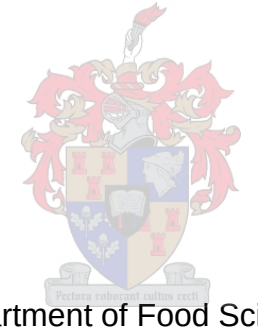
Effect of functional enzyme additives on the sensory and shelf-life properties of ready-to-eat breakfast cereals

by

Gaynor Adéle Daniels

Thesis presented in partial fulfilment of the requirements for the degree of

Master of Science in Food Science



Department of Food Science

Faculty of AgriSciences

Stellenbosch University

Supervisors

Prof. G.O. Sigge, Department of Food Science, Stellenbosch University
M. Muller, Department of Food Science, Stellenbosch University

April 2019

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2019

ACKOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to our Almighty God for granting me the opportunity to undertake a learning journey that will remain with me for the rest of my life.

To my husband, thank you for your love and encouragement. Thank you for all your invaluable support.

I am very fortunate to have had leaders that were supportive throughout the process of writing this report. Thank you to Professor Gunnar Sigge for always having the time and your most invaluable input. To Nina Muller, thank you for your kindness and motivation with my journey. You have guided me through this study of how to get to the finish line, but most of all, your guidance on how to deal with life itself and becoming my mentor, will forever stay in my heart.

Dr Greta Geldenhuys, thank you for your support and motivation in completing the sensory analysis of the study.

Ms Marieta van der Rijst, thank you for conducting the statistical analysis numerous times to complete the results.

Thank you to the staff from the sensory laboratory for their assistance in the sensory setup and testing. It is highly appreciated.

The financial support from Pioneer Foods is greatly appreciated.

Thank you to DSM for their invaluable technical support and for providing the samples for the products design.

NOTES

This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of two research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a chapter for elaborating a general discussion, recommendations and conclusions.

Language, style and format of referencing used are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable. Minor formatting changes have thus been made throughout the thesis to ensure consistency.

SUMMARY

The aim of this study was to test the sensory quality of a ready-to-eat breakfast cereal (RTEBC) fortified with two functional enzymes additives, i.e. Tolerase P® (TolP) and Tolerase L® (TolL), as well as the viability of these enzymes over a shelf-life period of 12 months. The purpose was also to establish whether fortification with Tolerase P® could enhance the mineral quality and, when using Tolerase L®, to determine the level of functional enzyme additives available to digest lactose for lactose intolerant individuals.

Three independent experimental batches of a RTEBC (100 kg per batch) were produced, and sub-samples of each of three batches were treated with TolL, with TolP and the third sub-sample was left untreated and thus functioned as a control. When comparing the two treated samples with the unfortified RTEBC in terms of full sensory profile (aroma, flavour and mouthfeel attributes) over the shelf-life period, the sensory profile of the TolL- and TolP-treated cereals were found to be comparable to that of the untreated control sample at the onset of shelf-life, as well as after 12 months of shelf-life. The sensory panel found that the fortified cereal samples did not differ significantly ($P>0.05$) from the unfortified cereal sample for the majority of the sensory characteristics, i.e. coarseness and toasted colour (dry cereal attributes) and toasted colour, malted aroma, toasted cereal flavour, sweet taste, bitter taste and mouthfeel (cereal attributes with added milk), indicating that the respective enzymes did not impact negatively on the sensory profile of this RTEBC during shelf-life. The minerals calcium, zinc and iron of the RTEBC fortified with TolP increased with 43.3%, 49.7% and 56.7%, respectively. This indicated the effectiveness of the functional enzyme TolP in the ready-to-eat breakfast cereal prior to consumption. Furthermore, the TolP content remained viable for a shelf-life period of 12 months. Based on the percentage activity of the functional enzyme in the base material, the enzyme activity of the TolL-treated RTEBC was found to be fully effective for a 9-month shelf-life period.

These results will find invaluable application in product development and marketing of RTEBC. The results will also add significantly to scientific information regarding the feasibility of fortification of RTEBC, specifically the effectiveness of functional enzyme additives for lactose intolerant individuals or the provision of sustained mineral nutrition for individuals that consume RTEBC on a regular basis.

OPSOMMING

The doel van die studie was om die sensoriese kwaliteit van ‘n kommersiële klaar-gaar ontbytgraan (KGOG), gefortifiseer met twee funksionele ensieme (Tolerase P® [TolP] en Tolerase L® [TolL]), te toets, asook om die effektiwiteit van die onderskeie ensieme tydens ‘n 12-maande rakleeftydstudie te bepaal. Verdere oogmerke was om vas te stel of TolP-fortifikasie die mineraalinhoud van die KGOG sou verhoog en of TolL-fortifikasie tot genoegsame funksionele ensiemvlakke sal lei om laktose effektief te verteer in laktose-intolerante individue.

Drie onafhanklike eksperimentele produksielotte KGOG is vir die studie geproduseer (100 kg per lot), waarna elke lot in drie gedeel is. Die eerste sub-lot is met die funksionele ensiem TolL behandel en die tweede sub-lot met TolP. Die derde sub-lot is met geen ensiem behandel nie en het dus gedien as ‘n kontrole. Na ‘n rakleeftydstudie van 12 maande het beide die behandelde en onbehandelde behandelings nie betekenisvol ($P>0.05$) van mekaar verskil in terme van sensoriese kwaliteit (growwe voorkoms en geroosterde kleur van die droë ontbytgraan, asook geroosterde kleur, mout aroma, geroosterde geur, soet smaak, bitter smaak en mondgevoel van die graan-melk mengsel) op maand 0 en maand 12 van die rakleeftydstudie nie. Die onderskeie ensieme het dus geen betekenisvolle effek op die sensoriese profiel van die KGOG tydens die 12-maande rakleeftydstudie gehad nie.

Die mineraalinhoud, dit is die kalsium-, sink- en ysterinhoud van KGOG gefortifiseer met TolP het onderskeidelik met 43.3%, 49.7% en 56.7% toegeneem. Hierdie verhoging van mineraalinhoud het die effektiwiteit van die funksionele ensiem TolP in KGOG effektief geïllustreer, veral gegewe die feit dat hierdie verhoging oor die 12-maande rakleeftyd standhoudend was. Gebasseer op die persentasie aktiwiteit van die funksionele ensiem TolL in die basismateriaal, is gevind dat die ensiemaktiwiteit van TolL ten volle funksioneel was vir nege maande van die totale 12-maande periode.

Hierdie resultate sal beslis betekenisvolle toepassing kan vind in produkontwikkeling en bemarking van KGOG. Dit sal ook effektief kan bydra tot die ontwikkeling van gefortifiseerde KGOG vir individue wat laktose-intolerant is of individue wat graag ‘n standhoudende mineraalinhoud-inname wil verseker tydens gereelde inname van KGOG.

TABLE OF CONTENTS

CHAPTER 1

Introduction

Globally consumers are increasingly aware of the importance of foods that they consume for breakfast. Ready-to-eat breakfast cereals (RTEBC) make a viable contribution towards the daily nutrition of children, adolescents and adults (Kent, 1983; Goglia *et al.,* 2010). The RTEBC's are classified as convenience foods, primarily due to the ease of preparation, making it extremely popular among breakfast cereal consuming individuals. Because of this increased popularity of RTEBC, there has been an increase in research to examine the nutritional quality thereof (Schwartz *et al.,* 2008). The consumption of breakfast cereals in South Africa has increased by more than 42.9% since 1999, both for RTEBC and breakfast cereals that require further preparation (Ronquest-Ross *et al.,* 2015). Although there is a lack of information available on the full nutritional quality of South African RTEBC, the breakfast cereal industry is becoming more competitive in providing sustainable nutrition to consumers.

There is currently an increased interest in the development and marketing of food products with nutrient-enhancing properties, i.e. food products that can improve the health and well-being of consumers (Yao *et al.,* 2011). According to consumer research results of the International Food Information Council (IFIC) of the United States of America (USA), the media, health professionals, family and friends are regarded as important sources of information on foods and food ingredients that can promote health (IFIC, 2011). Consumer's interest in the link between diet and health has increased consumer's interest in functional foods. Nutrient labelling is a viable form of informing consumers about health issues, particularly on how to sustain a healthy lifestyle. In South Africa the Department of Health (DOH) regulates the use of health claims on food labels, making it difficult for food manufacturing companies to inform consumers about food products with added functional properties (DOH, 2010).

In 2012 lactose intolerance was reported to be common in 78% Black South Africans (Labuschagne & Lombard, 2012). According to the Food and Allergy Consulting and Testing Services (FACTS, Cape Town, South Africa), >90% of the South African black population is lactose intolerant, 20-40% of the coloured population and only 20% of white South Africans (Personal communication, 2017, H. Steinman, Food and Allergy Consulting and Testing Services, Cape Town, South Africa). In the USA, the increase in the production of lactose-free dairy products can be ascribed to a shift towards organic food products (Reportlinker, 2018). In Europe there has been a decline in the consumption of cow's milk, most probably

as a result of the increased focus on lactose intolerance and symptoms associated with this disease. In a study to assess the consumption of milk and dairy products amongst Italian consumers in a specific region, it was found that 22% of the individuals tested do not drink milk, whilst 18.1% consume lactose-free milk. This study also found that consumers regard lactose-free milk as quite expensive, especially low income households (Zingone *et al.,* 2017). Apart from the production of lactose-free milk, the pharmaceutical industry has expanded on the production of lactase supplements for lactose intolerant individuals. An example of the latter is exogenous ß-galactosidase, an enzyme that is free of side effects and ideal to be added to a product during production or to be ingested as a supplement at meal-times (Ojetti *et al.,* 2010). There is thus a demand in the cereal industry for products with added functional enzyme additives such as lactase. In view of this, there is a potential for new product development with lactase supplementation, in particular an enzyme such as Tolerase L®, a lactose-degrading enzyme, which can be added to food as a functional ingredient (DSM Technologies, Heerlen, The Netherlands).

Specific mineral deficiencies are quite common in developing countries, however, some mineral deficiencies may also occur in developed countries where a diet high in fibre is the norm (Lopez *et al.,* 2002). Breakfast cereals, especially those produced from whole grains, are a valuable source of micronutrients (Coulibaly *et al.,* 2011). The minerals of importance in breakfast cereals are iron (Fe), zinc (Zn), calcium (Ca), magnesium (Mg) and phosporus (P) (Jacela *et al*., 2010). Iron deficiency is regarded as the most common micronutrient deficiency in developed and developing countries (Minihane & Rimbach, 2002). Most whole grain breakfast cereals furthermore contain phytic acid. Phytic acid, also known as inositol hexakisphosphate (IP_6), is the storage form of phosphorus in whole grains. A major disadvantage of phytic acid is that it binds the above-mentioned essential minerals, hampering their availability during absorption in the gastrointestinal tract (Coulibaly *et al.,* 2011). Over the past few years, public health authorities have become increasingly concerned about the nutritional quality of processed foods, particularly ready-to-eat breakfast cereals (Webster *et al.,* 2010). In a large, national food consumption survey conducted in South Africa in the 1990's, it was indicated that a large proportion of children aged 1-9 years consume food items such as maize, sugar, tea, whole milk and brown bread on a daily basis (Labadarios *et al.,* 1999). In this study dietary intake data indicated that these children's intake of calcium, iron, zinc, selenium, vitamins A, D, C and E, riboflavin, niacin, vitamin B_6, and folic acid was well below the recommended nutrient reference values (Labadarios *et al.,* 1999), indicating that there is a demand for functional enzyme additive fortification of RTEBC produced from whole grains, primarily to increase the bioavailability

of specific minerals. Tolerase P® (phytases) is a commercial enzyme produced for this purpose, i.e. to enhance the functional properties of RTEBC (DSM Technologies, Heerlen, The Netherlands).

Breakfast cereals are regarded as suitable vehicles to address issues on nutrient bioavailability. Breakfast cereals contribute approximately 46% of the energy intake in Africa, a continent where mineral nutrition is a significant concern (Blanco-Rojo & Vaquero, 2018). Fortification is convenient and could add to a healthy diet. In the South African food industry, the only product launched with additional functional ingredients is *Future Life® bran flakes.* This packaged breakfast cereal includes ten sachets of probiotics that the consumer can manually add to the cereal before consuming the product (FutureLife®, Durban, South Africa).

In view of the above, the aim of this study was to test the sensory and chemical quality of a ready-to-eat breakfast cereal fortified with two functional enzymes additives, Tolerase P® and Tolerase L®, as well as the viability of these enzymes on a month-to-month basis over a shelf-life period of 12 months. The aim was also to establish whether the fortification process with Tolerase P® could enhance the mineral quality of the final product and, when using Tolerase L®, to produce a ready-to-eat breakfast cereal that lactose intolerant consumers can consume with regular milk.

REFERENCES

Blanco-Rojo, R. & Vaquero, M.P. (2018). Iron bioavailability from food fortification to precision nutrition. A review. *Innovative Food Science and Emerging Technologies,* In press, doi.org/10.1016/j.ifset.2018.04.015.

Coulibaly, A., Kouakou, B. & Chen, J. (2011). Phytic acid in cereal grains: Structure, healthy or harmful ways to reduce phytic acid in cereal grains and their effects on nutritional quality. *American Journal of Plant Nutrition,* **1**, 1-22.

DOH (2010). Regulation 146 of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 [Act 54 of 1972]. Department of Health, Pretoria, South Africa.

Goglia, R., Spiteri, M., Ménard, C. & Volatier, J.L. (2010). Nutritional quality and labelling of ready-to-eat breakfast cereal: the contribution of French observatory of food quality. *European Journal of Clinical Nutrition.* **64**, S20-S25.

IFIC (2011). Functional foods. International Food Information Council Foundation, Washington, United States of America. http://foodinsight.org. Accessed September 2017.

Jacela, J.Y., DeRouchey, J.M., Tokach, M.D., Goodband, R.D., Nelssen, J.L., Renter, D.G. & Dritz, S.S. (2010). Feed additives for swine: Fact sheets - prebiotics and probiotics and phytogenics. *Journal of Swine Health and Production*, **18**, 87-91.

Kent, N.L. (1983). Technology of cereals. Pergamon Press, Oxford, UK.

Labadarios, D., Steyn, N.P., Maunder, E., MacIntryre, M., Gericke, G. & Swart, R. (1999). A National Food Consumption Survey (NFCS): South Africa. *Public Health Nutrition*, **8**, 533-543.

Labuschagne, I.L & Lombard, M.J. (2012). Understanding lactose intolerance and the dietary management thereof. *South African Family Practice,* **54**, 496-498.

Lopez, H.W., Duclos, V., Coudray, C. & Remesy, C. (2002). Minerals and phytic acid interactions: Is it a real problem? *International Journal of Food Science and Technology.* **37**, 727-739.

Minihane, A.M. & Rimbach, G. (2002). Iron absorption and the iron binding and antioxidant properties of phytic acid. *International Journal of Food Science and Technology.* **37**, 741-748.

Ojetti, V., Gigante, G., Gabrielli, M., Ainora, M.E., Mannocci, A., Lauritano, E.E., Gasbarrini, G. Y Gasbarrini, A. (2010). The effect of oral supplementation with *Lactobacillus reuteri* or tilactase in lactose intolerant patients. *European Review for Medical and Pharmacological Sciences*, **14**, 163-170.

Reportlinker (2018). Lactose free dairy products market (Global industry analysis (2012-2016) and opportunity assessment (2017-2027). New York, USA. https://www.prnewswire.com/news-releases/lactose-free-dairy-products-market-global-industry-analysis-2012---2016-and-opportunity-assessment-2017---2027-300677703.html

Ronquest-Ross, L.C., Vink, N. & Sigge, G.O. (2015). Food consumption changes in South Africa since 1994. *South African Journal of Science,* **10***,* 1-12.

Schwartz, M.B., Vartanian, L.R. & Wharton, C.M. (2008). Examining the nutritional quality of breakfast cereals marketed to children. *Journal of American Diet Association,* **108**, 702-705.

Webster, L.J., Dunford, K.E. & Neal, C.B. (2010). A systematic survey of the sodium contents of processed foods. *American Journal of Clinical Nutrition,* **91**, 413-420.

Yao, N., White, P.J. & Alavi, S. (2011). Impact of β-Glucan and other oat flour components on the physio-chemical and sensory properties of extruded oat cereal. *Journal of Food Science and Technology.* **46**, 651-660.

Zingone, F., Bucci, C., Lovino, P. & Ciacci, C. (2017). Consumption of milk and dairy products: Facts and figures. *Nutrition,* **33,** 322-325.

CHAPTER 2

Literature Review

1. BREAKFAST CEREAL INDUSTRY

In developing countries, particularly sub-Saharan Africa, local staple foods, i.e. cereals, legumes, cassava and potatoes are used to produce breakfast foods. According to Kent (1983) the most commonly eaten breakfast foods are cereals. Breakfast cereals can be classified as *hot breakfast cereals* and *cold breakfast cereals*. Hot breakfast cereals require some form of further cooking before consumption, whereas cold breakfast cereals (*ready-to-eat)* are usually consumed with the addition of milk (Tribelhorn, 1991). Hot cereals include porridge-type cereals, generally of maize or oat origin, however, sorghum-based porridges are also quite popular (BMI, 2012). Generally, cold breakfast cereals would come with whole-grain cereals, high-fibre cereals or pre-sweetened cereals. Cereals for children also form part of the cold breakfast cereal class (Grand View Research, 2018).

The South African breakfast cereal industry has been growing steadily since 1994 (BMI, 2012), with hot cereals currently holding 52.8% of the market and cold cereals 47.2% (Fig. 1). Furthermore, the Gauteng province consumes more breakfast cereal per annum than any of the other provinces in South Africa (Fig. 2). The fact that cold cereals, or so-called ready-to-eat cereals, form approximately 47% of the total breakfast cereal market can be attributed to many of factors, primarily convenience, but also the fact that cereals add significantly to a healthy diet (BMI, 2012).

A well-balanced breakfast adds significantly to adequate nutrient intake, as illustrated in numerous studies (Yan Want *et al.,* 1992). Should breakfast be skipped, food intake during the remainder of the day may be insufficient to meet the recommended daily nutrient reference values, especially that of micronutrients, vitamins and minerals, as well the important macronutrients such as fibre (Preziosi *et al.,* 1999).

Consumers tend to prefer a healthy lifestyle at breakfast (Kowtaluk, 2001; Mayo Clinic, 2009), thus contributing positively to research and development of breakfast cereals (BMI, 2012). According to the Global Breakfast Cereal Strategic Report (2017), there is a growing demand for healthy, natural and organic breakfast cereals (GBCS, 2017). In 2016 and 2017 numerous breakfast cereal manufacturing companies joined forces to combat the struggle against malnutrition in children. This constructive initiative was led by Future Life®, which supplied 650,000 meals to schoolchildren across South Africa. This product illustrated

innovative packaging designed to optimise the transport, storage, preparation and consumption of nutritious children's breakfast meals. This initiative consisted of a three-pillar approach to address the challenge of sustainability, whilst simultaneously tackling the broader societal issues of education and malnutrition (Insight Survey, 2017). This initiative also demonstrated that there is a demand in South Africa for sustainable and nutritious breakfast cereals.

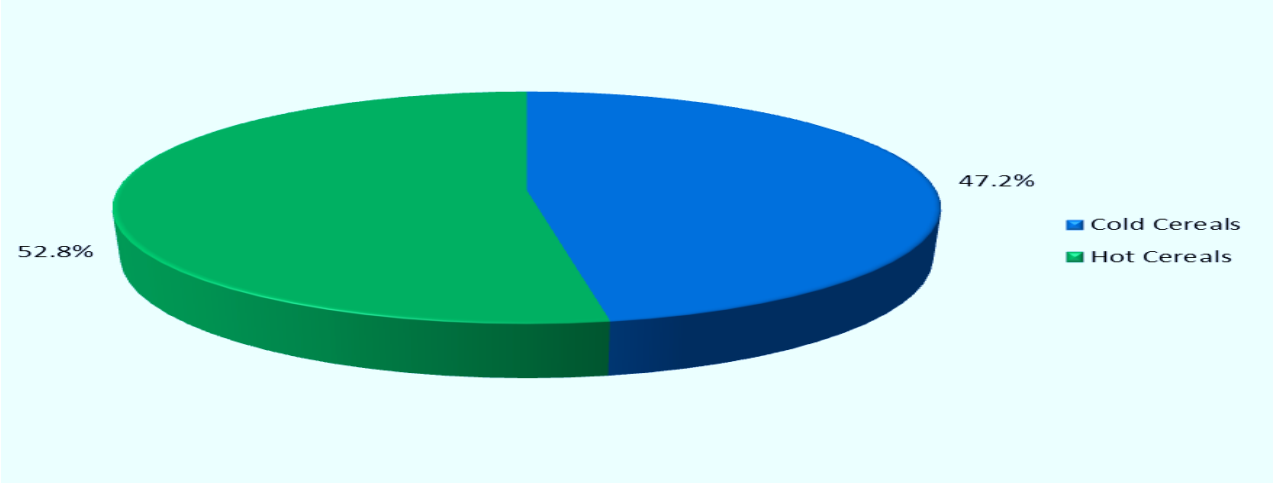


Figure 1 Market volume of hot and cold cereals in South Africa (BMI, 2012).

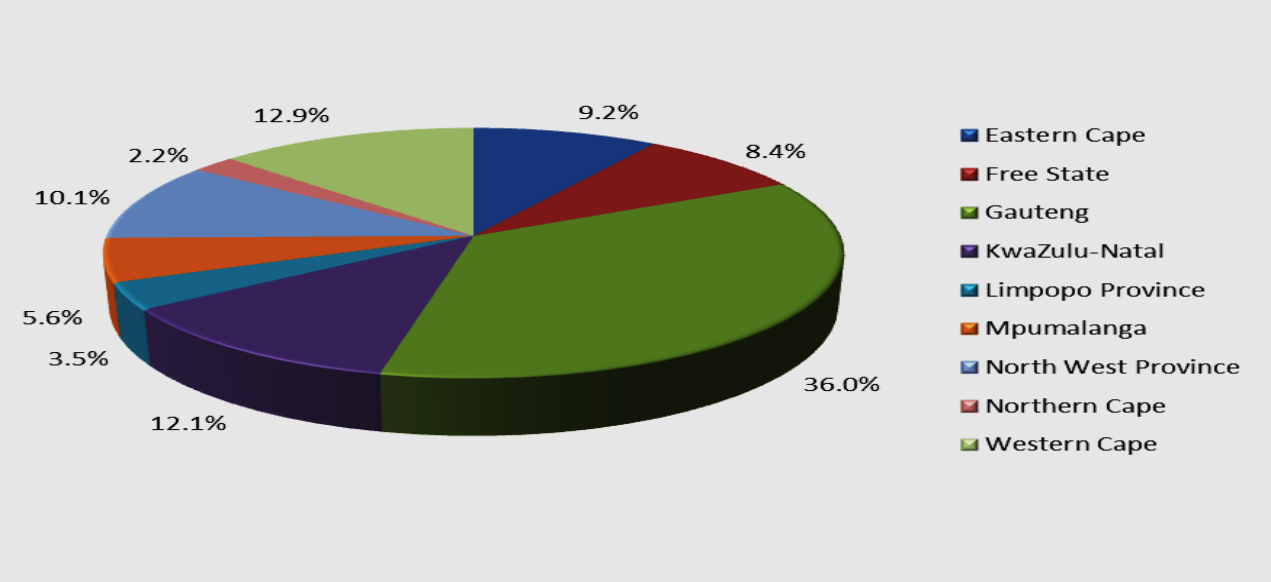


Figure 2 Regional distribution of breakfast foods in South Africa (BMI, 2012).

Ready-to-eat breakfast cereals served with milk are ideal vehicles for including functional enzyme additives such as lactases. Tolerase L®, a lactase functional enzyme additive, can be included in ready-to-eat breakfast cereal formulations in order for

consumers to enjoy the benefits of using regular milk for breakfast. Tolerase L® breaks down lactose in regular milk to benefit the lactose-intolerant consumer (DSM Technologies, Heerlen, The Netherlands).

Since 2003 it has been mandatory in South Africa for all bread flours and maize to be fortified with minerals and vitamin premixes to aid in malnutrition (Department of Health, 2003), thus making food fortification or food enrichment ideal for RTEBC, i.e. the process of adding micronutrients, i.e. minerals and vitamins, during food processing. Alternatively, a functional enzyme additive such as Tolerase P® could be used in cereal formulations to break down the phytic acid present in cereals, thereby releasing important minerals, such as iron, zinc and calcium (Gibson *et al*., 2010).

It is evident that there is enormous potential to develop ready-to-eat breakfast cereal products with added functional enzyme additives for the South African food industry. The challenges ahead for manufacturers of ready-to-eat breakfast cereal products with added functional enzyme additives include the labelling aspect thereof and how the product should be marketed to the consumer. The South African labelling regulations limit the extent to which the consumer can be persuaded to purchase a product. Furthermore, health claims are strongly regulated by the Department of Health, South Africa. The regulation on the labelling of foodstuffs (Department of Health, R146, 2010), under the division of general provisions, states that “the following information or declarations shall not be reflected on a label or advertisement of a foodstuff: the words "health" or "healthy" or other words or symbols implying that the foodstuff in and of itself or a substance of the foodstuff has health-giving properties in any manner including the name or trade name, except in the case of the fortification logo for food vehicles as determined by regulations made under the Act and regulation 51(2)” (Department of Health, R146, 2010).

There is thus a major opportunity in the market to develop affordable ready-to-eat breakfast cereals with functional enzyme additives that could breakdown phytic acid in cereals to provide adequate mineral nutrition or to digest lactose in milk when added to breakfast cereals (Ojetti *et al.,* 2010). The availability of lactose-free products in the food industry is reasonably limited. Although lactose-free milk is available to lactose intolerant consumers, some consumers prefer to use lactase supplements in order to consume a variety of dairy products that contain lactose instead of purchasing lactose-free milk, which is 50% more expensive than regular milk (Personal communication, 2017, M. Tredoux, Functional enzyme additives applications technologist, DSM Technologies (PTY) Ltd., Johannesburg, South Africa).

The purpose of this literature review is to provide the reader with a broad overview of the ready-to eat breakfast cereal industry and how industry addresses the challenges of the improvement of nutritional health. It should also provide the reader with some insight into the possibility of developing new products with added functional properties, not just for the ready-to-eat breakfast cereal industry, but for a range of different products such as "on-the-go" snacks. This literature review will provide insight into the concerns regarding lactose intolerance and associated clinical aspects. Phytic acid, naturally occurring in cereals, will also be discussed, particularly how mineral bioavailability is affected.

2. LACTOSE INTOLERANCE – PREVALENCE, GENETIC FACTORS AND LACTASE ENZYMES

2.1 Introduction

Breakfast cereals, especially ready-to-eat cereals are usually consumed with the addition of milk. Lactose, the main sugar present in milk, can be regarded as a problem for lactose-intolerant consumers. Seventy percent of the global population is lactose intolerant, if undiagnosed it can easily result in illness (Matthews *et al.,* 2005). In the United States of America (USA) the estimated number of individuals affected by lactose intolerance range between 30 and 50 million (NDDIC, 2005), whereas an estimated 75 million Americans have low intestinal lactase activity.

Lactose, a disaccharide present in mammalian milk, is vital for the nourishment of new-born babies (Matter *et al.,* 2012). Cow's milk also contains lactose and is regarded as a vital source of calcium for individuals of four years and older (Matter *et al.,* 2012). Lactose is hydrolysed by lactase into digestible sugars, glucose and galactose. The villi of the small intestine have cells, known as enterocytes, which are able to absorb these glycemic sugars (Swallow, 2003). Glucose and galactose are absorbed by the enterocytes using a specific transporter molecule. Low lactase activity can result in poor lactose digestion. Gastrointestinal problems develop if the ability of the gastrointestinal (GI) tract to digest lactose is not enabled with the presence of lactase. Lactose intolerance can be caused by a number of dietary factors, including the amount of lactose available, the amount of time it takes for the food to pass through the GI tract, beta-galactosidase consumed together with lactose (as in yoghurt) and regular dairy consumption. The diagnosis of lactose intolerance can be ascertained by using a breath-hydrogen or lactose-intolerance test (Matthews *et al.,* 2005). Treatment usually entails the avoidance of lactose-containing foods, consumption of

lactose-free foods or foods containing a functional ingredient, i.e. lactase enzymes added to foods (Enattah *et al.,* 2007).

2.2 Symptoms of lactose intolerance

Ineffective digestion of lactose can lead to symptoms of lactose intolerance, limiting the consumption of fresh milk (Swallow, 2003). Undigested lactose travels to the small intestine and large intestine, resulting in the emergence of symptoms of lactose intolerance (Matthews *et al.,* 2005). When lactose is not digested in the small intestine, it will pass through to the colon. In the colon lactose is fermented by colonic microorganisms, forming short chain fatty acids, as well as hydrogen, and potentially also methane and carbon dioxide (He *et al.,* 2006). The most common symptoms of lactose intolerance include abdominal pain, bloating, diarrhea and occasionally vomiting (Matthews *et al.,* 2005). Undigested lactose tends to increase the intestinal osmotic pressure and this draws electrolytes and water into the intestinal lumen, resulting in delayed digestion time and subsequently a loose stool (Heyman, 2006). Symptoms of lactose intolerance usually occur 30 min to 2 h after consuming lactose-containing foodstuffs (Rusynyk & Still, 2001). Table 1 indicates strategies for lactose-sensitive patients, i.e. strategies to minimise or avoid symptoms of lactose-intolerance.

2.3 Diagnosis of lactose intolerance

Early studies on the detection of poor milk sugar (lactose) digestion included the measuring of blood sugar levels after ingesting 50 g of lactose. A significant increase in blood glucose levels after 30 min would indicate high levels of lactose (Gugatschka *et al.,* 2005). More recently, lactase activity has been measured using intestine biopsies, however, this method is less sensitive than the lactose hydrogen breath test (Portincasa *et al*., 2008). The latter technique is presently regarded as the most reliable measure of lactose maldigestion (Shaw & Davies, 1999). The lactose hydrogen breath test involves taking 50 g lactose and measuring breath hydrogen levels over a period of 3 to 6 h, with <20 ppm of H_2 indicating lactose intolerance (Matthews *et al.,* 2005). There are quicker and easier methods of detecting the lactase gene such as genotyping, using real-time polymerase chain reaction assay (PCR). However, the latter method is not generally available in clinical practice (Gugatschka *et al.,* 2005)

Table 1 Therapeutic strategies and dietary management of lactose intolerance (Adapted from Brown-Esters *et al.,* 2012)

Factors affecting lactose digestion	Dietary management
Lactose dosage	Consume no more than a cup of milk at a time (12 g lactose)
Adaptation	Consuming lactose-containing foods on a daily basis enables colonic bacteria to develop increased ability to ferment lactose
Factors influencing gastrointestinal transit	Consume milk with meals rather than on its own
Yoghurt and other alternatives	Consume yoghurt with live cultures; lactose is in a digestible format. Hard cheeses are also better tolerated

2.4 Lactose intolerance: prevalence and types

In certain populations, high levels of lactase activity are maintained during adulthood (lactase persistence) (Matthews *et al.,* 2005). Although lactase non-persistence is the more common human phenotype, lactase persistence is believed to have occurred because of a selection process in the last 10,000 years, thereby sustaining dairy consumption in certain populations (Matthews *et al.,* 2005). Recent interest in lactase non-persistence and lactase persistence has focused predominantly on the molecular biological mechanisms regulating the maintenance or decline of intestinal lactase gene expression.

Enattah *et al.* (2002) reported the identification of single nucleotide polymorphisms (SNPs), -13910*C/T and -22018*G/A, upstream of the lactase phlorizin hydrolase (LCT) gene locus, which are in turn associated with lactase non-persistence or lactase persistence in Finnish families. The -13910*C/T and -22018*G/A SNPs are located within intron 13 and intron 9, respectively, of the adjacent MCM6 gene on chromosome 2q21. Furthermore, the -13910*T allele has shown to enhance transcription of lactase gene promoter-luciferase reporter constructs in intestinal $CaC0^{-2}$ cells (Olds & Sibley, 2003). Additional reports have stated that the -13910*T allele correlates well with lactase persistence in European individuals (Matter *et al.,* 2012).

Recently discovered polymorphisms, -3712*T/C, -13907*C/G, -13913*T/C, -13915*T/C, and -14010*G/G, associated with lactase non-persistence and lactase persistence, have recently been recognised in African and Saudi Arabian populations (Ingram *et al.,* 2009). The -13907*G, -13915*G and -14010*G/G were extensively found among African people, whereas the -13913*C was seldom found (Ingram *et al.,* 2007). The -13915*G and -3712*C variants were identified in Saudi Arabian populations (Enattah *et al.,* 2007). Furthermore, investigative efforts have focused on clarifying whether the various

lactase single nucleotide polymorphisms (SNPs) function to regulate lactase non-persistence and lactase persistence in adulthood. These molecular mechanisms have not been described fully.

A large percentage (>70%) of the global population have been diagnosed with lactase non-persistence, but not all individuals are intolerant to lactose as a number of nutritional and genetic factors play a role (Cavalli-Sforza, 1973). In some Asian countries almost 100% of all individuals are regarded as lactose intolerant, whereas in South America and Africa >50% of individuals are classified as being lactase non-persistent (De Vrese *et al.,* 2001). These global tendencies are shown in Fig. 3, illustrating that lactase non-persistence is regarded as the most common phenotype in humans (~70%) (Ingram *et al.,* 2009).

In subjects of mixed ethnicity, a lower prevalence of lactase non-persistence is observed, whereas a more regular prevalence is detected in singular ethnic populations (Johnson, 1981). The rate at which the lactase activity declines also varies according to ethnicity (Sahi *et al.,* 1983). It may take up to 18-20 years for Northern Europeans to reach low levels of lactase activity, whereas the Chinese and Japanese lose 80-90% of lactase activity within 3 to 4 years after weaning and Israelis and Asians lose 60-70% after >5 years after weaning (Matthews *et al.,* 2005).

Hypolactasia or lactase deficiency exists in three distinct forms, namely *primary lactase deficiency, secondary lactase deficiency* and lastly *congenital lactase deficiency.* Humans with extremely low lactase activity is diagnosed with congenital lactase activity. With only 40 cases reported to date, congenital lactase deficiency is an extremely rare form of lactose intolerance (Swallow, 2003). However, in all other cases, lactose intolerance is a lifelong disorder starting at the rejection of breast milk by an infant's digestive system at the first introduction to breast milk. Primary lactase deficiency, also referred to as adult-type hypolactasia, lactose maldigestion or lactase non-persistence (Heyman, 2006), occurs in the majority of lactose intolerant individuals, approximately 70-75% (Lomer *et al.,* 2007) where the function of the lactase enzyme is lost between the ages of 3 to 5 (Moore, 2003). The inability to digest lactose is classified as a normal physiological condition involving the function and activities of the human body (McBean & Miller, 1998; Stephenson & Latham, 1974; Swagerty *et al.,* 2002). Some individuals can, however, consume milk and dairy products without developing symptoms of discomfort, and they are thus not classified as lactose intolerant (Moore, 2003). Thus, the prevalence indicator of lactose intolerance in the world, as seen in Table 2, is not an accurate indicator due to the fact that most people with lactose intolerance can ingest lactose without experiencing intolerance symptoms (Johnson *et al.,* 1993; Suarez *et al.,* 1997; Moore, 2003).

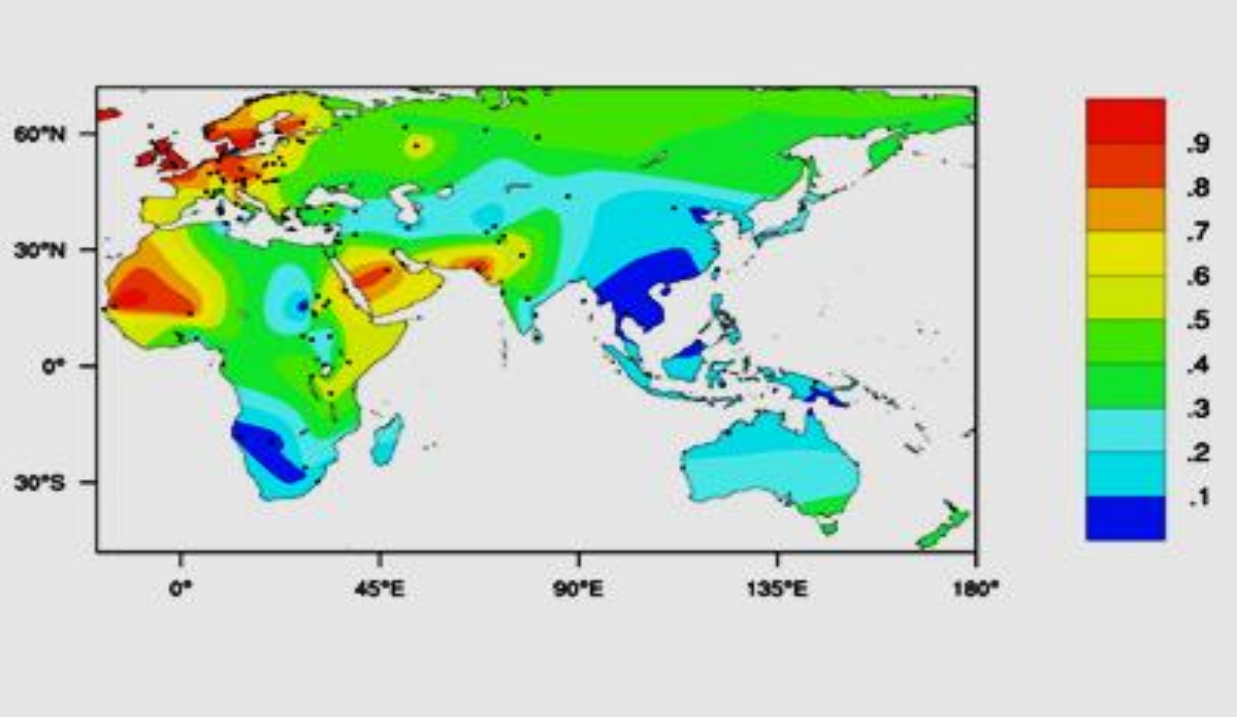


Figure 3 Worldwide frequency of lactase persistence, as assessed by lactose tolerance tests. Dots represent the data collections locations. The respective colours indicate frequencies of the lactase persistence phenotype (Adapted from Ingram *et al.,* 2009).

Table 2 Prevalence of primary lactase deficiency in various ethnic groups (Adapted from Swagerty *et al.,* 2002)

Group	Prevalence (%)
Northern Europeans	2-15
American whites	6-22
Central Europeans	9-23
Northern Indians	20-30
Southern Indians	60-70
Hispanics	50-80
Ashkenazi Jews	60-80
Blacks	60-80
American Indians	80-100
Asians	95-100

It has been postulated that lactase persistence in the remaining 25-30% of the global population is a result of genetic mutations, occurring thousands of years ago in communities where dairy products formed a significant part of their daily food intake (Simoons, 1978). This evolutionary advantage (McCracken, 1970) allowed communities to rely on milk as source of protein during poor harvest times (Simoons, 1978; Kretchmer, 1972; Beja-Pereira *et al.,* 2003).

Secondary lactase deficiency is the second form of lactase deficiency that occurs because of damage to the lining of the small intestine. This deficiency, usually the result of medication, irritable bowel syndrome, surgery or radiation therapy, is not permanent and can be over-turned with time (Savaiano & Levitt, 1987; Scrimshaw & Murray, 1988; Srinivason & Minocha, 1998).

2.5 Lactase enzymes: origin and properties

Lactase, present in the brush-border membrane of the intestinal absorptive cells, i.e. enterocytes, are responsible for the hydrolysis of the disaccharide lactose (Olds *et al.,* 2011). Lactase is a large glycoprotein with two active sites that is able to catalyse the hydrolysis of a variety of β-glucosides, including phlorizin, flavonoid glucosides (Nemeth *et al.,* 2003), as well as pyridoxine-5-β-D glucosides and β-galactosides in addition to lactose (Mackey *et al.,* 2002).

Lactase enzyme communication via the enterocytes takes place in the small intestine. Lactase is encoded by a single gene, LCT (lactase gene), i.e. a gene on chromosome 2q21 (Mantei et al., 1988; Harvey *et al.,* 1995). Lactase is primarily expressed in the jejunum area of the gastrointestinal tract (GI), i.e. similar to that of another digestive hydrolase enzyme,

sucrose-isomaltase (Newcomer *et al.,* 1978). During pregnancy lactase is expressed at low levels, whereas sucrose is expressed at high levels in the small intestine of early fetal life (Wang *et al.,* 1994). Humans are born with high levels of lactase expression, resulting in lactase persistence throughout adult life, i.e. a continued lactase activity (Swallow, 2003). However, genetically the process of lactase transcription can be reduced after weaning. This usually results in lessened production of lactase in the small intestine, and thus lactase non-persistance after weaning and during adult life, resulting in hypolactasia. Lactase persistence usually involves high levels of mRNA expression, resulting in continued lactase activity throughout adulthood, whereas lactase non-persistance involves low mRNA expression, usually resulting in low lactase activity that is typical of individuals suffering from lactose intolerance (Escher *et al.,* 1992; Sebastio *et al.,* 1989).

Due to lactose non-persistance of a large number of individuals world-wide, it is important that the functional properties and digestibility of milk and milk products be improved. One of the most promising and interesting studies of applications in the food industry is the use of lactase enzymes as functional ingredients (Pomeranz *et al.,* 1964). Widely, various microorganisms, animals and vegetables have also been found to be sources of lactase enzymes. However, the most promising commercial source of this enzyme are microorganisms, particularly, crude cell preparations of *Neurospora crassa*, *Saccharomyces fragilis* and *Lactobacillus helveticus (*Wierzbicki & Kosikowski, 1971); strains of thermophilic filamentous fungi (Sorensen & Crisan, 1974); mutant strain of *Aspergillus foetidus* (Borglum & Sternberg, 1972) and extracellular lactase produced by *Aspergillus oryzae* (Neuberg & Rosenthal, 1924). The extraction and isolation process of extracellular lactase from strains of *Aspergillus oryzae* is regarded as highly effective for industrial applications, much more than that of other taxonomic groups. Lactase produced from *Aspergillus oryzae* illustrates a higher thermal tolerance with optimum activity at lower pH ranges (Ogushi *et al.,* 1980; Takenishi *et al.,* 1983). It has also been illustrated that the intrinsic thermo-resistance of fungal β-galactosidases is very important in the industrial processing of milk and milk products, particularly the ability of the enzyme to stay viable after processing (Maciunska *et al.,* 1998).

2.6 The properties of Tolerase L® (lactases)

There are many lactase enzymes available in the pharmaceutical industry, most of which are found in tablet form. Depending on the brand of exogenous lactase, tablets are consumed approximately 15 min prior to consumption (Ojetti *et al.,* 2010). According to DSM Technologies tablets should be taken approximately 30 min before the consumption

of a ready-to-eat breakfast cereal, i.e. when consumed with lactose-containing milk. Lactose-digesting tablets are much less convenient than ready-to-eat breakfast cereals that already contain lactase, i.e. added during production (Personal communication, 2017, M. Kent, DSM Technologies (PTY) Ltd., Johannesburg, South Africa). The latter format of enzyme supplementation is already available for the international breakfast cereal industry, e.g. Tolerase® L that is manufactured by DSM Technologies, a global leader in food enzymes (Personal communication, 2017, M. Tredoux, Functional enzyme additives applications technologist, DSM Technologies (PTY) Ltd., Johannesburg, South Africa).

According to DSM Technologies (Heerlen, The Netherlands) Tolerase® L is classified as an acid lactase enzyme, it converts lactose into its sugars (glucose and galactose), is extracted from the fungus *Aspergillus oryzae* and works at a low pH range, i.e. 3.5-5.5. This low pH range is suitable and effective to digest lactose in the stomach and therefore ideal for the use as a dietary supplement in breakfast cereals. Tolerase® L is distributed in powder form for the food processing industry. It is a highly soluble powder and has a neutral taste. The quantity of Tolerase® L to be added to aid in digestion, depends of the amount of lactose consumed. A large meal remains in the stomach for longer period of time and thus requires less enzyme for full hydrolysis, whereas more enzyme is required for a light meal that travels faster through the stomach. A quantity of 2500 ALU (Acid Lactase Units) is recommended when 10-13 g of lactose is consumed. For very sensitive lactose-intolerant consumers, in need of a 100% conversion of lactose, 10000 ALU per meal is recommended.

3. PHYTIC ACID IN CEREALS – STRUCTURE, INTERACTIONS AND PHYTASES

3.1 Introduction

Cereal products, especially those produced from whole grains, are regarded as an excellent source of minerals. The minerals of significance in breakfast cereals are magnesium (Mg), zinc (Zn), iron (Fe) and calcium (Ca). The bioavailability of these minerals can be quite low, especially when they form insoluble complexes with phytic acid (Coulibaly *et al.,* 2011).

Phytic acid ($C_5H_{18}O_{24}P_6$), also known as inositol hexakisphosphate (IP_6), is the major storage form of phosphorus in cereals (Jacela *et al*., 2010). The amount of phytic acid in cereals is dependent on certain conditions, e.g. growing conditions, particularly soil and the use of fertilisers, harvesting techniques and age of the product in question. In grains such as wheat, millet, and barley phytic acid is formed primarily in the aleuron layer, whereas in corn it is formed in the germ. Phytic acid content of whole cereals varies from 0.5 to 2.0%, whereas the bran section usually has the highest phytate content (Coulibaly *et al.,* 2011).

The formation of insoluble salts, i.e. due to the strong ability of phytic acid to bind with minerals such as Zn, Ca, Fe and Mg, leads to the poor bioavailability of these minerals in human nutrition (Zhou & Erdman, 1995). There are numerous methods available to determine the phytic acid content of cereals. It has been advised that ion-exchange methods are not specific enough. These methods tend not to separate inositol hexakisphosphate from lower inositol phosphates, thus overestimating the phytic acid content in processed foods (Sandberg, 1995). In contrast, it has been demonstrated that high performance liquid chromatography (HPLC) is an effective method for the separation and determination of phytic acid and lower lower inositol phosphates in processed foods such as breakfast cereals (Burbano *et al.,* 1995).

In general, cereal grains contain a high percentage of carbohydrates, 70-80% starch, 15% protein and <5% lipids, minerals and vitamins (Coulibaly *et al.,* 2011). However, the nutritional quality of these nutrients remains inadequate: the bioavailability of important micronutrients tends to be low as a result of the presence of anti-nutritional factors, i.e. primarily phytic acid that is well able to reduce the bioavailability of important minerals. Various pre-treatment processing methods are available to improve the quality of the cereal grains, primarily to ensure bioavailability of specific nutrients (Nout, 1993).

3.2 Source and structure of phytic acid

Phytic acid, also known as phytate, is the storage form of phosphorus in cereals, legumes, seeds and nuts. It is also found in small amounts in specific fruits and vegetables such as berries and green beans (Marchner, 1997). Soil is usually treated with phosphorus-containing fertilisers where plant roots absorb the phosphorus, mainly as PO^{-3}, with a residual component as inorganic phosphorus (P) (Coulibaly *et al*., 2011). Inorganic phosphorus can link as single phosphate ester to the carbon chain (C-O-P) or it can link to another phosphate via a pyrophosphate bond.

The amount of phytic acid in plant seeds and grains, i.e. cereals and legumes range from 0.5 to 5% (Loewus, 2002) and is regarded as a common constituent of foods produced from plant seeds and grains (Coulibaly *et al*., 2011). During the development of seeds and grains, the plant cells accumulate components such as starch, protein and phytic acid. In cereals the activity of phytic acid is highest in the aleuron layer and scutulum, resulting in the formation of significant amounts phosphate, calcium, magnesium and potassium that are available for the common metabolic processes.

As previously mentioned, phosphorus is primarily stored in seeds as inositol hexakisphosphate, IP_6. The structure of phytic acid is shown in Fig. 4 (A). Figure 4 (B)

shows the structure of phytic acid where it forms complexes with minerals such as Zn, Ca, Fe and Mg. In humans, the phosphorus is not available for normal biochemical processes in the body, furthermore the bioavailability of the respective minerals is also much less, resulting in impaired nutrition.

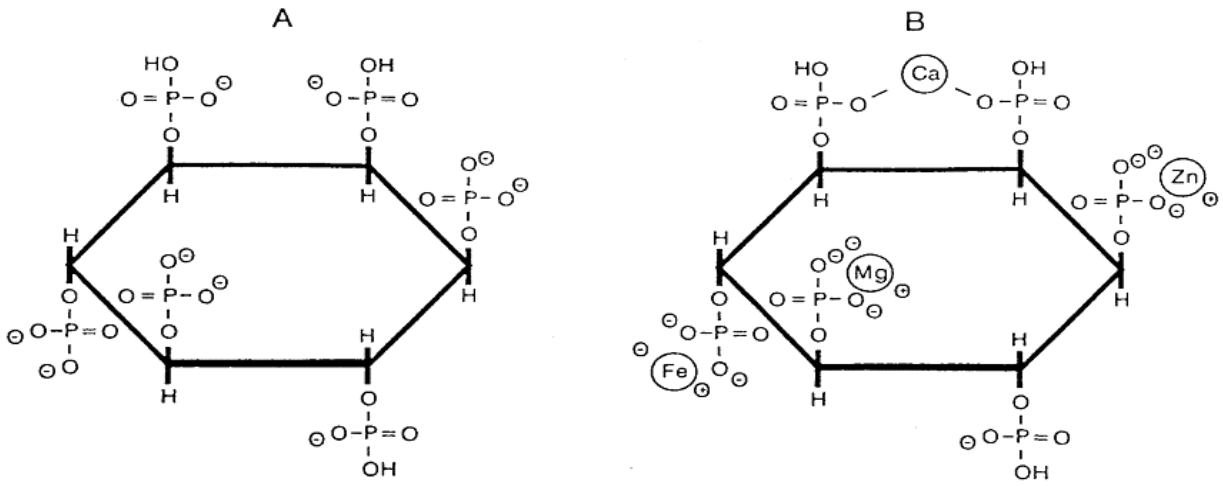


Figure 4 Structure of phytic acid (A) and the structure of phytic acid forming insoluble complex salts with minerals Fe, Mg, Zn and Ca (B) (Adapted from Mansbridge, 2016).

3.3 Phytic acid interactions with minerals

Phytic acid forms approximately 80% of the total percentage of phosphorus (P) in whole grains with the remaining P being represented by soluble organic phosphate and cellular phosphorus (Lopez *et al.,* 2002). Phytic acid has the ability to chelate metal ions, in particular the two trace minerals zinc (Zn), iron (Fe) and the major mineral calcium (Ca), resulting in salts with poor bioavailability in human nutrition (Coulibaly *et al.,* 2011).

3.3.1 Phytic acid and zinc interaction

Zinc is an important mineral involved in the immune system, the activation of enzymes and the growth of cells in the human body (Lopez *et al.,* 2002). Zinc deficiency is quite prevalent in developed countries because of an insufficient supply of Zn from the diet and significant blood losses, but also as a result of an increased requirement during pregnancy and lactation (Coulibaly *et al.,* 2011). Zinc deficiency can also be prevalent in developed countries, primarily due to the fact that phytic acid has the ability to bind Zn in whole grain cereals, making this mineral non-hydrolysable in the GI tract (Flanagan, 1984). The inhibitory effect of phytic acid on Zn can be predicted by the molar ratio of phytic acid to Zn. A molar ratio that exceeds that of 15:1, inhibits Zn absorption resulting in inadequate dietary

intake of Zn (Gibson *et al.,* 1997). Furthermore, in the presence of Ca, the inhibitory effect of phytic acid on Zn is increased even more due to the formation of Ca-Zn-phytic acid complexes in the gastro-intestinal tract making Zn even less bioavailable. It has been postulated that phytic acid and Ca/Zn ratios are better predictors of Zn bioavailability than phytic acid/Zn molar rations (Fordyce *et al.,* 1987).

By enriching foods with Zn, thereby increasing the amount of dietary Zn, the bioavailability of the mineral can be increased (Lopez *et al.,* 2002). When no inhibitory factors are present, absorption of dietary Zn can be >50%, even if the intake of Zn is relatively low. However, a high intake of Zn can result in lower absorption percentages, primarily due the fact that mineral ions carrying the same charge (i.e. Fe, Cu, Mg, Ca and Zn) tend to compete for absorption in the GI tract (Lopez *et al.,* 2002). There are means to enhance Zn bioavailability, i.e. by encouraging the intake of so-called enhancers during the consumption of cereals rich in phytic acid or by consuming fermented products (Lönnerdal, 2000). Fermented foods are acidic in nature (malic, acetic, lactic and citric acid) and these acids are able to form soluble complexes with Zn, thereby inhibiting the formation of insoluble complexes with phytic acid. Dietary proteins are also able to assist in Zn absorption in the GI tract as proteins inhibit the precipitation of Zn in the small intestine, while amino acids such as cysteine enhance the absorption of Zn via the mucosal cells (Sandström *et al.,* 1989). The solubility of Zn at the site where it is absorbed, has a major effect on its availability. Due to the low pH level of the stomach, Zn in foods is easily solubilised, whereas it binds to organic compounds at higher pH levels (Lopez *et al.,* 2002).

3.3.2 Phytic acid and iron interaction

World-wide Fe deficiency can be regarded as one of the major nutritional deficiency disorders. It affects most 1st and 3rd world populations, i.e. one in every three individuals (Hercberg *et al.,* 2001). This deficiency is the result of insufficient intakes of Fe, increased requirement of Fe during pregnancy, blood losses and lastly impaired absorption of Fe in the GI tract (Lopez *et al.,* 2002). Dietary factors also play a role in Fe absorption, as the source and quality of Fe is important. In terms of dietary source of Fe, heme-iron originates from animal foods and non-heme iron from plant foods. It seems that phytic acid particularly inhibits the absorption of non-heme iron in humans (Lopez *et al.,* 2002).

Phytic acid is known to decrease the solubility of Fe and thus the ability of Fe to be absorbed effectively in the GI tract when food products such as whole wheat bread is consumed (Brune *et al.,* 1992; Sandberg & Svanberg, 1991). Protein or acids (especially ascorbic acid), which can act as enhancing components, are both effective in inhibiting the

negative effect of phytic acid on the absorption of Fe in the GI tract (Reddy *et al.,* 1996; (Gillooly *et al.,* 1983). Ascorbic acid reduces the ferric iron to the ferrous state, thereby making it more absorbable in the small intestine. The latter is, however, affected by the amount of Fe and phytic acid (Hallberg *et al.,* 1989).

3.3.3 Phytic acid and calcium interaction

Calcium (Ca) bioavailability is influenced by intrinsic and extrinsic factors, the former include gender, age and whether a female is pregnant of breastfeeding, whereas the latter includes dietary variables that could affect Ca absorption, e.g. the percentage of ingested Ca, vitamin D, fat, lactose and phytic acid (Gueguen & Pointillart, 2000). It has been postulated that phytic acid reduces Ca absorption (Reinhold *et al.,* 1976), however, it has also been reported in literature that phytic acid can have an inhibitory effect on Ca absorption (Lönnerdal *et al.,* 1989; Rimbach *et al.,* 1995).

3.4 Effects of food processing on mineral and phytic acid interactions

Processes associated with cereal production such as kneading, soaking, cooking, fermenting, baking, toasting and extrusion can result in significant losses of phytic acid, however, the reduction of phytic acid during extrusion has resulted in differing results (Lopez *et al.,* 2002). Le Franćois (1988) indicated a loss of 25% of phytic acid, whereas Sandberg & Anderson (1988) indicated that extrusion cooking may lead to a substantial loss of phytic acid, primarily because of the negative effect of extrusion on the phytase enzyme activity. During extrusion cooking, the phytase enzymes in plant material can be deactivated (Le Franćois, 1988). Sandberg *et al* (1993), proposed that the reduction of the absorption of minerals in the GI tract, i.e. due to the resistance of phytic acid to digestion, should be calculated and rectified through mineral supplementation.

World-wide bread is regarded as one of the major staple foods (Lopez *et al.,* 2002). Supermarkets recommend that brown or whole wheat bread should form part of the basic food basket, primarily due to the health benefits of fibre and the potential of reducing the risk of lifestyle diseases that associate with diets low in fibre (Nävert *et al.,* 1985; Brune *et al.,* 1992). The fact that the phytic acid binds important minerals, and thus decrease their bioavailability, can be combatted by fortification with relevant minerals. It is mandatory in South Africa that all bread flours and maize are fortified with specific minerals, i.e. electrolytic iron and zinc oxide (Department of Health, R2003, 2010). During the commercial breadmaking process, the breakdown of phytic acid is facilitated by the action of phytase enzymes present in the dough, adding to the retention of important micronutrients

(MacKenzie-Parnell & Davies, 1986). It has been postulated that fermentation of wheat and rye bread dough can result in a significant reduction phytic acid, thereby improving the bioavailability of Zn (Fretzdorff & Brümmer, 1992). When individuals consume significant amounts of non-fermented whole cereal products in countries such as Iran and Turkey, Zn deficiencies have been observed (Reinhold *et al.,* 1976).

The breakdown of phytic acid can also take place during food processing of cereals due to the action of phytase enzymes from plants and yeasts or from other microorganisms during production processes such as soaking, malting, hydrothermal processing and lactic acid fermentation (Coulibaly *et al.,* 2011). To enhance increased mineral bioavailability by phytic acid degradation during food production, it is important to be aware of the optimal conditions for phytase enzymes responsible for phytic acid degradation (Lönnerdal *et al.,* 1989; Sandberg *et al.,* 1993).

3.5 Hydrolysis of phytic acid in the GI tract

There are a number of variables to consider when the breakdown of phytic acid takes place in the GI tract (Widdowson & Thrussell, 1951). In theory, phytic acid degradation might happen as a result of changes in enzyme secretion or due to the change in intestinal microorganism balance (Sandberg & Andlid, 2002).

3.6 Structure, properties and stability of phytase enzymes

Phytase, myo-inositol hexakisphosphate (1,2,3,4,5,6) phosphohydrolase, catalyse the fractional or complete removal of orthophosphates from phytic acid (inositol hexakishophotates) (Konietzny & Greiner, 2002). During hydrolysis phytase enzymes break down phytic acid into one molecule of inositol and six molecules of inorganic phosphate (Fig. 5). As mentioned, phytic acid is the storage form of phosphorus and inositol in cereals, legumes, and seeds (Coulibaly *et al.,* 2011). According Reddy *et al.* (1996), more than 60% of phosphorus content in plant material form part of phytic acid.

In order for phytic to be hydrolysed in the GI tract, the addition of the enzyme phytase is essential for the breakdown of phytic acid-linked phosphates. Phosphates that are excreted as part of undigested phytic acid are often re-used as waste material in the production of fertilisers (Greiner *et al.,* 1993).

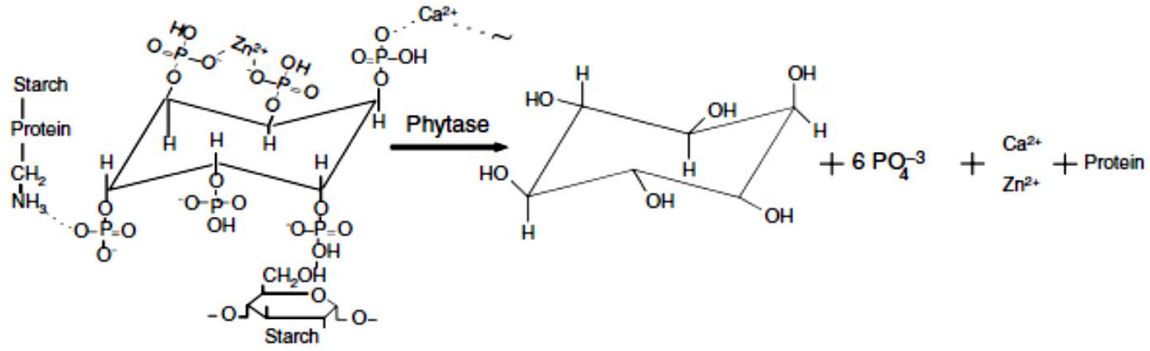


Figure 5 The hydrolysis of phytic acid, using the enzyme phytase and the formation of inositol, phosphate and other substances, i.e. metals, metal-binding enzymes and proteins (Adapted from Yao *et al.,* 2011)

As mentioned, phytase enzymes have an important function in human nutrition, i.e. as enzymes in the degradation of phytic acid during food processing, as well as in the human gut. Various types of phytase enzymes are available to reduce the amount of phytic acid in food. Biotechnologically-produced microbial phytases, currently commercially available for animal feed production, could potentially be used in food processing (Konietzny & Greiner, 2002). Phytases with the desired properties could also be cloned and inserted into plants, thereby yielding improved levels of phytase enzyme for increased hydrolysis in the GI tract. Table 3 shows a number of phytate-degrading enzymes that are capable of releasing orthophosphate from phytic acid, as well as other compounds (Konietzny & Greiner, 2002). These plant-derived phytic acid-degrading enzymes should be refined, however, one of the challenges in purifying phytic acid-degrading enzymes that originate from plants, is the separation of phytic acid-degrading enzyme from contamination by non-specific acid phosphatase (Konietzny *et al.,* 1994). Plant-derived phytases are less stable than phytases sourced from microorganisms. Phytases from microbial sources yield higher extracellular amounts obtained by filtrating specific cultures (Konietzny & Greiner, 2002).

To purify the phytate-degrading enzyme from *Aspergillus niger* NRRL 3135, a three-step process has been suggested, including ion-exchange chromatography. Research indicated that a recovery rate of >60% from *Aspergillus niger* NRRL 3135 require multiple purification steps (Gibson & Ullah, 1988). Phytase enzymes retrieved from *Escherichia coli,* using a five-step approach, require even more purifications (<10,000) and for *Escherichia coli* the recovery rate is minimal (<20%) (Greiner *et al.,* 1993). A six-step process, including butanol extraction, ethanol precipitation, ion-exchange chromatography and gel filtration has been used for the purification of intestinal phytate-degrading enzymes from rats. In this instance a recovery rate of 19% was achieved after >1000 purifications (Yang *et al.,* 1991).

3.6.1 Temperature, pH and protease stability of phytase enzymes

Microbial sourced phytase enzymes are more temperature- and pH-stable than phytase enzymes sourced from plants (Konietzny & Greiner, 2002). At pH levels <4 and >7.5, the stability of most plant enzymes decrease significantly, whereas that of microbial-sourced phytases tend to be stable at pH >8.0 and <3.0. Phytases sourced from plants are deactivated within minutes when exposed to temperatures >70˚C. In contrast, microbial-sourced phytase enzymes are more stable and can withstand prolonged incubation times at high temperatures. Phytase enzymes resistant to high temperatures have been isolated from *Aspergillus fumigatus* (Pasamontes *et al.,* 1997) and *Schwanniomyces castellii* (Segueilha *et al.,* 1992). Phytase enzymes isolated from *Aspergillus fumigatus* are reasonably resistant (10% loss) to high temperatures for short periods (90˚C for 20 min). Although the phytase enzymes sourced from *Aspergillus fumigatus* are not wholly thermostable, they have the ability to refold completely into a fully active conformation after denaturation (Wyss *et al.,* 1999).

Phillippy (1999) illustrated that phytates sourced from *Aspergillus niger* were quite stable in the presence of pepsin (a protein-degrading enzyme in stomach) or pancreatic enzymes (commercial mixtures of amylase, lipase, and protease), however, the corresponding enzymes from wheat were not stable. Furthermore, phytase enzymes sourced from *Aspergillus* is more resistant to trypsin than that from *Escherichia coli* (Rodriguez *et al.,* 1999).

3.6.2 Enzymatic degradation of phytic acid

Phytases or phytic acid-degrading enzymes are able to hydrolyse phytic acid (inositol hexakisphosphate, IP_6), i.e. catalyse the sequential release of phosphate from phytic acid, i.e. release the phosphorus as well as the dietary minerals (Cosgrove, 1966). Phytase enzyme activity is dependent on the type of food processing method used. It is also active in the GI tract, where optimal conditions exist for hydrolysis (Sandberg & Andlid, 2002). Different properties of a phytase enzyme should be considered when searching for the correct enzyme, i.e. stability at low pH levels and high temperatures, resistant to digestive proteolytic enzymes, easy cultivation and purification and lastly classified as non-allergenic and non-toxic (Sandberg & Andlid, 2002).

3.6.3 Sources of phytases

Four sources of phytase have been identified; 1) plant phytases isolated from range of plant sources, 2) microbial phytases (fungal and bacterial phytases sourced from bacteria and fungi), 3) phytases produced by the small intestine mucosal cells of pigs, and lastly 4) intestinal microbial phytases primarily found in pigs (Kumar *et al.,* 2010). This literature review will focus on the first two sources of phytase, i.e. plant- and microbial-derived phytases.

3.6.3.1 Plant-derived phytases

Only recently plant-derived phytase enzymes have been isolated, characterised and purified from a number of plant sources, i.e. rice, rape seed, soybean, maize, wheat and rye (Greiner *et al.,* 1993, 1997, 1998; Konietzny *et al.,* 1994; Greiner & Larrson-Almeiger, 1999). The degradation pathway of IP_6 associated with dried peas, was found to be different to the pathway of cereals (Skoglund *et al.,* 1997a), whereas the degradation pathway of IP_6 in oats, rye and barley was found to be similar to that in wheat (Skoglund *et al.,* 1997b).

3.6.3.2 Microbial-derived phytases

Fungi and bacteria are important sources of fungal and bacterial phytases. Phytase enzymes derived from microbial origin are important in food processing applications and fermentations, they are able to effectively degrade phytic acid and are also regarded as an important source of phosphorus (Kumar *et al.,* 2010).

Generally, yeasts have the ability to synthesize and secrete phytase enzymes. High performance liquid chromatography (HPLC) has been used to indicate phytase enzyme activity of yeasts species (Sandberg & Andlid, 2002). Microbial phytases, especially those common in baker's yeast, are quite unspecific and are able to hydrolise organic phosphorus sources other than phytic acid (Nayini & Markakis, 1983). The first report on the yeasts as source of phytase was as early as 1984 (Kumar *et al.,* 2010). Yeasts tested include >10 different *Saccharomyces cerevisiae* strains, other yeasts such as *Debarymyces hansenni, Rhodotorula rubra, Rh. Glutinis, S. boulardii,* as well as several tropical yeast species such as *Metschmikowia lochheadii, Candida drosphilae* and *Candida tolerans* (Andlid, 2000).

Table 3 Phytate-degrading enzymes purified close to uniformity (Adapted from Konietzny & Greiner, 2002).

Phytase source	Localisation
Aspergillus niger	Extracellular
Aspergillus terreus	Extracellular
Aspergillus oryzae	Extracellular
Bacillus subtilis	Extracellular
Bacillus amyloliquefaciens	Extracellular
Escherichia coli	Periplasmic
Klebsiella sp.	Cytoplasmic
Schwanniomyces castellii	Extracellular
Penicillium simplicissimum	Extracellular
Phaseolus aureus (mung bean)	Cotyledon
Glycine max. (soybean)	Cotyledon
Zea mays (maize)	Seed, Root
Lycopersicon esculentum	Root
Triticum spelta (spelt)	Seed
Secale cereale (rye)	Seed
Hordeum vulgare (barley)	Seed
Triticum aestivum (wheat)	Bran
Avena sativa (oat)	Seed
Vicia faba (faba bean)	Seed
Lupinus albus (lupine)	Seed
Allium fistulosum (scallion)	Leaves
Rats	Intestine

However, the most extensively investigated fungi genus is *Aspergillus* from which a number of commercialised phytase enzymes originated. Other phytase producing fungi species studied and investigated include *A. niger, A. ficuum* and *A. fumigatus*. Phytase enzymes from *Aspergillus* have been improved by applying genetic engineering (Wyss *et al.,* 1999).

Numerous phytase enzymes have also been isolated from bacteria such as *Escherichia coli*, *Bacillus subtilis, Klebsiella terringa, Pseudomonas spp*. (Greiner *et al.,* 1993, 1997). These bacteria are able to degrade phytic acid during growth through the production of extracellular phytases, however, research has demonstrated that this is not completely true for lactic acid bacteria (LAB) (Fredrikson *et al.,* 2002).

3.7 Properties of Tolerase P® (phytases)

Phytase supplementation has primarily been implemented in Europe, mainly by adding exogenous phytase during processing (Kumar *et al.,* 2010; Mittal *et al*., 2013). In the South African breakfast cereal industry ready-to-eat breakfast cereals are fortified with minerals to compensate for the loss of minerals during processing and to provide approximately 30% of the recommended daily intake of specific minerals: Fe, Zn, Ca and Mg (Personal communication, 2017, M. Kent, DSM Technologies (PTY) Ltd., Johannesburg, South Africa). Tolerase® P is a phytase enzyme which helps the body to absorb Fe, Zn, Ca, Mg and phosphorus from cereals and legume-based foods rich in phytate. Tolerase® P, supplied by DSM Technologies (Heerlen, The Netherlands), is a global leader in food enzymes (Personal communication, 2017, M. Tredoux, Functional enzyme additives applications technologist, DSM Technologies (PTY) Ltd., Johannesburg, South Africa). This commercial enzyme is a white, free-flowing powder. Tolerase® P, extracted from various microorganisms, can be added during food processing to directly break down phytic acid in ready-to-consume food commodities and to ensure that the minerals are bioavailable (intrinsic and/or added minerals). Tolerase® P can also be taken together with a phytic acid-rich meal. Tolerase P® (phytases) digests the anti-nutrient in the stomach and makes the added (fortified) minerals and intrinsic minerals available for absorption, thereby effectively addressing the challenge of Fe and Zn deficiency diseases in both developing and developed countries (Theodoropoulos *et al*., 2018).

4 CONCLUSIONS

This literature review is an attempt to illustrate the potential of lactase and phytase as viable ingredients in the production of RTEBC. It has shown the importance of and need for lactase to be added to ready-to-eat breakfast products, thereby potentially excluding the expensive lactose-free milk from a consumer's food basket. This review also emphasized the importance of adding phytase to RTEBC during processing, thereby ensuring optimum mineral availability. There are limited products of this kind on the market, as well as limited information on ready-to-eat breakfast cereals with functional enzyme additives. Furthermore, the South African labelling regulation is a major challenge for the marketing of these products.

5 REFERENCES

Andlid, T. (2000). Extracellular phytase activity expressed by Saccharomyces cerevisiae in relation to the pho gene family. *Tenth International Symposium on Yeasts*, Papendal, Arnhem, the Netherlands 2000. (Abstract) Symposium Book p. 223. Delft: Delf, University Press.

Beja-Pereira, A., Luikart, G., England, P.R., Bradley, D.G., Jann, O.C. & Bertorelle, G. (2003). Gene-culture coevolution between cattle milk protein genes and human lactase genes. *Nature Genetics*, **35**, 311-313.

BMI (2012). Online reference included in article [Internet document] URL www.fastmoving.co.za/breakfast/south-africa/FeedbackBreakfastFoods2012.pptx. Accessed 03/06/2017.

Borglum, G.B. & Sternberg. M.Z. (1972). Properties of a fungal lactase. *International Journal of Food Science and Technology*, **37,** 619.

Brown-Esters, O., McNamar, P. & Savaiano, D. (2012). Dietary and biological factors influencing lactose intolerance. *International Dairy Journal,* **22,** 98-103.

Brune, M., Rossander-Hulten, L., Hallberg, L., Gleerup, A. & Sandberg, A.S. (1992). Iron absorption from bread in humans: inhibiting effects of cereal fibre, phytate and inositol phosphates with different numbers of phosphate groups. *Journal of Nutrition*, **122,** 442-449.

Burbano, C., Muzquiz, M., Osagie, A., Ayet, A. & Cuadrado, C. (1995). Determination of phytate and lower inositol phosphates in Spanish legumes by HPLC methodology. *Food Chemistry,* **55**, 321-325.

Cavalli-Sforza L.L. (1973). Analytic review: some current problems of human population genetics. *American Journal of Human Genetics*, **25,** 82-104.

Cosgrove, D.J. (1966). The chemistry and biochemistry of inositol polyphosphates. *Reviews of Pure and Applied Chemistry*, **16,** 209-224.

Coulibaly, A., Kouakou, B. & Chen, J. (2011). Phytic acid in cereal grains: Structure, healthy or harmful ways to reduce phytic acid in cereal grains and their effects on nutritional quality. *American Journal of Plant Nutrition*, **1**, 1-22.

De Vrese, M., Stegelmann, A., Richter, B., Fenselau ,S., Laue, C. & Schrezenmeir, J. (2001), Probiotics-compensation for lactase insufficiency. *American Journal of Clinical Nutrition*, **73** (Suppl. 2), 421S-9S.

DOH (2003). Regulation 504 of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 [Act 54 of 1972]. Department of Health, Pretoria, South Africa.

DOH (2010). Regulation 146 of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 [Act 54 of 1972]. Department of Health, Pretoria, South Africa.

Enattah, N.S., Kuokkanen, M., Forsblom, C., Natah, S., Oksanen, A. & Jarvela, I. (2007). Correlation of intestinal disaccharides activities with the C/T-13910 variant age. *World Journal of Gastroenterology,* **13,** 3508-3512.

Enattah, N.S., Sahi, T., Savilahti, E., Terwilliger, J.D., Peltonen, L. & Jarvela, I. (2002). Identification of a variant associated with adult-type hypolactasia. *Nature Genetics*, **30,** 233-237.

Escher, J.C., De Koning, N., Van Engen, C., Arora, S. & Büller, H.A. (1992). Molecular basis of lactase levels in adult humans. *Journal of Clinical Investigation,* **89,** 480-83.

Flanagan, P.R. (1984). A model to produce pure zinc deficiency in rats and its use to demonstrate that dietary phytate increases the excretion of endogenous zinc. *Journal of Nutrition*, **114,** 493-502.

Fordyce, E.J., Forbes, R.M., Robbins, K.R. & Erdman, J.W. (1987). Phytate-calcium/zinc molar ratios: Are they predictive of zinc bioavailability? *International Journal of Food Science and Technology*, **52,** 440-444.

Fredrikson, M., Andlid, T., Haikara, A. & Sandberg, A.S. (2002). Phytate degradation by micro-organisms in synthetic media and pea flour. *Journal of Applied Microbiology,* **92,** 1-6.

Fretzdorff, B. & Brümmer, J.M. (1992). Reduction of phytic acid during bread making of whole-wheat breads. *Cereal Chemistry*. **69,** 266-270.

Gibson, D.M. & Ullah, A.H.J. (1988). Purification and characterization of phytase from cotyledons of germinating soybean seeds. *Archives of Biochemistry and Biophysics*, **260,** 503-513.

Gibson, R.S., Donovan, U.M. & Heath, A.L.M. (1997). Dietary strategies to improve the iron and zinc nutrition of young women following a vegetarian diet. *Plant Foods for Human Nutrition*, **51,** 1-16.

Gibson, R.S., Bailey, K.B., Gibbs, M. & Ferguson, E.L. (2010). A review of phytate, iron, zinc, and calcium in plant-based complementary foods used in low-income countries and implications for bioavailability. *Food and Nutrition Bulletin*, **31,** S134-S146.

Gillooly, M., Bothwell, T.H. & Torance, J.D. (1983). The effect of organic acids, phytates and polyphenols on iron absorption from vegetables. *British Journal of Nutrition*, **49,** 331-342.

GBCS (2017). Global Breakfast Cereal Strategic Report. PR News Wire. Retrieved from: https://www.prnewswire.com/news-releases/global-breakfast-cereals-strategic-

business-report-2017-2024---growing-preference-for-healthy-organic--all-natural-cereals-drives-steady-market-demand-300530908.html

Grand View Research (2018). Report on Breakfast Cereals. Grand View Research Inc., San Francisco, USA.

Greiner, R., Konietzny, U. & Jany, K.D. (1993). Purification and characterization of two phytases from *Escherichia coli. Archives of Biochemistry and Biophysics*, **303,** 107-113.

Greiner, R., Haller, E., Konietzny, U. & Jany, K.D. (1997). Purification and characterization of a phytase from Klebsiella terrigena. *Archives of Biochemistry and Biophysics*, **341,** 201-206.

Greiner, R., Konietzny, U. & Jany, K.D. (1998). Purification and properties of a phytase from rye. *Journal of Food Biochemistry*, **22,** 143-161.

Greiner, R. & Larsson-Alminger, M. (1999). Purification and characterization of a phytate-degrading enzyme from germinated oat (Avena sativa). *Journal of the Science of Food and Agriculture*, **79,** 1453-1460.

Greiner, R., Jany, K.D. & Larsson Alminger, M. (2000). Identification and properties of myo-inositol hexakisphosphate phosphohydrolases (phytases) from barley (*Hordeum vulgare*). *Journal of Cereal Science*, **31,** 127-139.

Gueguen, L. & Pointillart, A. (2000). The bioavailability of dietary calcium. *Journal of American College of Nutrition.* **19,** 119S-136S.

Gugatschka, M., Dobnig, H. & Fahrleitner-Pammer, A. (2005). Molecularly-defined lactose malabsorption, milk consumption and anthropometric differences in adult males. *Quarterly International Journal of Medicine*, **98,** 857-863.

Hallberg, L., Brune, M. & Rossander, L. (1989). Iron absorption in man: ascorbic acid and dose-dependent inhibition by phytate. *American Journal of Clinical Nutrition*, **49**, 140-144.

Harvey, C.A., Wang, Y. & Hughes L.A. (1995). Studies on the expression of intestinal lactase in different individuals. *American Journal of Human Genetics*, **36,** 28-33.

He, T., Priebe, M.G., Harmsen, H. J., Stellaard, F., Sun, X. & Welling, G. W. (2006). Colonic fermentation may play a role in lactose intolerance in humans. *Journal of Nutrition,* **136**, 58-63.

Hercberg, S., Preziosi, P. & Galan, P. (2001). Iron deficiency in Europe. *Public Health Nutrition*, **4,** 537-545.

Heyman, M.B. (2006). Lactose intolerance in infants, children, and adolescents. *Pediatrics*, **118,** 1279-1286.

Ingram, C.J.E., Elamin, M.F. & Mulcare, C.A. (2007). A novel polymorphism associated with lactose tolerance in Africa: Multiple causes for lactase persistence? *Human Genetics*, **120,** 779-788.

Ingram, C.J., Mulcare, C.A., Itan, Y., Thomas, M.G. & Swallow, D.M. (2009). Lactose digestion and the evolutionary genetics of lactase persistence. *Human Genetics,* **124,** 579-591.

Insight Survey (2017). B2B Market Research. Retrieved from: http://www.insightsurvey.co.za/wp-content/uploads/2017/10/Breakfast-Cereals-Industry-2017-Brochure-(2-Oct-2017).pdf

Jacela, J.Y., DeRouchey, J.M., Tokach, M.D., Goodband, R.D., Nelssen, J.L., Renter, D.G. & Dritz, S.S. (2010). Feed additives for swine: Fact sheets - prebiotics and probiotics and phytogenics. *Journal of Swine Health and Production*, **18,** 87-91.

Johnson, J. (1981). The regional and ethnic distribution of lactose malabsorption. In D. Paige, & T. Bayless (Eds.), Lactose digestion: Clinical and nutritional implications. Baltimore, MD, USA: John Hopkins University Press.

Johnson, A.O., Semenya, J.G., Buchowski, M.S. Enwonwu, C.O. & Schrimshaw, N.S. (1993). Correlation of lactose maldigestion, lactose intolerance and milk intolerance. *The American Journal of Clinical Nutrition,* **57,** 399-401.

Kent, M. (2014). DSM Technologies, Johannesburg, South Africa. Personal communication.

Kent, N.L. (1983). Technology of Cereals. Pergamon Press, Oxford, UK.

Konietzny, U., Greiner, R. & Jany, K.D. (1994). Purification and characterization of a phytase from spelt. *Journal of Food Biochemistry*, **18,** 165-183.

Konietzny, U. & Greiner, R. (2002). Molecular and catalytic properties pf phytate degrading enzymes (phytases). *International Journal of Food Science and Technology,* **37,** 791-812.

Kowtaluk, H. (2001). Food for today (9th ed). Tata McGraw Hill Publishing Company. New Delhi, India.

Kretchmer, N. (1972). Lactose and lactase. *Scientific American Journal*, **227**, 71-78.

Kumar, V., Sinha, A.K., Makkar, H.P.S. & Becker, K. (2010). Dietary roles of phytate and phytase in human nutrition: a review. *Food Chemistry*, **120,** 945-959.

Le François, P. (1988). Phytic acid and zinc contents of cereal products: relation to the manufacturing process. *Journal of Food Composition and Analysis*, **1,** 139-145.

Loewus, F.A. (2002). Biosynthesis of Phytate in Food Grains and Seeds. In: Food Phytate, Reddy, N.R. and S.K. Sather (Eds.). CRC Press, Boca Raton, Florida.

Lomer, M.C., Parkes, G. C., & Sanderson, J. D. (2007). Review article: lactose intolerance in clinical practice-myths and realities. *Alimentary Pharmacology and Therapy*, **27,** 93-103.

Lönnerdal, B., Sandberg, A.S., Sandström, B. & Kunz, C. (1989). Inhibitory effects of phytic acid and other inositol phosphates on zinc and calcium absorption in suckling rats. *Journal of Nutrition*, **119**, 211-214.

Lopez, H.W., Duclos, V., Coudray, C. & Remesy, C. (2002). Minerals and phytic acid interactions: Is it a real problem? *International Journal of Food Science and Technology,* **37**, 727-739.

Maciunska, J., Czyz, B. & Synowiecki, J. (1998). Isolation and some properties of β-galactosidase from thermophyllic bacterium *Thermus thermophylus. Food Chemistry,* **63,** 441-445.

MacKenzie-Parnell, J.M. & Davies, N.T. (1986). Destruction of phytic acid during home bread making. *Food Chemistry*, **22**, 181-192.

Mackey, A.D., Henderson, G.N. & Gregory, J.F. (2002). Enzymatic hydrolysis of pyridoxine-50-beta-D glucoside is catalysed by intestinal lactase-phlorizin hydrolase. *Journal of Biological Chemistry*, **277**, 58–64.

Mantai, N., Villa, M., Enzlen, T., Wacker, H., Boll, W., James, P., Hunziker, W. & Semenza, G. (1988). Complete primary structure of human and rabbit lactase-phlorizin hydrolase: implications for biosynthesis, membrane anchoring and evolution of the enzyme. *The EMBO Journal,* **7(9),** 2705-2713.

Marchner, H. (1997). *Mineral and Nutrition of Higher Plants*. 2nd edn., Academic Press, San Diego, USA.

Matter, R., Ferraz de Campos Mazo, D., Carrilho, F.J. (2012). Lactose intolerance: Diagnosis, genetic, and clinical factors. *Clinical and Experimental Gastroenterology*, **5**, 113-121.

Matthews, S.B., Waud, J. P., Roberts, A. G. & Campbell, A. K. (2005). Systemic lactose intolerance: a new perspective on an old problem. *Postgraduate Medical Journal*, **81**, 167-173.

Mayo Clinic (2009). "Why is breakfast the most important meal of the day?" http://www.mayoclinic.com/health/food-and-nutrition/AN01119. [September 2010].

McBean, L. D. & Miller, G.D. (1998). Allaying fears and fallacies about lactose intolerance. *Journal of the American Dietetic Association*, **98,** 671-676.

McCracken, R.D. (1970). Adult lactose tolerance [letter]. *Journal of the American Medical Association*, **13,** 2257-2260.

Mittal, A., Gupta, V., Singh, G., Yadav, A. & Aggarwal, N.K. (2013). Phytase, a boom in food industry. *Octa Journal of Biosciences,* **1**, 158-169.

Moore, B.J. (2003). Dairy foods: Are they politically correct? *Nutrition Today*, **38,** 82-90.

National Digestive Diseases Information Clearinghouse (NDDIC) (2005). Digestive diseases statistics. http://digestive.niddk.nih.gov/statistics/statistics.htm.

Nävert, B., Sandström, B. & Cederblad, A. (1985). Reduction of the phytate content of bran by leavening in bread and its effect on absorption of zinc in man. *British Journal of Nutrition*, **53,** 47-53.

Nayini, N.R. & Markakis, P. (1983). Effect of fermentation on the inositol phosphates of bread. *Journal of Food Science,* **48,** 262-263.

Nemeth, K., Plumb, G.W., Berrin, J.G., Juge, N. & Jacob, R. (2003). Deglycosylation by small intestinal epithelial cell β-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *European Journal of Nutrition,* **42,** 29-42.

Newcomer, A.D., Douglas, B. & McGill, M.D. (1966). Lactose tolerance tests in adults with normal lactase activity. *Gastroenterology,* **50,** 340-346.

Newcomer, A.D., McGill, D. B., Thomas, P. J., & Hofmann, A. F. (1978). Tolerance to lactose among lactase-deficient American Indians. *Gastroenterology*, **74,** 44-46.

Neuberg, G. & Rosenthal, D. (1924). Uber taka-lactase*. Biochemistry Department*, **45,**186.

Nout, M.J.R. (1993). Processed weaning foods for tropical climates. *International Journal of Food Science and Nutrition*, **43,** 213-221.

Ogushi, S. Yoshimoto, T. & Tsuru, D. (1980). Purification and comparison of two types of beta-galactosidase from *Aspergillus oryzae. Journal of Fermentation Technology,* **58,** 115-122.

Ojetti, V., Gigante, G., Gabrielli, M., Ainora, M.E., Mannocci, A., Lauritano, E.E., Gasbarrini, G.Y Gasbarrini, A. (2010). The effect of oral supplementation with *Lactobacillus reuteri* or tilactase in lactose intolerant patients. *European Review for Medical and Pharmacological Sciences*, **14**, 163-170.

Olds, L.C. & Sibley, E. (2003). Lactase persistence DNA variant enhances lactase promoter activity in vitro: functional role as a cis regulatory element. *Human molecular genetics,* **12,** 2333-2340.

Olds, L.C., Sibley, E. & Ahn, J.K. (2011). -13915 *G DNA polymorphism associated with lactase persistence in Africa interacts with Oct-1. *Human Genettics,* **129,** 111-113.

Phillippy, B.Q. (1999). Susceptibility of wheat and Aspergillus niger phytases to inactivation by gastrointestinal enzymes. *Journal of Agriculture and Food Chemistry,* **47,** 1385-1388.

Pomeranz, Y. (1964). Lactase 1. Occurrence and properties. *International Journal of Food Science and Technology,* **88,** 682.

Portincasa, P., Di Ciaula, A., Vacca, M., Montelli, R., Wang, D.Q. & Palasciano, G. (2008). Beneficial effects of oral tilactase on patients with hypolactasia. *European Journal of Clinical Investigation,* **38,** 835-844.

Preziosi, P., Galan, P., Deheeger, M., Yacoub, N., Drewnowski, A. & Hercberg, S. (1999). Breakfast type, daily nutrient intakes and vitamin and mineral status of french children, adolescents and adults. *Journal of American Clinical Nutrition*, **18,** 171-178.

Reddy, M.B., Hurrell, R.F., Juillerat, M.A. & Cook, J.D. (1996). The influence of different protein sources on phytate inhibition of nonheme-iron absorption in humans. *American Journal of Clinical Nutrition*, **63,** 203-207.

Reinhold, J., Faradji, B., Abadi, P. & Ismail-Beigi, F. (1976). Decreased absorption of calcium, magnesium, zinc and phosphorus by humans due to increased fibre and phosphorus consumption as wheat bread. *Journal of Nutrition*, **106,** 493-503.

Rimbach, G., Pallauf, J., Brandt, K. & Most, E. (1995). Effect of phytic acid and microbial phytase on Cd accumulation, Zn status and apparent absorption of Ca, P, Mg, Fe, Zn, Cu and Mn in growing rats. *Annals of Nutrition and Metabolism*, **39,** 361-370.

Rodriguez, E., Porres, J.M., Han, Y. & Lei, X.G. (1999). Different sensitivity of recombinant *Aspergillus niger* phytase (r-PhyA) and *Escherichia coli* pH 2.5 acid phosphatase (r-AppA) to trypsin and pepsin in vitro. *Archives of Biochemistry and Biophysics,* **365,** 262-267.

Rusynyk, R.A., & Still, C. D. (2001). Lactose intolerance. *Journal of the American Osteopathic Association*, **101,** S10-S12.

Sandberg, A.S., (1995). Determination of phytic acid. *In recent progress in the analysis of dietary fibre*. Luxembourg: European Commission.

Sandberg, A.S. & Andlid, T. (2002). Phytogenic and microbial phytases in human nutrition. *International Journal of Food Science and Technology,* **37,** 823-833.

Sandberg, A.S. & Andersson, H. (1988). Effect of dietary phytase on the digestion of phytate in the stomach and small intestines of humans. *Journal of Nutrition*, **118**, 469-473.

Sandberg, A.S. & Svanberg, U. (1991). Phytate hydrolysis by phytase in cereals: effects on in vitro estimation of iron availability. *Journal of Food Science and Technology,* **56,** 1330-1333.

Sandström, B., Almgren, A., Kivisto, B. & Cederblad, A. (1989). Effect of protein and protein source on zinc absorption in humans. *Journal of Nutrition*, **119,** 48–53.

Savaiano, D.A. & Levitt, M.D. (1987). Milk intolerance and microbe-containing dairy foods. *Journal of Dairy Science*, **70**, 397-406.

Scrimshaw, N. & Murray, E. (1988). Lactose tolerance and milk consumption. *Journal of Latin American Nutrition*, **38**, 543-567.

Sebastio, G., Villa, M., Sartorio, R., Guzzetta, V. & Poggi, V. (1989). Control of lactase in human adult-type hypolactasia and in weaning rabbits and rats. *American Journal of Human Genetics,* **45**,489-97.

Segueilha, L., Lambrechts, C., Baze, H., Moulin, G. & Galzy, P. (1992). Purification and properties of the phytase from *Schwanniomyces castelli. Journal of Fermentation Bioengineering,* **74,** 7-11.

Shaw, A.D. & Davies, G.J. (1999). Lactose intolerance: problems in diagnosis and treatment. *Journal of Clinical Gastroenterology,* **28,** 208-16.

Simoons, F.J. (1970). Primary adult lactose intolerance and the milking habit: a problem in biologic and cultural interrelations. II. A culture historical hypothesis. *American Journal of Digestive Diseases*, **15**, 695-710.

Simoons, F. (1978). The geographic hypothesis and lactose malabsorption. A weighing of the evidence. *American Journal of Digestive Diseases*, **11,** 963-980.

Skoglund, E., Carlsson, N.-G. & Sandberg, A.S. (1997a). Analysis of inositol mono- and diphosphate isomers using high-performance ion chromatography and pulsed amperometric detection. *Journal of Agriculture and Food Chemistry,* **45,** 4668-4673.

Skoglund, E., Larsen, T. & Sandberg, A.S. (1997b). Comparison between steeping and pelleting a mixed diet at different calcium levels on phytate degradation in pigs. *Canadian Journal of Animal Science,* **77,** 471-477.

Sorensen, S.G. & Crisan, E.V. (1974). Thermostable lactase from thermophyllic fungi. *Journal of Food Science and Technology*, **39,** 1184-1187.

Srinivasan, R. & Minocha, A. (1998). When to suspect lactose intolerance. Symptomatic, ethnic, and laboratory clues. *Postgraduate Medicine*, **104**, 109-111, 115-116, 122-123.

Stephenson, L. S. & Latham, M.C. (1974). Lactose intolerance and milk consumption: The relation of tolerance to symptoms. *American Journal of Clinical Nutrition*, **27**, 296-303.

Suarez, F. L., Savaiano, D., Arbisi, P., & Levitt, M.D. (1997). Tolerance to the daily ingestion of two cups of milk by individuals claiming lactose intolerance. *American Journal of Clinical Nutrition*, **65**, 1502-1506.

Swagerty, D.L., Walling, A. D., & Klein, R. M. (2002). Lactose intolerance. *American Family Physician*, **65,** 1845-1850.

Swallow, D.M. (2003). Genetics of lactase persistence and lactose intolerance. *Annual Review of Genetics*, **37,** 197-219.

Tajensihi, S., Watanabe, Y., Miwa, T. & Kobayashi, R. (1983). Purifiaction and some properties of β-galactosidase from Penicillium multicolor. *Agricultural and Biological Chemistry*, **47,** 2533-2540.

Theodoropoulos, V.C.T., Turattia, M.A., Ralf Greiner, R., Macedoc, G.A. & Pallonea, J.A.L. (2018). Effect of enzymatic treatment on phytate content and mineral bioacessability in soy drink. *Food Research International*, **108,** 68-73.

Tredoux, M. (2017). DSM Technologies (PTY) Ltd., Functional enzyme additives applications Technologist, Johannesburg, South Africa. Personal communication.

Tribelhorn, R.E. (1991). "Breakfast Cereals" In: *Handbook of Cereal Science and Technology*, Marcel Dekker, Incorporation, New York.

Wang, Y., Harvey, C., Rousset, M. & Swallow, D.M. (1994). Expression of human intestinal mRNA transcripts during development: analysis by a semi quantitative RNA polymerase chain reaction method. *Pediatrician Residence*. **36,** 514–21.

Widdowson, E.M. & Thrussell, L.A. (1951). The absorption and excretion of nitrogen, calcium, magnesium and phosphorus. In: *Studies of Undernutrition*, Wuppertal 1946-1949. *Medical Research Council Special Report 275,* 296–312. London: His Majesty's Stationery Office.

Wyss, M., Brugger, R. & Kronenberger, A. (1999). Biochemical characterization of fungal phytases (myoinositol hexakisphosphate phosphohydrolase): Catalytic properties. *Applied and Environmental Microbiology,* **65,** 367-373.

Yan Want, C., Reilly, C., Patterson, C., Morrison, E. & Tinggi, A. (1992). Contribution of breakfast cereals to Australian intake of trace elements. *Food Australia*, **44,** 70-72.

Yang, W.J., Matsuda, Y., Inomata, M. & Nakagawa, H. (1991). Developmental and dietary induction of the 920K subunit of rat intestinal phytase. *Biochimica et* Biophysica Acta *Journal*, **1075,** 83-87.

Yoa, M.Z., Zhang, Y.H., Lu, W.L., Hu, M.Q., Wang, W. & Liang, A.H. (2011). Phytases: Crystal structures, protein engineering and potential biotechnological applications. *Journal of Applied Microbiology* **11,** 1364-5072.

Zhou, R.J. & Erdman, J.W. (1995). Phytic acid in health and disease. *Critical Reviews in Food Science and Nutrition*, **35,** 495-508.

CHAPTER 3

Enzyme performance and mineral composition of a fortified ready-to-eat breakfast cereal treated with functional enzymes in a shelf-life study

ABSTRACT

The aim of this study was to conduct large-scale factory trials of a ready-to-eat breakfast cereal to evaluate the effect of added functional enzymes within a 12-month shelf-life study. Two functional enzymes, Tolerase L® (lactases) and Tolerase P® (phytases), were tested individually in the ready-to-eat breakfast cereal. The samples were produced, packed in the factory and put on shelf-life at ambient temperature (22ºC) for 12 months. Based on the percentage activity of the enzyme in the base material, the enzyme activity of the Tolerase L-treated samples was found to be acceptable within a 9-month shelf-life period, whereas the enzyme activity of the Tolerase P-treated samples was found to be acceptable for the full 12-month shelf-life period. The mineral content results of the Tolerase P-treated samples indicated that Tolerase P® enzyme activity remained sufficient throughout product shelf-life and that the increase in mineral composition could potentially exclude future fortification. The percentage increase in the mineral content for calcium, zinc and iron over a period of 12 months' shelf-life was found to be 43.3%, 49.7% and 56.7%, respectively. According to the South African labelling regulations, these results correspond to the percentage nutrient reference values required for individuals over the age of four years. The results of the enzyme activity of the Tolerase P-treated and Tolerase L-treated cereal samples could find valuable application in product development and marketing of ready-to-eat breakfast cereals.

***Keywords*:** Ready-to-eat breakfast cereal, functional enzymes, lactases, phytases, minerals.

1 INTRODUCTION

1.1 Lactose intolerance

Globally lactose intolerance has become increasingly common (Ingram *et al.,* 2009). In 1983 it was reported that 78% of the South African black population are lactose intolerant (Segal,1983), however, according to FACTS (South Africa), ca. 90% of the black population are lactose intolerant, 20-40% of the coloured population and 20% of white South Africans (Personal communication, 2015, H. Steinman, Food and Allergy Consulting and Testing Services, Cape Town, South Africa). Only 5% of the population in Europe are lactose intolerant, i.e. in contrast to the ca. 90% in Asian and African countries (Bulhões *et al.,* 2007).

A large percentage of individuals suffer from the *primary* lactase deficiency disease, hypolactasia, which manifests post-weaning between the ages of 3 and 5 years as a result of a natural loss of intestinal lactase (Moore, 2003; Heyman, 2006). Undigested lactose travels to the colon where it is fermented by the microorganisms to produce short chain fatty acids, hydrogen, methane, and carbon dioxide (Matthews *et al.,* 2005; He *et al.,* 2006), causing discomfort with symptoms such as diarrhoea, nausea, bloating and abdominal pain (Lomer *et al.*, 2007). Some individuals suffering from primary lactase deficiency are able to consume fresh milk and dairy products as part of their diet without illustrating symptoms typically associated with this disease (Moore, 2003). *Secondary* lactase deficiency is regarded as a deficiency of lactase as a result of damage to the inner lining of the small intestine as a result of medication, irritable bowel syndrome, surgery or radiation therapy (Savaiano & Levitt, 1987; Scrimshaw & Murray, 1988; Srinivason & Minocha, 1998). When individuals with lactose intolerance, primary or secondary, avoid lactose-containing dairy products, they could have a hampered quality of life due to diet restrictions and potential calcium deficiency (Swallow, 2011).

In the United States of America (USA) the demand for lactose-free dairy products has risen 20% per year since 2000 (Jelen & Tossavianen, 2003), resulting in lactose-free milk products (<1 g/L), milk products containing reduced levels of lactose (<5 g/L) and dairy-free milk products (Adhikari *et al.,* 2010; Trani *et al.,* 2017). A survey amongst Italian consumers indicate that 18.1% individuals drink lactose-free milk (Zingone *et al.,* 2017).

Lactose hydrolysis, using lactase as an additive in commercial lactose-free milk, increase lactose digestibility, thereby improving the functional properties of lactose–containing products. Although milk products supplemented with lactase are more expensive than regular milk due to the sensitive nature of this enzyme during shelf-life (Harju *et al.,* 2012), the use of lactase as functional enzyme additive during production, can be regarded

as a viable option. Lactase supplements are also available at pharmacies, giving the lactose-intolerant consumer the liberty of consuming dairy products. Tolerase L®, an enzyme used globally as functional ingredient in production, is able to convert lactose into glucose and galactose (Fig.1). This enzyme originates from the fungus *Aspergillus oryzae* and works at a low pH (3.5 – 5.5), thus making it ideal to digest lactose in the stomach. This enzyme, with its properties mentioned above, can therefore be regarded as a viable dietary supplements or digestive aid (Wyss *et al.,* 1999)

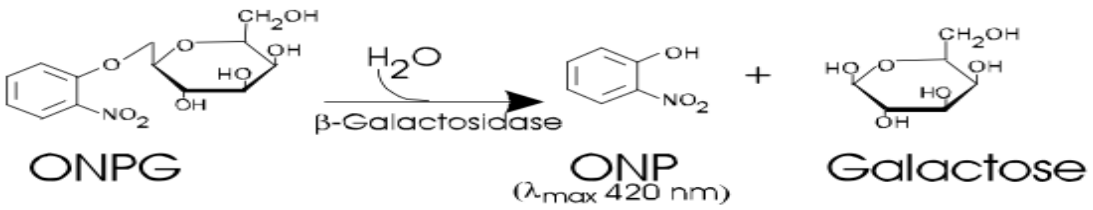


Figure 1 Reaction catalysed by β-galactosidase when *ortho*-nitrophenyl-β-galactoside (ONPG) is used as substrate (Adapted from Biotek® resources online).

A limited number of products supplemented with lactase during production exist, one example is instant oats breakfast shakes containing the enzyme lactase, however, breakfast cereals with functional enzyme additives, such as lactase have to date not been introduced in South Africa (Personal communication, 2015, M. Kent, DSM Technologies, Johannesburg, South Africa). Due to the restrictions on labelling of foodstuffs with health claims, the successful marketing of such products in terms of potential health benefits is quite limited (Regulation 146 of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 [Act 54 of 1972], DOH, 2010).

Phytic acid in cereals

In South Africa, the fortification of bread flour and maize meal is strongly regulated by the Department of Health (Regulation 146 of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 [Act 54 of 1972], DOH, 2010). The minerals of importance are iron (Fe), zinc (Zn), calcium (Ca) and phosphorus (P). The recommended nutrient reference values (NRV) for consumers four years and older for Fe and Zn are 15 mg per day and for Ca and P the NRV are 1,000 mg per day.

The main aim of fortification of high-fibre breakfast cereals is to compensate for the mineral loss during processing, but more importantly for the lessened bioavailability of specific minerals due to the fact that phytic acid naturally binds minerals, thereby making them biochemically less accessible to humans. Inadequate mineral intake can lead to

nutritional deficiencies (Hercberg *et al.,* 2001). Zinc is regarded as an essential trace element, it is involved in immune function, activation of enzymes and cell growth (Coulibaly *et al.,* 2011). Zinc deficiency is quite prevalent due to inadequate dietary supply or as a result of an increased requirement of Zn during growth, puberty, pregnancy and lactation (Coulibaly *et al.,* 2011). Globally Fe deficiency, i.e. anemia, is regarded as the most prevalent mineral deficiency (Hercberg *et al.,* 2001) and it affects a large proportion of individuals in developed, as well as developing countries. Iron deficiency is primarily caused by insufficient intake of Fe. A number of dietary factors can impact on Fe absorption, however, the source of Fe supplementation, as well as the composition of a meal significantly add to the efficacy of Fe absorption (Hercberg *et al.,* 2001). Calcium deficiency is related to the removal of Ca from bone, resulting in lower bone density and in turn osteoporosis (Grases *et al.,* 2000).

Apart from the fact that phytic acid in cereals forms insoluble complexes with the minerals Fe, Zn and Ca, phytic acid (inositol hexakisphosphate, IP_6), is the major storage form of phosphorus in grains and plant seeds (Coulibaly *et al.,* 2011). In agro-processing of cereals, phytase enzymes are used in animal feed to increase the potential bioavailability of phosphorus (Simon & Igbasan, 2002). It can also be added to breakfast cereals as a processing aid to facilitate the breakdown of mineral-phytic acid-complexes and ultimate release of important minerals, i.e. Zn, Fe and Ca (Jacela *et al*., 2010; Coulibaly *et al.,* 2011). The level of phytic acid in cereals can be variable as a result of factors such as growing conditions, type of fertiliser used, harvesting techniques, age of the product, processing methods, test methodologies (Srivasteva *et al*., 1955) and the pH of the product in question as the binding of Ca with phytic acid is pH-dependent (Zhou & Erdman, 1995). When phytic acid is hydrolysed by phytase, one molecule of inositol and six molecules of inorganic phosphate is formed. Fig. 2 illustrates the effect of phytase on phytic acid where minerals and other elements are released from the complex molecule.

The determination of phytase activity in commercial products is based on the calorimetrical quantification at 700 nm of free phosphorus released by the hydrolysis of phytate using ammonium molybdate as colour reagent (Sambrook *et al.,* 1989). Tolerase P® is a phytase enzyme which can ultimately assist the gastrointestinal tract in effectively absorbing Fe, Zn, Ca, Mg and P from cereal- and legume-based foods, thereby improving mineral nutrition (Personal communication, M. Kent, DSM Technologies, Johannesburg, South Africa).

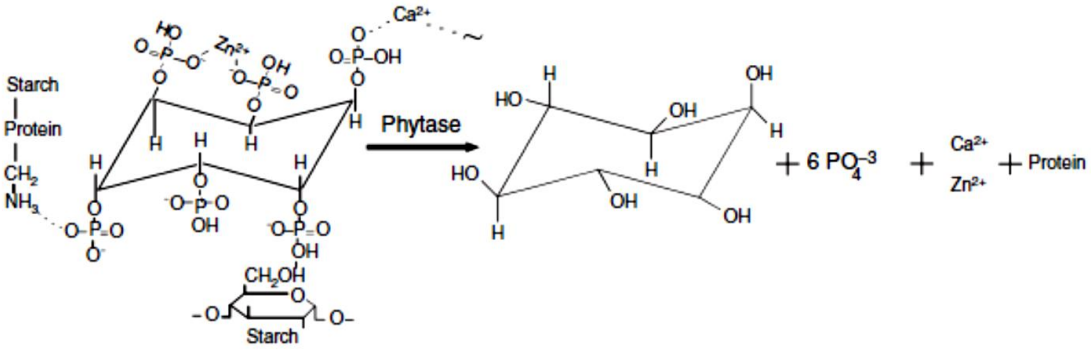


Figure 2 The hydrolysis of phytate by phytase into inositol, phosphate and other elements. The removal of phosphate groups by phytase results in the release of metals, metal-binding enzymes and proteins (Adapted from Yao *et al*., 2011).

In view of the above, the objective of this study was to determine the enzyme activity and shelf-life properties of Tolerase L® and Tolerase P® in the enzyme-fortified ready-to-eat breakfast cereal, as well as the percentage increase in the mineral composition of the product treated with Tolerase P® throughout a 12-month shelf-life period.

2 MATERIALS AND METHODS

2.1 Sample materials and compounds

For this research, three independent batches (100 kg per batch) of the breakfast cereal were produced at a central production facility (Wadeville, Johannesburg, South Africa). Each independent production batch was further divided into three lots, one lot per treatment. The first treatment, a control, comprised of untreated standard ready-to-eat breakfast cereal (existing formulation), for the second treatment a lactose digesting enzyme was added to the ready-to-eat breakfast cereal and for the third treatment a phytate digesting enzyme was added. The two enzymes used as functional additives for the respective enzyme-treated products were Tolerase L® and Tolerase P®, both commercialised by DSM Technologies, Heerlen, The Netherlands.

2.1.1 Formulation, production and storage of untreated samples

The formulation used for the production of the untreated samples is shown in Table 1. The formulation consisted of the two main ingredients, whole ground yellow maize flour and soya flour. A vitamin premix consisting of vitamin D_3, vitamin A and vitamin B_1, a slurry mixture consisting of sugar, salt, tri-calcium phosphate and chicory powder, as well as flavouring

and colourant (beta-carotene) was added to the two main ingredients to produce the ready-to-eat cereal, 100 kg in total. This basic formulation was produced in triplicate (3 x 100 kg).

The ingredients of the three batches were pre-weighed and mixed according to a standard production procedure (Fig. 3), i.e. the ground soya and maize, 50% of the vitamin mixture and premix (chicory powder, sugar, salt and tri-calcium phosphate). This mixture of dry ingredients was then divided manually (50:50) between the mixing tanks of autoclave A and autoclave B (Fig.4). Processing water was added to produce a slurry. This mixture was autoclaved for 20 min at 125ºC. After cooking, the beta-carotene and 50% of the flavouring was added. The automated system mixed the ingredients for a further 15 min, followed by roller drying of the slurry mix (Fig. 5), resulting in thin sheets of ready-to-eat cereal. Chicory powder and cereal flavouring were added to mask the natural, but negative aftertaste associated with soya flour (Personal communication, 2016, M. Doms, International Flavour and Fragrances, Johannesburg, South Africa). Thereafter the thin sheets were transferred to a holding bin where after it was crushed into small cereal flakes using a screw blender (Nauta mixer, Shanghai, China). The remainder of the flavouring, premix and vitamin mixture was added and the total mixture was mixed for a further 10 min. Finally, the product was sieved through a 5 mm sieve to ensure uniform distribution of flavour and to remove any lumps that might have formed during processing. The final product was packed in metallised foil (Nampak, Epping Industrial Park, Cape Town) via a form-fill and seal process and stored for 12 months in corrugated carton boxes in a temperature-controlled (22ºC) storage area. The metallised foil was used to keep out direct light which may influence the activity of the enzyme. The packed product went through a metal detection stage (aluminium, lead and copper) to check for any metal contamination.

2.1.2 Formulation, production and storage of samples treated with Tolerase L® (TolL)

The formulation used for the production of the Tolerase L-treated cereal samples was the same as for the untreated samples (Table 1), however, the only difference was the addition of the enzyme. The dosage of TolL (0.500%) was based on the treatment of TolL required to digest the lactose content of 200 mL milk added to each 50 g serving of cereal (Personal communication, M. Kent, DSM Technologies, Johannesburg, South Africa). The TolL was added during the manual ingredient addition stage (Fig. 3).

2.1.3 Formulation, production and storage of samples treated with Tolerase P® (TolP)

The formulation used for the production of the Tolerase P-treated cereal samples was the same as for the untreated samples (Table 1), again the only difference was that the enzyme

in question was added. The dosage of TolP (0.850%) was based on the treatment of TolP required to digest the phosphate content of each 50 g serving of cereal (Personal communication, M. Kent, DSM Technologies, Johannesburg, South Africa). The TolP was added during the manual ingredient addition stage (Fig. 3).

Table 1 Formulation of untreated ready-to-eat cereal

Ingredients	Quantity (kg)
Vitamin mixture	0.385
Premix consisting of	7.883
White sugar	3.995
Salt	0.586
Tri-calcium phosphate	1.332
Chicory powder	1.971
Beta-carotene as colourant	0.010
Cereal flavouring	0.096
Whole ground yellow maize flour	65.333
Soya flour	26.293
Total	**100.00**

2.2 Experimental layout and sampling for enzyme activity assay tests

As mentioned, there were three block replicates of each treatment (untreated, control sample; TolL-treated sample; TolP-treated sample). The TolL-treated samples were tested for lactase enzyme activity, whereas the TolP-treated samples were tested for phytase enzyme activity, both at the Department of Biochemistry, Stellenbosch University, South Africa. Analyses were conducted once a month over a 12-month period giving a total of 13 testing time-slots. Per block, observations over time were thus replicated measures of the same block (or batch), i.e. per treatment, three batches of each shelf-life month were analysed for enzyme activity. The experimental layout is indicated below (Table 2).

Table 2 Sample set-up for enzyme activity analysis of treated ready-to-eat breakfast cereal.

Sample identification	Shelf-life period (months)	Number of independent batches tested per month	Total number of samples submitted for testing over 13 time-slots
Cereal with enzyme treatment-TolP	0–12	3	39
Cereal without enzyme treatment (Control)	0–12	3	39
Cereal with enzyme treatment-TolL	0–12	3	39
Cereal without enzyme treatment (Control)	0–12	3	39

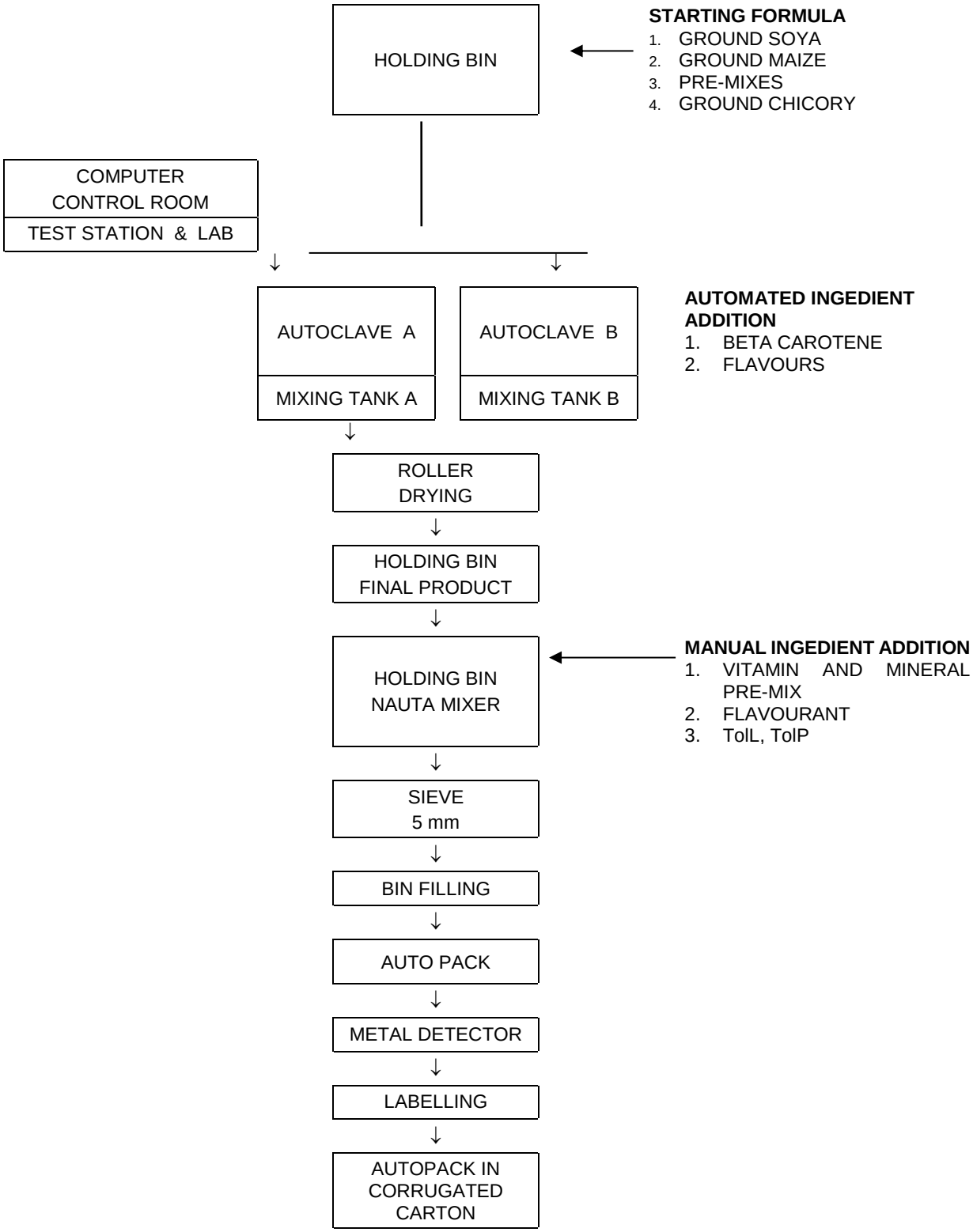


Figure 3 Automated production process in factory of ready-to-eat cereals: production of the untreated control samples, TolL-treated samples and TolP-treated samples.

Figure 4 Autoclave area where the slurry mix is produced after the addition of ingredients.

Figure 5 Roller driers in where the slurry mix is dried after the autoclave step.

2.3 Enzyme activity assay of TolL

2.3.1 Tolerase L® (β-Galactosidase) enzyme activity assay

β-Galactosidase (β-gal), also generally identified as lactase, is a hydrolase enzyme which catalyses the hydrolysis of lactose to form β-galactosides (Lomer *et al.,* 2007). This enzyme is also able to hydrolyse the ether bond of o-nitrophenyl β-D-galactopyranoside, a colourless compound, to yield o-nitrophenyl which absorbs light at 420 nm. This reaction can be monitored continuously by using an Ultraviolet-visible (UV) spectrophotometer (Sambrook *et al.,* 1989).

2.3.2 Soluble protein extraction

Water soluble proteins, including β-galactosidase and phytase, were extracted by liquid-solid extraction (22ºC). Five hundred milligrams of each cereal sample were suspended in 10 mL distilled water. This suspension was shaken vigorously at 2,000 rpm for 10 min using a bench top shaker (IKA® Vibramax VXR, Germany), followed by centrifugation at 3,500 x g. The supernatant, containing the water-soluble proteins, was used as the crude extract for all subsequent analyses without further dilution.

2.3.3 β-gal activity assay

β-galactosidase activity was determined by comparison to the enzyme standard (TolL). The assay was performed as described by Sambrook *et al*. (1989). One hundred microliter aliquots of each standard and sample were transferred to a 96-well microplate. The assay was initiated by the addition of 2x100 µL reaction buffer consisting of 200 mM sodium phosphate, pH 7.0, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol and 1.33 $mg.mL^{-1}$ *ortho*-Nitrophenyl-β-galactoside (ONPG). The absorbance of the mixtures was determined every 5 s at 25°C for a total of 2 min using a spectrophotometer running Biotek Gen5® data analysis software (Biotek Powerwave, BioTek Instruments, Winooski, Vermont, United States of America) at 420 nm. The data obtained were analysed using Excel software.

2.4 Enzyme activity assay of TolP

2.4.1 Tolerase P® (phytases) enzyme activity assay

The determination of phytase activity is a colorimetrical method to quantify the free phosphorus released by the hydrolysis of phytate using ammonium phosphomolybdate (Heinonen & Lahti, 1981).

2.4.2 Soluble protein extraction

Water soluble proteins, including phytase, were extracted by liquid-solid extraction performed at room temperature (22ºC). Five hundred milligrams of each cereal sample were weighed and suspended in 10 mL distilled water. This suspension was vigorously shaken at 2,500 revolutions per min (rpm) for 10 min using a benchtop shaker (IKA® Vibramax VXR, Germany), followed by centrifugation at 3,500 x g for 10 min. The supernatant, containing the water-soluble proteins, was used as the crude extract for all subsequent analyses without further dilution.

2.4.3 Phytase activity assay

The activity of phytase in different batches of cereal was determined by comparison of the absorbance of a sample to that of a potassium dihydrogen phosphate standard. The assay was performed by using a modified protocol to that of the Fujian Fuda Biotech Company (Fujian, China). Potassium dihydrogen phosphate stock solution (8.0 mol.L^{-1}) was prepared in deionised water with potassium dihydrogen phosphate crystals which were dried to a constant mass at 60°C. The stock solution was then serially diluted to working standards of 0.8, 1.6, 2.4, 3.2, 4.0 and 8.0 mol.L^{-1}.

Two hundred microliters of these standards were incubated at 37°C for 5 min after which 800µL of substrate solution (577.4 mg sodium phytate ($C_6H_6O_{24}P_6Na_{12}$) from rice, 574.2 mg sodium acetate (pH 5.0) in 100 mL diH_2O v.v^{-1}) was added. The solutions were mixed and incubated for exactly 30 min at 37°C at which time 1.0 mL 5% trichloroacetic acid was added as a stop reagent. Subsequently, 1 mL colour reagent (4 parts solution A [7.5 g ammonium heptamolydate ($N_6H_{24}MO_7O_{24}\cdot 4H_2O$], 22 mL of 98% sulphuric acid in 500 mL diH2O); 1 part solution B [2.7% ferrous sulphate]) was then added.

Vial contents were mixed and centrifuged for 10 min at 3,500 rpm before standing for 10 min at room temperature (22ºC). The absorbance of the standards (200 µL) was then measured in triplicate at 700 nm using a 96-well plate spectrophotometer (Biotek® Powerwave, BioTek Instruments, Winooski, Vermont, United States of America) under the control of the software package. After correcting the absorbance of the standards by subtraction of the value for a water control, the measurements were plotted against phosphate quantity (µmole). The obtained values were then divided by 30 min to quantify enzymatic units within the assay.

Taking extraction volume and cereal mass into account, phytase units (FTU) were used to calculate FTU.100 g^{-1} dry cereal. Furthermore, a reference curve, plotting micromole

liberated phosphate per min against mass Tolerase P® (g), the enzyme additive under investigation, was generated in precisely the same way.

2.5 Mineral content analysis of Tolerase P–treated samples

The mineral content of the TolP-treated samples were also tested. The minerals, calcium (Ca), zinc (Zn) and iron (Fe) were of interest in the TolP-treated samples. The sample sets used for the analysis of the mineral content in the TolP-treated samples are shown in Table 3. For each time slot, a composite sample of the three independent batches were prepared to test the minerals content. The samples were analysed in triplicate every four months over the 12-month shelf-life period. The samples were not tested at month 0 as the standard cereal mineral content was known.

The mineral content was determined using Method 2013.06 of the Association of Official Analytical Chemists (AOAC) and Agilent Application Note 5990-4539EN ICP-MS methods. Inductively coupled plasma mass spectrometry or ICP-MS was used as an analytical technique to determine the mineral content in the TolP-treated cereal samples (AOAC, 2016). As a composite sample was tested in triplicate for mineral content, only average content was calculated.

Table 3 Sample set for the mineral content analysis of a ready-to-eat breakfast cereal treated with TolP

Sample Identification	Shelf-life period (months)	Number of samples per batch per month	Total number of samples submitted for testing for the 3 time-slots
Cereal with enzyme treatment – Tolerase P	4, 8 & 12	3	9

3 RESULTS AND DISCUSSION

The shelf-life of the ready-to-eat breakfast cereal used in this study (without any added enzymes) is approximately 12 months. Breakfast cereals with added functional ingredients such as digestive enzymes usually have a shorter shelf-life (approximately 7 months), primarily to ensure that the functional ingredient's enzyme activity stays effective throughout the entire shelf-life period. The latter specifications usually form an integral part of the marketing strategy of companies that produce exclusive ranges of breakfast cereals focussing on specific health issues (Personal communication, 2017, J. Barendse, Breakfast

Cereals Brand Manager, Pioneer Foods (PTY) Ltd., Cape Town, South Africa). According to the suppliers of the enzymes used in this study (TolL & TolP) both enzymes should have an effective shelf-life, i.e. functionality of two years as an ingredient packaged on its own (Personal communication, 2015, M. Kent, DSM Technologies, Johannesburg, South Africa).

3.1 TolL-treated breakfast cereal

For this study, the existing formulation of the ready-to-eat breakfast cereal without enzyme additives was developed to have a shelf-life of 12 months. To ensure a sustained, adequate 12-month shelf-life of the TolL-treated breakfast cereal, it was decided to overdose the product with TolL at month zero, primarily because the shelf-life of the newly developed TolL-treated product was not known. According to literature (Portincasa *et al.,* 2008), the optimum quantity of the enzyme in a breakfast cereal should be 50 acid lactase units (ALU) per gram cereal. Thus, for a 50 g serving of TolL-treated breakfast cereal, the optimum quantity is defined as 2500 ALU.

The acid lactase units per gram dry cereal of the TolL-treated cereal samples for each month of the full shelf-life period (12 months) are shown in Fig. 6**.** The initial value at month 0 was quite high at 67.7 acid lactase units per gram dry cereal (ALU.g^{-1}). At the end of the 12-month shelf-life period, the ALU.g^{-1} value was found to be lower at 31.9, much lower than the prescribed optimum of 50 acid lactase units (ALU) per gram cereal. This decline in enzyme activity could be as a result of loss of enzymatic activity after shelf-life of 12 months. Between the shelf-life months of five and seven, there was a rapid decline in the acid lactase units per gram dry cereal from 62.3 to 39.1 (respectively), however, before and after five and seven months the decline was less and the enzyme thus seemed more stable or less active. The reason for this tendency is not clear. Between the shelf-life months five and six the breakfast cereal illustrates an optimum amount of TolL, i.e. 50 acid lactase units (ALU) per gram cereal, i.e. similar to the prescribed effective dosage, however, after six months the results indicate that the shelf-life and thus the activity of TolL declines (less than the prescribed optimum of 50 acid lactase units (ALU) per gram cereal).

The mean acid lactase units of the TolL-treated cereal samples (per 50 g serving), per shelf-life month, are illustrated in Fig. 7. The initial value at month 0 was found to be 3385 ALU.g^{-1}. At the end of the shelf-life period (12 months), the ALU.g^{-1} was found to be 1598. The optimum quantity of this enzyme required to digest lactose in 200 mL milk mixed with a 50 g serving dry cereal is 2500 ALU (Personal communication, M. Kent, DSM Technologies, Johannesburg, South Africa). Between shelf-life months five and six the breakfast cereal illustrated the latter optimum quantity, i.e. 3115 ALU at five months shelf-

life and 2358 ALU at six months shelf-life. Similarly, as indicated in Fig. 6, a shelf-life of five to six months results in an optimum quantity of enzyme for lactose digestion.

Studies by Portincasa *et al*. (2008) showed with the hydrogen breath test that 109 patients with hypolactasia and 25 patients with an inability to absorb lactose and treated with an oral lactase supplement, tilactase (3080 ALU.200 mL^{-1} milk), illustrated an effective decrease in lactose maldigestion symptoms (>80%). This research group's dosage levels correspond to the dosage levels required for TolL used in this study in ready-to-eat breakfast cereal, as seen in Fig. 7. In another study by Ojetti *et al.* (2010), 60 patients with hypolactasia were treated with a lactase supplement (4320 ALU.200 mL^{-1} milk). The results of the latter study also showed a significant reduction in symptoms associated with lactose intolerance.

The percentage enzyme activity of the TolL enzyme in the dry breakfast cereal is shown in Fig. 8. The enzyme activity of TolL at month zero was found to be 100%. At the end of the shelf-life (12 months), the enzyme activity was found to be 47.9%. Using the results of Figures six and seven, one could postulate that the ideal percentage enzyme activity should be between 62.5% and 70.82%, as indicated in Fig. 8 at months five and six, respectively. According to Semenza *et al*. (2001), only 50% of the initial enzyme activity of lactase is required to digest the amount of lactose found in 200 mL of milk. One could argue that the latter could indicate that the breakfast cereal treated with TolL could have a shelf-life that is stable up until 9 months where the percentage activity was found to be 53.9%.

3.2 TolP-treated samples

3.2.1 TolP enzyme activity

The initial phosphorus content of the breakfast cereal was calculated, i.e. 528 mg per 100 g dry cereal. Phosphorous is mainly stored in the form of phytic acid in cereal grains, and the phosphorus content (Mittal *et al*., 2013). The amount of TolP could be calculated using the initial phosphorus content present in the breakfast cereal. Thus, the quantity of enzyme required to digest the amount of phytic acid per 100 g dry cereal was calculated as 170 phytase enzyme units (FTU).

The mean values of the TolP-treated cereal samples enzyme activity are illustrated in Fig. 9. At month zero the FTU per 100 g dry cereal was found to be 187. At the end of the shelf-life (12 months), the FTU.$100g^{-1}$ dry cereal was found to be 259. Over the shelf-life period of 12 months, the activity of this enzyme increased rapidly. Our results illustrated that the quantity of the enzyme remains sufficient throughout the 12 months shelf-life of the

product. The latter positive outcome could be due to the metallised foil sheeting that excluded light and oxygen from the product for the duration of the shelf-life period, thereby conserving the enzyme.

3.2.2 Mineral content

The cereal used for this study aims to provide 30% of the NRV for Fe and Zn, and 60% of the NRV for the Ca per serving (50 g) of cereal, i.e. according to in-house product formulation specifications (Personal communication, 2017, J. Barendse, Breakfast Cereals Brand Manager, Pioneer Foods (PTY) Ltd., Cape Town, South Africa).

The initial mineral content of the untreated breakfast cereal was known as the breakfast cereal was tested for full nutritional content prior to the addition of TolP. The mineral content of the samples was analysed to determine the increase in mineral content over a period of 12 months' shelf-life.

The mineral content of the treated samples (mg.100 g^{-1} dry cereal) are shown in Fig's. 10, 11 and 12**,** where Ca, Zn and Fe quantities are seen, respectively. The results are illustrated over a 12-month period, with values in mg.100 g^{-1} dry cereal given every four months. The initial Ca content at month zero was known, i.e. 397 mg per 100 g dry cereal. At month 12, the Ca content was found to be 700 mg per 100 g dry cereal (Fig. 10). This indicates a 43.3% increase in the Ca content over a 12-month shelf-life period. The initial Zn content was found to be 8.3 mg per 100 g dry cereal at month 0. At month 12, the Zn content was found to be 16.5 mg (Fig. 11). The percentage increase for the Zn content over a period of 12 months is thus 49.7%. The Fe content at month 0 (Fig. 12), was found to be 9.1 mg per 100 g dry cereal. At month 12, the Fe content was found to be 21 mg per 100 g dry cereal, indicating a 57.1% increase in the Fe content over a period of 12 months' shelf-life. This increase of Ca, Zn and Fe, measured every four months, clearly indicates that the enzyme was active during the full 12-month shelf-life of the product. The mineral increase of Ca, Zn and Fe is thus due to the constant breakdown of phytic acid in the dry cereal by the TolP enzyme (phytases) in order to release the intrinsic minerals present in the cereal. Phytases have been widely used in animal feed to release intrinsic phosphorus for nutrition purposes and to minimise phosphorus pollution in animal waste (Yao *et al.,* 2011). These amounts for the different minerals provide more than the untreated cereal in terms of NRVs of individuals four years and older, i.e. according to the South African labelling regulations (DOH, 2010). For Ca, Fe and Zn, the TolP-treated breakfast cereal provides 70%, 140% and 110%, respectively. Thus, to achieve the NRVs for individuals four years and older,

fortification with mineral premixes of the breakfast cereal can clearly be excluded from the process when treated with TolP.

4 CONCLUSIONS

This research illustrated that TolP and TolL are both potential enzymes to be used in the food industry as the enzyme activity remains sufficient for the ascribed minimum shelf-life (seven months) of the product. The percentage activity of the TolL enzyme remains sufficient for lactose digestion for up to 9 months of the product's shelf-life based on the fact that only 50% of the enzymes activity is required for lactose digestion. The optimum quantity of TolL required for effective lactose digestion (2500 ALU.200 mL^{-1} milk) is thus justified by the illustrated studies.

The activity of the TolP enzyme also remains sufficient throughout shelf-life of the product for 12 months. Therefore, it could be deduced that the shelf-life of the dry cereal fortified with TolP, is 12 months, based on the activity of the enzyme. The content of the all minerals analysed increased throughout the shelf-life of the product treated with TolP. The percentage increase in the mineral content for Ca, Zn and Fe over a period of 12 months' shelf-life was found to be 43.3%, 49.7% and 57.1%, respectively. The increase in content of all the minerals is an indication that possible exclusion of fortification from the formulation could be implemented, with the cost saving of the addition of minerals. However, this aspect should be tested.

5 REFERENCES

Adhikari, K., Dooley, L.M., Chambers, E. & Bhumiratana, N. (2010). Sensory characteristics of commercial lactose free milks manufactured in the United States. *LWT Food Science and Technology,* **43**, 113-118.

Andlid, T. (2000). Extracellular phytase activity expressed by Saccharomyces cerevisiae in relation to the pho gene family. *Tenth International Symposium on Yeasts*, Papendal, Arnhem, the Netherlands 2000. (Abstract) Symposium Book p. 223. Delft: Delf, University Press.

AOAC (1995). Official Methods of Analysis, 15th ed. AOAC, Gaithesburg, MD.

Bulhões, A.C., Goldani, H.A.S. & Oliviera, F.S. (2007). Correlation between lactose absorption and the C/T-13910 and G/A-22018 mutations of the lactase phlorizin

hydrolase (LCT) gene in adult-type hypolactasia. *Brazilian Journal of Medical & Biological Research,* **40,** 1441-1446.

Coulibaly, A., Kouakou, B. & Chen, J. (2011). Phytic acid in cereal grains: Structure, healthy or harmful ways to reduce phytic acid in cereal grains and their effects on nutritional quality. *American Journal of Plant Nutrition*, **1**, 1-22.

Department of Health (2010). Regulation 146 of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 [Act 54 of 1972]. Department of Health, Pretoria, South Africa.

Grases, F., Prietro, R.M., Simonet, B.M. & March, J.G. (2000). Phytate prevents tissue calcification in female rats. *Biofactors,* **11**, 171-177.

He, T., Priebe, M.G., Harmsen, H. J., Stellaard, F., Sun, X. & Welling, G. W. *(*2006). Colonic fermentation may play a role in lactose intolerance in humans. *Journal of Nutrition,* **136**, 58-63.

Heinonen, J.K. & Lahti, R.J. (1981). A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Analytical Biochemistry,* **113**, 313-317.

Hercberg, S., Preziosi, P. & Galan, P. (2001). Iron deficiency in Europe. *Public Health Nutrition,* **4**, 537-545.

Heyman, M.B. (2006). Lactose intolerance in infants, children and adolescents. *Pediatrics,* **118**, 1279-1286.

Harju, M., Kallionen, H. & Tossavainen, O. (2012). Lactose hydrolysis and other conversions in dairy products: Technological aspects. *International Dairy Journal*, **22**, 104-109.

Ingram, C.J., Mulcare, C.A., Itan, Y., Thomas, M.G. & Swallow, D.M. (2009). Lactose digestion and the evolutionary genetics of lactase persistence. *Human Genetics,* **124,** 579-591.

Jacela, J.Y., DeRouchey, J.M., Tokach, M.D., Goodband, R.D., Nelssen, J.L., Renter, D.G. & Dritz, S.S. (2010). Feed additives for swine: Fact sheets-prebiotics, probiotics and phytogenics. *Journal of Swine Health and Production*, **18**, 87-91.

Jelen, P. & Tossavainen, O. (2003). Low lactose and lactose-free milk and dairy products – prospects, technologies and applications**.** *Australian Journal of Dairy Technology,* **58**, 161-165.

Lomer, M.C., Parkes, G. C. & Sanderson, J. D. (2007). Review article: Lactose intolerance in clinical practice-myths and realities. *Alimentary Pharmacology and Therapy*, **27**, 93-103.

Mansbridge, S.C. (2016). Strategies and efficacy of phytase supplementation and it's interaction with pharmaceutical zinc oxide in newly weaned pigs. PhD in Philosophy Thesis, Harper Adams University, United Kingdom.

Matthews, S.B., Waud, J. P., Roberts, A. G. & Campbell, A. K. (2005). Systemic lactose intolerance: a new perspective on an old problem. *Postgraduate Medical Journal*, **81**, 167-173.

Mittal, A., Gupta, V., Singh, G., Yadav, A. & Aggarwal, N.K. (2013). Phytase, a boom in food industry. *Octa Journal of Biosciences,* **1**, 158-169.

Moore, B.J. (2003). Dairy foods: are they politically correct? *Nutrition Today,* **38**, 82-90.

Ojetti, V., Gigante, G., Gabrielli, M., Ainora, M.E., Mannocci, A., Lauritano, E.E., Gasbarrini, G. Y Gasbarrini, A. (2010). The effect of oral supplementation with *Lactobacillus reuteri* or tilactase in lactose intolerant patients. *European Review for Medical and Pharmacological Sciences*, **14**, 163-170.

Portincasa, P., Di Ciaula, A., Vacca, M., Montelli, R., Wang, D.Q. & Palasciano, G. (2008). Beneficial effects of oral tilactase on patients with hypolactasia. *European Journal of Clinical Investigation,* **38** (11), 835-844.

Sambrook J., Fritsch, E.F. & Maniatis, T. (1989) Molecular Cloning: *A Laboratory Manual* 2nd edition, New York: Cold Spring Harbor Laboratory Press.

Savaiano, D.A. & Levitt, M. D. (1987). Milk intolerance and microbe-containing dairy foods. *Journal of Dairy Science*, **70**, 397-406.

Scrimshaw, N. & Murray, E. (1988). Lactose tolerance and milk consumption. *Journal of Latin American Nutrition*, **38**, 543-567.

Segal, I. (1983). Lactase deficiency in the South African black population. *American Journal of Clinical Nutrition,* **38,** 901-905.

Semenza, G, Auricchio, S. & Mantei, N. (2001). Small intestinal disaccharides. In: The Metabolic and Molecular Bases of Inherited Disease, 8th edition. Netherlands: SSIM and Kluwer Academic Publishers.

Simon, O. & Igbasan, F. (2002). In vitro properties of phytases from various microbial origins. *International Journal of Food Science and Technology,* **37**, 813-822.

Srivastava, B.N., Biswas, T.D. & Das, N.B. (1955). Influence of fertilizers and manures on the content of phytin and other forms of phosphorus in wheat and their relation to soil phosphorus. *Journal of the Indian Society of Soil Science*, **3**, 33-40.

Srinivasan, R. & Minocha, A. (1998). When to suspect lactose intolerance. Symptomatic, ethnic, and laboratory clues. *Postgraduate Medicine*, **104**, 109-111, 115-116, 122-123.

Swallow, D.M. (2011). Lactose intolerance. University College London, London, UK, 236-240.

Trani, A., Gambacorta, G., Loizzo, P., Cassone, A., Fascianno, C., Zambrini, A.V. & Faccia, M. (2017). Comparison of HPLC-RI, LC/MS-MS and enzymatic assays for the analysis of residual lactose in lactose-free milk. *Food Chemistry,* **233**, 385-390.

Wyss, M., Brugger, R. & Kronenberger, A (1999). Biochemical characterization of fungal phytases (myoinositol hexakisphosphate phosphohydrolase): Catalytic properties. *Applied and Environmental Microbiology,* **65**, 367-373.

Yao, N., White, P.J. & Alavi, S. (2011). Impact of β-Glucan and other oat flour components on physico-chemical and sensory properties of extruded oat cereal. *Journal of Food Science and Technology,* **46**, 651-660.

Zhou, R.J. &. Erdman, J.W. Jr. (1995). Phytic acid in health and disease. *Critical Reviews in Food Science and Nutrition*, **35**, 495-508.

Zingone, F., Bucci, C., Lovino, P. & Ciacci, C. (2017). Consumption of milk and dairy products: Facts and figures. *Nutrition,* **33**, 322-325.

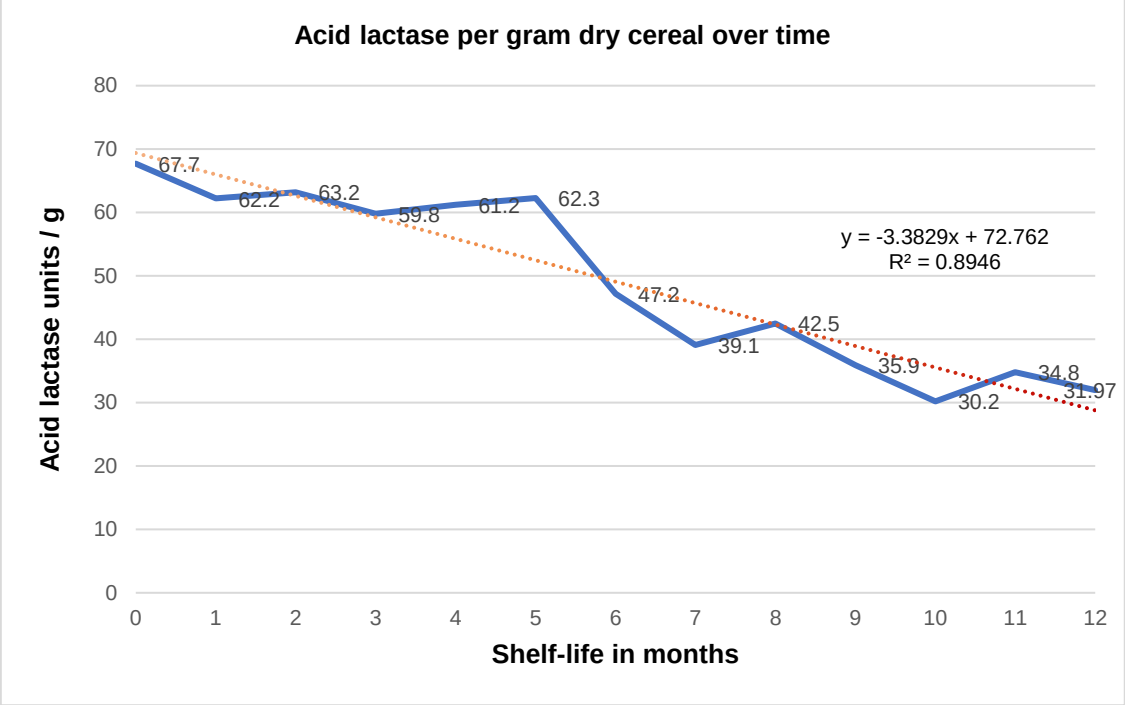


Figure 6 Acid lactase units per gram product over a period of 12 months shelf-life storage.

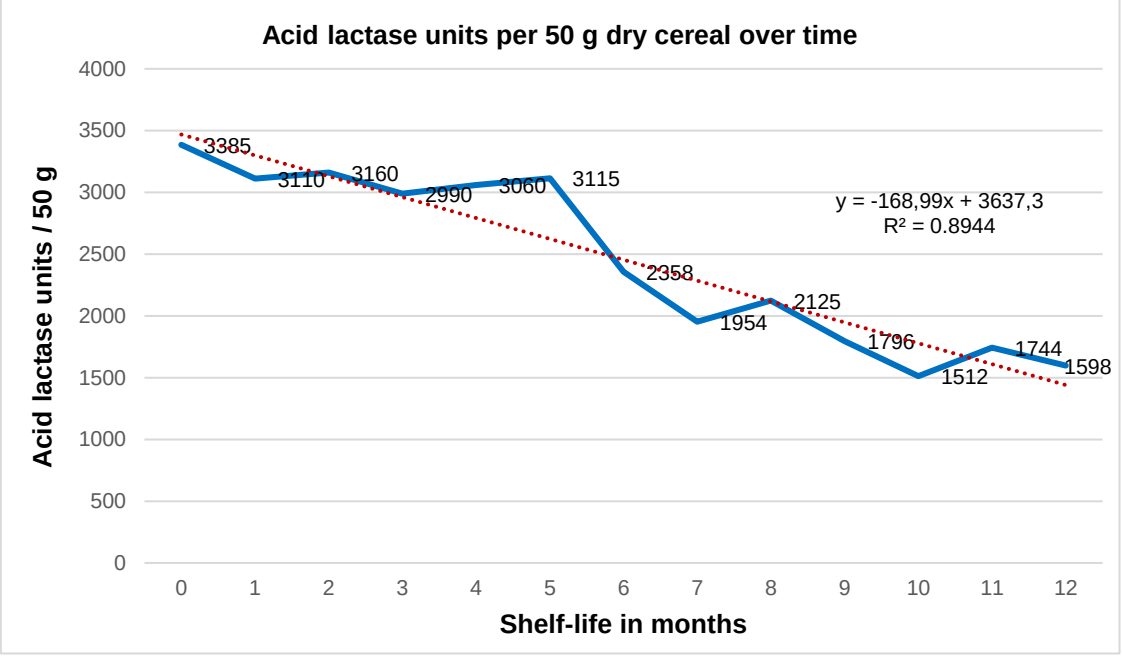


Figure 7 Acid lactase units per 50 gram serving of dry cereal over a 12 months shelf-life period.

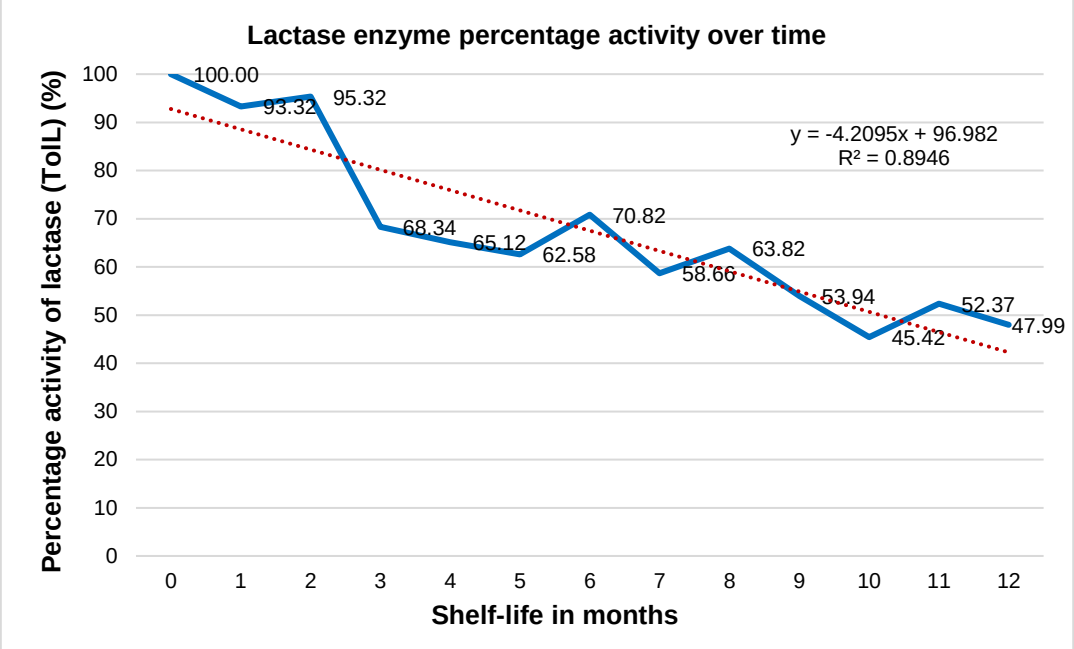


Figure 8 Percentage activity of lactase enzyme retained per storage month.

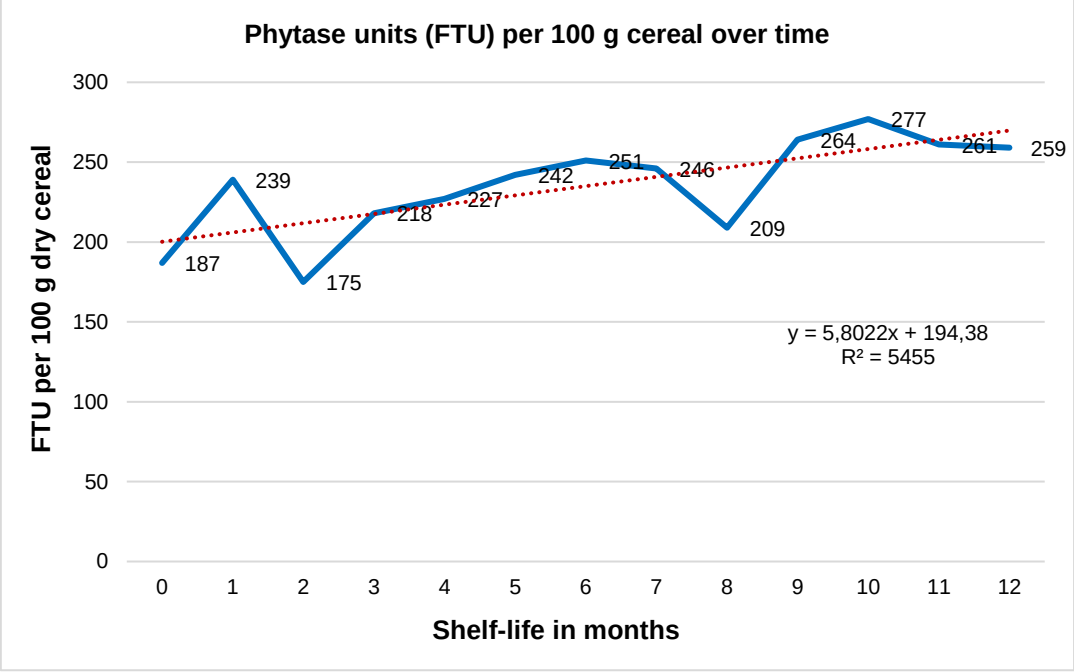


Figure 9 Phytase units per 100 gram dry cereal over a period of 12 months shelf-life storage.

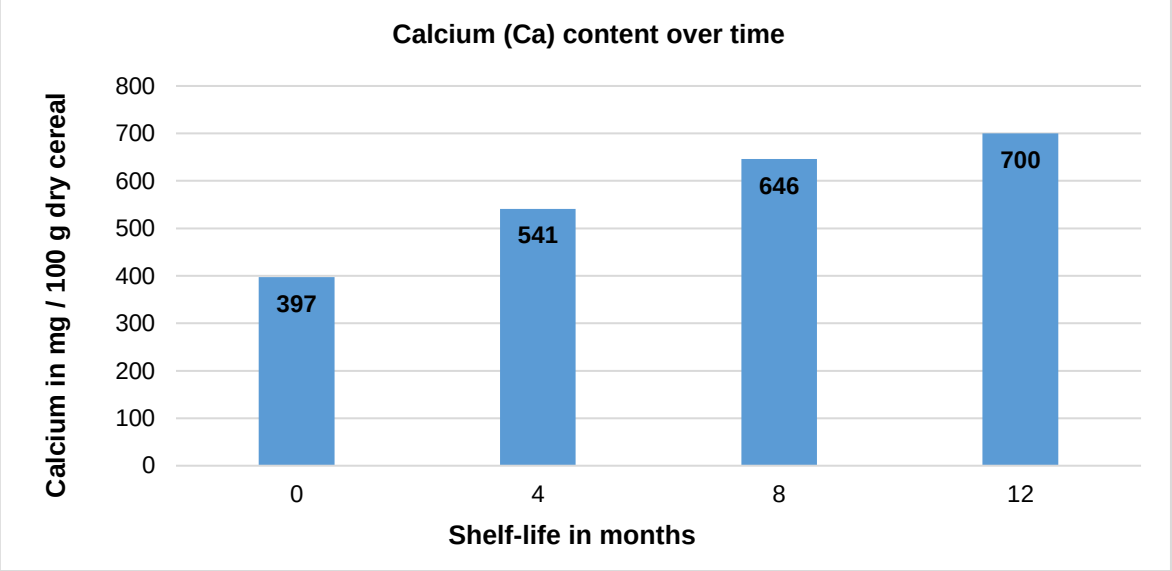


Figure 10 Calcium (Ca), average content of a ready-to-eat breakfast cereal over a period of 12 months shelf-life fortified with a phytase enzyme.

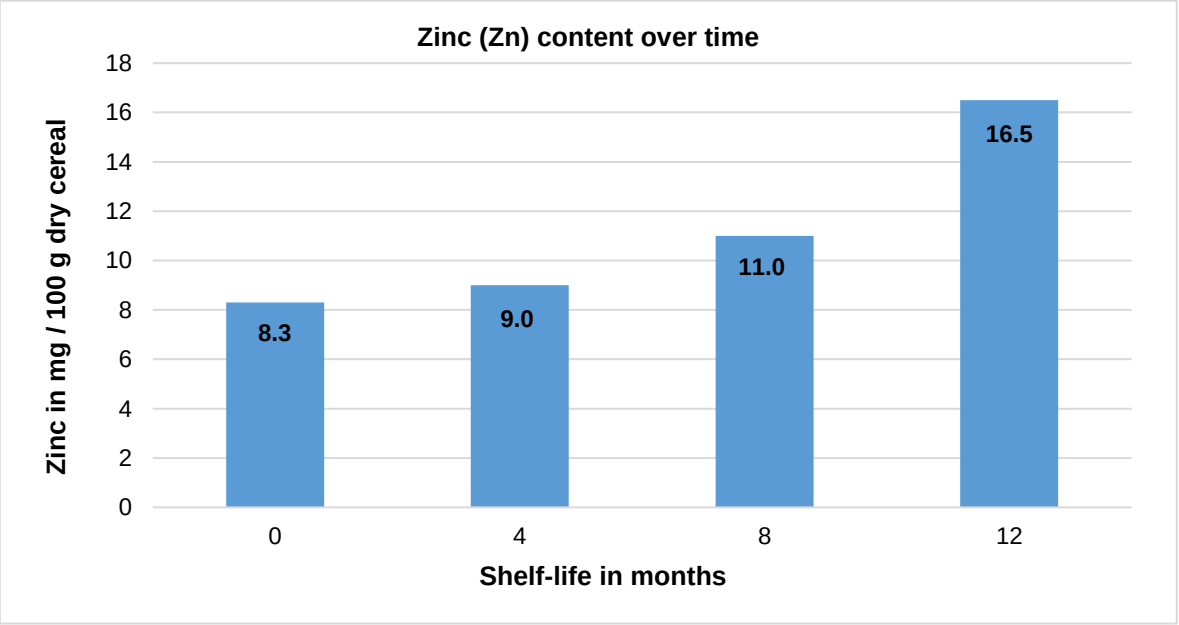


Figure 11 Zinc (Zn), average content of a ready-to-eat breakfast cereal over a period of 12 months shelf-life fortified with a phytase enzyme.

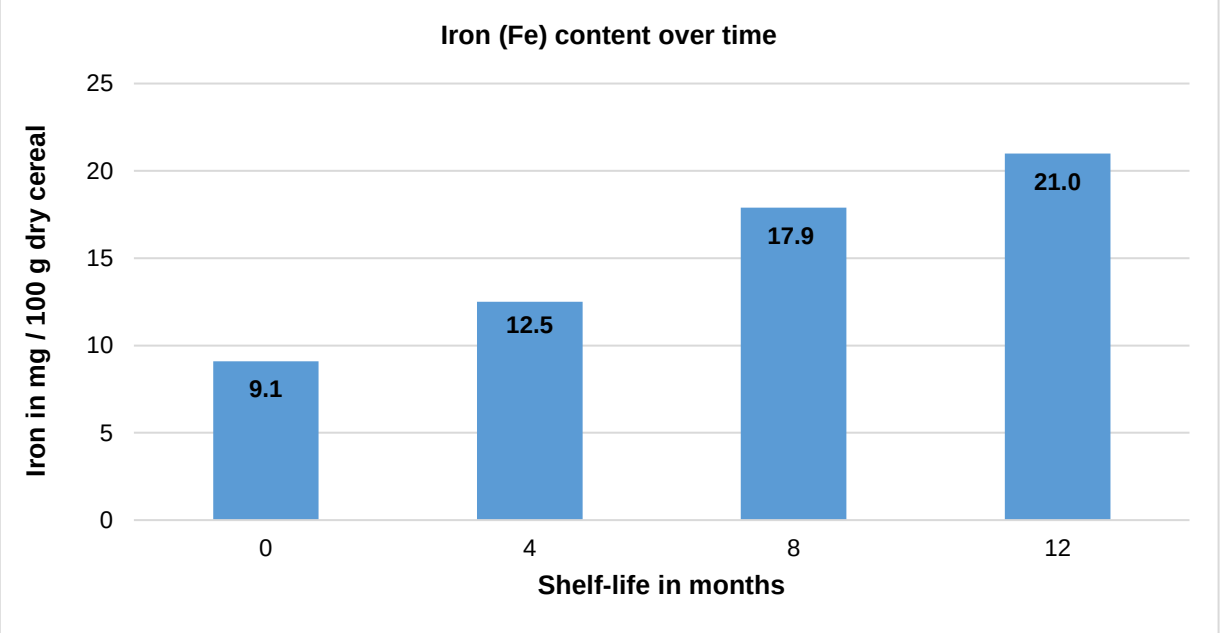


Figure 12 Iron (Fe), average content of a ready-to-eat breakfast cereal over a period of 12 months shelf-life fortified with a phytase enzyme.

CHAPTER 4

Sensory of ready-to-eat breakfast cereal treated with functional enzymes in a shelf-life study

ABSTRACT

No comprehensive sensory profiling results are available for ready-to-eat breakfast cereal fortified with functional enzyme additives. The aim of this study was to conduct descriptive sensory analysis in order to establish the sensory attributes that determine the shelf-life of the ready-to-eat breakfast cereal fortified with Tolerase L® (a lactose reducing enzyme) and Tolerase P® (a phytase enzyme). The sensory profile of breakfast cereal fortified with Tolerase L® was compared using samples with a shelf-life of zero months and 12 months. Similarly, the breakfast cereal fortified with Tolerase P® was evaluated conducting sensory analysis using samples with a shelf-life of zero months and 12 months. These four enzyme-treated samples were also compared to samples with no addition of enzymes at month zero and month 12. For Tolerase L®, no significant differences in terms of sensory profile were found between the freshly produced enzyme-treated and non-treated samples at month 0 and the enzyme-treated and non-enzyme treated samples at month 12. The results indicate that the lactose-reducing enzyme Tolerase L® had no negative effect on the sensory profile of the product over shelf-life. In contrast, limited significant differences were found in the sensory profile of samples treated with Tolerase P® during shelf-life. Tolerase P® resulted in a cereal and milk mixture with a slightly stiffer visual consistency, a slightly lower “toasted cereal” aroma and salty taste ($P \leq 0.05$), as well as a slightly higher “bran-like” residue ($P \leq 0.05$) on the palate after swallowing. These results of the ready-to-eat breakfast cereal fortified with functional enzyme additives can now be used for further sensory studies on a range of ready-to-eat breakfast cereals in the food industry.

***Keywords*:** Ready-to-eat breakfast cereals (RTEBC), Tolerase L-treated cereal, Tolerase P-treated cereal, Descriptive sensory analysis.

1 INTRODUCTION

In developing countries, particularly sub-Saharan Africa, breakfast meals for both adults and infants are based on local staple diets made from cereals, legumes, cassava and potatoes. However, the most widely consumed breakfast foods are cereals (Kent, 1983). Breakfast cereals can be produced from different processed grains such as maize, wheat, oats and barley and are usually consumed with milk or with yogurt, fruit or natural sweeteners such as honey (Tribelhorn, 1991). Breakfast cereals can be classified as *hot breakfast cereals* and *cold breakfast cereals*. Cold breakfast cereals (ready-to-eat) are usually consumed with the addition of milk. In contrast, hot breakfast cereals are classified as those that require further cooking, for example rolled oats and maize meal (Tribelhorn, 1991).

Ready-to-eat breakfast cereals (RTEBC) belong to the category of foods that usually have a long shelf-life, ideally 12 months (Howarth, 1994). The requirements for products with a longer shelf-life are quite different from those of products that have a much shorter shelf-life, for example, chilled foods, fresh milk and fresh fruit and vegetables which may only be stable for a number of days. Factors affecting shelf-life also differ from product to product; for example, deterioration during shelf-life will probably not affect the safety of a breakfast cereal but could have an impact on consumer satisfaction due to changes in sensory profile of the product (Howarth, 1994). The main drivers of product quality during shelf-life, i.e. when considering ready-to-eat breakfast cereals, are primarily sensory quality attributes such as appearance, flavour and texture. However, where ready-to-eat cereals products have been fortified with vitamins and/or minerals, or supplemented with enzymes to achieve added nutritional functionalities, sustained sensory quality is of importance and could pose a challenge (Claasen & Lawless, 1992; Howarth, 1994).

Interest in developing food products with added nutritional benefits has been driven by market potential for foods that could improve the health and well-being of individuals (Yao *et al*., 2011). An example of the latter is the addition of functional enzymes such as Tolerase L®, a lactase-based functional enzyme, to ready-to-eat breakfast cereals to address the dietary challenge of lactose intolerance (Chapter 3). It is, however, important to assess whether such enzymes impact negatively on the overall sensory profile of breakfast cereals, with or without the addition of milk. Adhikari *et al.* (2010) researched the sensory profile of commercial lactose-free milk, a growing industry segment in the USA, and established that lactose-free milk illustrated higher intensities of sweet taste, cooked milk flavour and chalkiness when compared to regular fresh milk. According to Harju *et al*. (2012) the increased sweetness associated with lactose-free milk provides an opportunity to food

manufacturers to reduce the level of added sugar in lactose-free dairy products. Harju *et al.* (2012) also illustrated that the quality of lactases can impact on the sensory quality of lactose-reduced and lactose-free milk products.

Phytic acid, quite common in high-fibre cereals and related products, are known to bind minerals such as iron (Fe), zinc (Zn), calcium (Ca) and magnesium (Mg), thus lowering the bioavailability thereof (Coulibaly *et al.,* 2011). As these minerals all play an important role in human health, especially for pre-school children and pregnant women in poor communities, high-fibre food commodities such as flour, bread and breakfast cereals are often fortified with minerals to enhance mineral bioavailability. Such fortification could potentially affect the sensory profile of the final product in terms of astringency, metallic aftertaste and colour (Gharibzahedi & Jafari, 2017). Tolerase P®, a phytase-based functional enzyme, can be added to ready-to-eat breakfast cereals to reduce the detrimental effect of phytic acid on mineral bioavailability and the potential development of mineral deficiencies (Chapter 3). Tolerase P®, however, has been difficult to market to the general consumer due to the complex regulatory environment in South Africa (Personal communication, 2017, M. Tredoux, Functional enzyme additives applications technologist, DSM Technologies (PTY) Ltd., Johannesburg, South Africa). Despite the scientific consensus on the beneficial role of phytases in human nutrition, South African health regulations limit the labelling thereof (DOH, 2010).

Consumer acceptance and purchase intent are driven by many factors, in particular when considering the large range of ready-to-eat breakfast cereals on the market (Albertson *et al.,* 2008). As consumer acceptance is driven by the sensory quality of the final product, it is important to measure the full sensory profile of newly developed food commodities to ensure that the products ultimately meet consumer expectations (Chapman *et al.,* 2001). Descriptive sensory analysis (DSA) is an ideal tool to establish the full sensory profile of a product, and can result in a comprehensive, robust data set (Stone *et al.,* 1974; Lawless & Heymann, 2010). This sensory profiling method has been used in processed foods, including extruded foods and ready-to-eat breakfast cereals (Stone & Sidel, 1998). Faller *et al.* (1998) used DSA to characterise the sensory attributes of corn-soy breakfast cereals.

As major breakfast cereal-producing companies usually have an embargo on the dissemination of product information, especially the sensory drivers of consumer preference, a limited number of descriptive terms are available to characterise the sensory profile of extruded cereals, such as crunchiness before and after milk addition, taste, sweetness, mouthfeel and consistency after milk addition. No shelf-life studies of ready-to-eat breakfast cereals fortified with functional enzyme additives have been conducted, i.e.

studies to establish the effect of these types of enzymes on the full sensory profile. In addition, the shelf-life of products containing functional enzyme additives is mainly determined through trial and error. Real-time shelf-life is often regarded as the only option and is usually responsible for successful launches in the food industry (Personal communication, 2017, J. Mohlala, Cereal Technologist, Pioneer Foods (PTY) Ltd., Cape Town, South Africa). In the South African food industry, the only product launched with additional functional ingredients currently is *Future Life® branflakes* with 10 sachets of probiotics that the consumer can manually add to the cereal before consuming the product (Personal communication, 2017, E. George, Cereal Group Executive, Pioneer Foods (PTY) Ltd., Cape Town, South Africa).

In view of the above, the objective of this study was to determine the full sensory profile of a ready-to-eat breakfast cereal fortified with functional enzyme additives using descriptive sensory analysis. This methodology was performed to estimate the shelf-life of the cereal in terms of sensory characteristics and if the functional enzymes would have an effect on the overall sensory profile of the products over the full shelf-life period of 12 months.

2 MATERIALS AND METHODS

2.1 Experimental layout and sample preparation

The ready-to-eat breakfast cereal (RTEBC) samples were produced as described in Chapter 3. The ingredients to produce the samples were the same as described in Chapter 3. The experimental layout for this research study is indicated in Table 1. As discussed in Chapter 3, the experimental design consisted of three cereal treatments and three independent block replicates per treatment. Samples to be tested over time were sourced from each of the block replicates. Sensory analysis was conducted on each of the three treatments at month 0, as well as at the end of shelf-life at month 12. The samples tested were: control treatment without enzymes at month 0 (NE-0) and at month 12 (NE-12); treatment with Tolerase P® at month 0 (TolP-0) and at month 12 (TolP-12) and treatment with Tolerase L® at month 0 (TolL-0) and at month 12 (TolL-12). The sensory analysis was conducted on 12 months instead of nine months, to determine if the current shelf-life of 12 months can be achieved with the addition of enzyme, in terms of taste and product delivery.

Sensory analysis of the six breakfast cereal treatments, indicated in Table 1, was replicated three times (Chapter 3). Eighteen samples were randomly selected for each of the treatment x replication sessions. Each RTEBC sample was served with a portion of full

cream milk (Clover®, South Africa) according to the RTEBC serving size (50 g cereal with 200 mL milk). Due to the limitation of space within the sensory booths, the serving size was proportionally decreased to 12.5 g cereal and 50 mL full cream milk.

2.2 Descriptive sensory analysis

Descriptive sensory analysis (DSA) was performed on the six breakfast cereal treatments as seen in Table 1**.** A panel of 10 judges, with previous experience of analysing the sensory quality of cereals, was selected. The panellists were trained in three 2-hour sessions according to the guidelines for generic descriptive sensory analysis, as described by Lawless and Heymann (2010). Food-based reference standards, illustrating the key sensory attributes associated with the ready-to-eat breakfast cereal being tested, were used to familiarise the panel with the attributes in question (Table 2). During each of the training sessions the panellists received six cereal samples (Table 1), as well as seven reference standards (Table 2).

After training, the panellists analysed the six treatments in three replicate sessions, one session per day. The panellists received the six treatments in a complete randomised order while seated in individual tasting booths fitted with the software programme, Compusense® five (Compusense, Guelph, Canada). The dry cereals were served in clear glass ramekins. The cereals were firstly analysed dry for two appearance attributes (Table 3). The full amount of the milk was thereafter added to each sample. After the addition of milk and resting the samples for 1 min, the panellists were instructed to evaluate the samples with added milk for eleven appearance, aroma, flavour, taste and mouthfeel attributes (Table 3). All samples were scored on unstructured line scales starting at 0 (indicating “low intensity”) and 100 (indicating “high intensity”) (Table 3). The sensory analysis sessions were conducted in a temperature- (21ºC) and light-controlled laboratory. In order to cleanse and refresh their palates between samples, the panellists received distilled water (21ºC) and water biscuits (Carr, UK).

2.3 Statistical procedures

The experimental design consisted of three treatments (Treatment without enzyme, treatment with Tolerase L® , treatment with Tolerase P®) and two shelf-life periods (Months 0 & 12). Each shelf-life time was a randomised block with three block replicates (batches) for each of the three treatments. After conducting DSA, Panelcheck software (Nofima, Ås,

Norway) was used to assess the reliability of the panel. SAS® software (version 9.2; SAS Institute Inc., Cary, USA) was also used to confirm panel consistency (Næs *et al.,* 2010) and normality of the data (Shapiro & Wilk, 1965). In the event of the Shapiro-Wilk test indicating a significant deviation from normality ($P \leq 0.05$), outliers were removed (Shapiro & Wilk, 1965). A final ANOVA was conducted to adjust for batch (block) effect. Levene's test for homogeneity indicated that variances between batches were equal for the two production times. Principle component analysis (PCA), using the correlation matrix, and discriminant analysis (DA) were performed to test the relationship between treatments and attributes. Multivariate analyses were conducted using XLStat software (Version 2017, Addinsoft, New York, USA).

3 RESULTS AND DISCUSSION

Breakfast cereals can be categorised as hot cereals that require further cooking or heating or ready-to-eat (cold) cereals that can be consumed without any further cooking or heating (Fast, 1990; Tribelhorn, 1991). All breakfast cereals are legally defined as foods obtained by swelling, grinding, rolling or flaking of a number of cereals which can all influence the product stability (Sharma & Caralli, 2004). It is thus important for the manufacturer to identify parameters most critical to consumer satisfaction and to ensure that product quality is preserved throughout shelf-life (Howarth, 1994). There are two main areas of interest when looking at stability of breakfast cereal quality, the stability of the overall sensory profile of the breakfast cereal, as well as the stability of additives (IFST, 1993).

In ready-to-eat breakfast cereals (RTEBC) the overall sensory profile refers to an array of positive attributes which are important for shelf-life purposes, such as appearance, flavour and texture attributes of a product as well as the vitamin and mineral content especially when a claim has been made on pack (Howarth, 1994), which should be sustained over shelf-life. Negative sensory attributes may develop during shelf-life, particularly the development of a rancid odour and bitter taste as a result of the onset of rancidity (Howarth, 1994) or a metallic aftertaste as a result of mineral fortification (Gharibzahedi & Jafari, 2017). Unsaturated fatty acids in breakfast cereals are prone to the development of rancidity during shelf-life. This quality control issue is usually controlled by using appropriate packaging and keeping the moisture content of the ready-to-eat breakfast cereal to a minimum of 4% (Howarth, 1994).

In a number of instances, RTEBC are fortified with additives, i.e. vitamins and minerals to enhance general consumer health and/or to address specific nutritional

deficiencies or to make a nutrient content claim (Howarth, 1994). In this study a RTEBC was fortified with minerals in such way that it could potentially be possible to make a nutritional claim. Nutritional content claims are legally binding and it is important for manufacturers to have valid evidence to substantiate these claims, particularly the stability of the nutritional compound throughout the shelf-life of the product (IFST, 1993).

The fortified RTEBC samples used in this study consisted mainly of maize and soya (Chapter 3). Globally, the expected shelf-life of this untreated RTEBC, i.e. without the addition of functional enzymes, is approximately 12 months (Azanha & Faria, 2005). Retailers expect the product to have 75% of its shelf-life remaining after the product has been put on shelf (Howarth, 1994). However, when functional enzyme additives are considered, the effect thereof on the above-mentioned sensory qualities of RTEBC should be considered during shelf-life studies. The functional enzymes Tolerase L® (TolL) and Tolerase P® (TolP) have been characterised as being odourless and tasteless and should, according to the suppliers, have no negative effect on the sensory profile of cereals during shelf-life (Personal communication, 2017, M. Tredoux, Functional enzyme additives applications technologist, DSM Technologies (PTY) Ltd., Johannesburg, South Africa). The main aim of this study was thus to determine the effect of the mentioned functional enzymes, TolL and TolP, on the sensory quality of the RTEBC in question, i.e. when compared to the untreated cereal over the full shelf-life period of 12 months, i.e. at onset and after 12 months.

The sensory profile of the untreated and treated RTEBC samples was evaluated without milk (dry cereal) and with the addition of milk at month 0 (fresh samples) and after 12 months of shelf-life, giving a total of six treatments (Table 1). It was not important to evaluate the sensory profile of samples in between shelf-life months 0 and 12, as the aim of the study was to achieve a shelf-life of 12 months for this specific RTEBC.

The mean intensity scores and significant differences for the respective aroma, flavour, taste and texture attributes of the six treatments are shown in Table 4. The two untreated samples at month 0 (NE-0) and month 12 (NE-12) did not differ significantly ($P>0.05$) for any of the 13 attributes, illustrating that the full sensory profile of the untreated, standard formula of this RTEBC did not change significantly during shelf-life and was thus stable. The standard formulation is formulated with a moisture content of 4% and packed in metallised foil to maintain a shelf-life of 12 months.

When comparing the sensory profile of the untreated and treated treatments (Table 4), i.e. the dry breakfast cereal without the addition of milk, there were no significant differences ($P>0.05$) between any of the six treatments for “coarseness” and “toasted colour”. This indicates that the addition of both functional enzymes, TolL as well as TolP,

had no significant effect at month 0 (directly after processing and packaging) or at the end of shelf-life at month 12 on flake size or toasted appearance of the dry cereal. The enzyme additives thus have no effect on the dry appearance of the RTEBC.

When considering changes in the sensory profile of the untreated and TolL-treated samples evaluated with the addition of milk (Table 4), it is clear that the functional enzyme TolL had no significant effect ($P>0.05$) on any of the product attributes at month 0 or at month 12, except for “malted” flavour. Although significant ($P\leq0.05$), the difference in the mean intensity of “malted” flavour in the untreated sample at month 0 (NE-0) and the TolL-treated sample at month 0 (TolL-0) and month 12 (TolL-12) can be regarded as extremely small. This small difference in “malted” aroma is probably due to irregularities in the processing of the RTEBC as a 100 kg base batch was produced to formulate the three cereal treatments.

However, when considering changes in the sensory profile of the untreated and TolP-treated samples evaluated with the addition of milk (Table 4), it is clear that the functional enzyme TolP had a variable effect on the sensory profile over the 12-month shelf-life period. When compared to the untreated samples (NE-0 and NE-12), TolP had no significant effect ($P>0.05$) on the treated samples at month 0 (TolP-0) or month 12 (TolP-12) when considering “toasted” colour, “toasted cereal” flavour, “malted” aroma and flavour, sweet taste, bitter taste and the mouthfeel attribute, smooth texture. These seven attributes are thus stable over time in the untreated sample, but also the sample treated with TolP over the entire shelf-life period of 12 months. In contrast, at the beginning of shelf-life (month 0), the untreated sample (NE-0) and treated sample (TolP-0) differed significantly ($P\leq0.05$) in “consistency”, with the sample treated with TolP (month 0) having slightly more stiff visual cereal-milk consistency than the untreated sample (NE-0). This could be due to a processing variable, which is known for this type of ready-to-eat breakfast cereal where the last 300 kg breakfast cereal packed from the Nauta® mixers have bigger flakes than the first 300 kg which, in contrast, usually have finer flakes. The latter usually results in quicker milk absorption and consequently a stiffer visual cereal-milk consistency (Personal communication, 2017, J. Mohlala, Cereal Technologist, Pioneer Foods (PTY) Ltd., Cape Town, South Africa). Similarly, at the end of shelf-life the untreated sample (NE-12) and the sample treated with TolP (TolP-12) differed significantly ($P\leq0.05$) in “toasted cereal” aroma, as well as bitter taste. The slightly lower “toasted cereal” aroma and bitter taste of the TolP-treated sample at the end of shelf-life could potentially be as a result of this enzyme masking these two attributes to a slight degree and thus resulting in a lower intensity for “toasted cereal” aroma and bitter taste (Personal communication, 2017, M. Tredoux, Functional

enzyme additives applications technologist, DSM Technologies (PTY) Ltd., Johannesburg, South Africa). According to Table 4 the intensity of the "bran-like" residue differed significantly (P≤0.05) between the untreated sample at month 12 (NE-12) and the TolP-treated sample at month 0 (TolP-0). The reason for this statistical difference is unclear, most probably it is a sample effect.

Principle component analysis (PCA) plots are ideal to demonstrate the multivariate relationship or association between all samples (scores) and sensory attributes (loadings) (Næs *et al*., 2010). The PCA bi-plot (Fig. 1) provides insight into the sensory attribute associations when comparing the different treatments over shelf-life, with the first principal component 1 (PC1/F1) explaining 45.2% of the variance and the second principal component (PC2) 27.2% of the variance. The sensory attributes illustrated on the right of PC1 associate equally strongly with both TolL-0 and TolL-12. The close proximity of these two treatments (TolL-0 and TolL-12) on the PCA plot indicates that the latter two TolL treatments were associated and thus did not largely change from month 0 to month 12 in terms of the tested sensory attributes, especially with regard to "malted" aroma and flavour, "toasted cereal" aroma and flavour, sweet taste and salty taste, "consistency" and "bran-like" residue of the cereal and milk mixture. The sensory attributes illustrated on the left of PC1 (Fig. 1), i.e. "toasted" colour and "coarseness" of the dry cereal and "toasted" colour and bitter taste of the samples evaluated with milk, associate with the treated samples, TolP-0, TolP-12, as well the untreated samples, NE-0 and NE-12. The proximity of these two treatment combinations, TolP-0 and TolP12, as well as NE-0 and NE-12 on the left of PC1 are not as close as that of TolL-0 and TolL12 on the right of PC1 (Fig. 1), most probably as a result of the significant changes in attribute intensity (Table 4) for **"**consistency" (NE-0 and TolP-0), "toasted cereal" aroma (NE-12 and TolP12) and salty taste (NE-12 and TolP-12) when analysing the respective treatments with the addition of milk.

Discriminant analysis (DA) enables the researcher to examine whether significant differences exist among groups of treatments in terms of predictor variables. DA also evaluates the accuracy of classification (Næs *et al.,* 2010). As shown in Fig. 2a the six treatments share similar attributes, and there is thus no real separation of treatments along PC1 (Fig. 2b). However, the three replications of the respective treatments were all classified correctly according to the DA classification table**,** i.e. the respective replications per treatment could be regarded as similar on the first linear discriminant dimension.

4 CONCLUSIONS

This research illustrated that the fortification of ready-to-eat breakfast cereals with functional enzymes, Tolerase L® and Tolerase P® does not have a disadvantageous effect on the shelf-life of the product in terms of all the sensory attributes evaluated. It can be concluded that the ready-to-eat breakfast cereals treated with the TolL and TolP have a stable shelf-life over a period of 12 months when considering the sensory profile of the products.

The attributes evaluated without the addition of milk (dry cereal) were found to have no significant differences between the six cereal treatments. It was also found that between the treatments at the respective shelf-life months (0 months and 12 months), there were no significant differences.

The results suggest that the addition of the two functional enzyme additives in similar ready-to-eat breakfast cereals will not affect the key sensory profile of the product over a shelf-life period of 12 months.

5 REFERENCES

Adhikari, K., Dooley, L.M., Chambers, E. & Bhumiratana, N. (2010). Sensory characteristics of commercial lactose free milks manufactured in the United States. *LWT Food Science and Technology,* **43**, 113-118.

Albertson, A.M., Anderson, G.H., Crockett, S.F. & Goebel, M.T. (2003). Ready-to-eat cereal consumption its relationship with BMI and nutrient intake of children aged 4 to 12 years. *Journal of American Diet Association,* **103,** 1613-1619.

Azanha, A.B. & Faria, J. A. F. (2005). Use of mathematical models for estimating the shelf-life of cornflakes in flexible packaging. *Packaging Technology and Science*, **18,** 171-178.

Chapman, K.W., Lawless, H.T. & Boor K.J. (2001). Quantitative descriptive analysis and principal component analysis for sensory characterization of ultra-pasteurized milk. *Journal of Dairy Science,* **84**, 12-20.

Claassen, M.R. & Lawless, H.T. (1992). A comparison of descriptive terminology systems for the sensory analysis of flavour defects in milk. *Journal of Food Science*, **57**, 596-621.

Coulibaly, A., Kouakou, B. & Chen, J. (2011). Phytic acid in cereal grains: Structure, healthy or harmful ways to reduce phytic acid in cereal grains and their effects on nutritional quality. *American Journal of Plant Nutrition*, **1**, 1-22.

Department of Health (2010). Regulation 146 of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 [Act 54 of 1972]. Department of Health, Pretoria, South Africa.

Faller J.F, Klein B.P, Faller J.Y. (1998). Characterization of corn/soy breakfast cereals by generalized procrustes analyses. *Cereal Chemistry,* **75**,904-908.

Fast, R.B. (1990). "Manufacturing Technology of Ready-To-Eat Cereals" In: *Breakfast cereals and how they are made.* American Association of Cereal Chemists, Minnesota.

Gharibzahedi, S.M.T. & Jafari, S.M. (2017). The importance of minerals in human nutrition: Bioavailability, food fortification, processing effects and nanoencapsulation. *Trends in Food Science & Technology,* **62,** 119-132.

Harju, M., Kallionen, H. & Tossavainen, O. (2012). Lactose hydrolysis and other conversions in dairy products: Technological aspects. *International Dairy Journal*, **22**, 104-109.

Howarth, J.A.K. (1994). Ready-to-eat breakfast cereals. In: *Shelf-life Evaluation of Foods*. (Edited by C.M.D. Man). Chapman and Hall, London, UK.

Institute of Food Science and Technology (1993). Good Manufacturing Practise. *Shelf life of Foods – Guidelines for its Determination and Prediction. The Institute of Food Science & Technology*, London.

Kent, N.L. (1983). Technology of Cereals, In: *Cereals of the world*. Pergamon Press, Oxford, England.

Lawless, H.T. & Heymann, H. (2010). *Sensory Evaluation of Food: Principles and Practices,* 2nd ed. New York, USA: Springer.

Næs, T., Brockhoff, P.B. & Tomic, O. (2010). *Statistics for Sensory and Consumer Science.* West Sussex, UK: John Wiley & Sons.

Stone, H.H. & Sidel, J. (1998). Quantitative descriptive analysis: developments, applications, and the future. *Journal of Food science and Technology* **52,** 48-52.

Tribelhorn, R.E. (1991). "Breakfast Cereals" In: *Handbook of Cereal Science and Technology*, Marcel Dekker, Incorporation., New York.

Shapiro, S.S. & Wilk, M.B. (1965). An analysis of variance test for normality (complete samples). Biometrika, **52**, 591-611.

Sharma, J.L. & Caralli, S. (2004). *A Dictionary of Food and Nutrition*. CBS Pub. New Delhi.

Stone, H.H., Sidel, J., Oliver, S., Woolsey A. & Singleton, R.C. (1974). Sensory evaluation by quantitative descriptive analysis. *Journal of Food science and Technology* **28**, 24-34.

Yao, N., White, P.J. & Alavi, S. (2011). Impact of β-Glucan and other oat flour components on physico-chemical and sensory properties of extruded oat cereal. *Journal of Food Science and Technology* **46:**6, 51-660.

Table 1 Sample sets for testing sessions evaluated in three sessions

Sample name	Sample description	No of independent replicates per treatment
NE-0	RTEBC without enzyme at month 0	3
TolL-0	RTEBC with Tolerase L at month 0	3
TolP-0	RTEBC with Tolerase P at month 0	3
NE-12	RTEBC without enzyme at month 12	3
TolL-12	RTEBC with Tolerase L at month 12	3
TolP-12	RTEBC with Tolerase P at month 12	3

NE = No Enzyme; TolL = Tolerase L®; TolP = Tolerase P®; RTEBC = Ready-to-eat breakfast cereal.

Table 2 Food-based reference samples used during training phase of DSA

Attribute	Food-based reference standards	Reference number
Toasted appearance, freshly produced RTEBC	Sample NE-0	1
Maize-like aroma	Bokomo Cornflakes	2
Soya-like aroma	Soya nuggets, Pioneer Foods	3
Toasted cereal aroma	Bokomo Cornflakes	4
Malted aroma	Horlicks Traditional, dry powder	5
Chemical aroma	Pioneer Foods, Vitamin premix	6
Bran-like residue	Snowflake Digestive Brand	7

Table 3 Definitions and scale details of each attribute used for DSA

Sensory attributes	Description	Scale	Abbreviations
Dry cereal attributes			
Coarseness, dry cereal	Visual evaluation of the coarseness of dry cereal	0 = Very fine 100 =Very coarse	Coarseness (D)
Golden colour, dry cereal	Visual evaluation of the colour of the dry cereal	0 = Very light yellow 100 =Golden toasted	ToastedColour (D)
Attributes for cereal with added milk			
Consistency of prepared cereal	Visual evaluation of the consistency of the prepared cereal, 1 min after the addition of milk	0 = Extremely runny 100 = Extremely stiff	Consistency (M)
Golden colour of prepared cereal	Visual evaluation of the colour of the cereal after milk addition	0 = Lightly toasted 100 = Golden toasted	ToastedColour (M)
Toasted cereal aroma	Aroma associated with the toasted cereal after milk addition	0 = Extremely bland 100 =Extremely intense	ToastedCerealAroma (M)
Malted aroma	Aroma associated with malted protein powder Horlicks® after addition of milk	0 = Extremely bland 100 = Extremely intense	MaltedAroma (M)
Toasted cereal flavour	Toasted cereal flavour associated with Corn flakes, i.e. prior to swallowing of cereal and milk	0 = Extremely bland 100 = Extremely intense	ToastedCerealFlavour (M)
Malted flavour	Flavour associated with malt prior to swallowing of cereal and milk	0 = Extremely bland 100 = Extremely intense	MaltedFlavour (M)
Sweet taste	Sweet taste prior to swallowing of cereal and milk	0 = Extremely bland 100 = Extremely sweet	SweetTaste (M)
Salty taste	Salty taste prior to swallowing of cereal and milk	0 = Extremely bland 100 = Extremely intense	SaltyTaste (M)
Bitter taste	Bitter taste prior to swallowing of cereal and milk	0 = Extremely bland 100 = Extremely intense	BitterTaste (M)
Mouthfeel	Mouthfeel of prepared cereal prior to swallowing	0 = Smooth texture 100 = Coarse texture	Mouthfeel (M)
Bran–like residue	Residual cereal left in the mouth after swallowing	0 = No residue 100 = High % residue left	BranLikeResidue (M)

Table 4 The average values (±SD[1]) of the sensory attributes evaluated from the six cereal samples

Attributes	**Means ± SD of treatments**						**LSD[2]**
	NE-0	**NE-12**	**TolL-0**	**TolL-12**	**TolP-0**	**TolP 12**	**P = 0.05**
Dry cereal attributes							
Coarseness (D)	19.98^{a} ± 0.03	19.99^{a} ± 0.06	19.97^{a} ± 0.06	20.0^{a} ± 0.00	19.98^{a} ± 0.03	19.98^{a} ± 0.03	0.08
Toasted colour (D)	79.88^{a} ± 0.10	79.83^{a} ± 0.15	79.88^{a} ± 0.10	79.72^{a} ± 0.15	79.79^{a} ± 0.22	79.79^{a} ± 0.22	0.35
Attributes for cereals with added milk							
Consistency (M)	31.68^{a} ± 1.13	33.30^{a} ± 0.87	33.63^{a} ± 0.74	33.57^{a} ± 1.13	34.10^{a} ± 1.64	32.35^{a} ± 1.51	2.44
Toasted colour (M)	48.71^{a} ± 0.59	49.15^{a} ± 0.48	48.82^{a} ± 0.51	48.83^{a} ± 0.50	49.18^{a} ± 0.42	49.20^{a} ± 0.48	0.58
Toasted cereal aroma (M)	88.73^{ab} ± 0.64	89.20^{a} ± 0.22	88.51^{ab} ± 0.86	89.10^{a} ± 0.66	88.97^{ab} ± 0.38	88.20^{b} ± 0.26	0.86
Malted aroma (M)	39.42^{a} ± 0.52	39.52^{a} ± 0.58	39.63^{a} ± 0.32	39.73^{a} ± 0.25	39.57^{a} ± 0.45	39.51^{a} ± 0.45	0.47
Toasted cereal flavour (M)	79.15^{b} ± 0.13	79.92^{ab} ± 0.70	79.93^{ab} ± 0.63	80.21^{a} ± 0.41	79.85^{ab} ± 0.84	79.49^{ab} ± 1.04	0.95
Malted flavour (M)	38.90^{b} ± 0.40	39.36^{ab} ± 0.13	39.71^{a} ± 0.24	39.52^{ab} ± 0.46	39.36^{ab} ± 0.20	39.27^{ab} ± 0.39	0.63
Sweet taste (M)	19.78^{a} ± 0.03	19.73^{a} ± 0.12	19.87^{a} ± 0.12	19.83^{a} ± 0.10	19.78^{a} ± 0.10	19.83^{a} ± 0.10	0.21
Salty taste (M)	10.02^{ab} ± 0.03	10.05^{a} ± 0.05	10.04^{ab} ± 0.03	10.02^{ab} ± 0.03	9.98^{ab} ± 0.03	9.97^{b} ± 0.08	0.07
Bitter taste (M)	6.81^{a} ± 0.42	7.37^{a} ± 1.09	6.89^{a} ± 0.89	6.89^{a} ± 0.19	7.05^{a} ± 0.68	7.10^{a} ± 0.36	1.12
Mouthfeel (M)	39.82^{a} ± 0.34	39.90^{a} ± 0.40	39.78^{a} ± 0.22	39.93^{a} ± 0.30	39.81^{a} ± 0.12	39.68^{a} ± 0.21	0.33
Bran–like residue (M)	29.58^{bc} ± 0.10	29.51^{c} ± 0.21	29.68^{abc} ± 0.15	29.73^{ab} ± 0.14	29.87^{abc} ± 0.12	29.68^{ab} ± 0.30	0.19

Means in rows with different superscripts are significantly different at P≤0.05. [1]SD (standard deviation); [2]LSD (least significant difference).

D = Samples evaluated without the addition of milk (dry cereal)

M =Samples evaluated with the addition of milk

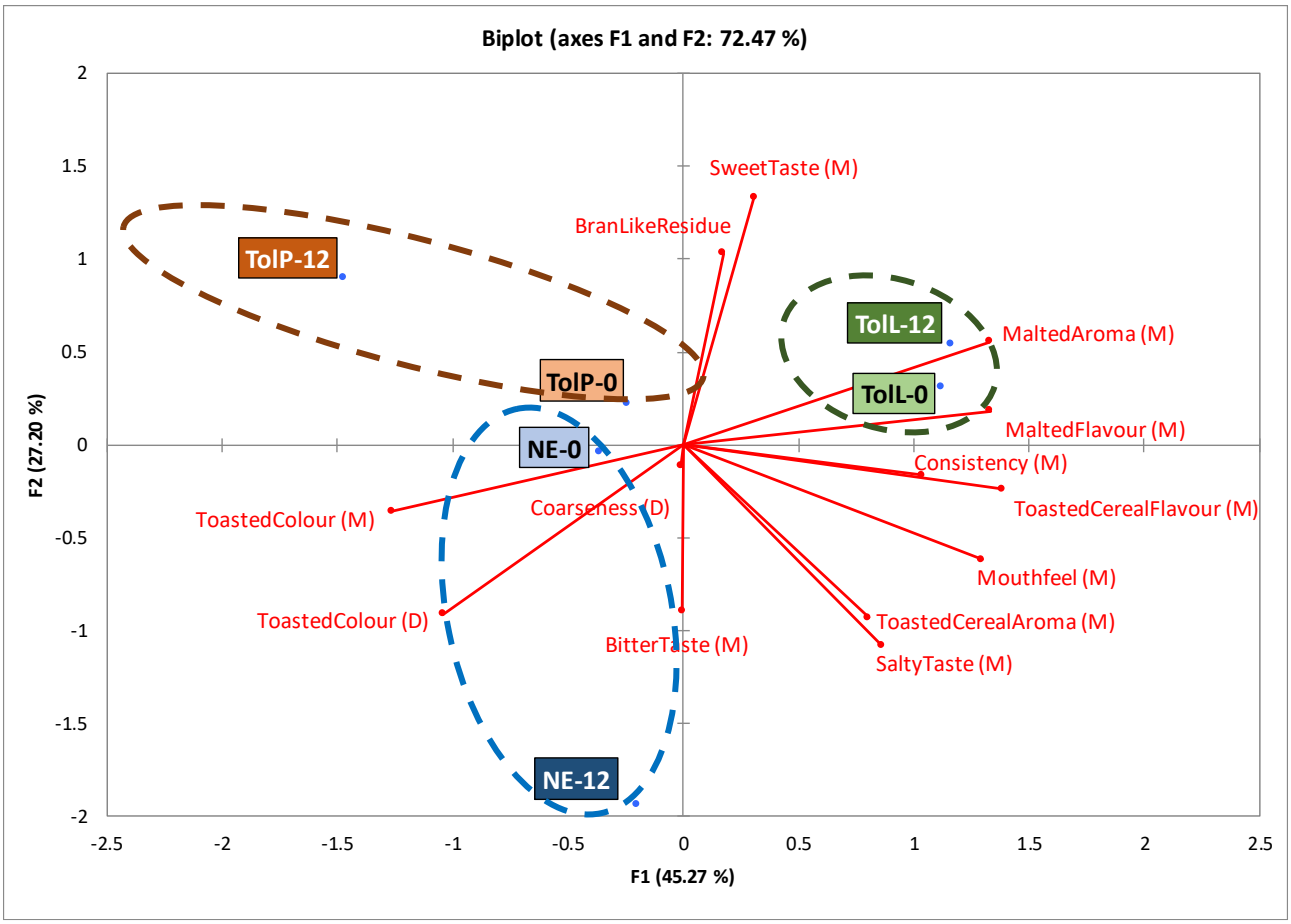
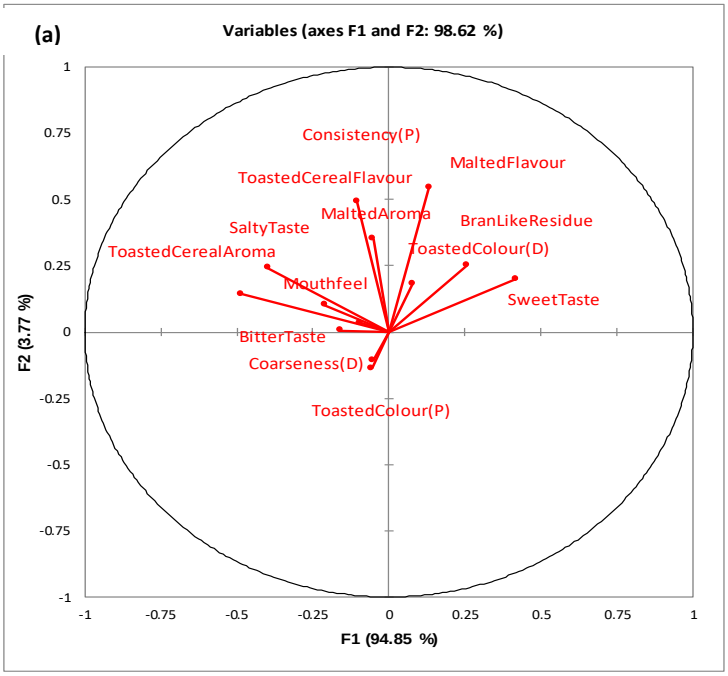
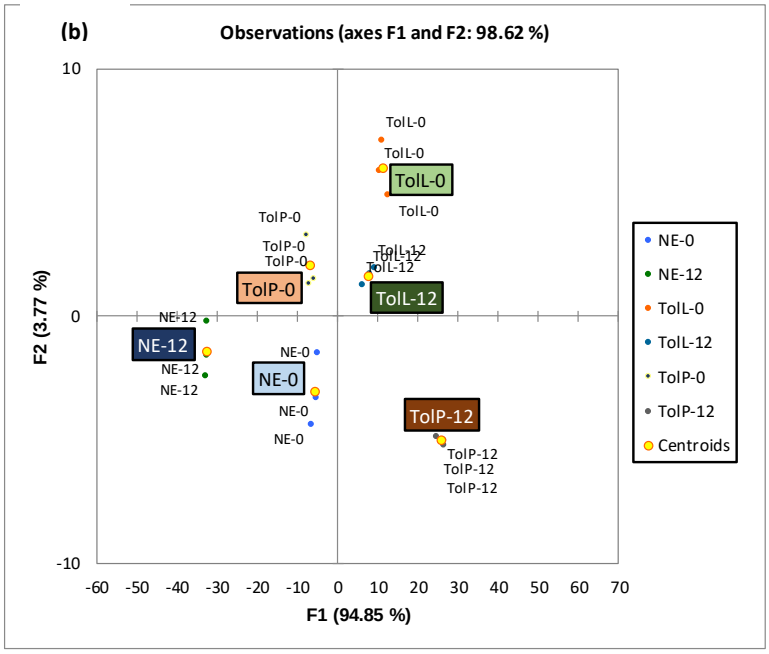


Figure 1 Principle component analysis bi-plot indicating the association of sensory attributes and treatments, two samples without enzymes (NE-0 at month 0, and NE-12 at the end of shelf-life after 12 months of storage). Treatment with Tolerase P® added at month 0 (TolP-0) and after 12 months of storage (TolP-12) and treatment with Tolerase L® added at month 0 (TolL-0) and after 12 months of storage (TolL-12).





	NE-0	NE-12	TolL-0	TolL-12	TolP-0	TolP-12	Total	%Correct
NE-0	3	0	0	0	0	0	3	100.00%
NE-12	0	3	0	0	0	0	3	100.00%
TolL-0	0	0	3	0	0	0	3	100.00%
TolL-12	0	0	0	3	0	0	3	100.00%
TolP-0	0	0	0	0	3	0	3	100.00%
TolP-12	0	0	0	0	0	3	3	100.00%
Total	3	3	3	3	3	3	18	100.00%

Figure 2 Discriminant analysis variables plot of sensory attributes of the six cereal samples **(a),** DA observations plot **(b)** and accompanying classification table illustrating percentage correctness of groupings of samples according to treatments.

CHAPTER 5

General discussion and conclusions

With an evolving global breakfast cereal industry, the opportunities for development of ready-to-eat breakfast cereal (RTEBC) products has increased significantly (Adhikari *et al.,* 2010). As part of product development programmes a number of private companies are currently actively sourcing new health-promoting ingredients as additives (Personal communication, 2017, E. George, Cereal Group Executive, Pioneer Foods (PTY) Ltd., Cape Town, South Africa). Ready-to-eat breakfast cereal consumers are also continuously searching for sustainable, affordable and health-promoting product options for their daily food intake (BMI, 2012). Furthermore, RTEBC consumers that are lactose intolerant are increasingly requesting lactose-free milk products, due to the global growing lactose intolerant population and for health reasons for both non-dairy and dairy consumers (McCarthy *et al.,* 2016). Although the variety of lactose-free milk products has increased in the food industry, this product is still regarded as quite an expensive commodity when considering the general consumer (Personal communication, 2017, E. George, Cereal Group Executive, Pioneer Foods (PTY) Ltd., Cape Town, South Africa). This provides opportunity for the ready-to-eat breakfast cereal manufacturing industry for the development of affordable cereals with added functional ingredients. Furthermore, the focus on specific health issues has shifted over the past few years as consumers have become more aware of the benefits of functional ingredients. Breakfast cereals with added functional ingredients such as digestive enzymes have a shorter shelf-life (approximately seven months), primarily to ensure that the functional ingredient's enzyme activity stays effective throughout the entire shelf-life period. Suppliers of functional enzymes stipulate that enzyme activity of functional ingredients could remain sufficient for two years without affecting the sensory profile of the final product negatively. The shorter shelf-life of current fortified ready-to-eat breakfast cereals opens up an opportunity for product development (Personal communication, 2017, J. Barendse, Breakfast Cereals Brand Manager, Pioneer Foods (PTY) Ltd., Cape Town, South Africa). The main drivers of sensory quality during shelf-life, i.e. when considering ready-to-eat breakfast cereals, are primarily sensory quality attributes such as appearance, flavour, aroma and texture (Howarth, 1994).

The aim of this study was to compare an existing RTEBC without enzyme treatment to two new ready-to-eat breakfast cereal products, each with an added functional enzyme additive, i.e. Tolerase L® (TolL) and Tolerase P® (TolP), respectively. A control breakfast

cereal, without enzyme treatment, was used as reference standard in order to compare the sensory and chemical characteristics of each of the fortified ready-to-eat breakfast cereals with that of the control sample.

The RTEBC fortified with TolL was tested for enzyme activity measured in acid lactase units (ALU) per 50 g cereal, as well as for percentage performance of the enzyme during shelf-life. It was found that the optimum activity of TolL in the ready-to-eat breakfast cereal of 2500 ALU.50g^{-1} was maintained throughout the product shelf-life up until 7 months, where after it declined quite rapidly. The maintenance of the enzyme's activity for a shelf-life of 7 months can be regarded as successful as the ascribed shelf-life for functional enzyme additive fortified products is currently at 7 months in the RTEBC industry. In addition to the enzyme activity measured in ALU, the percentage enzyme activity portrayed a different result in terms of shelf-life. According to Semenza *et al*. (2001) and Swallow (2003), only 50% of the initial enzyme activity of lactase is required to digest the amount of lactose found in 200 mL of milk. In the current study, the percentage enzyme activity of TolL at 9 months shelf-life was 53.9%, where after the percentage declined slowly to 47.9% at 12 months.

In the sensory analysis study where the RTEBC without enzyme at month 0 (NE-0) and 12 (NE-12) and the RTEBC with TolL at month 0 (TolL-0) and 12 (TolL-12) were compared, the samples were evaluated with and without the addition of milk. It was found that there were no significant differences between the following sensory attributes evaluated for the samples NE-0, TolL-0, NE-12 and TolL-12 over the 12-month period, i.e. "coarseness", "toasted" colour with and without milk, "toasted cereal" aroma and flavour, "malted" aroma, sweet taste, salty taste, bitter taste, smooth texture and "bran-like" residue. When only considering changes in the sensory profile of the untreated and TolL-treated samples evaluated with the addition of milk, it is clear that the functional enzyme TolL had no significant effect on any of the product attributes at month 0 or at month 12, except for "malted" flavour. This significant trend in "malted" flavour could probably be attributed to irregularities in the processing of the RTEBC as a 1000 kg base batch was produced to formulate the respective cereal treatments.

In the study with TolP, the quantity of the TolP enzyme remained adequate throughout the 12 months shelf-life of the product in terms of measured FTU.100 g^{-1} cereal, as prescribed by the supplier. It was, however, found that there was a 25% increase in the activity of TolP enzyme over the shelf-life of 12 months from 187 FTU.100 g^{-1} to >250 FTU.100 g^{-1} cereal. It is postulated that this increase could be due to indigenous phytases found in soybean flour, i.e. one of the major ingredients in this breakfast cereal (Skoglund

et al., 1997). The sensory profile of the untreated and TolP-treated samples evaluated with the addition of milk indicated that the functional enzyme TolP had a variable effect on the sensory profile over the 12-month shelf-life period. Most of the sensory attributes were found to be stable over the 12-month period, i.e. “toasted” colour, “toasted cereal” flavour, “malted” aroma and flavour, sweet taste, bitter taste and smooth texture. These sensory quality attributes are regarded by industry as the main drivers of consumer liking (Howarth, 1994), indicating that RTEBC treated with TolP would be accepted by the general consumer, i.e. in terms of it sensory profile.

The mineral content of the ready-to-eat breakfast cereal was known before the addition of TolP. It was found that over a period of 12 months shelf-life the minerals calcium, iron and zinc increased with 43.5%; 57.1% and 49.7%, respectively. This indicates that the TolP functional enzyme additive was effective in breaking down phytic acid and releasing the bound minerals. This result is a major advantage for the breakfast cereal industry, as nutritional supplementation can be excluded from breakfast cereals fortified with enzymes such as TolP.

Table 1 illustrates final product specifications of the current ready-to-eat breakfast cereal fortified with functional enzyme additives. These specifications indicate that the addition of functional enzyme additives had no effect on final product quality, i.e. moisture content, fraction size, flavour profile and aroma profile.

Table 1 Final product specifications of the current functional enzyme additive fortified ready-to-eat breakfast cereal

Measurement	**Specification**
Moisture (%)	<5%
Sieve fraction	Max 12% through 17 µm sieve
Flavour tested with / without milk	Acceptable when compared to approved standard
Aroma tested with / without milk	No rancid/off flavours

It can be concluded that the enzyme activity stayed relatively constant during shelf-life of ready-to-eat breakfast cereal fortified with the functional enzymes, TolL and TolP. An effective shelf-life of 7 months was achieved for TolL and 12 months for TolP, based on the enzyme activity of the respective functional enzymes. Furthermore, the treated RTEBC and untreated RTEBC did not differ significantly in terms of the overall sensory profile over the shelf-life period. These results illustrate that the functional enzyme additives do not impact negatively on the overall sensory profile of the ready-to-eat breakfast cereal used in the

study. TolP also resulted in an increased mineral content at the end of shelf-life, an important advantage for ready-to-eat breakfast cereals with a significant phytic acid content. This increase in mineral content could possibly exclude future fortification, thereby saving production costs.

Full scale consumer research should be conducted to determine the sensory drivers of consumer acceptability of ready-to-eat breakfast cereals fortified with functional enzymes tested in this study. This research also created a platform for future research. Adding functional enzyme additives to other products such as extruded breakfast cereals, muesli and breakfast bars could be investigated. Efforts such as this, could add significantly to the issues related to human nutrition and public health.

REFERENCES

Adhikari, K., Dooley, L.M., Chambers, E. & Bhumiratana, N. (2010). Sensory characteristics of commercial lactose free milks manufactured in the United States. *LWT Food Science and Technology,* **43**, 113-118.

BMI (2012). Online reference included in the article [Internet document] URL www.fastmoving.co.za/...breakfast...south-africa.../FeedbackBreakfastFoods2012.pptx. Accessed 03.06.2017

Howarth, J.A.K. (1994). Ready-to-eat breakfast cereals. In: *Shelf-life Evaluation of Foods*. (Edited by C.M.D. Man). Chapman and Hall, London, UK.

McCarthy, K.S., Parker, M., Ameerally, A., Drake, S.L. & Drake, M.A. (2016). Drivers of choice of fluid milk versus plant-based alternatives: What are consumer perceptions of fluid milk? *Journal of Dairy Science,* **100,** 6125-6138.

Semenza, G, Auricchio, S. & Mantei, N. (2001). Small intestinal disaccharides. In: *The Metabolic and Molecular Bases of Inherited Disease*, 8th edition. Netherlands: SSIM and Kluwer Academic Publishers.

Skoglund, E., Carlsson, N.G. & Sandberg, A.S. (1997). Analysis of inositol mono- and diphosphate isomers using high-performance ion chromatography and pulsed amperometric detection. *Journal of Agriculture and Food Chemistry*, **45**, 4668-4673.

Swallow, D.M. (2003). Genetics of lactase persistence and lactose intolerance. *Annual Review of Genetics*, **37**, 197-219.