value of 1 at 550 nm was measured, the cells were harvested through centrifugation at 10000 rpm for 10 minutes and washed once with sterile dH_2O. New cells were cultured each week to ensure viability of cells.

Pilot study

Cells were resuspended in equal volumes of sucrose (10% m/v). Aliquots of cells were transferred into separate flasks representing the different diet groups (PM-1 and PM-5). For freeze-drying, 50 ml of cells resuspended in sucrose (10% m/v) were frozen overnight in 250 ml Erlenmeyer flasks at -80 °C. The VirTis benchtop K (model: 6KBTEL-85, SP Scientific, United States) was used to freeze-dry the cells until dry.

Experimental trials

Bacterium in sucrose solution

For the bacterium in the sucrose experimental trial, every day 2 L of cells were harvested and resuspended in 40 ml of sucrose (10% m/v). Twenty ml aliquots of cells were transferred into separate flasks representing the different diet groups (PM-Feed and PM-Water).

Bacterium microencapsulated in calcium alginate beads

The extrusion method was used to encapsulate whole *P. marcusii* cells in calcium alginate beads (see Chapter 2) (Lin *et al*., 2016). Three litres of cells were harvested and resuspended in 200 ml of 2% alginate. The solution was then added drop-wise into 200 ml of 2% $CaCl_2$ solution using a 21G x 1.5" hypodermic needle and syringe. The $CaCl_2$ solution was constantly stirred at 150 rpm at room temperature. The resulting beads were then separated from the solution using a sieve and allowed to dry overnight in a laminar flow cabinet at room temperature. The beads were stored at room temperature in a dark container.

Hen weight and laying rate

The initial and final mean weight was measured for all diet groups in all feeding trials. The egg production rate of each group was calculated using the total number of eggs laid over the duration of the trial.

$$\text{Laying rate } (\%) = (\text{Total eggs laid})/(\text{Number of hens} \times \text{Number of days}) \times 100 \qquad (1)$$

Egg quality analysis

Eggs were collected every day following the acclimation period. Daily measurements included the following: whole egg weight, shell thickness, shell weight, yolk weight, yolk height, yolk colour and thick albumen height. The Haugh Unit (*HU*) was calculated using the formula:

$$HU = 100 \log(\mathrm{h} - 1.7w^{0.37} + 7.6) \qquad (2)$$

where h is the height of the albumen and *w* is the whole egg weight (Haugh, 1937). The shell thickness was measured using an electronic digital caliper (Fig. 4A). The height of the albumen and yolk was measured by using a Haugh meter (Fig. 4B). For measuring the colour of the yolk, the yolk was first separated from the albumen and placed into a plastic petri dish (90 mm x 15 mm). The colour was then measured using a yolk colour fan (DSM) (Fig. 4C).

Detection of *Paracoccus marcusii* in hen faecal matter

Hen faecal matter was collected from three hens per group. A dilution series (10^{-1} – 10^{-10}) was prepared in sterile saline solution (0.9% NaCl) and plated onto nutrient agar (2 g/L yeast extract, 5 g/L peptone, 1 g/L meat extract, 8 g/L NaCl and 15 g/L agar) (Biolab, South Africa). The plates were incubated at 26 °C for 4 days and analysed to detect the presence of *P. marcusii* (bright orange colony, Fig. 5).

Detection of potential food pathogens and *Paracoccus marcusii* in the egg content

Eggs were randomly selected during the trials from all diet groups and tested for the presence of potential food pathogens (*Salmonella, Listeria* and *E. coli*) (Gast, 1992) and *P. marcusii* in the egg content. The surface of the egg was sterilised by rolling the whole egg in 70% ethanol. The internal egg content was homogenised in a plastic petri dish (90 mm x 15 mm) and a dilution series of 10^{-1} – 10^{-5} was prepared in sterile saline solution (0.9% NaCl). The dilutions were plated in triplicate onto PALCAM-*Listeria*-selective agar (3 g/L yeast extract, 23 g/L peptone, 5 g/l NaCl, 1 g/L starch, 10 g/L mannitol, 0.8 g/L aesculin, 0.5 g/L glucose, 0.5 g/L ammonium iron(III) citrate, 0.08 g/L phenol-rot, 15 g/L lithiumchloride and 13 g/L agar) (Merck, Germany), SS agar (10 g/L lactose, 10 g/L peptone, 10 g/L NaCl, 8.5 g/L ox bile dried, 1 g/L ammonium iron(III) citrate, 8.5 g/L sodium thiosulfate, 0.025 g/L neutral red, 0.0003 g/L brilliant green and 12 g/L agar) (Merck, Germany), LEVINE-EMB-agar (10 g/L lactose, 10 g/L peptone, 0.4 g/L eosin yellow, 0.065 g/L methylene blue, 2 g/L di-potassium hydrogen phosphate and 13.5 g/L agar) (Merck, Germany) and nutrient agar (2 g/L yeast extract, 5 g/L peptone, 1 g/L meat extract, 8 g/L NaCl and 15 g/L agar) (Biolab, South Africa). The *Listeria,* EMB and SS agar plates were incubated for 2 – 3 days at 37 °C and the nutrient agar plates were incubated for 4 – 5 days at 26 °C.

Statistical analysis

Statistical analysis was performed using Statistica (version 13.0, Statsoft Inc., United States). One-way analysis of variance (ANOVA) was used for mean comparisons and Tukey's honest significant difference (HSD) was calculated where $P \leq 0.05$.

Results

Hen weight

Pilot study

The average body weight at the start of the trial was 1.77 kg (± 0.16) between all diet groups. There was no significant increase between all diet groups at the end of the trial (Table 3).

Experimental trials

At the start of the experimental trial, the mean body weight was 1.45 kg (± 0.11) between the diet groups. There was no significant difference in hen body weight at the start of the trial. The increase in body weight of the hens were consistent at the end of the trial with a significant difference ($P \leq 0.05$) only between the different maize groups (white or yellow maize), where the yellow maize groups gained more weight (1.97 kg ± 0.18) than the white maize groups (1.79 kg ± 0.15) (Fig. 6).

Laying rate

Pilot study

There was a significant difference ($P \leq 0.05$) detected in the hen laying rate between the sucrose control, PM-Freezedried, PM-1 and PM-5 groups compared to the control group. The control group had the lowest laying rate of 89.35% (± 9.98), followed by PM-Freezedried and PM-5 with 94.52% (± 6.75 and ± 7.23, respectively), sucrose control with 96.09% (± 6.08) and PM-1 with 96.45% (± 4.86) (Fig. 7, green columns).

Experimental trials

Bacterium in sucrose solution

A significant difference ($P \leq 0.05$) was detected between the yellow control, yellow sucrose control, white sucrose control and PM-Feed compared to the white control. The white control group had the lowest laying rate of 85.06% (± 4.31), followed by PM-Feed with 92.38% (± 3.64), white sucrose control with 92.59% (± 3.98), yellow control with 95.21% (± 5.06) and yellow sucrose control with 96.40% (± 1.53) (Fig. 7, red columns).

Bacterium microencapsulated in calcium alginate beads

There was a significant difference ($P \leq 0.05$) detected in the hen laying rate. The white control group performed the worst with a laying rate of 84.44% (± 1.92) compared to the white control beads (88.06% ± 5.80) and PM-Beads (90.22% ± 0.61). The yellow control group had the highest laying rate of 94.16% (± 2.10) (Fig. 7, purple columns).

Egg quality

Pilot study

There was a significant difference ($P \leq 0.05$) detected for all egg quality parameters measured (Table 4). PM-Freeze-dried and PM-1 had heavier egg weights (58.85 g ± 4.43 and 58.65 g ± 3.31, respectively) compared to the control groups (Control and Sucrose Control) and PM-5 (57.23 g ± 3.31, 57.91 g ± 2.90 and 56.72 g ± 4.44, respectively). PM-Freeze-dried (15.10 g ± 1.32), PM-1 (15.41 g ± 1.23) and PM-5 (15.02 g ± 1.36) had heavier yolk weights compared to the control and sucrose control (Fig. 8).

There was a significant increase ($P \leq 0.05$) in yolk colour for all bacterial treatments compared to the controls, where PM-5 had the highest yolk colour average of 7.53 (± 0.76) (Table 4). The yolk colour increased significantly after week 4 for PM-5 and PM-Freeze-dried from 7.46 (± 0.18) and 6.97 (± 0.27), respectively, to 8.12 (± 0.26) and 7.83 (± 0.67), respectively, in week 5 (Fig. 9). There was a slight increase in yolk colour for the control groups, but did not exceed the experimental treatments and seemed to stabilise after 4 weeks. After cessation of the experimental treatments the colour of the yolk decreased for all diet groups.

Experimental trials

Bacterium in sucrose solution

Five weeks into the trial there was no significant increase ($P \geq 0.05$) in the yolk colour for the PM-Water group. The treatment for the PM-Water group was stopped for the rest of the trial and no further data was collected.

There was no significant difference ($P \geq 0.05$) detected in albumen height and *HU* (Table 4). The yellow maize groups had heavier egg weights (57. 96 g ± 4.81 and 56.93 g ± 4.57) compared to the white maize groups (54.38 g ± 5.11 and 54.06 g ± 4.78), but the PM-Feed had a significantly ($P \leq 0.05$) heavier egg weight (55.54 g ± 4.68) compared to the white control groups (Fig. 10). There

was also a significant difference ($P \leq 0.05$) in yolk weight between the PM-Feed (13.67 g ± 1.15) compared to the white control (13.07 g ± 1.67) and white sucrose control (13.39 g ± 1.11). PM-Feed and yellow sucrose control had similar yolk weights (13.67 g ± 1.15 and 13.75 g ± 1.30, respectively), but the yellow control had a significantly heavier yolk weight of 13.95 g (± 1.40) (Fig. 8).

In terms of yolk colour, there was a significant difference ($P \leq 0.05$) between the yellow maize diet groups compared to the white maize diet groups. A significant difference was also detected between the PM-Feed (3.69 ± 1.23) compared to the white control (1.00 ± 0.00) and white sucrose control (1.00 ± 0.00) (Table 4). Three weeks after starting with the experimental treatments the yolk colour for the yellow control and yellow sucrose control stabilised (Fig. 11). The yolk colour for PM-Feed increased significantly after 4 weeks to 2.58 (± 0.73) and stabilised after 7 weeks at 3.78 (± 0.82). The highest number obtained in PM-Feed was a 4 on the yolk colour fan (Fig. 12).

Bacterium microencapsulated in calcium alginate beads

There was no significant difference ($P \leq 0.05$) detected in egg size, yolk height, albumen height and *HU* (Table 4). The egg weight and yolk weight of PM-Beads were slightly heavier than the white control and white control beads, but not significantly.

A significant difference ($P \leq 0.05$) was detected in yolk colour between the PM-Beads compared to the white control and white control beads. After 3 weeks the yolk colour had increased significantly for PM-Beads (2.88 ± 0.75) compared to the white maize groups (1.00 ± 0.00) (Fig. 13). The PM-Beads yolk colour did not exceed the yellow control group (6.43 ± 0.75) after 3 weeks.

Detection of *Paracoccus marcusii* in hen faecal matter

There were no *P. marcusii* colonies detected on any of the faecal dilution plates (Fig. 14). All plates were clear at the highest dilution of 10^{-10}.

Detection of potential food pathogens and *Paracoccus marcusii* in the egg content

None of the potential food pathogens (*E. coli, Listeria* and *Salmonella*) were detected in any of the egg contents plated out. There were also no *P. marcusii* colonies on any of the nutrient agar plates. All the plates were clear at the lowest dilution of 10^{-1}.

Discussion

The increase in demand for the use of feed additives and colourants in poultry farming to enhance egg yolk colour has prompted the application of using a carotenoid producing bacterium. For most consumers, the colour of food indicates the quality and freshness of the product (Clydesdale, 1993). Carotenoids have long been used as feed additives to generate good quality food products that meet the demands of the consumer and also hold a health benefit for the animal (Breithaupt, 2007). Plants, algae, fungi and bacteria produce carotenoids, but only a few are of industrial importance (Ambati *et al.,* 2014). Some microorganisms can be used to produce carotenoids economically. One such microorganism, *Paracoccus marcusii*, produces astaxanthin naturally (Harker *et al.*, 1998). In a previous study, De Bruyn (2013) has shown that *P. marcusii* can be used as an additive for fish to enhance skin pigmentation. This same principle can be applied in poultry feed to enhance egg yolk colour. Hens are not able to synthesise carotenoids and need to consume pigments through their feed (Surai *et al*., 2001 and Bortolotti *et al*., 2003). In this study we aimed to evaluate the potential whole cell application of *P. marcusii* to be used as a feed additive for laying hens to enhance egg yolk colour.

This study has shown that *P. marcusii* has the potential to be used in the poultry industry as a feed additive. In all of the feeding trials there were no negative effect on the weight of the hen, the laying rate or the overall quality of the egg observed. In some cases, the experimental groups performed better than the control groups i.e. laying rate, egg weight, yolk weight and colour. There seems to be a significant increase in laying rate between all the experimental diets compared to their control. In the experimental trials, the white control diets had a significantly lower ($P \leq 0.05$) laying rate than the yellow control diets and the experimental diets (Fig. 7). It is suspected that the white maize used in this study might not have provided enough energy to the hen to produce eggs on a daily basis. Some studies have indicated that a difference in protein, sugar and starch content between maize varieties can directly influence the layer hens' performance (Moore, 2007). White and yellow maize are believed to be similar in nutritional composition. However, composition can be affected by maize hybrid type, geographical growing site, harvesting maturity, plant density and soil nitrogen fertilisation (Zeidan *et al*., 2006, Moore, 2007, Idikut *et al*., 2009 and Raymond *et al*., 2009). The added bacterium, sucrose or calcium alginate in the experimental diets might have provided additional energy and, therefore, they performed significantly better than the white control diets. Even in the pilot study where all hens were fed a commercially available feed containing yellow maize, all the experimental diets performed significantly better than the control. These findings are in contrast to a previous study. Walker *et al.* (2012) fed various concentrations of alga biomass to laying hens to determine the effect on the quality of the egg and yolk colour change. They found that the added

alga biomass had no effect on the laying rate of the hen and all diet groups had a mean laying rate above 90% (Walker *et al*., 2012).

There is a definite increase in yolk weight and whole egg weight in all feeding trials where the experimental diets had heavier eggs compared to the control groups. There seems to be no correlation between egg weight and yolk weight. Some diet groups had a lighter egg weight compared to the controls, but had a heavier yolk weight. This can indicate that even if the whole egg is not heavier, the yolk weight increased with dosage of *P. marcusii*. However, these findings are in contrast to previously reported studies. Some studies have shown that by adding probiotics, antibiotics or bacterial enzymes, such as xylanase, to the feed of hens had no effect on any of the quality parameters, including egg and yolk weight (Yalçin *et al*., 2002; Yörük and Bolat, 2003; Yörük *et al*., 2004; Mahdavi *et al*., 2005 and Yang *et al*., 2006).

The *HU* values were not significantly different between the diet groups in the experimental trials. These results are in agreement with previous studies. The supplementation of vitamins C and E (astaxanthin precursors) or algae biomass had no undesirable effect on the *HU* of different experimental treatments (Franchini *et al*., 2002 and Walker *et al.,* 2012).

After the pilot trial it was clear that a diet free of all pigments was needed to effectively evaluate the pigmentation effect of *P. marcusii*. In the experimental trials, there was a significant increase in yolk colour compared to the white control. A higher dosage of *P. marcusii* resulted in a higher yolk colour change. The intestinal cells of the hen easily absorb natural sources of carotenoids. These pigments are transported to the yolk once it is released from the feed content (Surai *et al*., 2001 and Bortolotti *et al*., 2003). Different carotenoids have different deposition rates in eggs because of the bioavailability of esterified or free forms of carotenoids (Bowen *et al*., 2002). To be able to compete with a yellow maize diet a higher dosage of *P. marcusii* is needed. However, this was not possible in this study because of culturing limitations. The egg quality results indicate that whole *P. marcusii* cells can be used as a pigmentation source without the need for downstream processing to break the cells. However, higher cell concentrations are still needed to increase yolk colour.

This study further looked at the ability of *P. marcusii* to survive the hen's digestive tract. There are several reasons why no *P. marcusii* colonies were detected in the faecal sample dilutions. *Paracoccus marcusii* is an aerobic bacterium with optimum growth at 26 °C and a pH between 6 – 7 (see Chapter 2). One reason could be that the cells were able to colonise the gastrointestinal tract (GIT), but this is highly unlikely as the established microbiota will prevent any unknown

microorganisms from colonising the GIT (Brisbin *et al*., 2008). The whole GIT is also predominantly anaerobic and also highly acidic at the start of the intestinal tract (pH 2.5 – 3.5) in the proventriculus and gizzard (Gauthier, 2002 and Yeoman *et al*., 2012). The bacterium is, therefore, unlikely to survive the GIT and is most probably completely digested in the stomach. Further studies are needed to determine the effect of *P. marcusii* on the microbial diversity of the hen's GIT.

Some well-known food pathogens associated with chicken egg products are *E. coli, Listeria* and *Salmonella* (Gast, 1992). It was, therefore, necessary to determine if these organisms are present in the egg contents. None of the dilution plates had any growth on them. It is possible that the microorganisms were not viable anymore or the colony forming units were too low to detect. However, we did not expect to find any of the pathogens or *P. marcusii* in the egg content as previous studies have shown that the internal egg only gets contaminated when it comes into contact with the outer shell where trace amounts of the pathogens might be present and if the pathogens are present in the immediate environment of the hen (De Reu *et al*., 2005; Mallet *et al*., 2006 and Jones *et al*., 2011). None of the hens in all the trials were sick and the way the cages are designed prevents the egg from coming into contact with faecal matter on the floor of the house that might contain these pathogens (De Reu *et al*., 2005; Mallet *et al*., 2006 and Svobodová and Tůmová, 2014). The surroundings of the hen must be kept clean to prevent the potential contamination of food pathogens.

Conclusion

The findings in this study demonstrate the potential use of *Paracoccus marcusii* as a feed additive to enhance yolk colour. *P. marcusii* significantly increased the yolk colour in all experimental trials compared to the white maize diet groups and there is also an increase in whole egg and yolk weight. There was no negative effect on the overall egg quality. *Paracoccus marcusii* can, therefore, be used as a feed additive to enhance yolk colour in laying hens. It is important for future studies to determine the optimum dosage needed and the type of association between the bacterium and the hen.

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Figures

Figure 1 – Hen cages with sliding cage doors and slanted floors for easy collection of the eggs.

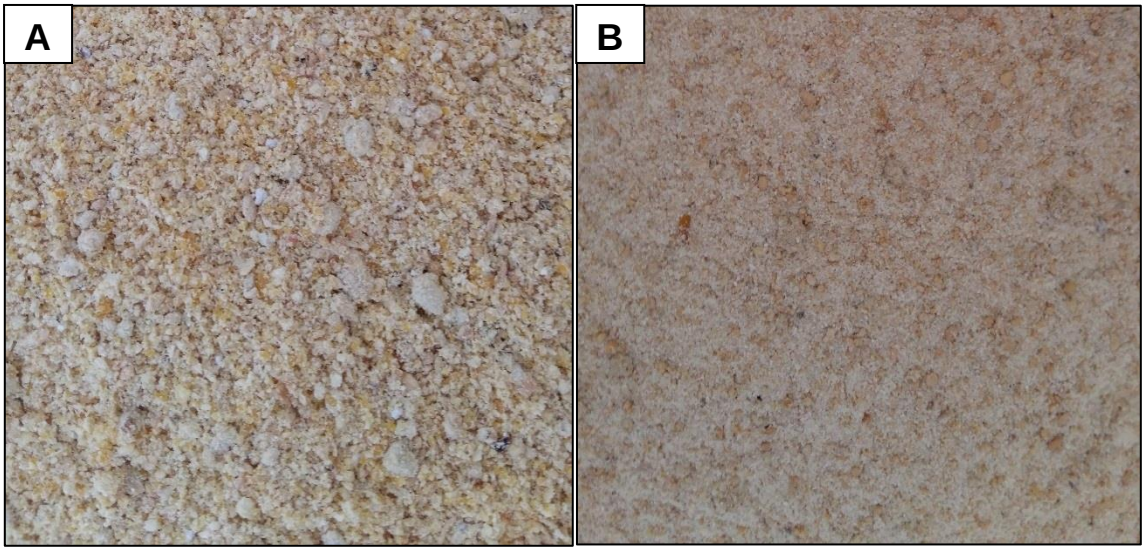

Figure 2 – Mixed feed used in the feeding trials containing either **A.** yellow maize or **B.** white maize.

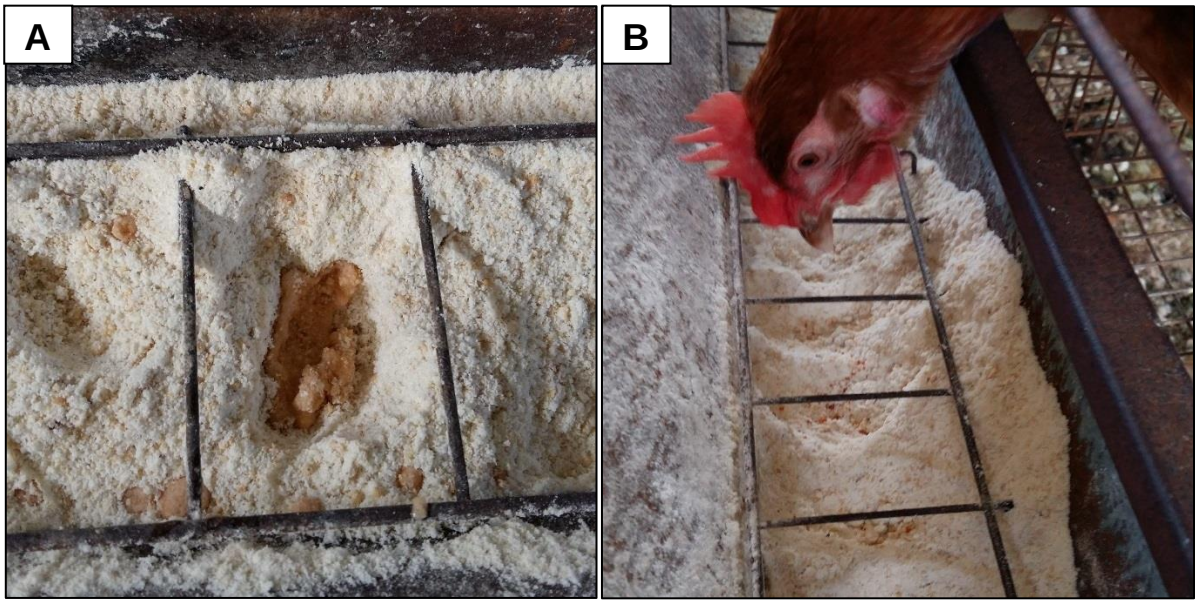

Figure 3 – **A.** *Paracoccus marcusii* in sucrose (10% m/v) dripped on feed. **B**. *Paracoccus marcusii* in calcium alginate beads sprinkled over feed.

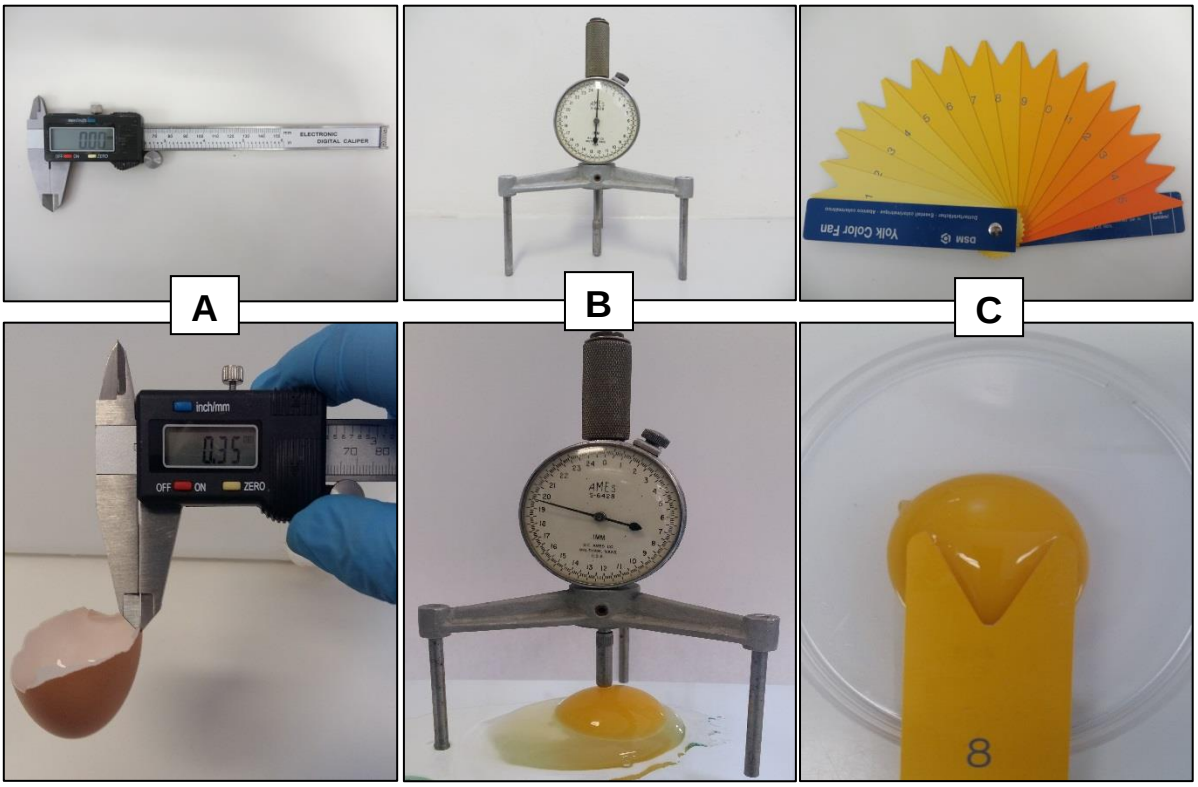

Figure 4 – **A.** Electronic digital caliper. **B.** Haugh meter. **C.** Yolk colour fan.

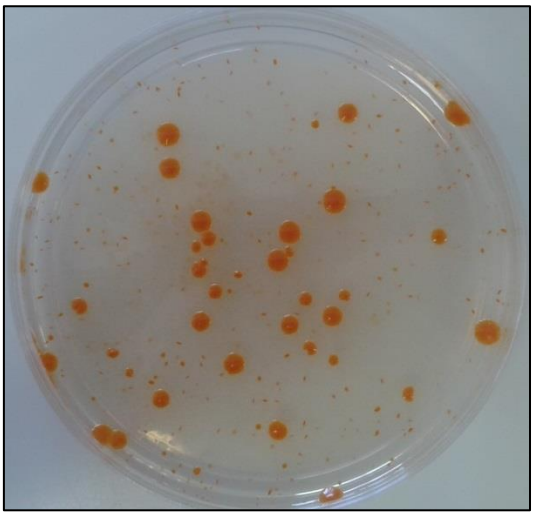

Figure 5 – Bright orange colony characteristic of *Paracoccus marcusii* on a nutrient agar plate.

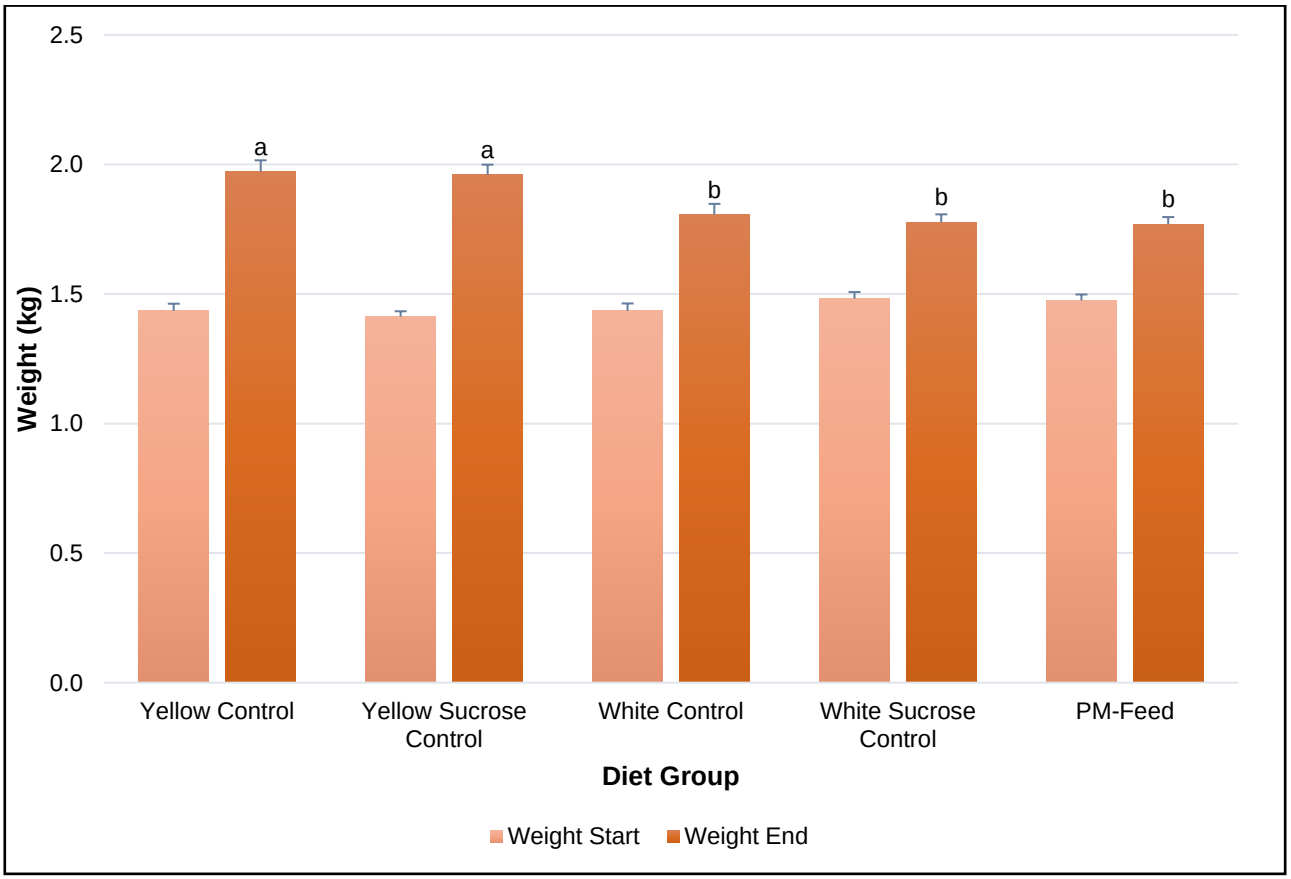

Figure 6 – Mean body weight of the hens at the start and the end of the experimental trial.

*Letters a and b indicates a significant difference at a confidence level of 95%, where $P \leq 0.05$.

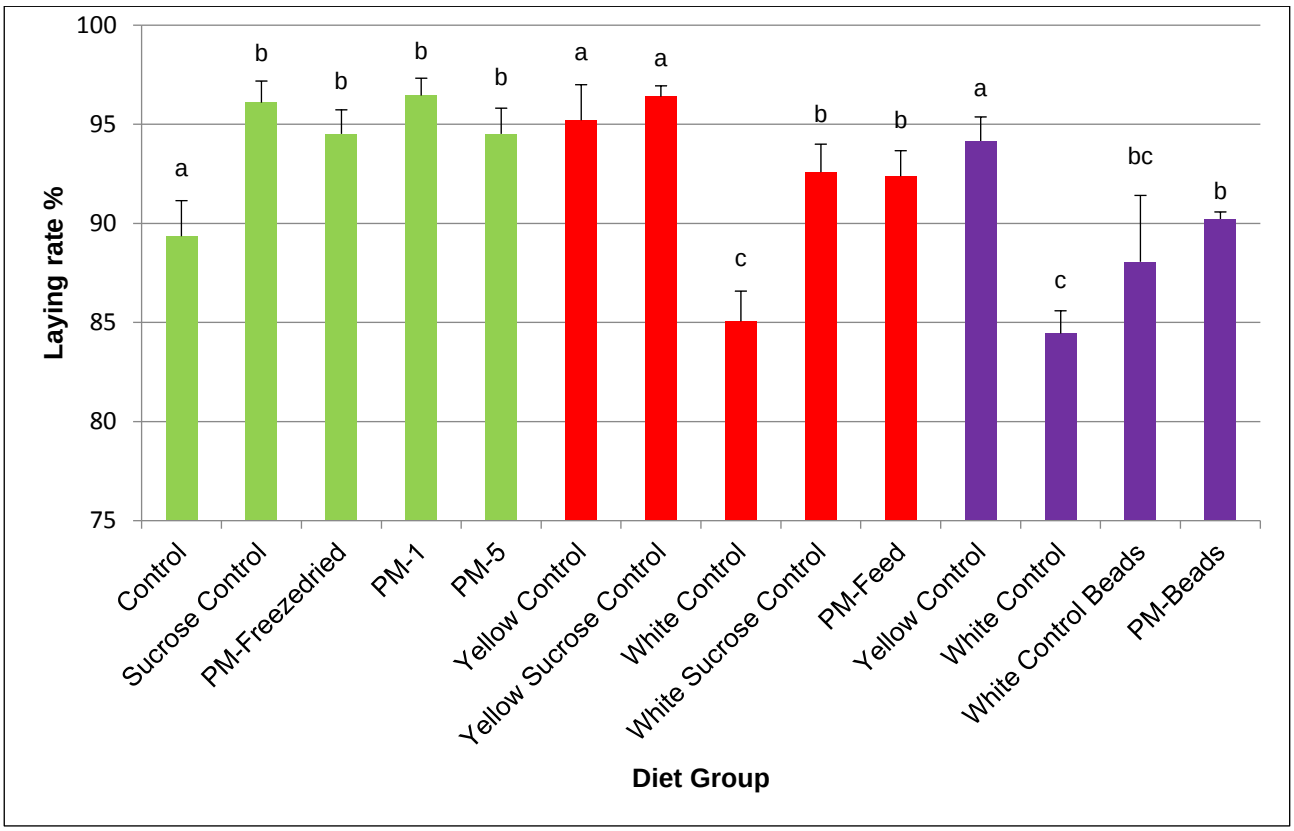

Figure 7 – Mean laying rate for all feeding trials where green is the pilot study, red is the bacterium in sucrose trial and purple is the bacterium in calcium alginate beads trial.

*Letters a-c indicates a significant difference at a confidence level of 95%, where $P \leq 0.05$.

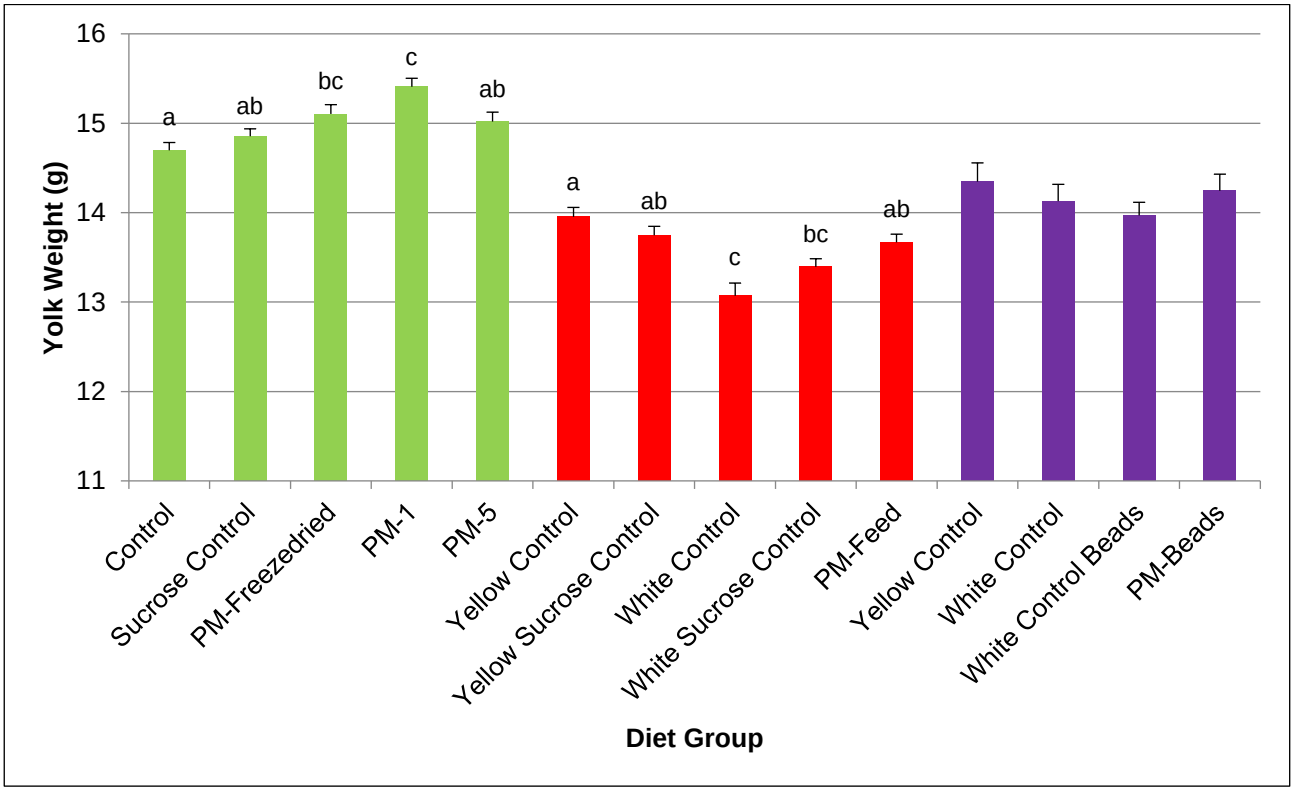

Figure 8 – Mean yolk weight for all feeding trials where green is the pilot study, red is the bacterium in sucrose trial and purple is the bacterium in calcium alginate beads trial.

*Letters a-c indicates a significant difference at a confidence level of 95%, where $P \leq 0.05$.

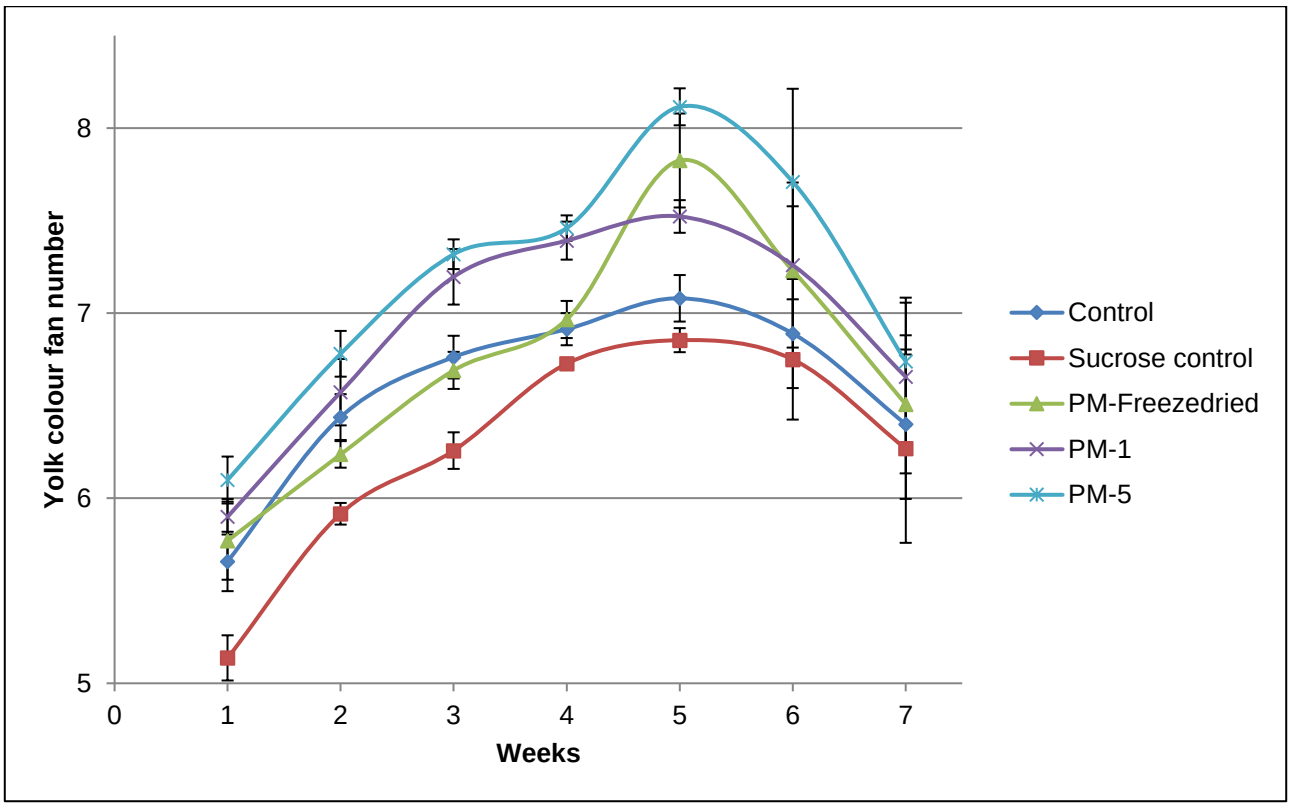

Figure 9 – Yolk colour change over a 7-week period in the pilot study.

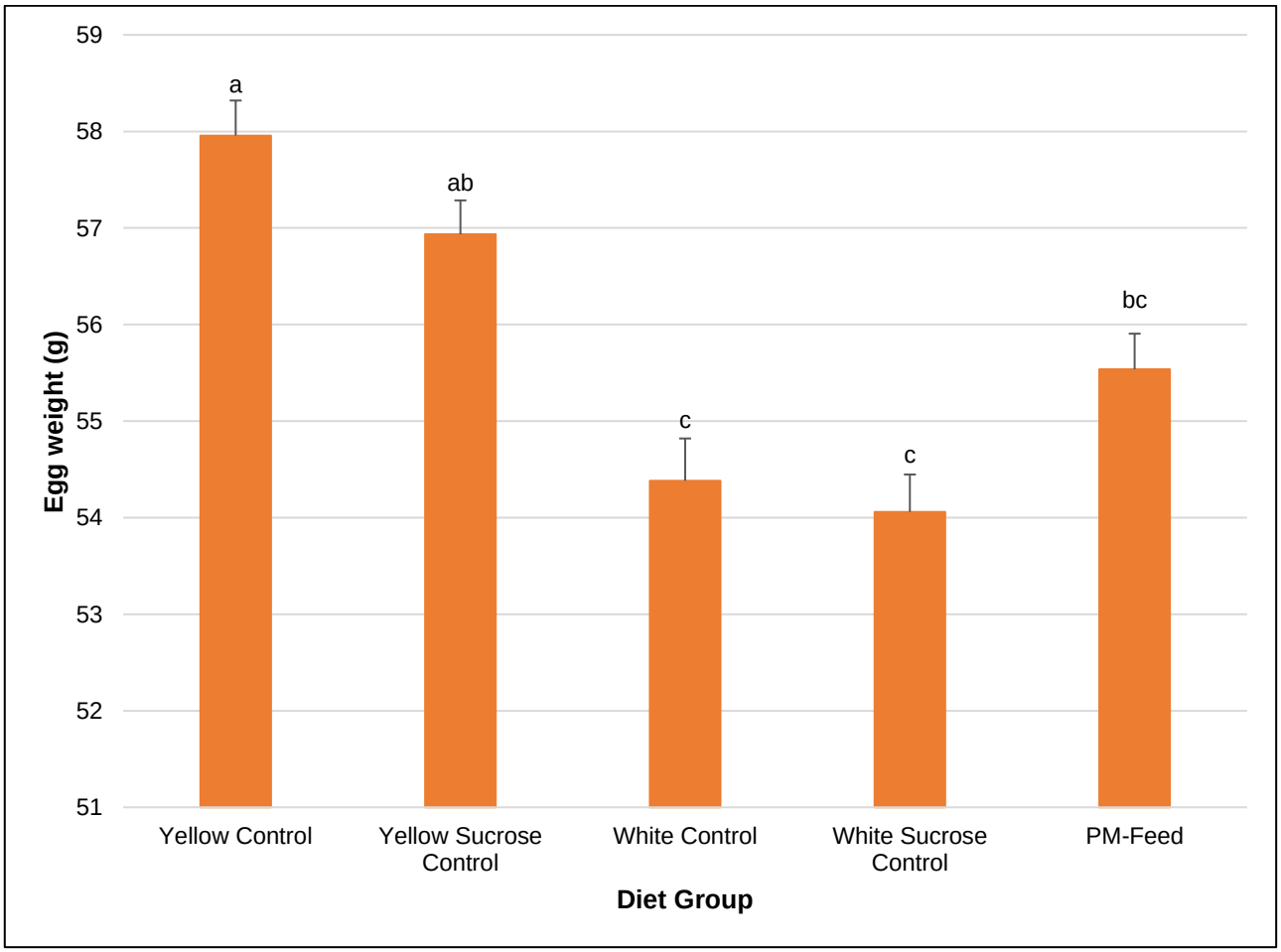

Figure 10 – Mean egg weight for bacterium in sucrose solution trial.

*Letters a-c indicates a significant difference at a confidence level of 95%, where $P \leq 0.05$.

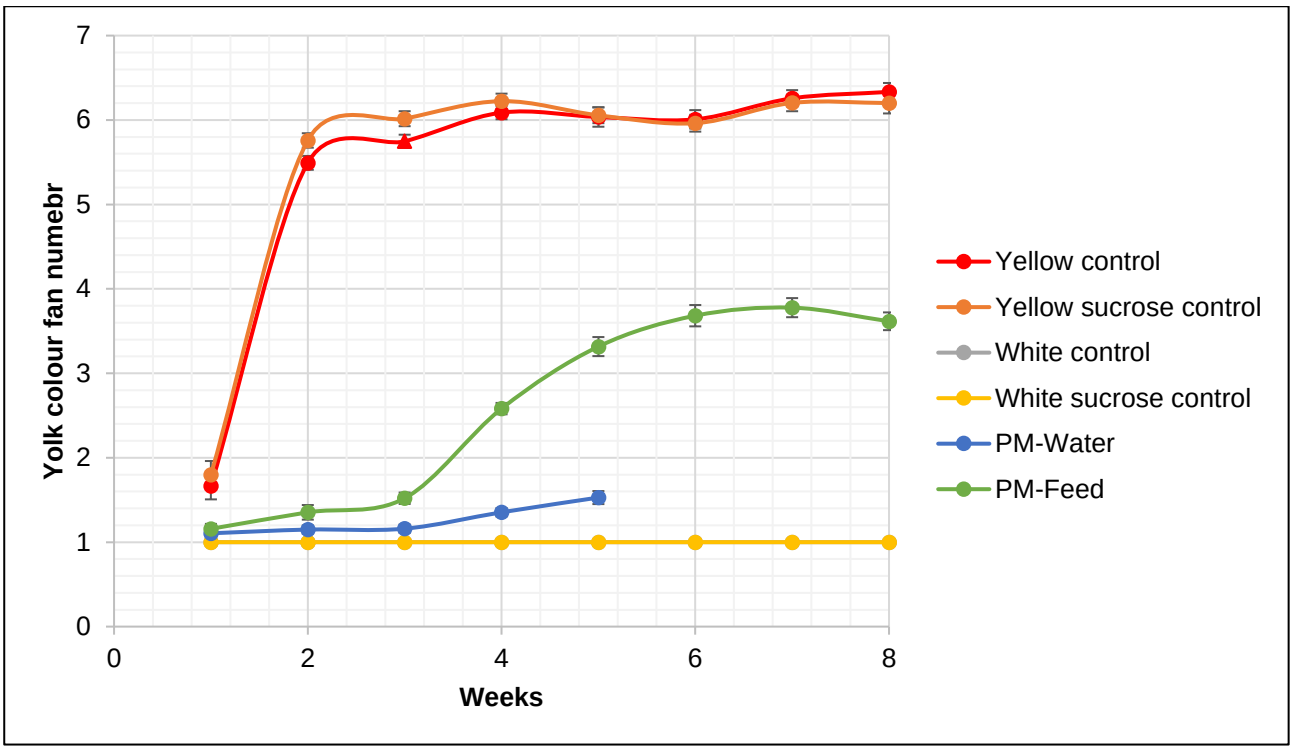

Figure 11 – Yolk colour change over an 8-week period in the bacterium in sucrose solution trial.

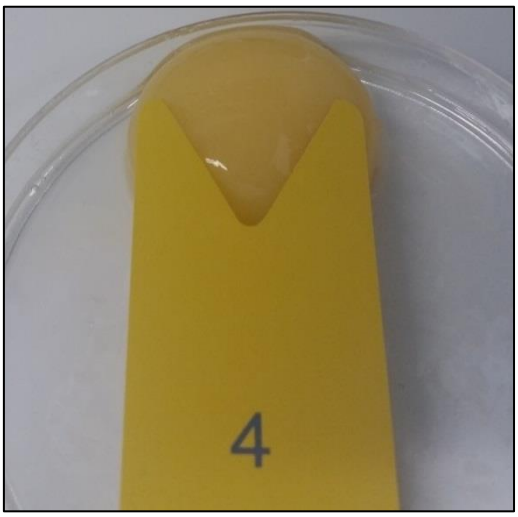

Figure 12 – Highest yolk colour obtained in PM-Feed. Yolk colour increased from a 1 to a 4.

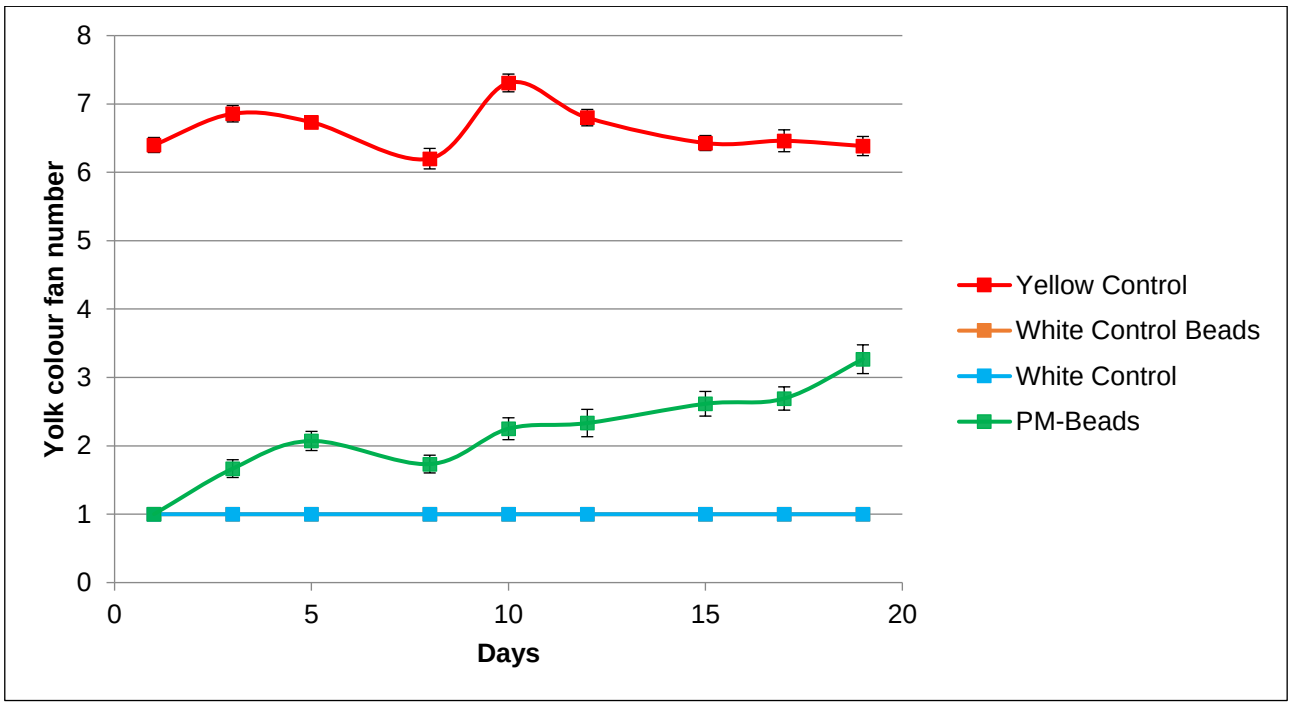

Figure 13 – Yolk colour change over a 3-week period in the bacterium in calcium alginate beads trial.

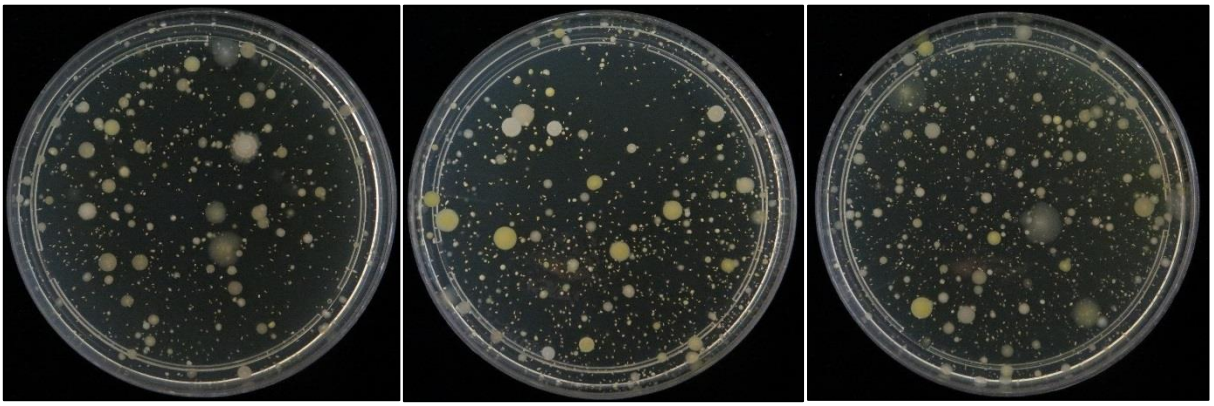

Figure 14 – Hen faecal dilution plates.

Tables

Table 1 – Layer hen feed composition

Ingredient	Prelay		Peaking	
	%	Weight (kg)	%	Weight (kg)
Maize (White or yellow)	65,806	658,063	63,338	633,379
Soybean Full Fat	7,088	70,876	9,479	94,789
Soybean 46	18,790	187,896	18,790	187,901
DL Methionine	0,109	1,092	0,203	2,033
L-Threonine	-	-	0,001	0,014
Vitamin and Mineral Premix	0,150	1,500	0,150	1,500
Limestone	5,975	59,747	5,961	59,607
Salt (NaCl)	0,262	2,616	0,263	2,629
Monocalcium Phosphate (MCP)	1,596	15,956	1,593	15,929
Sodium bicarbonate	0,225	2,253	0,222	2,218
Total	**100**	**1000**	**100**	**1000**

Table 2 – Diet groups for all feeding trials and their dosages

Feeding Trial	Treatment number	Diet group name	Dosage
Pilot study	1	Control	None
	2	Sucrose	1 ml sucrose (10% m/v)
	3	PM-F	5 ml freeze-dried cells *
	4	PM-1	1 ml live cells **
	5	PM-5	5 ml live cells **
Experimental trials			
Bacterium in sucrose	1	Yellow Maize Control	None
	2	Yellow Maize + Sucrose Control	1 ml sucrose (10% m/v)
	3	White Maize Control	None
	4	White Maize + Sucrose Control	1 ml sucrose (10% m/v)
	5	PM-Feed	50 ml live cells **
	6	PM-Water	50 ml live cells **
Bacterium in calcium alginate beads	1	Yellow Maize Control	None
	2	White Maize Control	None
	3	White Maize + Beads Control	300 mg calcium alginate beads
	4	PM-Beads	500 mg calcium alginate beads with bacterium ***

*Cells were lyophilised in sucrose (10% m/v) and resuspended in sterile dH_2O before feeding
**1.4×10^8 CFU/ml
***Each 500 mg of beads contained about 200 ml of *P. marcusii* with a cell density of 1.4×10^8 CFU/ml

Table 3 – Mean body weight at the start and end of the pilot study

Diet groups	Weight before (kg)	Weight after (kg)
Control	1.70 ± 0.15	1.76 ± 0.16
Sucrose Control	1.74 ± 0.14	1.77 ± 0.16
PM-Freezedried	1.81 ± 0.16	1.80 ± 0.18
PM-1	1.79 ± 0.17	1.87 ± 0.14
PM-5	1.82 ± 0.18	1.82 ± 0.19
P value	0.44	0.60

Table 4 – Egg quality parameters of all feeding trials

Feeding Trial	Diet Groups	Egg size (mm)	Egg Weight (g)	Yolk Height (mm)	Yolk Weight (g)	Yolk Colour	Albumen Height (mm)	Haugh Unit
Pilot study 7 weeks	Control	54.65 ± 1.78 ab	57.23 ± 3.31 ab	17.77 ± 0.89 ab	14.70 ± 1.08 a	6.89 ± 0.52 b	6.56 ± 1.41 bc	83.53 ± 10.88 ab
	Sucrose Control	55.29 ± 1.61 c	57.91 ± 2.90 bc	17.61 ± 0.71 a	14.86 ± 0.99 ab	6.57 ± 0.74 a	6.19 ± 1.48 ab	80.79 ± 9.79 ab
	PM-Freezedried	55.37 ± 1.45 c	58.85 ± 4.43 c	17.91 ± 0.85 b	15.10 ± 1.32 bc	7.01 ± 1.19 b	6.76 ± 1.03 c	84.25 ± 6.38 b
	PM-1	54.91 ± 1.76 bc	58.65 ± 3.31 c	17.94 ± 0.98 b	15.41 ± 1.23 c	7.33 ± 0.64 c	6.51 ± 1.40 abc	82.38 ± 8.90 ab
	PM-5	54.17 ± 1.81 a	56.72 ± 4.44 a	17.57 ± 0.88 a	15.02 ± 1.36 ab	7.53 ± 0.77 c	6.14 ± 1.28 a	80.78 ± 8.0 a
	P value	**	**	**	**	**	**	**
Bacterium in sucrose trial 12 weeks	Yellow Control	55.03 ± 1.79 a	57.96 ± 4.81 a	18.96 ± 1.03 a	13.95 ± 1.40 a	6.20 ± 0.80 a	8.72 ± 0.99	93.65 ± 5.03
	Yellow Sucrose Control	54.56 ± 1.85 ab	56.93 ± 4.57 ab	18.78 ± 0.79 ab	13.75 ± 1.30 ab	6.12 ± 0.81 a	8.67 ± 0.98	93.63 ± 5.07
	White Control	54.12 ± 1.84 b	54.38 ± 5.11 c	18.34 ± 0.80 d	13.07 ± 1.67 c	1.00 ± 0.00 c	8.75 ± 1.06	94.63 ± 5.52
	White Sucrose Control	54.56 ± 2.15 ab	54.06 ± 4.78 c	18.43 ± 0.77 cd	13.39 ± 1.11 bc	1.00 ± 0.00 c	8.87 ± 1.13	95.22 ± 5.69
	PM-Feed	54.85 ± 2.24 a	55.54 ± 4.68 bc	18.62 ± 0.73 bc	13.67 ± 1.15 ab	3.69 ± 0.84 b	8.84 ± 1.23	94.61 ± 6.50
	P value	**	**	**	**	**	0.48	0.04
Bacterium in calcium alginate beads trial 3 weeks	Yellow Control	55.26 ± 2.06	58.28 ± 4.28	18.81 ± 0.77	14.35 ± 1.32	6.43 ± 0.75 a	8.94 ± 0.98	94.60 ± 5.27
	White Control	55.66 ± 2.45	56.34 ± 4.57	18.65 ± 0.56	14.13 ± 1.14	1.00 ± 0.00 c	8.51 ± 1.03	95.22 ± 5.06
	White Control Beads	55.35 ± 2.38	55.74 ± 5.20	18.38 ± 0.79	13.97 ± 0.95	1.00 ± 0.00 c	8.94 ± 0.76	93.05 ± 4.18
	PM-Beads	55.15 ± 1.53	56.54 ± 3.18	18.70 ± 0.84	14.25 ± 1.19	2.88 ± 0.75 b	8.72 ± 0.99	93.99 ± 4.87
	P value	0.72	0.07	0.09	0.52	**	0.13	0.23

Values are means ± standard deviations. Letters a-d in the same column indicates a significant difference at a confidence level of 95%, where **$P \leq 0.05$.

Chapter 4

The effect of prolonged feeding of *Paracoccus marcusii* on the microbial community of the laying hen's gastrointestinal tract

Abstract

The microbiome of any living organism is important for maintaining the overall health and function of the host. *Paracoccus marcusii*, first isolated from the gastrointestinal tract of the South African abalone (*Haliotis midae*), has shown promise to be used as a feed additive to laying hens to enhance pigmentation in egg yolks. The aim of this study was, therefore, to determine the effect of prolonged feeding of *P. marcusii* on the microbial community structure of the duodenum and caeca. Hens were fed different diets, namely a positive control (yellow maize), two negative controls (white maize; white maize with sucrose (10% m/v)) and experimental group (*P. marcusii* in sucrose (10% m/v)). After 12 weeks, duodenum and caeca samples were taken, DNA extracted and analysed with ARISA-PCR. The microbial community structure of the duodenum indicated no significant difference between the different diet groups and there were high similarities between groups. However, the microbial community structure of the caeca shifted with a significant difference observed between the experimental group and the control groups. Since no mortalities were recorded and no hens showed signs of poor health, it is safe to assume that *P. marcusii* did not have a negative effect on the hens' overall health and function.

Introduction

The microbiome is important in animal health and production as it plays an important role in maintaining the overall health of the host (Gong *et al*., 2002a; 2002b; 2007, Torok *et al*., 2008 and Stanley *et al*., 2012). The poultry microbiome is a major contributor to zoonotic infections worldwide (Scallan *et al*., 2011). The gastrointestinal tract (GIT) of poultry differs from mammals. It is much shorter and the type of microorganisms that will colonise are primarily influenced by the environment surrounding the hen (Oakley *et al*., 2014). The transit digestion times are shorter than that of mammals, but just as efficient and can be explained by the complex microbiome in the gut that is characterised by specialised communities (McWhorter *et al*., 2009 and Sergeant *et al*., 2014). The short digestion time of less than 3.5 hours selects for bacteria that grows fast and that can adhere to the mucosal layer in the gut (Table 1) (Gauthier, 2002 and Hughes, 2008). However, digestion is longer in the caeca (12 to 20 hours) and is an ideal habitat for a more dense and diverse microbial community (Rehmen *et al*., 2007 and Pan and Yu, 2014). Some of the major phyla found in the caeca include Bacteroidetes, Firmicutes, Proteobacteria and Archaea (Gong *et al*., 2002a; Saengkerdsub *et al*., 2007a; 2007b and Qu *et al*., 2008). These microorganisms are important for urea recycling and fermentation of carbohydrates (Sergeant *et al*., 2014).

Antibiotics have been used in poultry feed since the late 1940's to treat diseases and was first documented by feeding cyclotetracycline fermentation waste as an inexpensive source of vitamins (Stokstad and Jukes, 1950). Sub-therapeutic levels of antibiotics (STAT) enhances the growth of the hens and has been widely used in the USA and other countries for more than 50 years (Chapman *et al*., 2010). Additionally, STAT is also used as a tool to reduce the risk of foodborne diseases by reducing the number of pathogenic microorganisms (Gustafson and Bowen, 1997). Unfortunately, as with all antibiotics used in feed nutrition, there is a risk of transferring antibiotic resistant genes to human pathogens. STATs act non-specifically and affects a wide range of microorganisms. In the late 1960's it was already suggested that using STATs could lead to an increase in genes available to enhance antibiotic resistance in human pathogens (Swann, 1969) as several pathogens are able to colonise the GIT of both chickens and humans (Johnson *et al*., 2007 and 2008). In later years, metagenomic surveys have revealed an abundant source of resistant genes, almost four times more, in the poultry GIT of STAT treated hens. These genes encode for resistance to antibiotics commonly used in poultry feed (virginamycin, neomycin sulphate, tylosin, streptomycin, penicillin, chlortetracycline, oxytetracycline and erythromycin) as well as resistance to cobalt, cadmium and zinc (Qu *et al*., 2008; Danzeisen *et al*., 2011 and Zhou *et al*., 2012). Currently, legal steps are being taken to remove the use of STATs in poultry feed in the USA and is already banned within the European Union (Van Immerseel *et al*., 2002 and Yeoman *et al*., 2012). Other methods are needed

to limit the increase of pathogenic microorganisms and this can include the use of probiotics and prebiotics (Van Immerseel *et al.*, 2004 and 2009).

Probiotics and prebiotics are commonly used in poultry feed to promote the growth of bacteria that are beneficial in the GIT and to inhibit the growth of pathogenic microorganisms (Van Immerseel *et al.*, 2004 and 2009). Poultry probiotics have been extensively studied. More than a few bacilli and lactobacilli strains isolated from a healthy hen's gut have effectively inhibited the growth of some known pathogens, including *E. coli* (Molnár *et al.,* 2011), *Campylobacter* (Pascual *et al.,* 1999) and *Salmonella* (Nakphaichit *et al*., 2011). Not only are these potential probiotics able to reduce the number of pathogens, but these studies have also revealed an increase in hen body weight and feed efficiency (Oakley *et al*., 2014). Prebiotics are complex ingredients added to poultry feed that serves as a nutrient source for beneficial bacteria already present in the GIT. There are two well-known prebiotics. Galactooligosaccharides (GOS) promotes the growth of bifidobacteria in broiler hens and fructooligosaccharides (FOS) decreases *Salmonella* counts in laying hens (Donalson *et al*., 2008 and Jung *et al*., 2008).

With the exclusion of antibiotics from animal feed, it is becoming more of a reality to formulate feed that might have a beneficial effect on the gut microorganisms. The function and composition of gut microorganisms are possible influenced by the dietary components of the feed and this change in composition of gut microorganisms affects the animal's health, wellbeing and food safety (Rehman *et al*., 2007 and Hammons *et al*., 2010). There are several factors of feed that effects the intestinal health of poultry, including non-starch polysaccharides (NSP), physical texture and the form of feed (Yegani and Korver, 2008). NSP, such as arabinoxylans and β-glucans, are a major group of anti-nutritional compounds present in feed ingredients like maize (corn) (Iji, 1999 and Yegani and Korver, 2008). NSP is able to increase the transit digestion time of the GIT allowing for a more efficient digestion of nutrients and enables microbial colonisation and activity (Waldenstedt *et al*., 2000 and Yegani and Korver, 2008). Hammons *et al*. (2010) studied the influence of different variations in poultry feed on the microbial composition of the crop. They found that a diet high in wheat had an effect at strain level compared to a standard diet of corn/soymeal rations even though there was no significant difference observed in species composition (Hammons *et al*., 2010).

With the relative sensitive nature of the microbial community of the GIT to shift with a change in diet type, composition or additives, it is important to note what the impact might be of other microorganisms as a feed additive. The Gram-negative bacterium, *Paracoccus marcusii,* is an astaxanthin producing bacterium and has the potential to be used as a feed additive for laying hens

to enhance egg yolk colour. Therefore, the aim of this study was to determine the potential effect of *P. marcusii* on the microbial community of the GIT, specifically the duodenum and caeca.

Materials and Methods

Experimental layout, hens and feed

The experimental layout, hens and feed is described in Chapter 3. Only hens from the experimental trial with sucrose were used for this part of the study to determine the effect of prolonged feeding of *P. marcusii* on the gut of the hen. The Yellow Maize Control group served as the positive control to compare to the unknown effect of the white maize diets. The abattoir is also located at the poultry unit of Mariendahl Experimental Farm, Stellenbosch University. The abattoir consisted of two rooms. One room for the stun and bleed of the hens and the other for the dissection. The rooms were cleaned with a high-pressure hose before and after the slaughter (Ethical clearance protocol number: SU-ACUD15-00088).

Collection of intestinal samples

At 28 weeks of age, after completion of the experimental trial described in Chapter 3, 10 hens were randomly selected from the following groups: Yellow Control, White Control, White Sucrose Control and PM-Feed (Table 2 and 3). The hens were stunned to render them insensible to pain and bled within 10 seconds of stun. The whole duodenum (Fig. 1B) and caecum pouches (Fig. 1C) were dissected and placed in separate sterile 50 ml Falcon tubes and immediately placed on ice. The duodenum was selected because it is where most carotenoids are absorbed (Yeum and Russell, 2002) and the caeca is the fermentation chambers where digestion is the longest (Rehman *et al*., 2007). Dissecting scissors and gloves were cleaned with ethanol (70% v/v) before dissecting the different gut samples and after use on each hen to minimize contamination between samples. The gut contents were discarded and the gut samples rinsed twice with sterile saline solution (0.9% NaCl). All samples were cleaned and DNA was extracted within 24 hours of dissection.

DNA extractions

For the DNA extraction of the gut samples, glass beads (Sigma Aldrich, South Africa) and 20 ml of sterile saline solution was added to the Falcon tube. This aided in the homogenisation of each sample on a vortex (Vortex-2 Genie, Model: G560E, Scientific Industries, Inc., United States) for 10 minutes. Total genomic DNA was extracted from the homogenised duodenum and caeca using

the ZR Fungal/Bacterial DNA extraction kit™ (Zymo Research Corp., USA). The success of the DNA extraction was confirmed on an Ethidium Bromide (EtBr) stained 1% agarose gel and visualised under Ultra Violet (UV) light. The extracted DNA was then used in further analysis for ARISA-PCRs.

ARISA-PCR and analysis

The Automated Ribosomal Intergenic Spacer Analysis (ARISA) was used to characterise the bacterial communities from the two different gut samples. This method estimates community composition and microbial diversity by amplifying the highly conserved region (Internal Transcribed Spacer, ITS) between the 16S and 23S rRNA genes. The natural variability in length of these ITS regions infer diversity with the different lengths representing different OTUs (Operational Taxonomic Units). The PCRs were done using a fluorescently labelled (6-carboxy-fluorescein) forward primer (ITSF-FAM-(5'-GTCGTAACAAGGTAGCCGTA-3')) and the reverse primer (ITSReub-(5'-GCCAAGGCATCCACC-3')) (Cardinale *et al*., 2004). The reaction mixture (a total of 10 µl) contained the following components: 4.1 µl ddH_2O, 5 µl KapaTaq ready-mix (Kapa Biosystems, South Africa), 10 pmol of the forward and reverse primers and 0.5 µl genomic DNA. The PCR was carried out using the following conditions: 95 °C for 5 minutes (1 cycle), followed by 36 cycles of 95 °C for 45 seconds, 56 °C for 50 seconds and 72 °C for 70 seconds and a final cycle at 72 °C for 7 minutes. The presence of DNA was confirmed on an Ethidium Bromide (EtBr) stained 1% agarose gel and visualised under UV light.

The PCR products obtained were sent to the Central Analytical Facility (CAF) at Stellenbosch University. An automated Genetic Analyser ABI 3010XI was used for capillary analysis using the Liz 1200 size standard for bacteria. The raw data was used to generate electropherograms using different fluorescent intensities and fragment lengths and analysed using Gene Mapper® Version 5 Software (Applied Biosystems, United States). After performing size calling per the applicable size marker, the genotypes table was exported to Microsoft Excel (2016) (Microsoft Corporation, United States) for further analysis. Peak height was preferred over peak size.

To test similarities between samples, the Whittaker similarity index (S_w) was calculated using the following formula:

$$S_W = \sum_{i=1}^{n} \left(\frac{|bi1 - bi2|}{2} \right)$$

where the b1 and b2 variables represent the percentage contributed to the *i*th OTU of two samples. Each sample was compared in a pairwise manner to generate a distance matrix. The Whittaker

similarity index ranges on a scale from 0 to 1, where 0 is completely similar and 1 is completely dissimilar. Multi-Dimensional Scaling (MDS) scatterplots were drawn and the similarities and/or dissimilarities were analysed using Ward's method to calculate dendrograms using Statistica, version 13.0 (Statsoft Inc., USA).

Analysis of similarity (ANOSIM) on the dissimilarities matrix was also performed using R, version 3.3.1 (The R Foundation for Statistical Computing, Austria) for between group comparisons. ANOSIM compares between group dissimilarity means to within group dissimilarity means. R-values range on a scale from -1 to 1, where a value closer to 1 suggests dissimilarities between groups, 0 suggest an even distribution of low and high ranks between and within groups and a value less than 0 suggest that dissimilarities are greatest within groups and not between groups (Clarke, 1993).

The Shannon diversity index (*H'*) was calculated using the following formula:

$$H' = -\sum pi \, log_e \, pi$$

where the index is defined as the negative sum of each OTUs proportional abundance (p_i) multiplied by the natural log of its proportional abundance. Shannon diversity index is a quantitative measurement that reflects species evenness and abundance. The more unequal the abundances of the type, the larger the weighted geometric mean of the p_i values and the smaller the Shannon index. Shannon index approaches 0 when all abundances are concentrated to one type.

Statistical analysis

Statistical analysis was performed using Statistica, version 13.0 (Statsoft Inc., United States). One-way analysis of variance (ANOVA) was used for mean comparisons of the Shannon diversity index and total number of OTUs observed. Tukey's honest significant difference (HSD) was calculated where $P \leq 0.05$.

Results

ARISA-PCR analysis

Duodenum

There was no distinct pattern observed between the bacterial community structures of the different diet groups on the MDS scatterplots (Fig. 2). The dendrograms indicated distinct cluster patterns (Fig. 3). However, when considering the different diet groups, no distinct patterns were formed. This is further supported by the Shannon diversity index (Table 4). There was no significant difference ($P \geq 0.05$) between the Shannon diversity index of the different diet groups. The index ranges between 2.14 (± 0.42) and 2.59 (± 0.29). The number of OTUs observed ranges between 21.44 (± 6.13) and 28.60 (± 3.34) (Table 4). A significant difference ($P \leq 0.05$) was observed where the PM-Feed diet group had the lowest number of OTUs compared to the White Control diet group that had the highest number of OTUs.

The R-values of the communities from the duodenum indicated significant differences ($P \leq 0.05$) between diet groups, where the R-value of PM-Feed was significantly different to White Control (R-value – 0.175), as well as White Sucrose Control (R-value – 0.145) (Table 5). A significant difference ($P \leq 0.05$) was also observed between the White Control and White Sucrose Control (R-value – 0.122). No significant difference ($P \geq 0.05$) was observed for the Yellow Control diet group compared to the other diet groups.

Caeca

Distinct patterns were observed on the MDS scatterplots between the bacterial community structures of the different diet groups (Fig. 4). This is supported by the dendrogram indicating two distinct clusters (Fig. 5). The first cluster consists of PM-Feed and some control samples and the second cluster consists of the remaining control samples. However, there is no significant difference ($P \geq 0.05$) between the Shannon diversity index of the different diet groups (Table 4). The Shannon diversity index ranges between 2.45 and 3.03, where the PM-Feed had the lowest index and the White Sucrose Control had the highest index. A significant difference ($P \leq 0.05$) was observed in the total number of OTUs (Table 4). The White Sucrose Control diet group had the highest number of OTUs (38.00) compared to the PM-Feed with the lowest number of OTUs (28.30).

The R-values of the caeca communities indicated a significant difference ($P \leq 0.05$) between the PM-Feed diet group compared to all the control diet groups with the highest dissimilarity between the PM-Feed and White Control diet group (R-value – 0.435) (Table 5). A negative R-value was also measured between White Control and White Sucrose Control (R-value – 0.014).

Discussion

The Gram-negative bacterium, *P. marcusii*, has shown promise to be used as a feed additive in poultry feed to enhance egg yolk colour (Chapter 3). This species is able to produce astaxanthin, a xanthophyll carotenoid with several health benefits that include improved blood flow and boosting the immune system (Harker *et al*., 1998 and Yamashita, 2015). Previously, *P. marcusii* has been used as a feed additive for fish (De Bruyn, 2013) and as a potential probiotic for *Apostichopus japonicas* (juvenile sea cucumber) (Yan *et al*., 2014). Therefore, we aimed to determine the effect of prolonged feeding of *P. marcusii* on the microbial community structure in the GIT of laying hens.

The chicken GIT microbial diversity has previously been studied using both culture-dependent and culture-independent studies. These studies have revealed a simpler microbial community starting in the small intestines which becomes increasingly complex and more diverse further down the intestinal tract to the caeca (Gong *et al*., 2002a; 2002b, and Lu *et al*., 2003). This was also true in this study and was evident in the Shannon diversity index and total number of OTUs measured (Table 4). In the duodenum, which is the start of the small intestines, the mean index ranged between 2.14 and 2.59. The index increased in the caeca and ranged between 2.45 and 3.03. The total number of OTUs observed also increased from the duodenum (21.44 – 28.6) to the caeca (28.30 – 38.00). In both the Shannon diversity index and the total number of OTUs of the duodenum and caeca, the PM-Feed diet group had the lowest mean, but significance was only observed for the OTUs compared to the control diet groups. Surprisingly, the White Sucrose Control diet group had the highest number of OTUs in the caeca (38.00) followed by the White Control and Yellow Control with 33.80 and 29.40, respectively. The lower OTU observed for the Yellow Control compared to the White Control was unexpected as the only difference in feed was the maize type (Table 3) and white and yellow maize are similar in nutritional value. Therefore, there should be a similar microbial composition in terms of total OTUs and Shannon diversity index. However, there are some studies that indicated a difference in starch, protein and sugar content between maize varieties that directly influenced layer hen performance. This is affected by maize hybrid type, the nitrogen fertilisation of the soil, geographical growing site, plant density and harvesting maturity (Zeidan *et al*., 2006, Moore,

2007, Idikut *et al*., 2009 and Raymond *et al*., 2009). This difference in nutritional composition will have a direct effect on the microbial community structure as previously reported (Rehman *et al*., 2007 and Hammons *et al*., 2010). The effect of sucrose in the White Sucrose Control diet group was not unexpected as sucrose is made up of glucose and fructose, both of which are commonly used in poultry feed as prebiotics to enhance the growth of bifidobacteria and inhibit the growth of possible food pathogens (Donalson *et al*., 2008 and Jung *et al*., 2008). However, it is unknown why this was not the same with the PM-Feed diet group that also contained a sucrose dosage.

There was no similarity in microbial community structure of the duodenum between the different diet groups (Fig. 2 and 3). Although there was a significant R-value between PM-Feed compared to White Control and White Sucrose Control and between White Control compared to White Sucrose Control, all R-values were close to 0, suggesting an even distribution of low and high ranks between and within groups (Table 5). The short transit digestive time of the duodenum of 5 – 8 minutes only allows for rapidly growing bacteria and bacteria that can adhere to the mucosal layer of the gut (Hughes, 2008). *Paracoccus marcusii* grows relatively slow (replication time of ± 12 hours, see Chapter 2) and will, therefore, not be able to colonise in the small intestines.

For the caeca, distinct patterns and clusters formed between the PM-Feed diet group compared to the control groups (Fig. 4 and 5). There was no similarity in the community structure between the different control diet groups. Therefore, the different maize types (yellow or white) and added sucrose did not have an influence on the microbial community structure of the caeca. However, the community structure between the White Control and White Sucrose Control were more similar than both diet groups compared to the Yellow Control (Fig. 5). Significant R-values was observed between the PM-Feed diet group compared to the White Control, White Sucrose Control and Yellow Control with the highest dissimilarity of 0.435 between PM-Feed and White Control (Table 5). It is suspected that the longer digestion time of 12 – 24 hours in the caeca might be the reason for observing a change in community structure compared to the shorter digestion time of 5 – 8 minutes in the duodenum (Gauthier, 2002). The caeca are also known as the fermentation chambers for the important utilisation of carbohydrates not easily digested by the hen (Sergeant *et al*., 2014).

Paracoccus marcusii will most likely not be able to survive the conditions in the GIT of the hen. *Paracoccus marcusii* is an aerobic bacterium with an optimum growth at 26 °C and a pH between 6 – 7 (see Chapter 2). The whole GIT is predominantly anaerobic and also highly acidic at the start of the intestinal tract (pH 2.5 – 3.5) in the proventriculus and gizzard (Gauthier, 2002). Therefore, it

is suggested that the digested cells could have released by-products that influenced the microbial composition in the caeca where it could have been utilised by a specific group of microorganisms.

When considering the different results, it is reasonable to assume that *P. marcusii* did not influence the diversity or similarity of the microbial community structure in the duodenum of the hens. However, there is a shift in community structure of the caeca. The shift in the community structure of the caeca could also be explained by the different maize types. However, all R-values for the control groups were close to 0, indicating a similar distribution of high and low ranks between and within groups and no significant difference ($P \geq 0.05$) was observed. We can also assume that the shift in community structure was not negative as no mortalities were recorded during the trial and all hens in the experimental diet appeared to be in excellent health. Signs typically associated with poor health in hens include diarrhoea, depression and unwillingness to move.

Conclusion

This study has shown the effect of feeding *Paracoccus marcusii* to laying hens on the microbial community structure of the gastrointestinal tract over a prolonged period of time. The microbial community structure of the duodenum was similar between the different diet groups, but there was a shift in community structure of the caeca. The change in microbial composition did not have an influence on the overall health of the hen as no signs of disease were observed. Future studies are needed to determine which microorganisms were affected by the added bacterium in the hen's diet and if this change in microbial composition will influence food health safety and the production performance of the hen.

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Figures

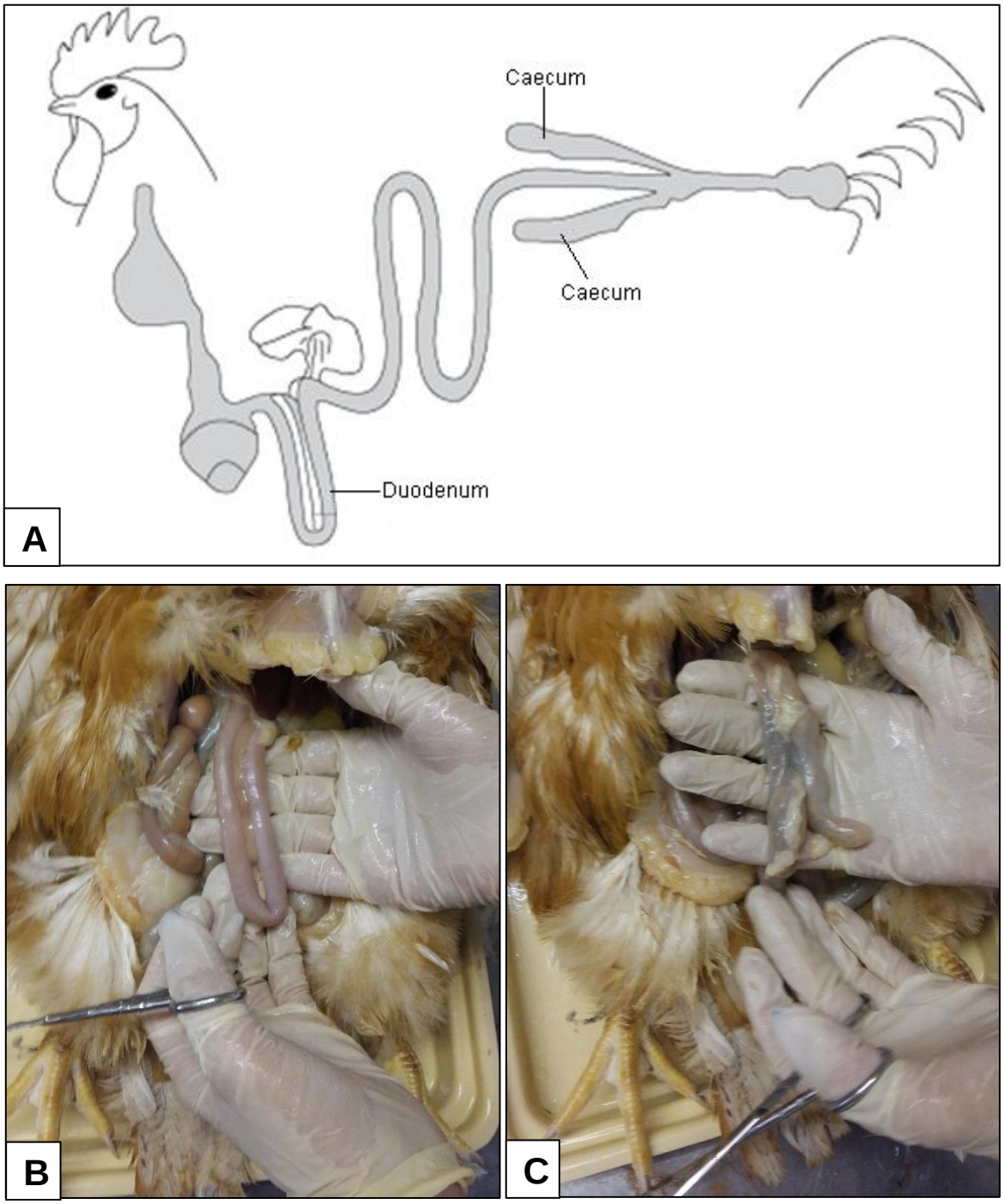


Figure 1 – **A.** Diagram indicating location of gastrointestinal tract samples taken. **B.** Duodenum. **C.** Caeca.

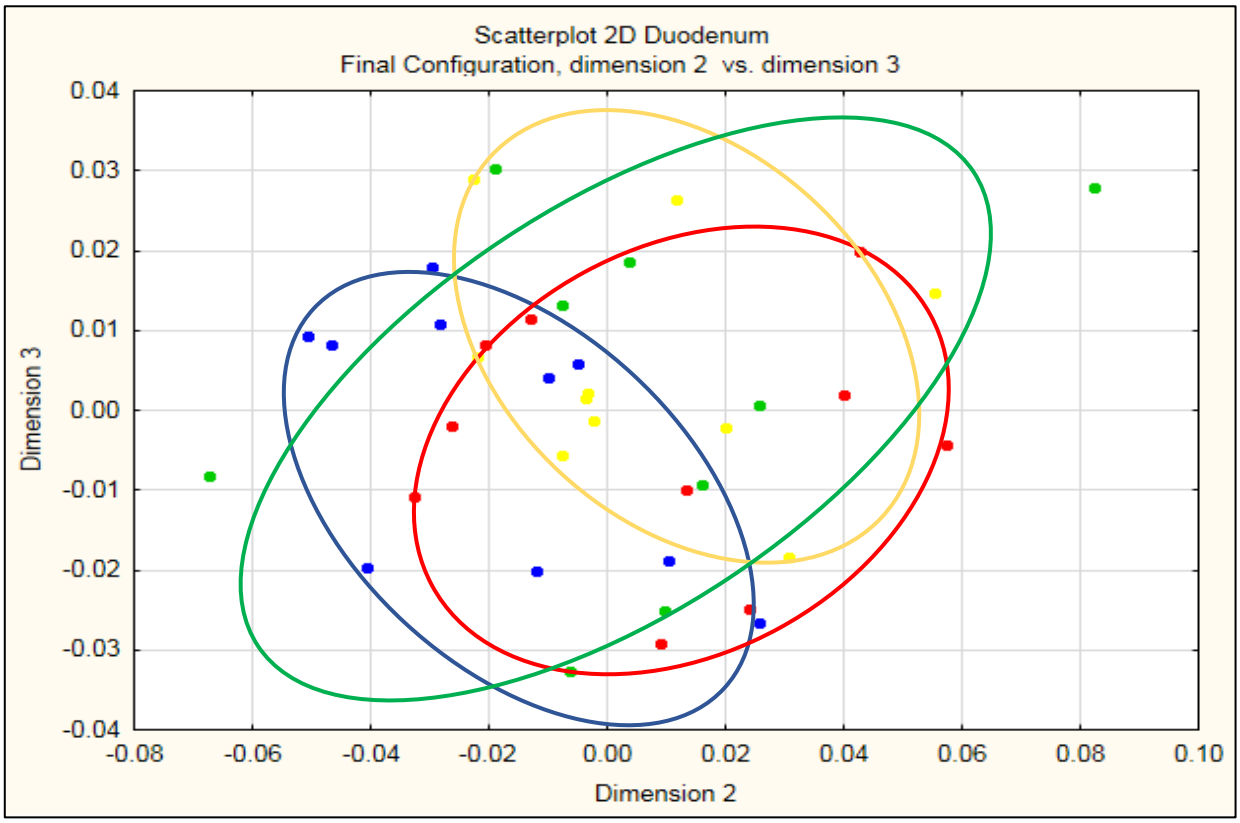


Figure 2 – Multi-Dimensional Scaling scatterplot representing the microbial community of the different diet groups in the duodenum, where red is Yellow control, yellow is White Control, blue is White Sucrose Control and green is PM-Feed.

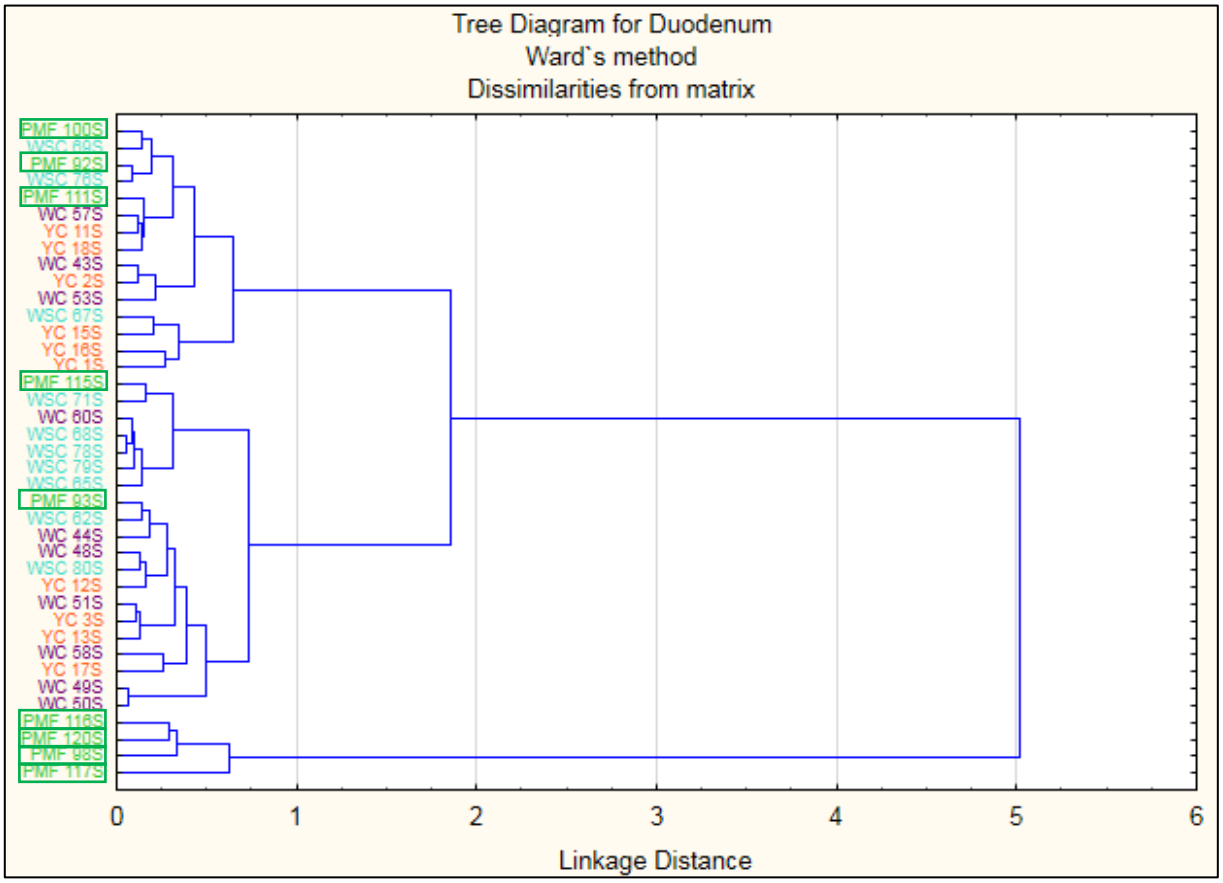


Figure 3 – Dendrogram of bacterial community structure of the duodenum of chicken fed different diets. YC – Yellow Control. WC – White Control. WSC – White Sucrose Control. PMF – PM-Feed.

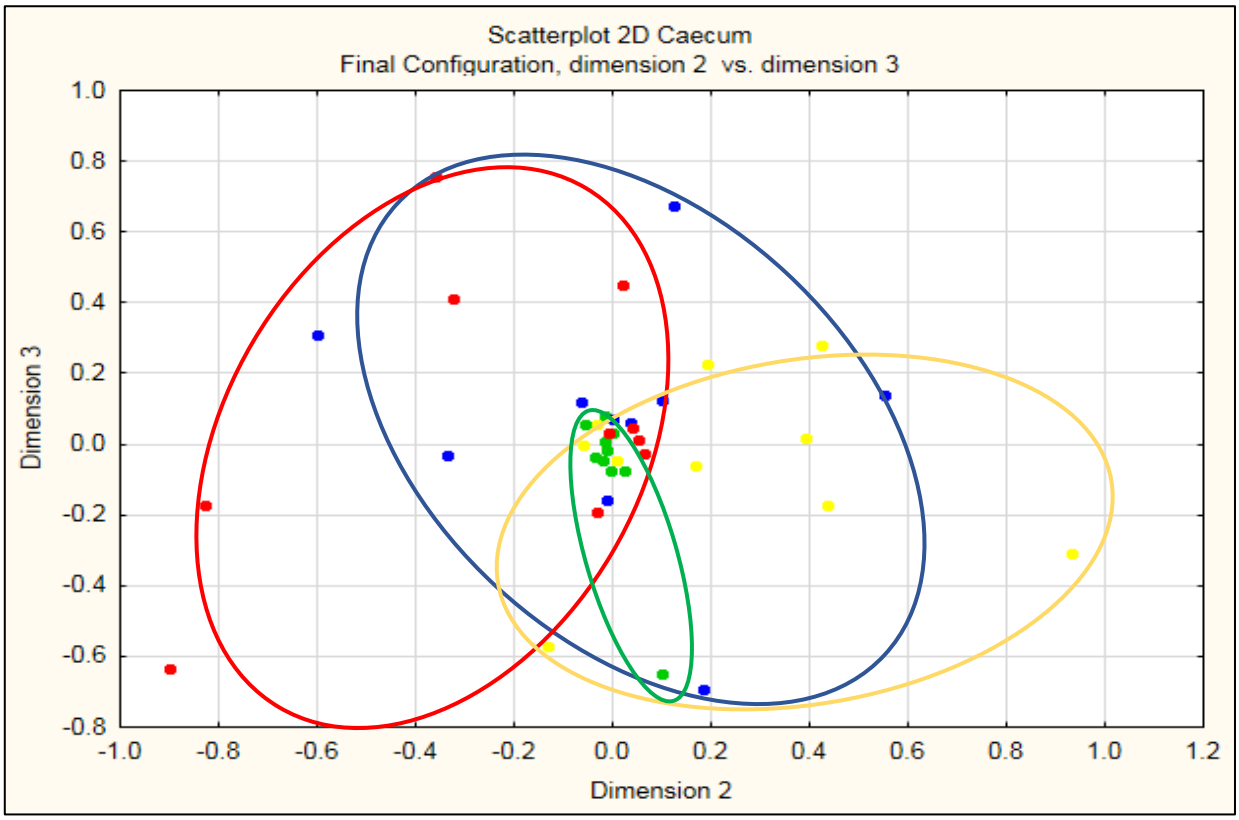


Figure 4 – Multi-Dimensional Scaling scatterplot representing the microbial community of the different diet groups in the caeca, where red is Yellow control, yellow is White Control, blue is White Sucrose Control and green is PM-Feed.

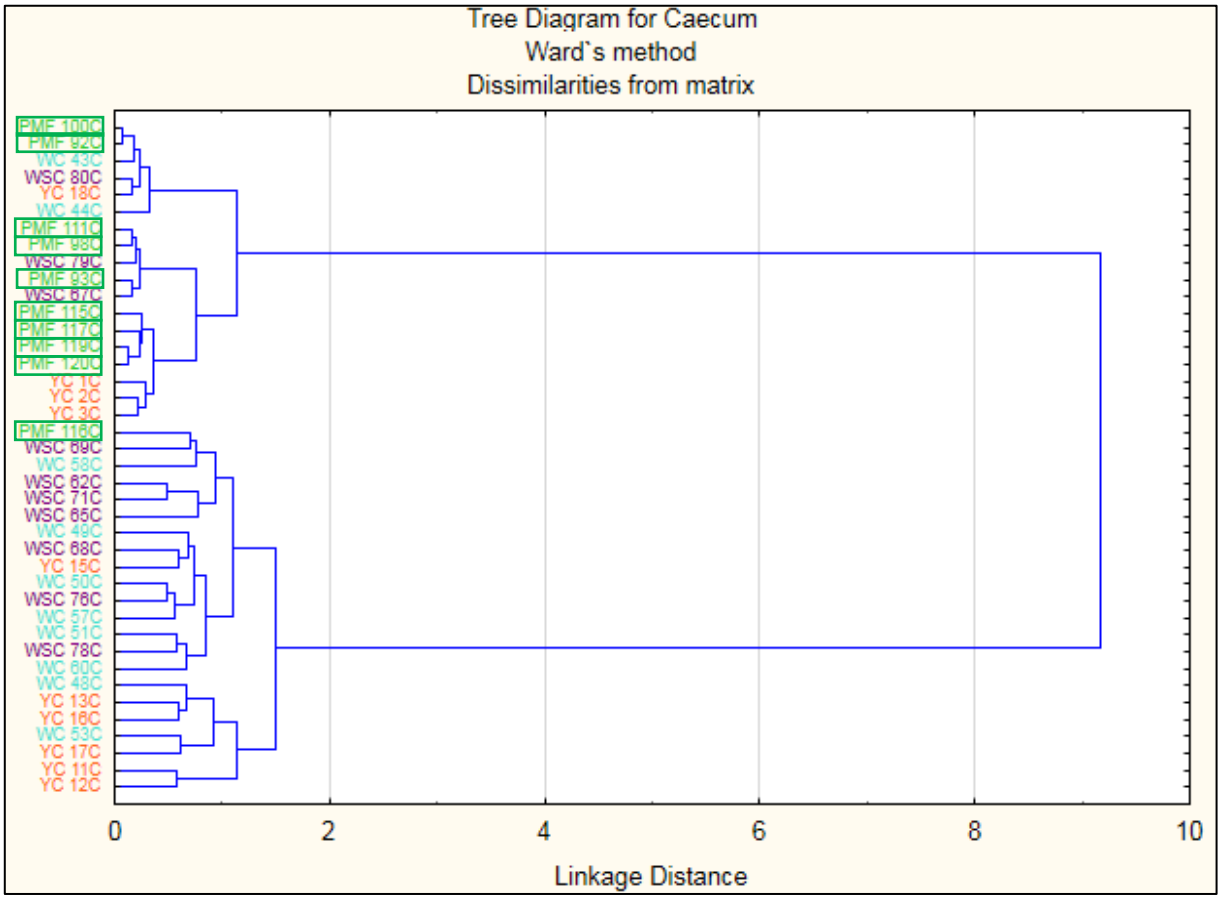


Figure 5 – Dendrogram of bacterial community structure of the caeca of chicken fed different diets. YC – Yellow Control. WC – White Control. WSC – White Sucrose Control. PMF – PM-Feed.

Tables

Table 1 – Digestive transit time and pH in the poultry GIT
(Gauthier, 2002)

GIT segment	Digestive transit time (min)	pH
Crop	50	5.5
Proventriculus/Gizzard	90	2.5 – 3.5
Duodenum	5-8	5 – 6
Jejunum	20-30	6.5 – 7
Ileum	50-70	7 – 7.5
Caeca	12 – 24 hours	7 – 8

Table 2 – Diet groups chosen for microbial community analysis

Treatment number	Diet group name	Dosage
1	Yellow Maize Control	None
2	White Maize Control	None
3	White Maize + Sucrose Control	1 ml sucrose (10% m/v)
4	PM-Feed	50 ml live cells *

*1.4 x 10^8 CFU/ml

Table 3 – Layer hen feed composition

Ingredient	Prelay		Peaking	
	%	Weight (kg)	%	Weight (kg)
Maize (White or yellow)	65,806	658,063	63,338	633,379
Soybean Full Fat	7,088	70,876	9,479	94,789
Soybean 46	18,790	187,896	18,790	187,901
DL Methionine	0,109	1,092	0,203	2,033
L-Threonine	-	-	0,001	0,014
Vitamin and Mineral Premix	0,150	1,500	0,150	1,500
Limestone	5,975	59,747	5,961	59,607
Salt (NaCl)	0,262	2,616	0,263	2,629
Monocalcium Phosphate (MCP)	1,596	15,956	1,593	15,929
Sodium bicarbonate	0,225	2,253	0,222	2,218
Total	**100**	**1000**	**100**	**1000**

Table 4 - Shannon diversity index and number of OTUs observed for each diet group

Diet Group	Shannon diversity (SD)	Number of OTUs
Duodenum		
PM-Feed	2.14 ± 0.42	21.44 ± 6.13 b
White Control	2.59 ± 0.29	28.6 ± 3.34 a
White Sucrose Control	2.20 ± 0.38	23.6 ± 4.45 ab
Yellow Control	2.27 ± 0.43	24.3 ± 4.79 ab
***P* value**	0.065	**0.017**
Caeca		
PM-Feed	2.45 ± 0.60	28.30 ± 7.83 bc
White Control	2.92 ± 0.60	33.80 ± 8.89 ab
White Sucrose Control	3.03 ± 0.52	38.00 ± 7.63 a
Yellow Control	2.65 ± 0.42	29.40 ± 6.02 ab
***P* value**	0.086	**0.028**

*Values are mean ± standard deviation. Letters a-c in the same column indicates a significant difference at a confidence level of 95%, where ***P* ≤ 0.05.

Table 5 - Analysis of similarity (ANOSIM) between different diet groups

Duodenum				
Diet Group	PM-Feed	White Control	White Sucrose Control	Yellow Control
PM-Feed		**0.019**	**0.036**	0.142
White Control	0.175		**0.043**	0.103
White Sucrose Control	0.145	0.122		0.073
Yellow Control	0.068	0.079	0.110	
Caeca				
Diet Group	PM-Feed	White Control	White Sucrose Control	Yellow Control
PM-Feed		**0.001**	**0.009**	**0.033**
White Control	0.435		0.445	0.251
White Sucrose Control	0.304	-0.014		0.335
Yellow Control	0.189	0.022	0.011	

*The R-values are indicated in the lower left part of the table. The *P* values are indicated in the upper right part and where there is significance (*P* ≤ 0.05) is indicated in red and bold.

Concluding remarks

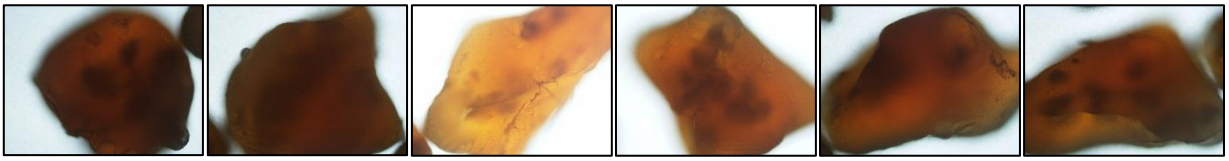
The colour of any food product is important to consumers. In poultry farming a new trend is emerging to use natural sources of colourants to enhance pigmentation of egg yolks. Previous studies have focused on adding a carotenoid-producing microalga or yeast to the feed of poultry. However, the use of whole microalga or yeast cells had no significant effect on pigmentation of the yolks. Therefore, this study aimed to determine if whole *Paracoccus marcusii* cells can be used as a pigmentation source to enhance egg yolk colour without the need to extract the pigment from the cell. This bacterium is able to produce astaxanthin naturally under certain growth conditions. In Chapter 2, the highest concentration of astaxanthin obtained with *P. marcusii* was 24.25 µg/g dry cell weight. The storage stability of the bacterium with astaxanthin was improved through microencapsulation in calcium alginate beads. After 3 weeks, only 30% of the astaxanthin content was lost compared to lyophilisation in sucrose with a total loss of 85%.

The pigmentation effect of *P. marcusii* fed to laying hens was evaluated in Chapter 3. All trials indicated a significant increase ($P \leq 0.05$) in yolk colour when compared to the white control groups with no negative effect on the overall quality of the egg. There was also an increase in whole egg and yolk weight observed. These results indicated the possibility of using whole *P. marcusii* cells as a feed additive. After the feeding trials, it was important to evaluate the effect of *P. marcusii* on the microbial community structure of the gastrointestinal tract (Chapter 4). Gut samples were taken from the duodenum, where carotenoids are absorbed, and from the caeca, where digestion is the longest. The results obtained indicated no significant difference in microbial composition of the duodenum. However, there was a shift in microbial composition of the caeca. There were no mortalities during the trials and all hens were in good health. It was, therefore, concluded that *P. marcusii* is safe to be used as a feed additive as it did not have a negative effect on the microbial composition of the gut or the overall performance and health of the hen as no signs of disease were detected. A preliminary economical evaluation was also done to determine the feasibility of the developing product for the poultry industry (see Addendum). Based on the analysis done, it was determined that microencapsulated *P. marcusii* is too expensive and, therefore, not feasible for poultry farmers at this stage.

For future research it is important to further optimise the production and storage stability of astaxanthin by *P. marcusii*. It is also important to determine the optimal dosage for satisfactory pigmentation effect and the possible long-term effect of the shift in microbial composition in the caeca. It is also essential to find other alternative and low-cost sources of peptone to lower the total production costs.

Addendum

A preliminary economic assessment of a potential feed additive for laying hens: *Paracoccus marcusii* microencapsulated in calcium alginate beads



Abstract

Astaxanthin is a high value compound used in aquaculture and poultry as a colouring agent. Over the years the focus has been on the production costs of astaxanthin by the microalga, *Haematococcus pluvialis* and yeast, *Xanthophyllomyces dendrorhous*, and the use thereof as a feed additive with little focus on astaxanthin producing bacteria. Therefore, we aimed to evaluate the economic value of a developing product of microencapsulated *Paracoccus marcusii* in calcium alginate beads. A total of 84 L of bacterium could be cultured each month on a small-scale production. Based on the analysis, the total cost is estimated at R2912.88/month for 210 g of calcium alginate beads containing *P. marcusii*. This is too expensive and not feasible for the poultry industry. Possible solutions to lower production costs is the use of alternative sources of peptone (soybean peptone), large-scale production and increasing the concentration of bacterium microencapsulated in calcium alginate beads.

Introduction

Astaxanthin is a very valuable compound with an estimated market value of about $250 million per year (Milledge, 2011). Commercial astaxanthin is currently dominated by synthetic astaxanthin, because of its relatively cheaper production cost of around $1000/kg (± R15 000/kg) and a market value above $2000/kg (± R30 000/kg) (Olaizola, 2003 and Milledge, 2011). Over the years synthetic and natural astaxanthin has been used as a colouring agent and as a feed additive in poultry to enhance egg yolk colour and in aquaculture for a rosier flesh in fish (Choubert and Heinrich, 1992; Lorenz and Cysewski, 2000; Guerin *et al*., 2003; Storebakken *et al.*, 2004 and Johnson *et al*., 2003). Even though synthetic astaxanthin is a stable source for larger quantities, some concerns have been raised about the food safety and little is known about its biological functions. In 1986, Newsome was the first to raise the concern of using carcinogenic petrochemicals to produce synthetic astaxanthin. There are several methods to produce synthetic astaxanthin. This includes the reaction between two C_{15}-phosphonium salts with C_{10}-dialdehyde (Wittig reaction) (Widmer *et al*., 1981), canthaxanthin hydroxylation (Bernhard *et al*., 1984) and lutein isomerisation to zeaxanthin from marigold and then oxidised to astaxanthin (Schloemer and Davis, 2001). Synthetic astaxanthin also consist of a mixture of three isomers (3R, 3'R), (3R, 3'S) and (3S, 3'S) in a ratio of 1:2:1, respectively (Schiedt *et al*., 1988 and Higuera-Ciapara *et al*., 2006). Figure 1 illustrates the mechanisms of production of synthetic astaxanthin.

Currently, *Haematococcus pluvialis* is used for the industrial scale production of natural astaxanthin. This process has been developed and optimised since the late 1990s (Lorenz and Cysewski, 2000). The astaxanthin yield from this microalga ranges between 1.5% - 3% of its dry cell weight and is achieved through nutrient deprivation, higher temperatures and/or high levels of light in the growth phase known as the 'reddening phase' (Lorenz and Cysewski, 2000). Extraction of astaxanthin from the cell is achieved through centrifugation to harvest all the cells, followed by drying and milling of the cells (Schmidt *et al*., 2011). The microalga exclusively produce the isomers (3R, 3'R) or (3S, 3'S) (Higuera-Ciapara *et al*., 2006). The market value for astaxanthin produced by *H. pluvialis* is around $7000/kg (± R100 000) (Li *et al*., 2011).

Astaxanthin production by the yeast *Xanthophyllomyces dendrorhous* is simpler than the microalga in terms of a faster growth rate and higher cell density that can be achieved. However, the astaxanthin yield with *X. dendrorhous* is lower than the microalga with concentrations ranging between 50 – 350 µg/g dry cell weight (Chandi and Gill, 2011). Factors that have been tested to increase astaxanthin production included temperature, pH, oxygen, nutrients and light. Unlike

H. pluvialis, yeast only produce the (3, 3'R) isomer of astaxanthin (Nguyen, 2013). For extraction of astaxanthin, like the microalga, the yeast cells must be milled or enzymatically digested to retrieve the astaxanthin (Johnson *et al*., 1977 and 1980). The market price for yeast astaxanthin is around $2500/kg (± R37 500), a lot cheaper than microalga and almost the same as synthetic astaxanthin (Li *et al*., 2011).

Currently, bacteria are not used as a natural source of astaxanthin for industrial production. There is a possibility to reduce costs because of the characteristics of bacteria which include a thinner cell wall which eliminates the need to crack the cell wall for extraction, faster growth rate and use of a simpler media and growth conditions compared to yeast and microalga. Therefore, the aim of this study is to provide a preliminary economic assessment of the small-scale production costs of a developing product of microencapsulated *P. marcusii* calcium alginate beads for poultry feed.

Cost Analysis

Method of production process at small-scale

Growth conditions, media and equipment

In Chapter 2, the production and storage stability of astaxanthin by *P. marcusii* was optimised. In short, *P. marcusii* was cultured in 2 L Schott bottles containing 1 L of specialised medium (10 g/L bacteriological peptone, 5 g/L yeast extract, 3% NaCl and pH 7 – 8). Schott bottles were incubated at 26 °C for 4 – 7 days. Cells were harvested through centrifugation at 10000 rpm for 10 minutes and washed once with sterile dH_2O. Every 3 L of harvested cells were resuspended in 200 ml of 2% sodium alginate. Using a 21G x 1.5" hypodermic needle and syringe, the solution was added drop-wise to 200 ml of 2% $CaCl_2$ solution under constant stirring of 150 rpm. The gel-like beads were separated from the $CaCl_2$ solution using a sift and dried overnight in a laminar flow cabinet at room temperature. Every 200 ml of sodium alginate and bacterium produced 7.5 g of dried beads. One gram of calcium alginate beads contained approximately 400 mg of dry cell weight and 9.65 ppm of astaxanthin.

Total needed per hen

During the feeding trial in Chapter 3, there was a significant increase in yolk colour compared to the white maize controls after 3 weeks when calcium alginate beads containing *P. marcusii* was fed to

laying hens. Each hen was given 500 mg of calcium alginate beads every day that contained approximately 4.8 ppm astaxanthin.

Analysis of operational and fixed capital investments

All costs are based on working parameters and actual quotes or purchased prices. Table 1 summarises all operational and fixed costs according to a small-scale production of 84 L per month. Given the facilities available at Stellenbosch University for a small-scale production of *P. marcusii* beads, the total production cost is estimated at R2912.88/month. Eighty-four litres of culture will produce 210 g of calcium alginate beads/month and a total of 420 hens can be fed with the beads produced. The estimated cost for every hen is R6.94 per day and R194.32 per month. Of this, 64.8% of the total cost is for the specialised medium, followed by electrical appliances (28.3%) and microencapsulation (6.9%).

Solutions to consider

There are several possibilities to reduce production costs of the beads. More than 50% of the total cost is for the use of bacteriological peptone (R1522.58 of R2912.88). Peptone and yeast extract are important components of the medium as these compounds form the building blocks from which biomolecules, such as astaxanthin, are produced (see Chapter 2) (Chougle and Singhal, 2012). There are some alternative media components that can be used. One example is the use of soybean products. There are several products available that can be used, including soybean peptone, soybean meal and soybean protein isolate, of which soybean protein isolate is the most inexpensive (R0.13/g). Large-scale cultivation methods might also reduce the total costs and a higher concentration of bacterium could be added to the sodium alginate solution. Future research is needed to determine if the bacterium is able to use soybean as a carbon and nitrogen source to grow and produce astaxanthin, what the lowest amount in g/L of yeast extract and peptone are needed, what the highest concentration of bacterium is that can be added to the sodium alginate solution without losing product consistency and also if a large-scale cultivation will reduce costs.

Conclusion

The *Paracoccus marcusii* microencapsulated in calcium alginate beads is an effective egg yolk colourant and is stable under different environmental conditions. For this reason, an economic assessment was done to determine the feasibility of the product for poultry farmers. Currently, the production cost alone to produce enough beads for one hen is R194.32/month (R6.94/day) of which almost 65% of the total cost is for the growth media. This is too expensive and not feasible for poultry farmers. There are several ways to decrease production costs that include the use of soybean protein isolate or related products, large-scale cultivation and increasing the concentration of bacterium microencapsulated in calcium alginate beads.

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Figures

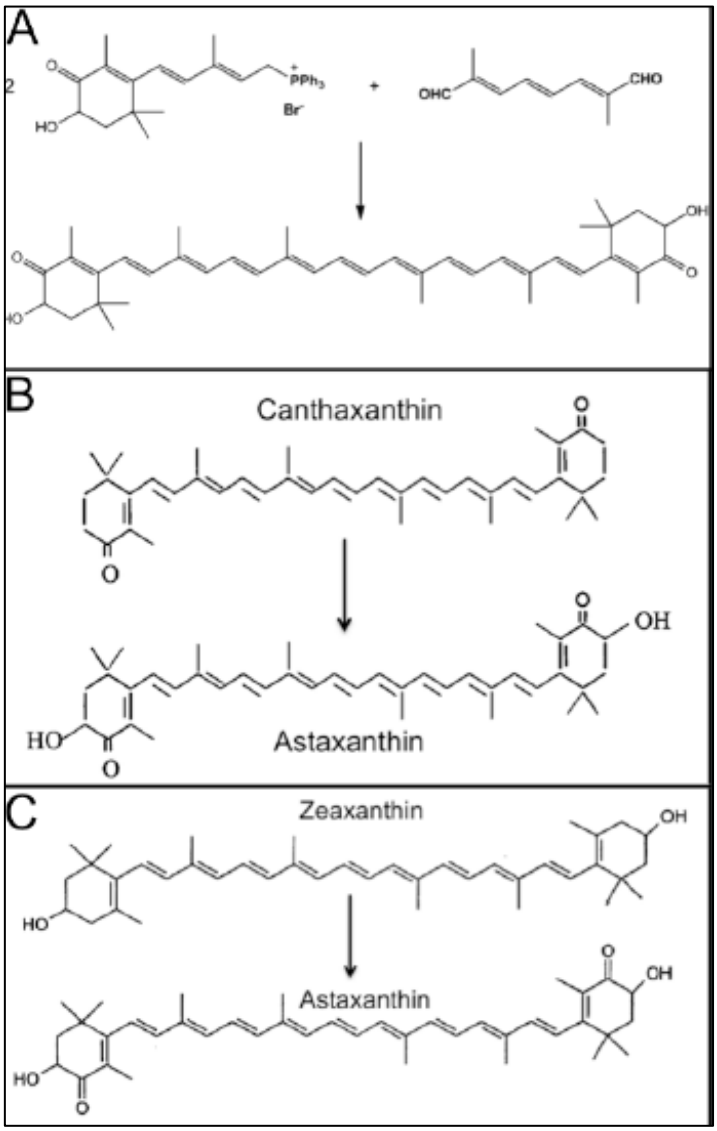

Figure 1 – Synthetic reactions to produce astaxanthin. **A.** Wittig reaction. **B.** Canthaxanthin hydroxylation. **C.** Zeaxanthin oxidation (taken from Nguyen, 2013).

Tables

Table 1 - Summary of fixed and operational costs for 1 month

	Unit (g)	Unit costs (R)	R/g	Total needed (g)	Cost (R)
				84 L	
Media components					
Yeast extract	500	344.28	0.69	420	194.91
	2500	2736	1.09		
	5000	3442.8	0.69		
	25000	11601.78	0.46		
Bacteriological peptone	500	906.3	1.81	840	1522.58
	1000	3239.88	3.24		
NaCl	500	79.8	0.16	2520	170.93
	5000	339.15	0.07		
Microencapsulation in calcium alginate					
				5.6 L	
Sodium alginate	1000	1915.2	1.92	112	168.54
	5000	7524	1.50		
$CaCl_2$	500	142.5	0.29	112	31.92
	1000	1774.2	1.77		
	5000	3399.48	0.68		
Equipment					
	kW	**h/week**	**R/kWh**		**Cost (R)**
Bench-top shaker	1.1	120	1.24		654.72
Centrifuge	5.52	2			54.76
Magnetic stirrer	0.698	6			20.77
Laminar flow cabinet	0.525	36			93.74
				R Total	**2912.88**

Green highlighted rows are the cheapest in R/g and was used in further calculations
* Total of 210 g calcium alginate beads produced
** 420 hens can be fed with 210 g of calcium alginate beads