© 2017 Shashank Gaur

LIPID-BASED NUTRIENT SUPPLEMENT TO ADDRESS CHILD UNDERNUTRITION AND ENTERIC PARASITIC INFECTIONS IN INDIA

BY

SHASHANK GAUR

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Food Science and Human Nutrition
with a concentration in Food Science
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

Doctoral Committee:

Professor Nicki J. Engeseth, Chair
Assistant Professor Juan E. Andrade, Director of Research
Professor Hao Feng
Assistant Professor Youngsoo Lee

Abstract

Undernutrition is an outcome of insufficient food intake and repeated infectious diseases. Lipid-based nutrient supplements (LNS) are calorie and nutrient dense food products that are suitable to complement most food rations due to their long shelf life and convenience. Although available in other markets, staple-based LNS for India do not exist. More importantly, no currently available LNS formulations address the other immediate causes of undernutrition such as parasitic infections and gut inflammation in children. The objective of this study was to develop advanced LNS 2.0 with improved shelf stability, consumer acceptability, and antiparasitic activity via added oregano essential oil (OEO) bioactives.

LNS were formulated using Indian staple dairy ingredients, and optimized using Response Surface Methodology (RSM) via face centered composite design with four factors and three coded levels: antioxidant (ascorbyl palmitate; at 0.0, 0.01, 0.03%), emulsifier (soy lecithin; at 0.5, 1.0, 1.5%), omega-3 (flaxseed oil (FO); at 0, 5, 10%) and accelerated storage temperature and time (23 and 40±2°C; at 0, 3, 6 months). Consumer acceptability was assessed using a 9-point hedonic scale among Indian mothers and students. Next, a dual modality *in vitro* cell culture model was employed to investigate the effect of OEO and its main bioactive carvacrol on prevention of parasite *C. parvum* invasion and infection of HCT-8 cells. Finally, β-Cyclodextrin (β-CyD) encapsulation of OEO and carvacrol (1:1 molar) was utilized to mask the potent flavor of bioactives, and achieve controlled intestinal delivery of bioactives, measured using triangle sensory test and 2-phase *in-vitro* digestion model, respectively.

Optimal levels to maximize storage time and flaxseed oil and minimize oxidation were found as 0.02% antioxidant 1.5% emulsifier, and 4.9% FO. LNS formulations were found

acceptable with or without FO similar to other commercial supplements. OEO and carvacrol were found to reduce relative *C. parvum* infectivity in a dose-dependent manner to 55.6 ± 10.4% and 45.8 ± 4.1% at 60 and 30 μg/mL of OEO and CV, respectively. Lastly, β-CyD complexes of OEO and CV were found significantly stable ($p<0.05$) through the gastric and intestinal phase enabling their potential release in colon via fermentation by colonic microflora. Triangle tests revealed no significant difference in color, smell, and taste between LNS with and without β-CyD-OEO complexes. In conclusion, staple-based LNS functionalized with β-CyD-OEO complex were feasible and can potentiate their application in addressing undernutrition and parasitic infections in at-risk populations.

Acknowledgements

It always seems impossible until it's done! - Nelson Mandela

Coming to the University of Illinois, Urbana-Champaign was one of the best and the most difficult decision of my life. As any other international graduate student, my life was filled with uncertainties related to education funding, housing, my project, and future jobs. Nevertheless, there was an excitement of meeting new people, learning from World-renowned scientists, conducting some impactful research, and living up to my parents' dreams and hope. Sure enough, this has been challenging, and after countless falls and throw outs of disappointments and joys, I am finally at the threshold of writing this note of appreciation for all who motivated and supported my success and failures and wished me well throughout my career.

First of all, I would like to express my sincere gratitude to my advisor and my role model, Dr. Juan Andrade for his patience, motivation, guidance, and immense support throughout my PhD education. His contributions extend far beyond giving advice on my research, and I will always miss his unique sense of humor and occasional discussions on topics beyond the usual research. I am deeply indebted to him for turning me into an experienced scientist, which will help me throughout my life.

I would also like to thank my committee members, Dr. Nicki Engeseth, Dr. Hao Feng and Dr. Youngsoo Lee, for their consistent support, timely guidance, candid suggestions, and constructive criticisms, all of which helped me stay on track and achieve the research goals on time. I express my profound gratitude to Dr. Theresa Kuhlenschmidt and Dr. Mark Kuhlenschmidt their kind support, candid suggestions, and lab assistance and facilities extended during the course of this endeavor.

My special thanks go to Emely Lopez who has helped me in almost everything in the lab. I owe her greatly for her unreserved help, support and lasting friendship. I humbly express my wholehearted thanks to my labmates: Dr. Ross Peterson, Richard Bukenya, Liz Sloffer, Nawaf Alruwaili, Anna Waller and Jennifer Lotton, and also to Dr. Andrade's family: Dr. Jeannette Andrade, and kids, Justine and Jules, for their timely motivation and moral support, and for giving me some of the best memories of my life. I am also thankful to my friends at Chai Town A Cappella for reviving my passion for music and for giving me lifetime memories of singing, traveling, recording, and performing in and around the United States.

I would like to acknowledge and thank the Indian Council of Agricultural Research International Fellowship, the US Department of Agriculture, the College of ACES Office of International Programs, and Abbott Nutrition, for providing financial, research, travel, and assistantship support during my education. I profusely thank our collaborators Dr. P.R. Patel, Dr. Dharmendra Shukla, Dr. Ankur Ojha and Dr. Falguni Pathak, faculty members at Mansinhbhai Institute of Dairy and Food Technology, Mehsana and Dudhsagar Dairy, Mehsana, India, for their contribution to our research and its application. I also gratefully acknowledge the help rendered by the office staff of the Department of Food Science and Human Nutrition.

On a personal note, I wish to express my gratitude to my affectionate and lovable father, mother and my brother, for their constant encouragement, prayers, love, care and affection showered upon me in the pursuit of my educational ambition for which I am eternally grateful.

Table of Contents

List of Tables viii
List of Figures ix
Chapter 1: Introduction 1
1.1 Malnutrition in India 1
1.2 Long-term Goal, Research Objective, and Hypothesis 4
1.3 Significance and Rationale 5
1.4 Thesis Structure 6
1.5 Figure 7
1.6 References 8
Chapter 2: Literature Review 10
2.1 Undernutrition 10
2.2 Undernutrition and Parasitic Infections 12
2.3 Current Interventions in India 14
2.4 Lipid-based Nutrition Supplements 15
2.5 Omega-3 Fortification 16
2.6 *Cryptosporidium parvum* Characteristics and Reproduction 17
2.7 Oregano Essential Oil 19
2.8 Encapsulation 21
2.9 Tables and Figures 24
2.10 References 34
Chapter 3: Omega-3 Fortified Lipid-based Nutrient Supplement – Development, Characterization and Consumer Acceptability 41
3.1 Abstract 41
3.2 Introduction 43
3.3 Materials and Methods 44
3.4 Results 50
3.5 Discussion 52
3.6 Conclusions 57
3.7 Tables and Figures 59
3.8 References 67

Chapter 4: Effect of Oregano Essential Oil and Carvacrol on *Cryptosporidium parvum* Infectivity in HCT-8 Cells 71
4.1 Abstract 71
4.2 Introduction 72
4.3 Materials and methods 74
4.4 Results 77
4.5 Discussion 78
4.6 Conclusion 82
4.7 Table and Figures 83
4.8 References 88
Chapter 5: Functionalization of Lipid-based Nutrient Supplement with β-Cyclodextrin Inclusions of Oregano Essential Oil: Preparation, Characterization and Sensory Evaluation 91
5.1 Abstract 91
5.2 Introduction 93
5.3 Materials and Methods 96
5.4 Results 102
5.5 Discussion 103
5.6 Conclusions 107
5.7 Tables and Figures 108
5.8 References 116
Chapter 6: Summary and Future Directions 120
Appendix A: Institutional Review Board Approval Notice 123
Appendix B: Institutional Review Board Approved Consent Form 124
Appendix C: Institutional Review Board Approved Consent Form in Gujarati 126
Appendix D: Demographic Information Questionnaire 129
Appendix E: Template for Participant Receipt 131
Appendix F: Ballot Template for Consumer Acceptability Test 132
Appendix G: Ballot Template for Ranking Test 134
Appendix H: Ballot Template for Discriminatory Sensory Tests (Triangle Tests) 135

List of Tables

Table 1. Programs to address undernutrition in India…………………………………………… 24

Table 2. Difference between LNS and RUTF…………………………………………………... 25

Table 3. Clinical trials on LNS supplementation in children…………………………………… 26

Table 4. Clinical trials on omega-3 fatty acid fortified LNS supplementation in children……… 27

Table 5. Characteristics of α-, β- and γ- Cyclodextrins………………………………………… 27

Table 6. Recipe formulation for LNS…………………………………………………………… 59

Table 7. RSM factor variables and levels………………………………………………………. 60

Table 8. Fatty acid profiles (Area %) of various LNS products………………………………… 60

Table 9. Results of face-centered central composite design for optimization………………….. 61

Table 10. Estimated coefficients of the fitted quadratic equation for different responses based on t-statistic………………………………………………………………………………………… 62

Table 11. Literature on activity of oregano essential oil (OEO) and carvacrol (CV) against parasites………………………………………………………………………………………. 83

Table 12. Recipe formulation for 100 g LNS with or without β-CyD-OEO………………….. 108

Table 13. CV recovery, surface CV and encapsulation efficiency of β-CyD inclusions of CV and OEO……………………………………………………………………………………… 109

List of Figures

Figure 1. Undernutrition/infection cycle…………………………………………………………… 7

Figure 2. Forms of undernutrition…………………………………………………………… 28

Figure 3. Waterlow classification of degree of undernutrition………………………………… 28

Figure 4. Weight for age distribution curve for children (<3 years) in India compared to global reference population………………………………………………………………………….. 29

Figure 5. Conceptual model of effects of undernutrition throughout the life cycle……………. 29

Figure 6. UNICEF conceptual framework of relations between basic, underlying and immediate causes to maternal and child undernutrition and its short-term and long-term consequences….. 30

Figure 7. Infection-undernutrition vicious cycle…………………………………………….. 30

Figure 8. Undernutrition and parasitic infection interaction………………………………….. 31

Figure 9. Pictures of Intestinal parasites…………………………………………………… 31

Figure 10. Pictures of *Anganwadi* centers in India, hot cooked rice and pulse meal, kitchen setting to cook those meals and take-home rations…………………………………………………… 31

Figure 11. Examples of currently available small, medium and large quantity LNS…………… 31

Figure 12. Life cycle of *Cryptosporidium parvum*………………………………………....... 32

Figure 13. Chemical structure of Carvacrol and Thymol…………………………………….. 32

Figure 14. Structures of α-, β- and γ- cyclodextrins………………………………………….. 33

Figure 15. Molecular complex of β-CyD-CV and β-CyD-thymol……………………………… 33

Figure 16. Process flowchart for preparation of Indian LNS…………………………………… 63

Figure 17. Response surface plots for peroxide value and lipid separation…………………….. 64

Figure 18. Effect of accelerated storage on water activity, moisture content, hardness, and vitamin C content of LNS…………………………………………………………………………... 65

Figure 19. Consumer acceptability test of several nutrition supplement products among women and student participants…………………………………………………………………. 66

Figure 20. Schematic of *in vitro* assessment of *C. parvum* growth and invasion………………. 84

Figure 21. Images of Live/ Dead cell assay………………………………………………….. 85

Figure 22. Effect of OEO and CV on relative *C. parvum* % growth at various doses………….. 86

Figure 23. Effect of OEO and CV on relative *C. parvum* % growth at a representative dose of 60 µg/mL OEO and 30 µg/mL CV with or without complete medium replacement containing a fresh preparation of bioactives after 24 h, during the 48 h incubation………………………………… 87

Figure 24. Major phenolics in oregano essential oil…………………………………………… 109

Figure 25. Procedure to prepare β-CyD inclusions of CV and OEO………………………….. 110

Figure 26. Scanning electron microscope images of β-CyD empty complex, β-CyD-CV and β-CyD-OEO……………………………………………………………………………………… 111

Figure 27. Stability of CV and OEO during the simulated digestion conditions……………… 112

Figure 28. Effect of digestive enzymes on digestive stability of CV and OEO……………….. 112

Figure 29. Bioaccessibility of OEO and CV from LNS samples………………………………. 113

Figure 30. Bioaccessibility of β-CyD-CV and β-CyD-OEO with or without LNS under *in-vitro* gastro-intestinal digestion conditions……………………………………………………….. 114

Figure 31. Bioaccessibility of β-CyD-CV and β-CyD-OEO under in-vitro gastro-intestinal digestion conditions………………………………………………………………………………. 114

Figure 32. Triangle test results showing no significant differences between LNS with or without β-CyD-OEO in students and women participants……………………………………………… 115

Chapter 1: Introduction

1.1 Malnutrition in India

Child undernutrition is a major cause of morbidity and mortality in young children (<6 years) and remains pervasive in low and middle-income countries such as India. Nearly half of all deaths in children under 5 are attributable to undernutrition, translating into an unnecessary loss of about 3 million children per year (UNICEF/WHO/World Bank Group, 2016). In India, undernutrition afflicts 1 in 2 children, where 43% (57 million) and 48% (68 million) of children under five are underweight and stunted, respectively (NFHS, 2007). About 9 million children in India suffer from severe acute malnutrition, which is defined as severe wasting with visual nutritional edema - very low weight-for-height or <-3 Z-scores of the median WHO growth standards. A larger number, 20 million, suffer from moderate acute malnutrition, which is defined as wasting with low weight-for-height or >-3 and <-2 Z-scores of the median WHO growth standards or mid-upper arm circumference between 11 and 12.5 cm. A significant 5 million children die every year as a direct or indirect result of undernutrition (NFHS, 2007). Undernutrition has severe consequences on academic performance, future work productivity, potential income and susceptibility to chronic diseases (Black et al., 2013). According to the UNICEF framework, the two immediate causes of undernutrition are inadequate dietary intake (quantity and quality of food) and repeated infectious diseases such as parasitic infections (Black et al., 2008). Both of which are interrelated and are found to impair the immune system interdependently, forming a vicious infection-malnutrition cycle (Hughes and Kelly, 2006) (Figure 1)

Problem

Nutrition Gaps - In India, supplementary feeding programs are widely used to address undernutrition in children from 6 – 72 months via the Integrated Child Development Service. Under these programs the children are entitled supplementary nutrition via hot cooked meal at *Anganwadi Centers* (day care centers) and/or take home rations such as mixed flours (*sattu*), sweets (*sheera*), and porridge (*upma*), or sometimes raw ingredients such as rice and pulses (ICDS, 2014). Although such interventions have been useful in improving some health outcomes, increasing children enrolment and reducing gender bias, there are still issues with nutritional adequacy and consumption compliance due to the low acceptability of products, and additional food preparation requirements (Lokshin et al., 2005). Moreover, some food rations contain insufficient nutrients to meet program goals (Bhagwat et al., 2014; Varma et al., 2007). Thus, a low cost, nutritious, palatable, ready to use food prepared using staple ingredients that could be included in the daily meals of children at schools or supplementary feeding centers in India is needed.

Repeated enteric parasitic infection and gut inflammation - Enteric parasitic infections are prevalent in children (<5 years) living in low-income settings, especially in countries such as India, owing to inadequate sanitation, hygiene and healthcare system and their less developed immune systems (Scrimshaw, 1994). These infections alter the epithelial integrity and weaken the immune system in children resulting in reduced nutrient digestion and absorption, chronic gut inflammation, iron deficiency anemia, protein-energy malnutrition, and reduced growth and cognitive development (Katona and Katona-Apte, 2008). This is further worsened by the existing increased levels of proinflammatory prostaglandins in Indian populations due to the high ω6:ω3 ratios of the vegetarian diets (30:1 to 70:1) (Abedi and Sahari, 2014). The lower ω6:ω3

ratio is recommended not only for normal growth, cell functioning, and immune function, but also for adequate anti-inflammatory response (Brenna et al., 2015; Calder, 2006). Thus, interventions focused on both providing adequate nutrition and addressing parasitic infections and gut inflammation in children are needed to effectively address the multietiological problem of undernutrition (Egger et al., 1990).

Opportunity

Indian lipid-based nutrient supplement - Lipid-based nutrient supplements (LNS) are fortified food products, containing concentrated nutrients (energy, protein, essential fatty acids, and micronutrients) and are suitable to complement most food rations, due to their longer shelf life and convenience (Arimond et al., 2013). Existing LNS such as Nutributter®, PlumpyDoz® and PlumpySup® (Nutriset, 2015) have been tested and proven successful in improving growth outcomes in children (Adu-Afarwuah et al., 2007; Phuka et al., 2008). Nonetheless, they have not been accepted in India mainly due to problems associated with high cost, poor acceptability, foreign import, safety and sustainability (Arie, 2010). Similar peanut-based LNS were not well accepted by pregnant and lactating women in Bangladesh (Ali et al., 2013), and by undernourished children in India (Dube et al., 2009). The World Food Program (WFP) and local doctors have expressed an urgent need for development of indigenous and low-cost LNS to address undernutrition in India (Gupta et al., 2006)

LNS specifications on nutritional composition, processing and microbiological standards provided by World Food Program (WFP) and other organizations allow easy manipulation of ingredients so these can be modified to staple ingredients from India, with lower ω6:ω3 fatty acid

ratio, and even functionalized to include antiparasitic ingredients (Jelensperger, 2016; Chaparro and Dewey, 2010).

1.2 Long-term Goal, Research Objective, and Hypothesis

The long-term goal of this project is to holistically address the multi-etiological problem of undernutrition through functional foods designed to provide benefits beyond the basic nutrition. The objective of this research, which is the next step towards accomplishing this long-term goal, is to develop an advanced LNS 2.0, which is nutritious, stable, and highly palatable, complemented with the effective dose of cyclodextrin-included oregano essential oil (OEO) to address the immediate causes of undernutrition including dietary intake of quality nutrients and gut inflammation originated from parasitic infections in children. The central hypothesis is that OEO and CV will reduce the *Cryptosporidium parvum (C. parvum)* invasion of HCT-8 cells *in vitro* and inclusion of bioactives in β-cyclodextrin (β-CyD) complexes will improve their stability, enabling their functional activity in the colon, as evaluated in simulated digestion conditions. The hypothesis will be tested by investigating three specific aims:

Specific Aim 1. *Development of an Omega-3 fortified lipid-based nutrient supplement using staple ingredients from India*. The working hypothesis is twofold: 1) Response surface methodology (RSM) optimization will pinpoint the adequate levels of flaxseed oil, antioxidant and lecithin ingredients in LNS, which will result in lower oxidation during storage. 2) LNS products with flaxseed oil (10% of the final weight) will have similar consumer acceptability to the other existing LNS and supplementary feeding products in India, among Indian mothers and students.

Specific Aim 2. *Evaluate the effectiveness OEO and CV against C. parvum using the HCT-8 cell model*. The working hypothesis is that OEO and its principal component CV will reduce *C. parvum* invasion and infection at concentrations well tolerated by the HCT-8 cell monolayers.

Specific Aim 3. *Preparation of β-cyclodextrin (β-CyD) inclusion complexes of CV and OEO, in vitro characterization of digestive stability and bioaccessibility, and sensory evaluation of LNS with β-CyD-OEO complexes.* The working hypothesis for this aim is twofold: 1) β-CyD inclusion will mask the potent unpleasant flavor of OEO and CV; and, 2) β-CyD inclusions limit the release of OEO or CV after 2-phase simulated *in vitro* digestion conditions, due to the absence of microflora during these phases.

1.3 Significance and Rationale

This study has both basic and applied significance in LNS research and development, natural bioactives, and parasitology and global health initiatives. In the field of LNS, these studies will provide a basis for systematic development, characterization and optimization of LNS with a focus on a particular target population. In the field of natural bioactives and parasitology, these studies will contribute to our limited knowledge of the use of CV and OEO in foods to prevent parasitic infections. In the field of global health, these studies will expand our ability to create functional LNS products that can include components that better address the immediate causes of undernutrition in low-income countries as well as to instigate interest of stakeholders to further the research in the area of functional LNS.

The rationale for the long-term goal of this research is that LNS designed with Indian ingredients and functionalized with OEO can holistically address the challenging problem of

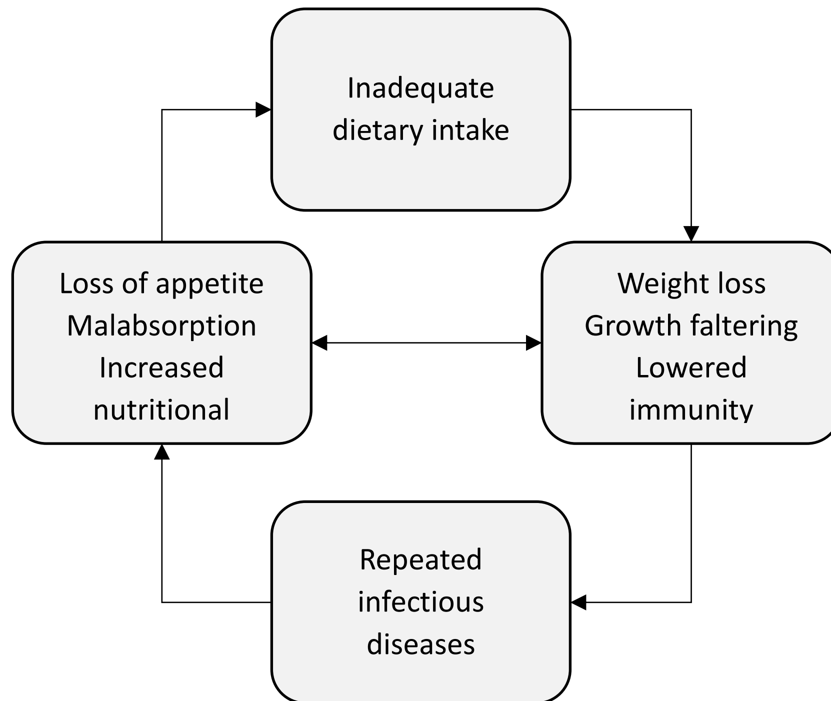
undernutrition in India via food-based approach. The rationale for assessing the effect of CV and OEO on *C. parvum* in Aim 2 is that it will enable the establishment of an effective dose needed to reduce enteric parasitic infections. The rationale for characterization and inclusion of OEO and CV in β-CyD in Aim 3 is that such inclusions will mask the potent flavor and enable controlled delivery of bioactives in the lower gut, where parasites usually reside.

1.4 Thesis Structure

The thesis is organized into six chapters. Chapter 1 and Chapter 2 are composed of an introduction to the thesis and a detailed literature review, respectively, which provide a background of each area of research relevant to the dissertation research included herein. This information gives the reader sufficient background knowledge to understand the theoretical basis of the conducted research. Chapter 3, describes the development of an Omega-3 fortified LNS using staple ingredients from India, which is nutritious, stable and acceptable among Indian populations (Gaur et al., 2017). These data from Chapter 3 partially satisfy the Aim 1 and provide a basic formula for further studies. The research in Chapter 4 describes for the first time the dose-dependent effect of OEO and CV on the *C. parvum* invasion and infection of HCT-8 cells. These data support the hypothesis of Aim 2 and provides a basis for extrapolation of an effective dose of OEO to be added to LNS basic formula. Chapter 5 contributes to completing the overall objective of this dissertation creating an LNS 2.0 through the incorporation of OEO in β-CyD inclusions to mask the potent flavor of OEO and enable controlled targeted delivery of antiparasitic OEO in the lower gut, where the enteric parasites usually reside. Chapter 6 summarizes the findings from each of the previous chapters and concludes with the potential future directions of this research.

1.5 Figure

Figure 1. Undernutrition/infection cycle (Schaible and Kaufmann, 2007).



1.6 References

Abedi E, Sahari MA. 2014. Long-chain polyunsaturated fatty acid sources and evaluation of their nutritional and functional properties. Food Sci. Nutr. 2: 443–63.

Ali E, Zachariah R, Shams Z, Manzi M, Akter T, Alders P, et al. 2013. Peanut-based ready-to-use therapeutic food: how acceptable and tolerated is it among malnourished pregnant and lactating women in Bangladesh? Matern. Child Nutr. 11: 1028–1035.

Arie S. 2010. Hungry for profit. BMJ 341: c5221.

Arimond M, Zeilani M, Jungjohann S, Brown KH, Ashorn P, Allen LH, et al. 2013. Considerations in developing lipid-based nutrient supplements for prevention of undernutrition: experience from the International Lipid-Based Nutrient Supplements (iLiNS) Project. Matern. Child Nutr. 11: 31–61.

Bhagwat S, Sankar R, Sachdeva R, Joseph L, Sivaranjani. 2014. Improving the nutrition quality of the school feeding program (Mid day meal) in India through fortification: a case study. Asia Pac. J. Clin. Nutr. 23 Suppl 1: S12–9.

Black RE, Allen LH, Bhutta ZA, Caulfield LE, de Onis M, Ezzati M, et al. 2008. Maternal and child undernutrition: global and regional exposures and health consequences. Lancet 371: 243–60.

Black RE, Victora CG, Walker SP, Bhutta ZA, Christian P, de Onis M, et al. 2013. Maternal and child undernutrition and overweight in low-income and middle-income countries. Lancet 382: 427–51.

Brenna JT, Akomo P, Bahwere P, Berkley J a, Calder PC, Jones KD, et al. 2015. Balancing omega-6 and omega-3 fatty acids in ready-to-use therapeutic foods (RUTF). BMC Med. 13: 1–4.

Calder PC. 2006. n-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 83: S1505–1519.

Chaparro CM, Dewey KG. 2010. Use of lipid-based nutrient supplements (LNS) to improve the nutrient adequacy of general food distribution rations for vulnerable sub-groups in emergency settings. Matern. Child Nutr. 6 Suppl 1: 1–69.

Dube B, Rongsen T, Mazumder S, Taneja S, Rafiqui F, Bhandari N, et al. 2009. Comparison of Ready-to-Use Therapeutic Food with cereal legume-based khichri among malnourished children. Indian Pediatr. 46: 383–388.

Egger RJ, Hofhuis EH, Bloem MW, Chusilp K, Wedel M, Intarakhao C, et al. 1990. Association between intestinal parasitoses and nutritional status in 3-8-year-old children in northeast Thailand. Trop. Geogr. Med. 42: 312–23.

Gaur S, Sloffer EM, Ojha A, Patra F, Shukla D, Engeseth NJ, et al. 2017. Omega-3-Fortified Lipid-Based Nutrient Supplement. Food Nutr. Bull.: 037957211770123.

Gupta P, Shah D, Sachdev HPS, Kapil U. 2006. National workshop on “Development of guidelines for effective home based care and treatment of children suffering from severe acute malnutrition”. Indian Pediatr. 43: 131–9.

Hughes S, Kelly P. 2006. Interactions of malnutrition and immune impairment, with specific reference to immunity against parasites. Parasite Immunol. 28: 577–88.

ICDS. 2014. Supplementary Nutrition Programme, Government of India.

Jelensperger C. 2016. Technical Specifications for Lipid-based Nutrient Supplement - Medium Quantity.

Katona P, Katona-Apte J. 2008. The interaction between nutrition and infection. Clin. Infect. Dis. 46: 1582–8.

Lokshin M, Das Gupta M, Gragnolati M, Ivaschenko O. 2005. Improving Child Nutrition? The Integrated Child Development Services in India. Dev. Change 36: 613–640.

NFHS. 2007. National Family Health Survey (NFHS-3), 2005–06: India: Volume I. Mumbai.

Schaible UE, Kaufmann SHE. 2007. Malnutrition and Infection: Complex Mechanisms and Global Impacts. PLoS Med. 4: e115.

Scrimshaw N. 1994. The consequences of hidden hunger for individuals and societies. Food Nutr. Bull. 15: 3–24.

UNICEF/WHO/World Bank Group. 2016. Levels and Trends in Child Malnutrition. Glob. Database Child Growth Malnutrition 12: 154–155.

Varma JL, Das S, Sankar R, Mannar MGV, Levinson FJ, Hamer DH. 2007. Community-level micronutrient fortification of a food supplement in India: a controlled trial in preschool children aged 36-66 mo. Am. J. Clin. Nutr. 85: 1127–33.

Chapter 2: Literature Review

2.1 Undernutrition

Undernutrition, as defined by UNICEF, is an outcome of insufficient food intake (hunger) and repeated infectious diseases (Black et al., 2008). Undernutrition occurs in various forms including underweight, stunting (low height for age), wasting (low weight for height) and micronutrient deficiency (iron, folic acid, vitamin A, zinc and iodine below healthy threshold) (Figure 2). Undernutrition is opposite of overnutrition, which is an outcome of excessive intake of energy and/or nutrients and includes conditions such as overweight obesity and non-communicable diseases such as heart disease, stroke, diabetes and cancer. Young children (<5 years) are most vulnerable to undernutrition and nearly half of all deaths in children under 5 are attributable to undernutrition, translating into an unnecessary loss of about 3 million children a year (UNICEF/WHO/World Bank Group, 2016). Undernutrition is a key underlying factor for under 5 mortality due to greater susceptibility to infections and slow recovery from illness, creating a vicious lethal cycle of worsening illness and deteriorating nutritional status. In addition, it has severe consequences on academic performance, future work productivity, potential income and susceptibility to chronic diseases (Black et al., 2013).

Stunting (height for age below -2z scores of the median WHO growth standards), wasting (weight for height below -2z scores of the median WHO growth standards) and underweight (weight for age below -2z scores of the median WHO growth standards) are the most used anthropometric indicators of child undernutrition and are priority global public health problems. Figure 3 shows Waterlow's classification of the degree of undernutrition (Waterlow et al., 1977).

According to recent surveys, 159 million and 50 million children are affected by stunting wasting worldwide, respectively (IFPRI, 2016). Approximately 800,000 deaths are lost due to wasting (60% of which are attributable to severe wasting) and over one million due to stunting (Black et al., 2013). Reportedly, wasting and stunting result in loss of 64.6 and 54.9 million Disability Adjusted Life Years (DALYs) respectively, and account for 14.8% and 12.6% of the total global DALYs for children under five (Black et al., 2008). In India alone, undernutrition afflicts 1 in 2 children, where 57 million (43%) children under 5 are underweight and 63 million (48%) stunted (NFHS 2005-06). Weight for age distribution curves for children (<3 years) in India falls to the left of the global reference population as shown in Figure 4 (Bhutia, 2014). Nine million children suffer from severe acute malnutrition (severe wasting with visual nutritional edema - very low weight for height, below -3z scores of the median WHO growth standards), 20 million from moderate acute malnutrition (wasting – low weight-for-height, between -3 and -2 z-scores of the median WHO growth standards or mid-upper arm circumference (MUAC) between 11 cm and 12.5 cm) and 5 million die every year as a direct or indirect result of undernutrition (NFHS 2005-06). In India, child undernutrition is declining and partial findings from recent surveys (2015-16) show reduction to 38.4% stunted, 21% wasted, 7.5 % severely wasted and 35.7% underweight children under 5 (NFHS, 2016). Although, there is a downward trend in child undernutrition in India and stunting and wasting rates have declined in the past decade, it still carries the largest burden of undernutrition.

Undernutrition often starts *in utero* and can have severe consequences throughout the life cycle and can span generations. For instance, undernutrition during pregnancy can have serious implications for the developing fetus and for future infant's morbidity and mortality risk,

vision and cognitive development. Figure 5 shows various nutrition problems, causes and consequences at various stages of life. The first 1000 days of child's life are crucial to avoid chronic stunting and prevent irreversible damage to child's brain development and physical growth, leading to reduced mental capacity, greater susceptibility to infection and disease and long-term loss of earning potential. Thus, proper nutrition and healthcare are essential during the formative years of the child.

There are several causes of undernutrition at different societal levels as recognized by the United Nations Children's Fund (UNICEF) conceptual framework. (Figure 6). Basic causes are influenced by the structure and processes of societies, underlying causes by households and communities and immediate causes by individuals (Black et al., 2008). Immediate causes include inadequate intake of nutrients and/or repeated infections that could further increase nutrient requirements and malabsorption of nutrients consumed, resulting in a vicious infection-undernutrition cycle (Figure 7). In this thesis our focus is on the immediate causes of undernutrition and other causes are discussed in detail by Black et al. (2008).

2.2 Undernutrition and Parasitic Infections

Immediate causes of undernutrition, i.e. inadequate dietary intake and repeated infectious diseases, such as parasitic infections, are closely related and both are found responsible to impair the immune system interdependently (Figure 8) (Hughes and Kelly, 2006; Cunnigham-Rundles S, 2008). Intestinal parasitic infection is considered as a global public health problem by the World Health Organization (WHO), afflicting 3.5 billion people and causing clinical morbidity to around 450 million people globally (WHO, 1998). Although it affects all ages and socio-

economic status, parasitic infections are more prevalent in children (<5 years) living in low-income settings, owing to inadequate sanitation, hygiene and healthcare system and their less developed immune systems (Scrimshaw, 1994). Enteric parasites result in reduced nutrient digestion and absorption (Northrop-Clewes et al., 2001), chronic gut inflammation (Wang, 2008), iron deficiency anemia (Farid et al., 1969), protein-energy malnutrition (Muniz-Junqueira and Queiróz, 2002), reduced growth (Stephenson et al., 1993) and reduced cognitive development (Olness, 2003) in children. Thus, interventions focused on providing nutritional foods to children are not sufficient to tackle the multietiological problem of malnutrition (Egger et al., 1990).

Common parasites responsible for morbidity and mortality in children are helminths such as *Ascaris lumbricoides* (roundworm), *Trichiuris trichiuria* (whipworm), *Ancylostoma duodenale*, and *Necator americanus* (hookworms) and protozoans such as *Giardia intestinalis*, *Entamoeba histolytica*, *Cyclospora cayetanensis*, and *Cryptosporidium* spp (Figure 9).

Subclinical infection with enteric pathogens is common in developing countries, even in the absence of diarrhea (Kotloff et al., 2013a). Repeated frequent exposure to enteric pathogens due to faeco-oral transmission results in a change of gut structure and function, characterized by villous atrophy and chronic inflammation of the small intestine resulting in environmental enteric dysfunction (EED). This leads to modest malabsorption and increased intestinal permeability (Keusch et al., 2013). Intestinal permeability can result in translocation of microbial products from the gut lumen to the systemic circulation, where they can trigger chronic inflammation suppressing IGF-1(Prendergast and Humphrey, 2014).

2.3 Current Interventions in India

Various government run interventions exist to address undernutrition in India as listed in Table 1. Through Integrated Child Development Services (ICDS) the beneficiaries' i.e. pregnant and lactating women and young children, are entitled to supplementary nutrition in the form of morning snack (milk/banana/egg/fruits/micronutrient fortified foods) and a hot cooked meal at *Anganwadi* centers (Figure 10). In addition, take-home rations (THR) sattu, sheera, upma or sometimes raw ingredients such as rice and pulses are provided to supplement the daily meals of children aging from 6 to 72 months. Otherwise healthy children under 6 years receive 12-15 g protein and 500 cal energy and undernourished children at a level of 800 kcal, 20 g of protein (ICDS, 2014). Beyond providing supplementary nutrition, *Anganwadi* centers are responsible for growth monitoring and identification of moderatly and severely underweight children, nutrition and health counseling, pre-school education, and often monthly immunization, micronutrient supplementation, deworming, and antenatal care.

The foods and take home rations offered through government welfare programs have been successful in providing nutrition, improving some health outcomes, increasing children enrolment and reducing gender bias. However, they have significant problems with consumption compliance due to the poor acceptability of products, and additional cooking requirements for women, which leads to the ingredient use for other purposes and consumption of lower quantities than those prescribed. Moreover, some food rations contain insufficient nutrients to meet program goals (Varma et al., 2007; Bhagwat et al., 2014).

2.4 Lipid-based Nutrition Supplements

Lipid-based nutrient supplements (LNS) are fortified ready to use food products, containing concentrated nutrients (energy, protein, essential fatty acids, and micronutrients) and are suitable to complement most food rations (Arimond et al., 2013). LNS are different from ready to use therapeutic foods (RUTFs) as outlined in Table 2 and are used for different applications ranging from prevention to treatment depending upon the quantity of LNS used.

The examples listed in Figure 11 are peanut-based RUTF or LNS, first formulated in 1996 and patented (US 6346284 B1) by André Briend, a French pediatric nutritionist. These products are now commercialized by the French company, Nutriset. Products are thick pastes made from peanut butter, sugar, vegetable oil, milk powder, and a vitamin/mineral premix with an aim to provide energy, proteins, and other nutrients that are needed by malnourished children to recover and resume their growth. The low water activity is suitable to store packaged product for up to two years at ambient temperatures.

Existing LNS such as Nutributter®, PlumpyDoz® and PlumpySup® (Nutriset, 2015) (Figure 11) have not been accepted in India mainly due to problems associated with higher cost, poor acceptability, foreign import, safety and sustainability (Arie, 2010). Similar peanut-based ready to use therapeutic foods (RUTF) were not well accepted by pregnant and lactating women in Bangladesh (Ali et al., 2013), and by malnourished children in India (Dube et al., 2009). The World Food Program (WFP) and local doctors have expressed an urgent need for the development of indigenous and low-cost LNS to address severe and moderate undernutrition in India (Gupta et al., 2006). LNS have been used for complementary feeding to address acute malnutrition in children and have had mixed results as listed in Table 3:

2.5 Omega-3 Fortification

Omega-3 long chain polyunsaturated fatty acids are essential fatty acids with an array of biological effects in human health and disease. The three principal omega-3 fatty acids are alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) and are important for a number of bodily functions, including muscle activity, brain development, blood clotting, digestion, cell division and overall growth (Yashodhara et al., 2009). They also play an important role in the regulation of tissue inflammation and are associated with inhibition and/or stimulation of cytokine production. Enteric infection, a trigger for inflammatory response, and intestinal inflammation are very common in the malnourished children in India, resulting in malabsorption of nutrients (Gupta et al 2011). Omega 6: omega 3 ratio is recommended to be 3:1, not only for normal growth, cell functioning and immune function but also for adequate inflammatory: anti-inflammatory response (Simopoulos, 2002; Simopoulos, 1991; Brenna et al., 2015). Currently, no specifications for omega 6: omega 3 ration exists for LNS which allows high n-6 polyunsaturated fatty acid (PUFA) content and low n-3 PUFA.

Indian population generally being vegetarian have ω6:ω3 ratios of 30:1 to 70:1, which results in increased levels of proinflammatory prostaglandins in the body (Abedi and Sahari 2014). Flaxseed oil is a good source of ALA omega 3 PUFA and have better conversion rates to PUFA EPA (anti-inflammatory agent to balance omega 6 arachidonic acid) and DHA (essential for neural development) in vegetarian populations (Welch et al., 2010), and has anti-inflammatory properties (Caughey et al., 1996).

Recently, there has been an increased interest in improving the omega-3 fatty acid profile of conventionally used peanut based LNS formulations, which are rich in omega-6 linoleic acid (LA) but contain negligible omega-3 alpha linolenic acids (ALA)(Brenna et al., 2015; Hsieh

et al., 2015; Jones et al., 2015) (Table 4). In a recent study of children with SAM, a two-month supplementation with RUTF high in oleic acid and with a 1:1 LA:ALA ratio increased circulating EPA and DHA and reduced arachidonic acid compared to standard RUTF with LA/ALA ratio of 53:1(Hsieh et al., 2015). In another study, a peanut-based RUTF fortified with FO significantly increased plasma EPA and docosapentaenoic acid, but not DHA among children suffering from SAM (Jones et al., 2015) These results support the hypothesis that if omega-3 PUFA oral delivery is increased, changes in their accumulation will be reflected in the circulation of malnourished individuals.

2.6 *Cryptosporidium parvum* Characteristics and Reproduction

Cryptosporidiosis, caused by a protozoan parasite of the genus *Cryptosporidium*, is the second largest cause of diarrheal disease and death in infants after rotavirus, (Kotloff et al., 2013b). It is the leading cause of infectious diarrhea in Indian children with positive rates of 1.1 to 18.9% (Ajjampur et al., 2008). It is excreted in the feces of an infected host in the form of an oocyst and is mainly transmitted from person to person, animals and indirectly through the environment (water and food)(Daniels et al., 2016). The thick-walled oocyst is environmentally resistant and does not decay in the environment as well as in most disinfection processes, making it highly resilient (Smith et al., 2005).

Humans are most frequently infected with *C. hominis* and *C. parvum* and both anthroponotic and zoonotic cycles have been documented in the life cycle of these parasites (Smith et al., 2005). *C. parvum*, a monoxenous apicomplexan mucosal parasite, has gained wide recognition due to its association with a severe diarrheal disease that affects mostly infants and children who are immunocompromised and undernourished. *C. parvum* does not multiply outside

of the host and completes all stages of its development (asexual and sexual) within a single, live host, however, it could only complete its asexual cycle *in vitro*. *C. parvum* infection initiates after ingestion of thick-walled oocysts, which are inactive in the stomach and upper intestine, but excyst in the gastrointestinal tract to release four infective sporozoites (Figure 12). These motile sporozoites then attach to the receptors on the apical membrane of host epithelial cell, mediated by carbohydrate: lectin interactions, and induce reorganization of host-cell actin cytoskeleton and protrusion of the host-cell membrane around the sporozoite to form a parasitophorous vacuole. The proteins involved in the parasite attachment to host cells are either present on the surface or exocytosed from the parasite via specialized secretory vesicles; micronemes, rhoptries, and dense granules (O'Hara and Chen, 2011). Here, the organism remains intracellular but extracytoplasmic. The internalized sporozoite further matures and undergoes series of asexual and sexual reproduction (schizogony) to produce oocytes. The two morphologic forms of the oocysts are thin-walled oocysts (asexual stage), which excyst within the same host (causing self-infection), whereas the thick-walled oocysts (sexual stage) are shed into the environment from fecal excretion (Chen et al., 2002).

Studies on genome analysis of *C. parvum* have revealed that the parasite relies solely on the host for nutrient acquisition as it lacks enzymes for Kreb's cycle and depends largely on glycolysis for energy requirements. It also lacks the capacity for the *de novo* synthesis of amino acids, fatty acids and nucleosides. It is speculated that *C. parvum* encodes an extensive array of transporters to transfer and acquire nutrients from the host (Xu et al., 2004).

As discussed earlier, undernutrition and parasitic infections are interrelated and can have an additive negative effect on growth outcomes of undernourished children. This relationship was found true and additive in undernourished suckling mice infected with *C. parvum* oocytes during

the first 2 weeks of life, analogous to the first postnatal year in humans. Undernutrition intensified *C. parvum* infection and mucosal damage in mice, conversely, *C. parvum* infection solely caused undernutrition and growth reduction in nourished mice. A synergistic effect between infection and undernutrition was seen in infected undernourished mice which exhibited higher oocyst shedding, greater mucosal damage in terms of blunted villi and deeper crypts, increased mucosal inflammatory TNF-α and IFN-γ intestinal levels, higher mRNA expression and severe enteropathy compared to nourished uninfected mice (Coutinho et al., 2008).

There are a few compounds with some, albeit limited, anticryptosporidial activity among them are paromomycin, nitazoxanide, azithromycin in combination with paromomycin, roxithromycin, and protease inhibitors often used in highly active antiretroviral therapy (Gargala, 2008). Currently, only one FDA-approved, moderately effective drug, nitazoxanide (Alinia®) (Miyamoto and Eckmann, 2015), is available for treatment of *Cryptosporidium* infection. Due to increasing resistance and severity of side effects, new alternative bioactives are currently under examination (Anthony *et al.*, 2005).

2.7 Oregano Essential Oil

Plant-derived essential oils, which are lipophilic, volatile plant compounds, primarily composed of monoterpenes and sesquiterpenes, have been used since ancient history and have seen a rapid resurgence in the recent years (Little and Croteau, 1999; Kelly et al., 2005). Essential oils of thyme, clove, sage, turmeric, oregano are known to exhibit antibacterial and antiparasitic activity (Anthony et al., 2005). Oregano Essential oil (OEO), also known as the Mediterranean miracle, Sathra in Ayurveda or Himalayan Marjoram in India, is known to possess antibacterial,

antiviral, antifungal, antiparasitic and antioxidant activities (Burt, 2004). OEO is extracted through steam distillation, cold solvent extraction, supercritical fluid extraction or microextraction of plants from *Origanum* genus, of which *Origanum vulgare* subsp. *hirtum* is the most common varied due to its high quality and yield (Kintzios, 2002). In India, oregano is found in the temperate Himalayas from Kashmir to Sikkim. The principal components responsible for the bioactivity of OEO are carvacrol (CV) and thymol (Burt, 2004) (Figure 13).

OEO and CV are considered as generally recognized as safe (GRAS) by FDA for food use and are also included in the Council of Europe in the list of chemical flavorings that can be added to foods (De Vincenzi et al., 2004). In addition, they are found to be non-toxic at the high dose in a short term study (Force et al., 2000). Most of the knowledge on OEO and CV effectiveness against parasites, their safety, and other side effects has been generally acquired through word of mouth or folklore. As of our knowledge, no literature or data is available on its potential bioactive use in a food supplement.

The mode of action of OEO and its constituents is not clear but is attributed to the presence of hydroxyl group in the phenolic compounds carvacrol and thymol (Ultee et al., 2002) and their hydrophobicity (Burt, 2004), which allows the essential oils to penetrate the cell membrane and reduce parasitic infection by affecting cytoplasmic metabolic pathways or organelles (Santoro et al., 2007). In addition, monoterpenes such as CV can modify the calcium-dependent protein kinase 1 (CDPK1) and affect the Ca^{2+} mediated signaling of the parasite, required for invasion, differentiation and regulation of other vital functions (Nagamune et al., 2008; Murphy et al., 2010). In addition, the hydrophobicity (Burt, 2004) and presence of hydroxyl group in CV and thymol (Ultee, Bennik, & Moezelaar, 2002), may allow the phenols to penetrate

the cell membrane and reduce parasitic infection by modulating cytoplasmic metabolic pathways such as ATP synthesis (Turina et al., 2006; Santoro et al., 2007).

2.8 Encapsulation

Incorporation of OEO or its constituents in food is a challenging task due to its high volatility, instability, interaction with food ingredients, and potent flavor. These compounds have extremely low flavor threshold, evaporate easily, and decompose and oxidize during formulation, processing and storage due to exposure to heat, pressure, light, or oxygen (Beirão-da-Costa et al., 2013; Hosseini et al., 2013). Moreover, their therapeutic effect against parasites might be limited by their rapid metabolism and instability during digestion. Thus, a small fraction of the oral dose, if any, reaches the lower gut, where these parasites reside (Kohlert et al., 2002; Kohlert et al., 2000). Encapsulation is a widely used technique for improving stability, controlled delivery and reducing the unpleasant odor and taste (Wang et al., 2009; McClements et al., 2009). Materials such as chitosan nanoparticles (Hosseini et al., 2013), tween 80 (Bhargava et al., 2015), milk powders (Baranauskienė et al., 2006), gelatin/sucrose microparticles (Beirão et al., 2004), sorghum/rice starch (Almeida et al., 2013), liposomes (Liolios et al., 2009), and cyclodextrins (Guimarães, Oliveira, Alves, Menezes, Serafini, Araújo, et al., 2015) have been used to improve functionality and stability of OEO or carvacrol.

Of all the available materials, cyclodextrin is the one of the few materials that is GRAS certified by FDA and Food Safety Standards Authority of India and has been reported successful in improving stability and solubility of essential oils, masking of unpleasant odor and taste and achieving controlled gut delivery (Wang et al., 2009; McClements et al., 2009) (Szente and Szejtli, 2004). In addition, cyclodextrins have been shown to treat *C. parvum* infections in lambs and

calves at a dose of 500 mg/Kg (Castro-Hermida et al., 2001; Castro-Hermida et al., 2002; Castro-Hermida et al., 2000).

Cyclodextrins (CyD) (α, β, and γ) are cyclic oligosaccharides with six to eight glucose units joined by α-1,4 glycosidic bonds and have a rigid structure with a hydrophilic outer surface and a singular hydrophobic cavity due to the absence of hydroxyl group (Szente and Szejtli, 2004) (Figure 14). CyDs are produced from starch (e.g., corn, potato, maize, and wheat.) and related *α*-1,4-glucans via enzymatic conversion – degradation and cyclization – using CyD glycosyltransferase (CGTase) and, partly, *α*-amylases. Due to their unique structure, CyDs are used to form inclusion complexes with hydrophobic compounds. CyDs are neither hydrolyzed nor absorbed in the stomach and small intestine, but are broken into small mono- and oligo-saccharides in the large intestine by colonic bacteria (approx. 1011 – 1012 CFU/mL), especially *Bacteroides*, using them as sole carbon source and by the stimulation of cyclodextranase activity by exposure to CyDs (Antenucci and Palmer, 1984)(Sinha and Kumria, 2001). This makes them excellent carriers for localized drug delivery to the colon, as they remain intact through the stomach deliver colon specific drugs, specifically targeted to gut parasites such as *C. parvum*. There are no toxic effects associated with oral administration of CyDs. These are not absorbed across the gastrointestinal tract due to their chemical structure (large number of hydrogen donors and acceptors), their molecular weight (>972 Da), and their low octanol/water partition coefficient (approx. log $P_{o/w}$ between -3 and 0) (Irie and Uekama, 1997; Matsuda, 1999). β- and γ-CyDs have been previously used as food ingredients in chocolate and mayonnaise for emulsification, grape drinks, coffee, ginseng extract, soybean lecithin for taste masking, and in instant tea drink for preserving flavor (Szente and Szejtli, 2004).

Selection of the form of CyD depends on two factors, its approval status as a food ingredient (α and β are not specified and γ – 5mg/Kg) (Marques, 2010), and its formation constant (K_f) as listed in Table 5 (Kfoury et al., 2016a). The higher the formation constant better the stability, due to the complementarity (of aromatic ring) between the guest molecule and the CyD cavity. The recommended drug properties for enhancing the drug-complex stability are high drug hydrophobicity (log P>2.5), low drug solubility (typically< 1 mg/mL), low dose (< 100 mg), and low CD: drug ratio (< 2:1). CV with drug hydrophobicity of log $P_{o/w} = 3.52$ and solubility 0.11 g/L shows an excellent potential to be included in Cyd complexes (Ben Arfa et al., 2006).

The most common stoichiometry of lipophilic molecule such as carvacrol and CyD complex reported in the literature is 1:1, meaning one molecule of CV forms a complex with one CyD molecule (Loftsson et al., 2005). This is also observed through molecular modeling study (Figure 15), where guest CV or thymol penetrate the CyD via their methyl moiety and the isopropyl group pointing to the secondary wider rim, to form most stable inclusion complexes (Kfoury et al., 2016a).

The complexation between a guest molecule and CyD molecules occurs in four steps. 1. Displacement of polar water molecules from the apolar CyD cavity. 2. Formation of hydrogen bonds as the displaced water returns to the pool. 3. Reduction of the repulsive interactions between the hydrophobic guest molecule and the aqueous environment. 4. Increase in hydrophobic interactions as the guest inserts itself into the apolar CyD cavity (Del Valle, 2004).

2.9 Tables and Figures

Table 1. Programs to address undernutrition in India.

Program	Organization	Service	Beneficiary	Reference
Integrated Child Development Scheme (ICDS) – since 1975	Government of India – *Anganwadi* Centers	Supplementary foods Pre-school education Health and Nutrition Education Health Services	Children under age 6 (34 million) Pregnant and Lactating Mothers (7 million)	http://icds-wcd.nic.in/icds/
Midday meal (MDM) scheme in schools – since 1995	Government of India ISKCON Food Relief Foundation Akshaya Patra Foundation	Freshly hot cooked plant-based meals – 300 Cal + 8 to 12 g protein	School going children age (6-14) (1.3 million)	http://mdm.nic.in/
National Rural Health Management (NRHM) Reproductive Child Health (RCH-II) – since 2013	Government of India	Antenatal care, counseling, micronutrient supplementation, immunization, postnatal care, counseling, deworming, health checkup	Pregnant and Lactating Mothers Children (0-6 years)	http://nrhm.gov.in/nhm/about-nhm.html
Kishori Shakti Yojana	Government of India	Supplementary Nutrition, Iron Folic Acid supplementation, vocational training of adolescent girls	Adolescent Girls (11-18 years)	http://wcd.nic.in/kishori-shakti-yojana

Other programs – Total Sanitation Campaign (Access to sanitation facilities), National Rural Drinking Water Program (Access to safe drinking water), Sarva Siksha Abhiyan (knowledge dissemination on nutrition in schools)

Table 2. Difference between LNS and RUTF (Dewey and Arimond, 2012).

LNS	RUTF
Used for prevention (when provided in small quantity) or treatment (when provided in large quantity) of wasting and stunting which are outcomes of MAM	Used for treatment of severe acute malnutrition
Provided in smaller quantities (20-50 g/d)	Provided in large quantities (200 – 300 g/d)
Used for "home-fortification" and to complements the foods already consumed by children	Temporarily replaces most or all other foods besides breast milk
Relatively low cost	Relatively high cost
Example –	Example –
Nutributter – LNS Small Quantity (108 kcal/d for 4 – 6 weeks)	Plumpy'Nut (200 kcal/kg body wt./d for 6 to 8 weeks)
Plumpy'Doz – LNS Medium Quantity (247 kcal/d)	
Plumpy'Sup – LNS Large Quantity (75 kcal/kg body weight/d for 6 – 8 weeks)	

Table 3. Clinical trials on LNS supplementation in children.

LNS	Country	Design	Subjects (number)	Duration	Impact	Reference
LNS 50 and LNS 25 g/day	Malawi	Randomized, controlled, single-blind trial	6 month (n=182) healthy rural infants	12 months	Reduction in incidence of severe stunting	(JC Phuka et al., 2009; Phuka et al., 2008)
LNS (25-75 g/d)	Malawi	Randomized, controlled, parallel-group, investigator-blind	6-17 month old underweight infants	12 weeks	Not conclusive	(Kuusipalo et al., 2006)
Ready to use LNS	Niger	Blanket distribution	Under 3 (n=60,000)	6 month distribution	Reduced severe wasting	(Defourny et al., 2009)
Nutributter	Ghana	Randomized, controlled	6-12 months (n=313)	12 month	Reduced prevalence of iron deficiency and improved growth	(Adu-Afarwuah et al., 2008; Adu-Afarwuah et al., 2007)
Fortified Soybean-maize-sorghum RUCF	Democratic Republic of Congo	Randomized, controlled	6 months (n=1383)	6 months	No reduction of stunting or underweight	(Bisimwa et al., 2012)
LNS	Haiti	Randomized, controlled	6-11 months (n=589)	6 months intervention and 6 months follow-up	Improved linear growth of children	(Iannotti et al., 2014)
Peanut-/soy-based fortified spread	Malawi	Randomized, Controlled	6 months (n=240)	18 months	Improved weight gain	(Lin et al., 2008)
LNS (280 kcal) supplemented with corn-soy blend	Malawi	Randomized, Controlled	6 months (n=240)	12 months	Non-conclusive	(Mangani et al., 2015)
Ready to use fortified spread	Malawi	Randomized	6-18 months (n=176)	12 weeks	Not conclusive	(J Phuka et al., 2009)

Table 4. Clinical trials on omega-3 fatty acid fortified LNS supplementation in children

LNS	Omega 3 source	Country	Design	Subjects	Duration	Impact	Reference
High-oleic RUTF	High-oleic peanut, palm oil, and linseed oil	Malawi	Prospective, randomized, double-blind	141 children with SAM (6-59 months)	4 weeks	Improved DHA and EPA levels in plasma	(Hsieh et al., 2015)
RUTF	Flaxseed oil and Fish oil	Kenya	Randomized controlled trial	60 children (6-50 months)	3 months	Fish oil RUTF increased n-3 PUFA status whereas Flaxseed oil had no effect on DHA	(Jones et al., 2015)

Table 5. Characteristics of α-, β- and γ- Cyclodextrins

Characteristics	α –	β –	γ –
No. of glucose units	6	7	8
Cavity diameter (nm)	0.47	0.60	0.75
Height of torus (nm)	0.79	0.79	0.79
Approval status as food ingredient	NS	NS	5 mg/kg
Formation constant with carvacrol (K_f) M^{-1}	454	2620	999
Formation constant with thymol (K_f) M^{-1}	107	1467	233

Figure 2. Forms of undernutrition (Black et al, 2008).

Figure 3. Waterlow's classification of the degree of undernutrition (Waterlow et al., 1977).

Weight for height \ Height for age	Normal (> - 2 SD HAZ)	Stunted (< -2 SD HAZ)
Normal (> -2 SD WHZ)	Normal	Stunted
Wasted (< -2 SD WHZ)	Wasted	Stunted and wasted

Figure 4. Weight for age distribution curve for children (<3 years) in India compared to global reference population (Taken from Bhutia, 2014).

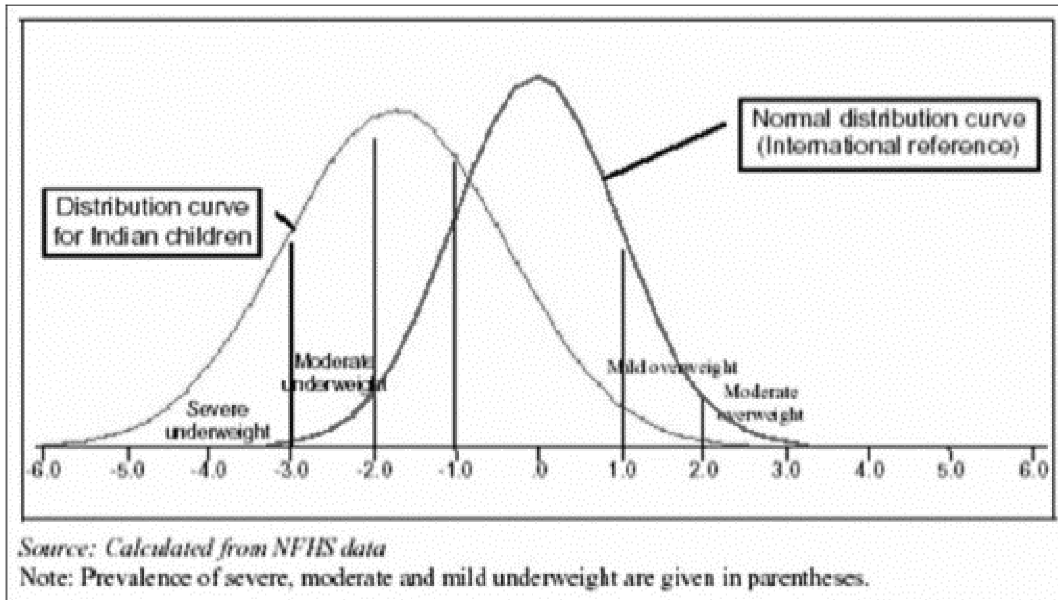


Figure 5. A conceptual model of effects of undernutrition throughout the life cycle (Adapted from ACC/SCN, 2000).

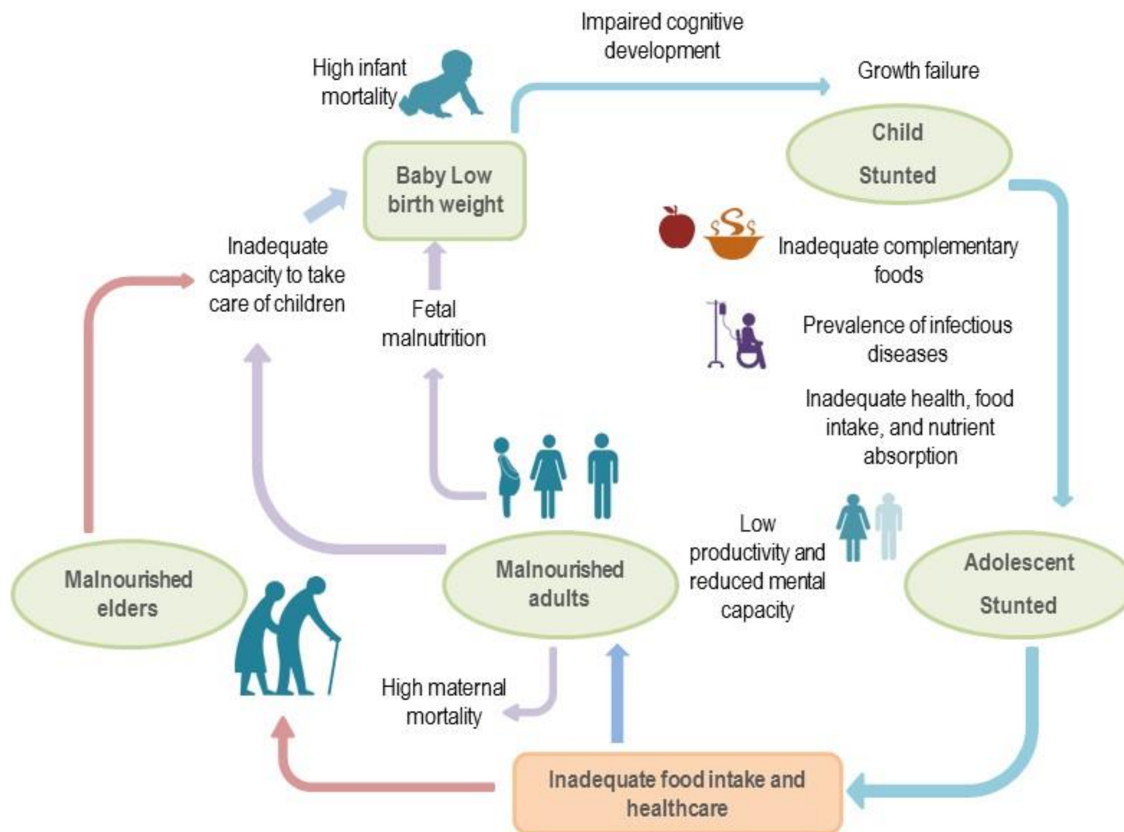


Figure 6. UNICEF conceptual framework of relations between basic, underlying and immediate causes to maternal and child undernutrition and its short-term and long-term consequences (Black et al. 2008).

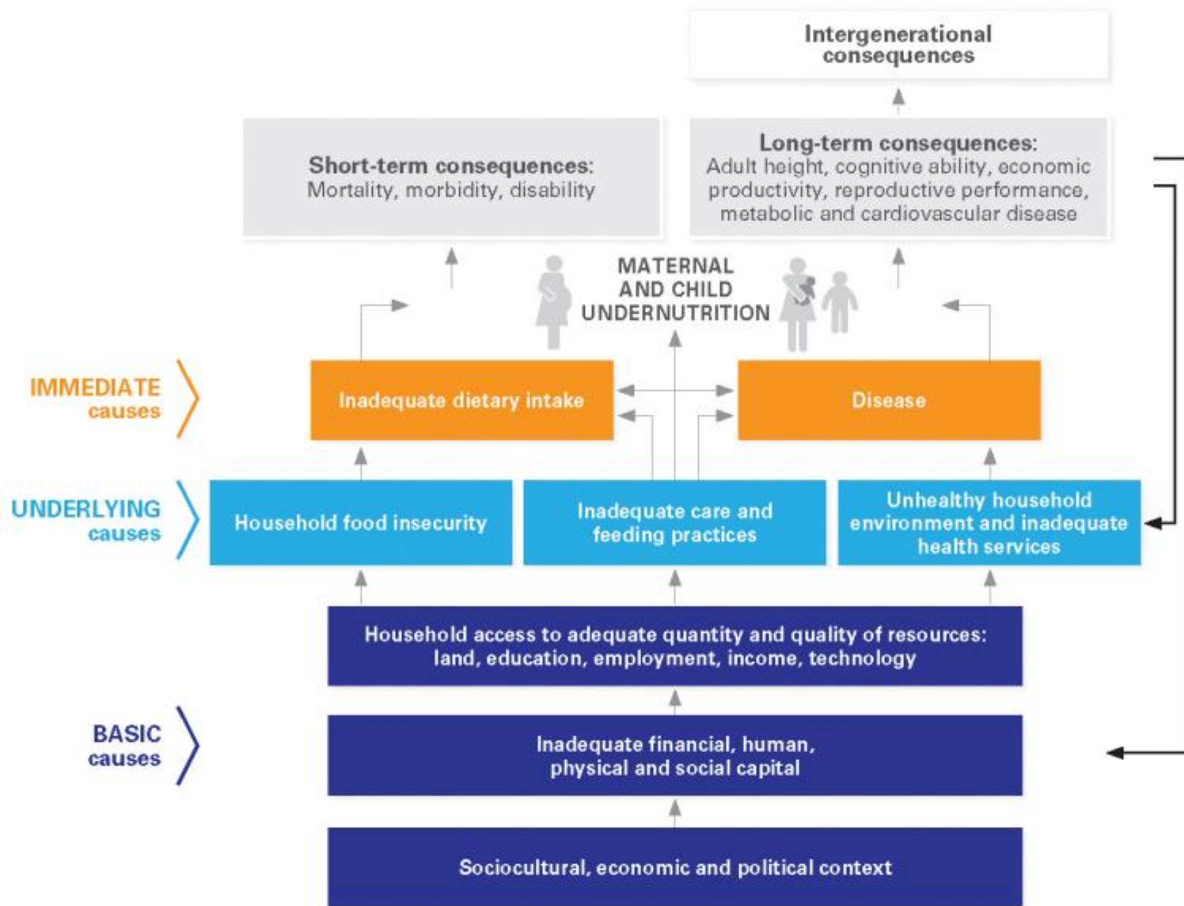


Figure 7. Infection-undernutrition vicious cycle (Katona and Katona-Apte, 2008)

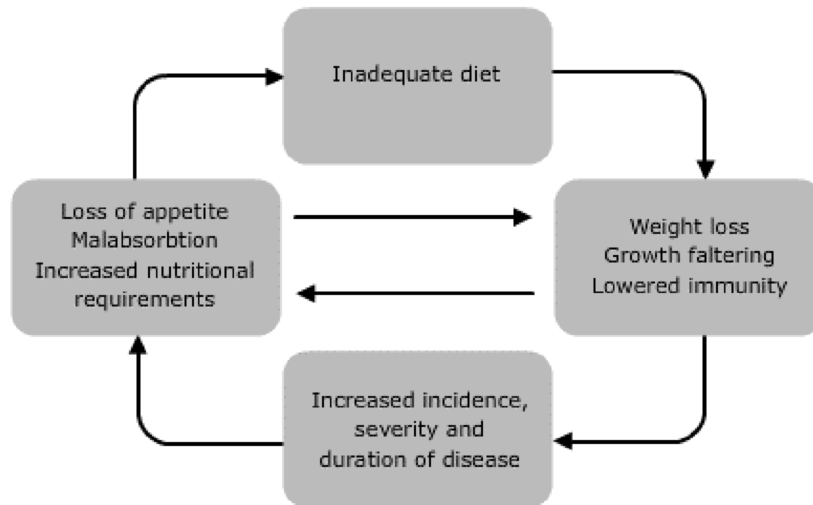


Figure 8. Undernutrition and parasitic infection interaction (Cunnigham-Rundles, 2008)

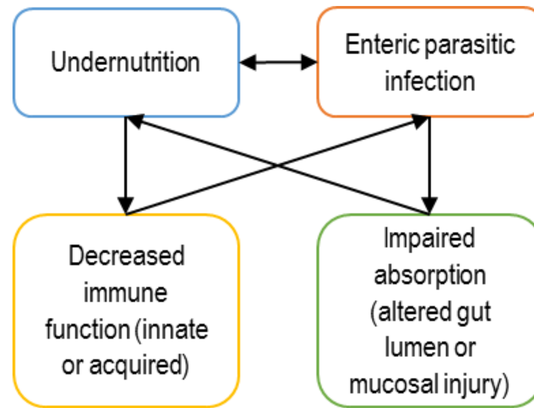


Figure 9. Intestinal parasites (left to right), protozoans; *Cryptosporidium parvum* and *Giardia lambia*, and helminths; *Ascaris lumbricoides* (roundworm) and *Ancylostoma duodenale* (hookworm).

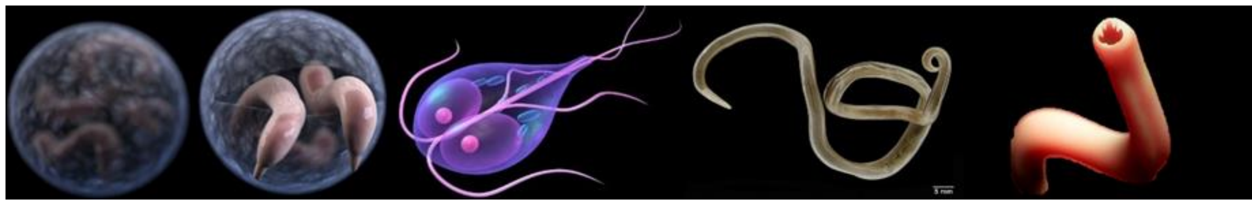


Figure 10. *Anganwadi* centers in India, hot cooked rice and pulse meal, kitchen setting to cook those meals and take-home rations (left to right).

Figure 11. Examples of currently available small, medium and large quantity LNS (Source: http://www.nutriset.fr/en/homepage-nutriset.html).

Figure 12. Life cycle of *Cryptosporidium parvum* (Adapted from Center for Disease Control and Prevention http://www.cdc.gov/parasites/crypto/biology.html).

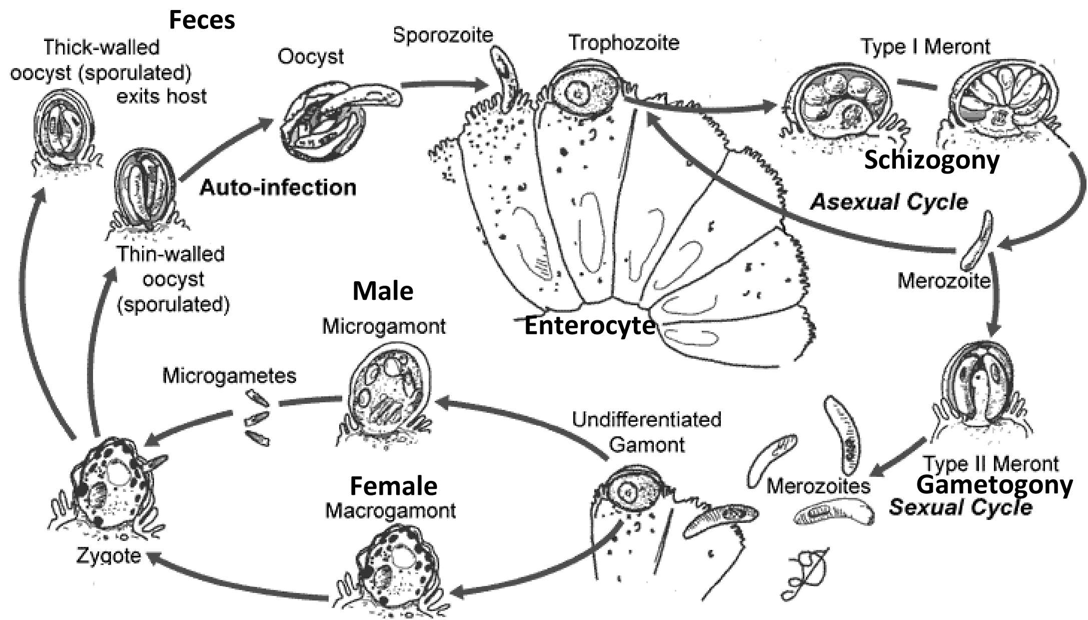


Figure 13. Chemical structure of carvacrol (left) and thymol (right).

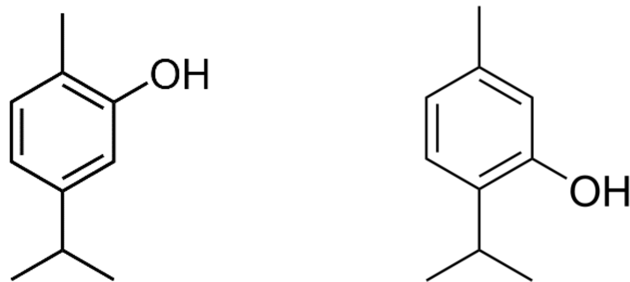


Figure 14. Structures of α-, β- and γ- Cyclodextrins

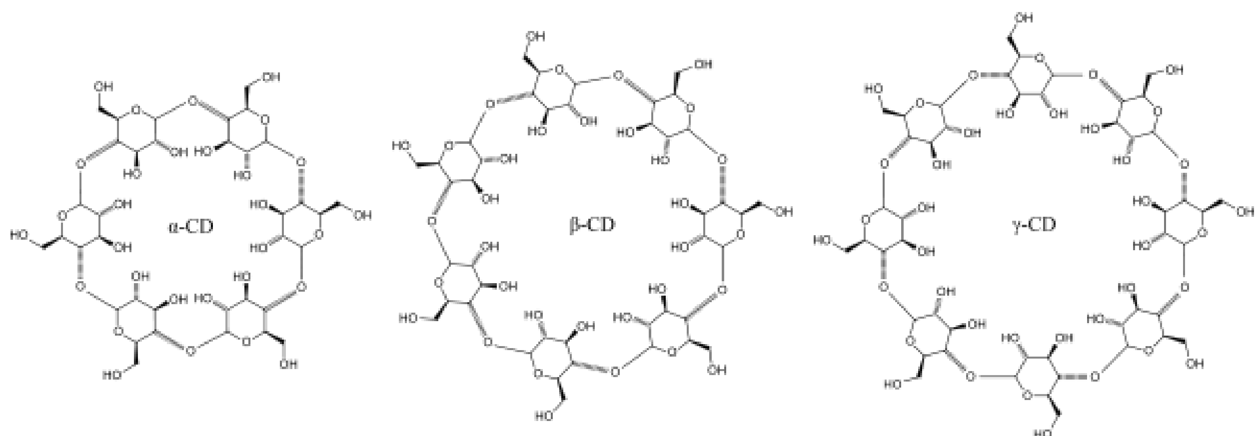
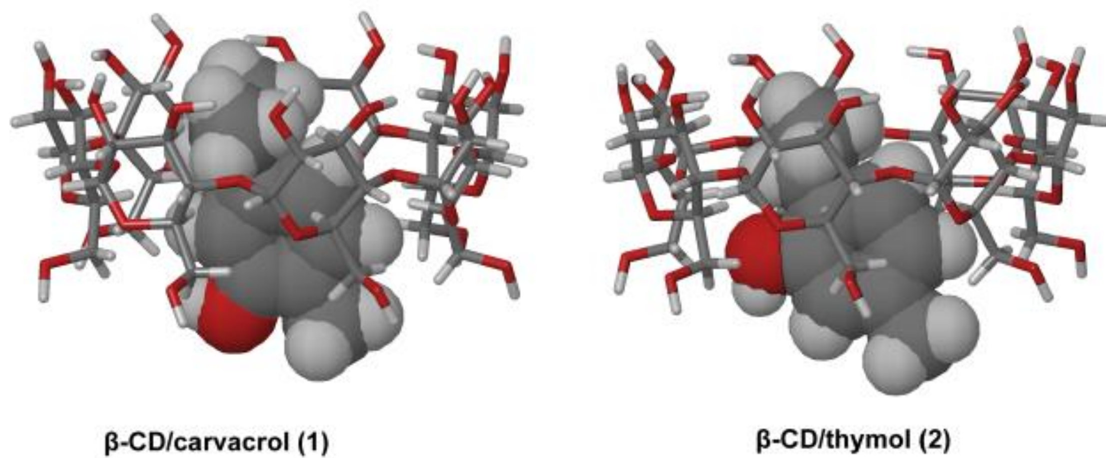


Figure 15. Molecular complex of β-CyD-CV and β-CyD-thymol (Taken from Kfoury et al., 2016)



2.10 References

ACC/SCN. 2000. Fourth Report on the World Nutrition Situation. Geneva: ACC/SCN in collaboration with IFPRI.

Adu-Afarwuah S, Lartey A, Brown KH, Zlotkin S, Briend A, Dewey KG. 2008. Home fortification of complementary foods with micronutrient supplements is well accepted and has positive effects on infant iron status in Ghana. Am. J. Clin. Nutr. 87: 929–38.

Adu-Afarwuah S, Lartey A, Brown KH, Zlotkin S, Briend A, Dewey KG. 2007. Randomized comparison of 3 types of micronutrient supplements for home fortification of complementary foods in Ghana: Effects on growth and motor development. Am. J. Clin. Nutr. 86: 412–420.

Ajjampur SSR, Sankaran P, Kang G. 2008. Cryptosporidium species in HIV-infected individuals in India: an overview. Natl. Med. J. India 21: 178–84.

Ali E, Zachariah R, Shams Z, Manzi M, Akter T, Alders P, et al. 2013. Peanut-based ready-to-use therapeutic food: how acceptable and tolerated is it among malnourished pregnant and lactating women in Bangladesh? Matern. Child Nutr. 11: 1028–1035.

Almeida AP, Rodríguez-Rojo S, Serra AT, Vila-Real H, Simplicio AL, Delgadilho I, et al. 2013. Microencapsulation of oregano essential oil in starch-based materials using supercritical fluid technology. Innov. Food Sci. Emerg. Technol. 20: 140–145.

Antenucci RN, Palmer JK. 1984. Enzymatic degradation of α- and β-cyclodextrins by Bacteroides of the human colon. 32: 1316–1321.

Anthony J-P, Fyfe L, Smith H. 2005. Plant active components - a resource for antiparasitic agents? Trends Parasitol. 21: 462–8.

Ben Arfa A, Combes S, Preziosi-Belloy L, Gontard N, Chalier P. 2006. Antimicrobial activity of carvacrol related to its chemical structure. Lett. Appl. Microbiol. 43: 149–54.

Arie S. 2010. Hungry for profit. BMJ 341: c5221.

Arimond M, Zeilani M, Jungjohann S, Brown KH, Ashorn P, Allen LH, et al. 2013. Considerations in developing lipid-based nutrient supplements for prevention of undernutrition: experience from the International Lipid-Based Nutrient Supplements (iLiNS) Project. Matern. Child Nutr. 11: 31–61.

Baranauskienė R, Venskutonis PR, Dewettinck K, Verhé R. 2006. Properties of oregano (Origanum vulgare L.), citronella (Cymbopogon nardus G.) and marjoram (Majorana hortensis L.) flavors encapsulated into milk protein-based matrices. Food Res. Int. 39: 413–425.

Beirão S, Duarte C, Pinheiro AC, Bourbon AI, Teresa A, Martins MM, et al. 2004. The Effect of the Matrix System in the Delivery and In-Vitro Bioactivity of Microencapsulated Oregano Essential Oil. : 2004–2007.

Beirão-da-Costa S, Duarte C, Bourbon AI, Pinheiro AC, Januário MIN, Vicente A a., et al. 2013. Inulin potential for encapsulation and controlled delivery of Oregano essential oil. Food Hydrocoll. 33: 199–206.

Bhagwat S, Sankar R, Sachdeva R, Joseph L, Sivaranjani. 2014. Improving the nutrition quality of the school feeding program (Mid day meal) in India through fortification: a case study. Asia Pac. J. Clin. Nutr. 23 Suppl 1: S12–9.

Bhargava K, Conti DS, da Rocha SRP, Zhang Y. 2015. Application of an oregano oil nanoemulsion to the control of foodborne bacteria on fresh lettuce. Food Microbiol. 47: 69–73.

Bhutia DT. 2014. Protein energy malnutrition in India: the plight of our under five children. J. Fam. Med. Prim. care 3: 63–7.

Bisimwa G, Owino VO, Bahwere P, Dramaix M, Donnen P, Dibari F, et al. 2012. Randomized controlled trial of the effectiveness of a soybean-maize-sorghum-based ready-to-use complementary food paste on infant growth in South Kivu, Democratic Republic of Congo. Am. J. Clin. Nutr. 95: 1157–1164.

Black RE, Allen LH, Bhutta ZA, Caulfield LE, de Onis M, Ezzati M, et al. 2008. Maternal and child undernutrition: global and regional exposures and health consequences. Lancet 371: 243–60.

Black RE, Victora CG, Walker SP, Bhutta ZA, Christian P, de Onis M, et al. 2013. Maternal and child undernutrition and overweight in low-income and middle-income countries. Lancet 382: 427–51.

Brenna JT, Akomo P, Bahwere P, Berkley J a, Calder PC, Jones KD, et al. 2015. Balancing omega-6 and omega-3 fatty acids in ready-to-use therapeutic foods (RUTF). BMC Med. 13: 1–4.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods--a review. Int. J. Food Microbiol. 94: 223–53.

Castro-Hermida JA, Freire-Santos F, Oteiza-Löpez AM, Ares-Mazás E. 2000. Unexpected activity of beta-cyclodextrin against experimental infection by Cryptosporidium parvum. J. Parasitol. 86: 1118–20.

Castro-Hermida JA, González-Losada Y, Freire-Santos F, González-Warleta M, Mezo-Menéndez M, Ares-Mazás E. 2002. Efficacy of beta-cyclodextrin against experimental cryptosporidiosis in neonatal lambs. J. Parasitol. 88: 185–7.

Castro-Hermida JA, Quílez-Cinca J, López-Bernad F, Sánchez-Acedo C, Freire-Santos F, Ares-Mazás E. 2001. Treatment with beta-cyclodextrin of natural Cryptosporidium parvum infections in lambs under field conditions. Int. J. Parasitol. 31: 1134–7.

Caughey GE, Mantzioris E, Gibson R a, Cleland LG, James MJ. 1996. The effect on human tumor necrosis factor alpha and interleukin 1 beta production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. Am. J. Clin. Nutr. 63: 116–122.

Chen X-M, Keithly JS, Paya C V, LaRusso NF. 2002. Cryptosporidiosis. N. Engl. J. Med. 346: 1723–1731.

Coutinho BP, Oriá RB, Vieira CMG, Sevilleja JEAD, Warren CA, Maciel JG, et al. 2008. Cryptosporidium Infection Causes Undernutrition and, Conversely, Weanling Undernutrition Intensifies Infection. J. Parasitol. 94: 1225–1232.

Daniels ME, Smith WA, Schmidt W-P, Clasen T, Jenkins MW. 2016. Modeling Cryptosporidium and Giardia in Ground and Surface Water Sources in Rural India: Associations with Latrines, Livestock, Damaged Wells, and Rainfall Patterns. Environ. Sci. Technol. 50: 7498–7507.

Defourny I, Minetti A, Harczi G, Doyon S, Shepherd S, Tectonidis M, et al. 2009. A Large-Scale Distribution of Milk-Based Fortified Spreads: Evidence for a New Approach in Regions with High Burden of Acute Malnutrition D Tomé, editor. PLoS One 4: e5455.

Dewey KG, Arimond M. 2012. Lipid-based nutrient supplements: how can they combat child malnutrition? PLoS Med. 9: e1001314.

Dube B, Rongsen T, Mazumder S, Taneja S, Rafiqui F, Bhandari N, et al. 2009. Comparison of Ready-to-Use Therapeutic Food with cereal legume-based khichri among malnourished children. Indian Pediatr. 46: 383–388.

Egger RJ, Hofhuis EH, Bloem MW, Chusilp K, Wedel M, Intarakhao C, et al. 1990. Association between intestinal parasitoses and nutritional status in 3-8-year-old children in northeast Thailand. Trop. Geogr. Med. 42: 312–23.

Farid Z, Patwardhan VN, Darby WJ. 1969. Parasitism and Anemia. Am J Clin Nutr 22: 498–503.

Force M, Sparks WS, Ronzio R a. 2000. Inhibition of enteric parasites by emulsified oil of oregano in vivo. Phyther. Res. 14: 213–214.

Gargala G. 2008. Drug treatment and novel drug target against Cryptosporidium. Parasite 15: 275–81.

Guimarães AG, Oliveira MA, Alves R dos S, Menezes P dos P, Serafini MR, Araújo AA de S, et al. 2015. Encapsulation of carvacrol, a monoterpene present in the essential oil of oregano, with β-cyclodextrin, improves the pharmacological response on cancer pain experimental protocols. Chem. Biol. Interact. 227: 69–76.

Gupta P, Shah D, Sachdev HPS, Kapil U. 2006. National workshop on "Development of guidelines for effective home based care and treatment of children suffering from severe acute malnutrition". Indian Pediatr. 43: 131–9.

Hosseini SF, Zandi M, Rezaei M, Farahmandghavi F. 2013. Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: preparation, characterization and in vitro release study. Carbohydr. Polym. 95: 50–6.

Hsieh J-C, Liu L, Zeilani M, Ickes S, Trehan I, Maleta K, et al. 2015. High Oleic Ready-to-Use Therapeutic Food Maintains Docosahexaenoic Acid Status in Severe Malnutrition. J. Pediatr. Gastroenterol. Nutr. 61: 1.

Hughes S, Kelly P. 2006. Interactions of malnutrition and immune impairment, with specific reference to immunity against parasites. Parasite Immunol. 28: 577–88.

Iannotti LL, Dulience SJL, Green J, Joseph S, Francois J, Antenor M-L, et al. 2014. Linear growth increased in young children in an urban slum of Haiti: a randomized controlled trial of a lipid-based nutrient supplement. Am. J. Clin. Nutr. 99: 198–208.

IFPRI. 2016. From promise to impact ending malnutrition by 2030. Washington, DC.

Irie T, Uekama K. 1997. Pharmaceutical Applications of Cyclodextrins. III. Toxicological Issues and Safety Evaluation. J. Pharm. Sci. 86: 147–162.

Jones KD, Ali R, Khasira M a, Odera D, West AL, Koster G, et al. 2015. Ready-to-use therapeutic food with elevated n-3 polyunsaturated fatty acid content, with or without fish oil, to treat severe acute malnutrition: a randomized controlled trial. BMC Med. 13: 93.

Kelly JP, Kaufman DW, Kelley K, Rosenberg L, Anderson TE, Mitchell AA. 2005. Recent Trends in Use of Herbal and Other Natural Products. Arch. Intern. Med. 165: 281.

Keusch GT, Rosenberg IH, Denno DM, Duggan C, Guerrant RL, Lavery J V., et al. 2013. Implications of Acquired Environmental Enteric Dysfunction for Growth and Stunting in Infants and Children Living in Low- and Middle-Income Countries. Food Nutr. Bull. 34: 357–364.

Kfoury M, Landy D, Ruellan S, Auezova L, Greige-Gerges H, Fourmentin S. 2016. Determination of formation constants and structural characterization of cyclodextrin inclusion complexes with two phenolic isomers: carvacrol and thymol. Beilstein J. Org. Chem. 12: 29–42.

Kintzios SE. 2002. Oregano : the genera Origanum and Lippia. Taylor and Francis.

Kohlert C, van Rensen I, März R, Schindler G, Graefe EU, Veit M. 2000. Bioavailability and pharmacokinetics of natural volatile terpenes in animals and humans. Planta Med. 66: 495–505.

Kohlert C, Schindler G, März RW, Abel G, Brinkhaus B, Derendorf H, et al. 2002. Systemic availability and pharmacokinetics of thymol in humans. J. Clin. Pharmacol. 42: 731–7.

Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. 2013a. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382: 209–222.

Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. 2013b. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382: 209–222.

Kuusipalo H, Maleta K, Briend A, Manary M, Ashorn P. 2006. Growth and Change in Blood Haemoglobin Concentration Among Underweight Malawian Infants Receiving Fortified Spreads for 12 Weeks. J. Pediatr. Gastroenterol. Nutr. 43: 525–532.

Lin CA, Manary MJ, Maleta K, Briend A, Ashorn P. 2008. An energy-dense complementary food is associated with a modest increase in weight gain when compared with a fortified porridge in Malawian children aged 6-18 months. J. Nutr. 138: 593–8.

Liolios CC, Gortzi O, Lalas S, Tsaknis J, Chinou I. 2009. Liposomal incorporation of carvacrol and thymol isolated from the essential oil of Origanum dictamnus L. and in vitro antimicrobial activity. Food Chem. 112: 77–83.

Little DB, Croteau RB. 1999. Biochemistry of Essential Oil Terpenes. In Flavor Chemistry. Boston, MA: Springer US, pp. 239–253.

Loftsson T, Hreinsdóttir D, Másson M. 2005. Evaluation of cyclodextrin solubilization of drugs. Int. J. Pharm. 302: 18–28.

Mangani C, Maleta K, Phuka J, Cheung YB, Thakwalakwa C, Dewey K, et al. 2015. Effect of complementary feeding with lipid-based nutrient supplements and corn-soy blend on the incidence of stunting and linear growth among 6- to 18-month-old infants and children in rural Malawi. Matern. Child Nutr. 11: 132–143.

Marques HMC. 2010. A review on cyclodextrin encapsulation of essential oils and volatiles. Flavour Fragr. J. 25: 313–326.

Matsuda H. 1999. Cyclodextrins in transdermal and rectal delivery. Adv. Drug Deliv. Rev. 36: 81–99.

McClements DJ, Decker EA, Park Y. 2009. Controlling lipid bioavailability through physicochemical and structural approaches. Crit. Rev. Food Sci. Nutr. 49: 48–67.

Cunnigham-Rundles S, Moon A, Mcneeley DF. 2008. Malnutrition and Host Defense. In: Duggan C, Watkins JB, walker WA eds. Nutrition in Pediatrics: Basic Science and Clinical Application, 4th ed. London: BC: Decker, Hamilton. 261-271.

Miyamoto Y, Eckmann L. 2015. Drug Development Against the Major Diarrhea-Causing Parasites of the Small Intestine, Cryptosporidium and Giardia. Front. Microbiol. 6: 1208.

Muniz-Junqueira MI, Queiroz EFO. (2002). Relationship between protein-energy malnutrition, vitamin A, and parasitoses in living in Brasília. Rev. Soc. Bras. Med. Trop. 35: 133–41.NFHS. 2016. National Family Health Survey (NFHS-4), 2016-16: India. Mumbai.

Northrop-Clewes CA, Rousham EK, Mascie-taylor CGN, Lunn PG, Al NET. 2001. Anthelmintic treatment of rural Bangladeshi children : effect on host physiology, growth , and biochemical status 1 – 3. : 53–60.

O'Hara SP, Chen X-M. 2011. The cell biology of cryptosporidium infection. Microbes Infect. 13: 721–30.

Olness K. 2003. Effects on brain development leading to cognitive impairment: a worldwide epidemic. J. Dev. Behav. Pediatr. 24: 120–30.Phuka J, Thakwalakwa C, Maleta K, Cheung YB, Briend A, Manary M, et al. 2009. Supplementary feeding with fortified spread among moderately underweight 6-18-month-old rural Malawian children. Matern. Child Nutr. 5: 159–170.

Phuka JC, Maleta K, Thakwalakwa C, Cheung YB, Briend A, Manary MJ, et al. 2008. Complementary feeding with fortified spread and incidence of severe stunting in 6- to 18-month-old rural Malawians. Arch. Pediatr. Adolesc. Med. 162: 619–26.

Phuka JC, Maleta K, Thakwalakwa C, Cheung YB, Briend A, Manary MJ, et al. 2009. Postintervention growth of Malawian children who received 12-mo dietary complementation with a lipid-based nutrient supplement or maize-soy flour. Am. J. Clin. Nutr. 89: 382–390.

Prendergast AJ, Humphrey JH. 2014. The stunting syndrome in developing countries. Paediatr. Int. Child Health 34: 250–65.

Santoro GF, das Graças Cardoso M, Guimarães LGL, Salgado APSP, Menna-Barreto RFS, Soares MJ. 2007. Effect of oregano (Origanum vulgare L.) and thyme (Thymus vulgaris L.) essential oils on Trypanosoma cruzi (Protozoa: Kinetoplastida) growth and ultrastructure. Parasitol. Res. 100: 783–90.

Scrimshaw N. 1994. The consequences of hidden hunger for individuals and societies. Food Nutr. Bull. 15: 3–24.

Simopoulos a P. 1991. Omega-3 fatty acids in health and disease and in growth and development. Am. J. Clin. Nutr. 54: 438–63.

Simopoulos a. . 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed. Pharmacother. 56: 365–379.

Sinha VR, Kumria R. 2001. Polysaccharides in colon-specific drug delivery. Int. J. Pharm. 224: 19–38.

Smith H V, Nichols RAB, Grimason AM. 2005. Cryptosporidium excystation and invasion: getting to the guts of the matter. Trends Parasitol. 21: 133–42.

Stephenson LS, Latham MC, Adams EJ, Kinoti SN, Pertet A. 1993. Physical fitness, growth and appetite of Kenyan school boys with hookworm, Trichuris trichiura and Ascaris lumbricoides infections are improved four months after a single dose of albendazole. J. Nutr. 123: 1036–46.

Szente L, Szejtli J. 2004. Cyclodextrins as food ingredients. Trends Food Sci. Technol. 15: 137–142.

Ultee A, Bennik MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. Appl. Environ. Microbiol. 68: 1561–8.

UNICEF/WHO/World Bank Group. 2016. Levels and Trends in Child Malnutrition. Glob. Database Child Growth Malnutrition 12: 154–155.

Del Valle EMM. 2004. Cyclodextrins and their uses: a review. Process Biochem. 39: 1033–1046.

Varma JL, Das S, Sankar R, Mannar MGV, Levinson FJ, Hamer DH. 2007. Community-level micronutrient fortification of a food supplement in India: a controlled trial in preschool children aged 36-66 mo. Am. J. Clin. Nutr. 85: 1127–33.

De Vincenzi M, Stammati A, De Vincenzi A, Silano M. 2004. Constituents of aromatic plants: carvacrol. Fitoterapia 75: 801–4.

Wang Q, Gong J, Huang X, Yu H, Xue F. 2009. In vitro evaluation of the activity of microencapsulated carvacrol against Escherichia coli with K88 pili. J. Appl. Microbiol. 107: 1781–8.

Wang L. 2008. Helminth infections and intestinal inflammation. World J. Gastroenterol. 14: 5125.

Waterlow JC, Buzina R, Keller W, Lane JM, Nichaman MZ, Tanner JM. 1977. The presentation and use of height and weight data for comparing the nutritional status of groups of children under the age of 10 years. Bull. World Health Organ. 55: 489–98.

Welch A a, Shakya-shrestha S, Wareham NJ, Khaw K. 2010. Dietary intake and status of n 2 3 polyunsaturated fatty acids in a population of fish-eating and non-fish-eating meat-eaters , vegetarians , and vegans and the precursor-product ratio of a -linolenic acid to long-chain n 2 3 polyunsaturated fatty acids : Am. J. Clin. Nutr. 3: 1040–51.

Xu P, Widmer G, Wang Y, Ozaki LS, Alves JM, Serrano MG, et al. 2004. The genome of Cryptosporidium hominis. Nature 431: 1107–1112.

Yashodhara BM, Umakanth S, Pappachan JM, Bhat SK, Kamath R, Choo BH. 2009. Omega-3 fatty acids: a comprehensive review of their role in health and disease. Postgrad. Med. J. 85: 84-90.

Chapter 3: Omega-3 Fortified Lipid-based Nutrient Supplement – Development, Characterization and Consumer Acceptability[1]

3.1 Abstract

Background: Lipid-based nutrient supplements (LNS) with balanced omega-6/omega-3 fatty acid ratio are beneficial for undernourished children. Incorporating omega-3 PUFA sources to LNS formulations is challenging due to storability, oxidative stability, and off-flavor issues resulting in reduced shelf-life and poor consumer acceptability

Objective: To systematically develop an omega-3 fortified LNS formulation, using flaxseed oil, by optimizing process variables based on stability parameters, characterizing the physicochemical properties, and evaluating consumer acceptability.

Methods: LNS formulations were designed to yield approximately 452 kcal, 13.2 g of protein, 30 g of fat, and 53 g of carbohydrates. Response surface methodology (RSM) with four-factor-three-level: omega-3 fatty acid source (flaxseed oil) (0-10%), antioxidant (ascorbyl palmitate: 0.0-0.03%), emulsifier (soy lecithin: 0.5-1.5%), and storage time (0-6 months) was employed to optimize LNS's functionality and storage stability. Factor effects were evaluated for peroxide value, oil separation, water activity, moisture content, hardness, and vitamin C content of samples. Consumer acceptability was assessed using a nine-point hedonic scale.

---

[1] Reprinted, with permission, from: Gaur, S., Sloffer, E. M., Ojha, A., Patra, F., … Andrade, J. E. (2017). Omega-3-Fortified Lipid-Based Nutrient Supplement. *Food and Nutrition Bulletin*, 037957211770123.

Results: Flaxseed oil (FO) was the most significant factor ($p<0.05$) in reducing storage stability in terms of peroxide value (0 to 7.75 mEq/kg fat) after six months. Reduction in vitamin C (10%) and water activity whereas an increase in lipid separation were observed after six months. Optimal levels to maximize storage time and FO, and minimize oxidation were 0.02% antioxidant, 1.5% emulsifier, and 4.9% FO. 5% FO addition resulted in omega-6/ omega-3 (LA/ALA) fatty acid ratio to 2.6. Indian women and students accepted LNS formulations with or without FO similar to other commercial supplements.

Conclusions: An LNS with reduced omega-6/omega-3 PUFA ratio was successfully formulated and optimized for ingredients and stability using RSM, which resulted in recommendations for specific amounts of antioxidant, emulsifier, and FO to obtain an increased storage time with lower oxidation. A 20-g serving size of this formula provides 90 kcals and can contribute a significant quota of micronutrients and essential fatty acids to children with moderate acute malnutrition.

3.2 Introduction

Moderate acute malnutrition (MAM) afflicts 33 million children worldwide, of which 20 million children live in India alone (WHO, 2013). MAM is characterized by weight for height score between -3 and -2 Z-scores compared with a reference population, or by Mid-Upper Arm Circumference (MUAC) < 12.5 cm (WHO, 1999). Supplementary feeding programs are widely used to tackle MAM and avoid mortality by reducing deterioration into severe acute malnutrition (SAM), despite insufficient evidence on their effectiveness (Annan, Webb, & Brown, 2014). In India, MAM is addressed through take-home rations such as mixed flours (*sattu*), sweets (*sheera*), and porridge (*upma*), or sometimes raw ingredients such as rice and pulses. Although such interventions have been useful in improving some health outcomes, there are still issues with nutritional adequacy and consumption compliance due to the low acceptability of products, and additional food preparation requirements (Varma *et al.,* 2007; Bhagwat *et al.,* 2014).

Lipid-based nutrient supplements (LNS) are ready to use energy and nutrient dense food products that are suitable to complement most food rations, especially due to their longer shelf life and convenience. Existing LNS have been widely successful in treating SAM among at-risk children and has instigated the development of similar or improved alternative foods for addressing MAM to overcome the issues with existing supplementary feeding program (Dewey & Arimond, 2012).

In the past two years, there has been an increased interest in improving the omega-3 fatty acid profile of conventionally used peanut based formulations, which are rich in omega-6 linoleic acid (LA) but contain negligible omega-3 alpha linolenic acids (ALA) (Brenna *et al.,* 2015; Hsieh *et al.,* 2015; Jones *et al.,* 2015). Balanced Omega-6/Omega-3 polyunsaturated fatty acids (PUFA)

are beneficial for children suffering from SAM or MAM, as omega-3 ALA serve as precursors for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which play a significant role in modulation of tissue inflammation, immune function, and overall neural development (Calder, 2006). In addition, omega-3 PUFA modulates intestinal inflammation, common in malnourished children, that results in malabsorption of nutrients from the diet (Gupta *et al.,* 2011; Teitelbaum & Allan Walker, 2001). Currently, there are no specifications for omega-6/omega-3 PUFA ratio in LNS, however, the importance of balanced PUFAs in LNS is now well recognized (Brenna *et al.,* 2015).

Incorporating omega-3 PUFA sources to LNS formulation is challenging due to storability, oxidative stability, and off-flavor issues resulting in reduced shelf-life and poor consumer acceptability (Ganesan, Brothersen, & McMahon, 2014). The aim of this study was to systematically develop an omega-3 fortified LNS formulation using flaxseed oil by optimizing process variables based on stability parameters, characterize its physicochemical properties, and evaluate its consumer acceptance. This process will result in a stable and acceptable complementary spread that is highly palatable, cost-effective, locally produced and that provides required micronutrients, which could be included in the daily meals of children at schools or supplementary feeding centers in India.

3.3 Materials and Methods

Ingredients. Ingredients included chickpea flour (Spicy World, Houston, TX), soy flour (Bob's Red Mill, Milwaukee, OR), maltodextrin (Honeyville, Inc, Brigham City, UT), FO (Spectrum Naturals, Inc, Melville, New York), clarified butter (ghee; AMUL, Anand, India), skim

milk powder (Barry Farm, Cridersville, OH), soybean oil (Crisco, Orville, OH), coconut oil (Spectrum Naturals, Inc, Melville, New York), palm oil shortening (Crisco, Orville, OH), vitamin mineral mix (Watson Inc, West Haven, CT), soy lecithin (Now, Bloomingdale, IL), antioxidant ascorbyl palmitate (Spectrum Chemical, Gardena, CA), and cocoa powder (Hershey's, Hershey, PA). LNS A20 was donated by USAID, and *Sattu Maavu* was procured from a commercial supplier in India. Three-layer minipouches (PAKVF4) were purchased from IMPAK Corporation (Los Angeles, CA).

Chemicals. Hexane, acetic acid, potassium iodide, sodium thiosulphate, starch solution and sand were procured from Fisher Scientific. Chloroform, metaphosphoric acid, ethylenediaminetetraacetic acid, sodium phosphate monobasic and L-ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO).

Recipe formulation. Recipes for LNS were designed using ESHA Food Processor Diet Analysis and Fitness Software (ESHA Research v. 10.10.0) and the Indian nutrient database (Gopalan *et al.*, 1989) to yield approximately 13% protein (i.e. dairy, chickpea, soy), 40% carbohydrate (i.e. sucrose, maltodextrin), 38% fat (different blends of ghee, soybean oil, coconut oil and flaxseed oil), and 3% complete vitamin and mineral mix (Table 6). FO was incorporated at 5% and 10% level by replacing soybean oil to prevent any textural changes, as they have a similar viscosity (800 mPa.s) and specific gravity (0.91 at 25 ºC) (El-Waseif *et al.*, 2013). Formulations were designed to meet the World Food Program (WFP) LNS technical specifications (Nguyen & Bouzambou, 2014) and specifications described by Chaparro and Dewey (2010). Ready to use therapeutic food (RUTF) specifications were obtained from MSF/UNICEF (Caron, 2012) and Commercial Item Description (2012) for standards not listed, but specified by Nguyen & Bouzambou (2014).

General LNS preparation. Figure 16 illustrates the preparation of LNS. Weighed flours were toasted at 110 °C for 120 min in a forced convection hot air oven (Mechanical Oven; Precision Scientific, Fisher Scientific, Marietta, OH). Flours were added to other dry ingredients and mixed (2 min); this constituted the dry mix. Variations in the content of antioxidant and emulsifier were made at this point. The dry mix was added to a wet grinder (PG503, Premier Wonder), and contents were mixed (3 min) to ensure homogenous mixing and size reduction. The fats and oils were brought to room temperature (23±2 °C) and mixed in a glass bowl for 2 min. Variations in the amount of flaxseed oil were made at this point. The lipid mixture was added to dry contents in a wet grinder and blended for 5 min.

Packaging and storage. The product was filled into 3-layer minipouches and heat-sealed under nitrogen (99.99% pure) using a Minipack America Sealer (MVS 31 – XP) with the following settings vacuum 50%, gas ratio 20% and time 2.5 s. Sealed pouches were held at 40±2 °C for 6 months for accelerated shelf life testing equivalent to 12 months at room temperature (~30 °C).

Water activity. Sample water activity was measured using an Aqualab water activity meter (AOAC, 1980). Sample (1 g) was weighed into 15 mL disposable sample cups and inserted into the instrument (Aqualab 4TE, Decagon Devices Inc., USA).

Peroxide value. Peroxide value of samples was measured in triplicate using a modified method from AOCS (2004), as described by Crowe & White (2001).

Moisture content. Modified oven drying method (sand pan technique) was used for the estimation of moisture content (Bradley *et al.*, 2010). Aluminum metal dishes with lids containing 10 g of sand were dried along with a glass rod for 1 h. The dish was weighed to the nearest 0.0001

g after cooling in a desiccator. Sample (5 g) was added to the dish and thoroughly mixed with sand using the glass rod. Samples were dried in a hot air oven for 5 h at 95°C.

Lipid separation. Lipid separation was measured in triplicate using a modified method of Hinds *et al.,* (1994) and Perlman (1999). Pouches were thoroughly and uniformly massaged for 2 min before opening. A 30 g sample was carefully transferred to a 50 mL centrifuge tube. To simulate natural separation, tubes were centrifuged at a low-speed (300 ×*g*) at 22°C (Sorvall ST 16R with TX400 swinging bucket rotor, Thermo Sci.) for 3 min. A digital Vernier caliper was used to determine the height of lipid layer separated at the top of the sample.

Texture analysis. Sample hardness was measured using a texture analyzer (TA-XT2; Texture Technologies Corp., Scarsdale, NY) following a method adapted from Ahmed & Ali (1986). Pouches extracted from incubator were uniformly massaged for 2 min and 50 g sample was placed in Petri dishes (15 cm ∅ by 1.5 cm height) in triplicates. Samples were left undisturbed for 1 h and temperature was monitored using an infrared thermometer. During analysis, each sample dish was secured to the base. The probe (50 mm diameter, flat surface) was moved at a crosshead speed of 0.5 mm/s with 4 mm penetration. Hardness was measured as the peak force (N) of the first compression cycle.

Vitamin C determination. The method of Tarrago-Trani *et al.,* (2012) with modifications was used for extraction and measurement of vitamin C. The sample size used was 0.2 g and the extraction buffer was 1 mM EDTA/4% meta-phosphoric acid. Separation and quantification of vitamin C were carried out by reverse phase separation and UV detection (PDA at 265 nm) using a Waters HPLC with an isocratic pump system. An ISIS C-18 column (150 × 4.6 mm, 3 µm particle size; Supelco-Sigma, St. Louis, MO) was equilibrated with a mobile phase consisting of 25 mM

sodium phosphate buffer, pH 3.0 and pumped at 0.4 mL/min. Vitamin C was quantified using HPLC grade external standards. Results were expressed as mg of vitamin C per g samples.

Microbiological analysis. Microbial analysis was conducted for samples (25 g) at 0 and 6 months for total viable count (ISO 4833-1:2013) and *Salmonella* (ISO 6579). Coagulase test was done for *S. aureus* in 10 g sample.

Response surface methodology for formula optimization. RSM, which includes the design of experiments, selection of levels of variables, fitting mathematical models, and optimization of variables, was applied in this study (Nwabueze, 2010). Face-centred central composite design for four independent variables (i.e. antioxidant, emulsifier, storage time, and FO content) at three levels was used for the experimental plan, resulting in 30 combined experiments. Responses associated with moisture content, water activity, peroxide value, lipid separation, hardness, and vitamin C content were recorded. For each response, its relationship with another variable was determined by fitting data to the second-order reaction using Equation 1.

$$Y=\beta_0+\sum_{i=1}^{4}\beta_i X_i+\sum_{i=1}^{4}\beta_{ij} X_i^2+\sum_i\sum_j \beta_j X_i X_j+\varepsilon$$

Equation 1

Where Y is the predicted response; β_0 is a constant; β_i is the linear coefficient; β_{ij} is the quadratic coefficient β_{ij} is the interaction coefficient of variables i and j; and X_i and X_j are the independent variables.

The statistical adequacy of the model was tested using the Fisher test value (F-ratio), the coefficient of determination (R^2), and Lack of Fit (LOF) test. To visualize the relationship between the response and the experimental levels for each factor and to deduce the optimum conditions,

2D and 3D response surface plots were generated. Table 7 shows the respective low and high levels for the factors coded.

Consumer acceptability. Research protocols using human subjects were approved by the Institutional Review Board at the University of Illinois, Urbana–Champaign (UIUC). The study was conducted in collaboration with the Mansinhbhai Institute of Dairy and Food Technology (MIDFT), Mehsana, India. Participants rated the characteristics of samples presented on a 9-point hedonic scale (ISO 11136:2014) from dislike extremely to like extremely. Further, participants ranked the samples from 1 to 4, where 1 was the top-ranked sample. Eighty-six students (18-24 y) and twenty-five women (18-45 y) with young children (1-6 years) at home, otherwise healthy (no diarrhea, cold or fever) were randomly recruited from MIDFT and within a 20-km radius from MIDFT, respectively. Written consents were obtained from subjects. All the documents presented to women were translated into the local language (Gujarati). The participants were trained and instructed in local language by the team. Samples evaluated were: LNS without flaxseed oil (LNS w/o FO), LNS with flaxseed oil (10% FO; LNS w FO), LNS A-20 and *Sattu Maavu* (a commercially available nutrition supplement in India). The responses from students were recorded on a visual analog scale using Qualtrics online (Online Survey Software & Insight Platform) and from women on paper-based forms. For students, the products were evaluated based on taste, color, sweetness, smell, stickiness, texture, appearance and overall acceptability; whereas for women, the characteristics were taste, color, smell, mouthfeel, appearance and overall acceptability.

LNS samples were prepared based on ingredients shown in Table 6. LNS w FO was prepared by replacing soybean oil with FO at a level of 10% of the final weight. LNS-A20 was prepared based on the recipe developed by Briggs *et al.,* (2007) using: maltodextrin, soybean oil, confectionary sugar, non-fat milk powder, whey protein concentrate, cream powder, lecithin, pea

protein isolate, vitamin/ mineral premix, salt, and antioxidants. *Sattu Maavu* was obtained locally and contains millet (finger and pearl), corn, wheat, barley, mung bean, groundnut, cashews, sago, and cardamom. Samples were prepared in one-kg batches at the Food and Dairy Technology Lab at MIDFT. Coded samples (2±0.5 g) maintained at 25±2°C were presented at random to the participants in clear disposable sensory cups under well-lit conditions. Water (~25±2°C) and crackers were provided for rinsing between samples. All testing sessions were scheduled between 11:00 AM and 1:00 PM.

Statistical Analyses. Design Expert v7.0 was used for experimental design, generation of RSM plots and optimization. The variables were analyzed using the General Linear Model (GLM) procedure, and means were compared using Tukey's Studentized Range Test and considered statistically different at $p \leq 0.05$. A Full Factorial Completely Randomized Design was used to study the effect of independent variables using GLM procedure. The normality assumption was tested by the Shapiro–Wilk test. Data from consumer acceptability test with women were analyzed with Kruskal-Wallis test using npar1way function in SAS and the Dunn's test for post hoc mean analyzes. All statistical analyses were performed using SAS 9.4. The Friedman rank sum test was performed to classify samples based on ratings (O'Mahony, 1986). The results are presented as means ± SD.

3.4 Results

Development of LNS product. Each 100 g of LNS provided 452 kcal, 13.2 g of protein, 30 g of fat, and 53 g of carbohydrates. Flaxseed oil incorporation at 5% and 10% level reduced the omega-6/ omega-3 (LA/ALA) fatty acid ratio to 2.6 and 1.4 compared to 5.8 in control LNS with

0% FO (Table 8). All formulas of the LNS showed a higher proportion of saturated fatty acids and a lower omega-6/omega-3 ratio than the LNS-A20 counterpart (Table 8).

Responses to RSM. Results for the following response variables: water activity, lipid separation, peroxide value, hardness, moisture content, and vitamin C are presented in Table 9. The estimated regression coefficients of the fitted model are given in Table 10. ANOVA indicated that responses Y_2, Y_3, Y_4, and Y_6 were significant at $p \leq 0.001$, and model Y_1 was significant at $p \leq 0.05$. Also, all the coefficients of determination (R^2) were greater than 0.73, except for moisture. Thus, more than 73% of the total variations provoked by the experimental conditions could be explained by the models fitted to the regression curve.

Only significant terms in second order polynomials were used to generate three-dimensional RSM plots and two-dimensional line plots. Peroxide values ranged from 0 to 7.75 mEq/kg fat and no oxidation was determined in samples at 0 and 3 months (Figure 17A). Slight oxidation was detected in the samples containing 5 and 10% FO at 6 months. Storage time and FO content had a significant factor and interaction effect. Lipid separation ranged from 0 to 2.27 mm and was affected by storage time and emulsifier content (Figure 17B). An interaction was observed between lipid separation and these two variables. Fresh samples did not show oil separation. Water activity decreased in samples from 0 to 3 months (Figure 18A). No further changes in water activity were observed after 3 months. Moisture content remained the same throughout the study, ranging from 0.0476 to 0.1657 % (Figure 18B). Only storage time had an effect on hardness (N), in which the force (ranging from 10.93 to 45.31 N) required to penetrate the samples decreased during storage (Figure 18C). Vitamin C content declined in samples from 0 to 3 months in storage, but it remained the same after 3 months (Figure 18D).

Conditions for optimum response. For optimization, multiple regression equations were solved to maximize FO content, storage time, and minimize oxidation, using a multiple response method called desirability. The solution to obtain a storability of 3.61 months was a product of mixing ingredients containing 0.02% antioxidant, 1.49% soy lecithin, and 4.87% FO.

Microbiological testing. At 0 and 6 months, the total viable count of samples was 100 ± 25 CFU/g against the tolerance of 10,000 CFU/g. LNS samples were negative for *Salmonella* and *S. aureus* (coagulase test).

Consumer acceptability. Demographic results showed that a large proportion (72%) of the women was involved in farming and cattle rearing businesses. Almost all women (92%) provided unfortified homemade complementary foods to their children, which mostly consisted of grains, pulses, fats, and, in some occasions, fruits.

Consumer acceptability ratings from both subject groups and for all LNS samples were above 6.0 ("like slightly") on the 9-point hedonic scale. Only *Saatu Maavu* received acceptability ratings below 6.0. Results from acceptability tests can be found in the Supplementary materials, Figures 19a and 19b. Among sensory attributes, clear differences in acceptability ratings were observed for taste, mouthfeel, and appearance. For most sensory attributes, LNS samples were accepted above *Sattu Maavu*. Subjects accepted LNS w FO to a lesser extent in terms of taste and smell than the other LNS samples. Both women and students consistently ranked LNS w/o FO and LNS-A20 as number one and two (data not shown) compared to the other products. *Sattu Maavu* was consistently ranked last ($p \leq 0.05$).

3.5 Discussion

An omega-3 fortified LNS product was designed using staple ingredients and optimized using RSM with the objective to improve omega-3/omega-6 PUFA balance and to serve as a take home ration or complement the meals provided at supplementary feeding centers (*Anganwadi*) in India. A 20-g serving size of this formula provides 90 kcals and can contribute a significant quota of micronutrients and essential fatty acids to children with MAM. Flaxseed oil (FO), a vegetarian source of omega-3 PUFA, was selected for its high content of ALA, the precursor of long chain omega-3 EPA and DHA, and for its additional benefits beyond energy, such as anti-inflammatory, antithrombotic and hypolipidemic effects (Takahata *et al.,* 1998). Other ingredients such as chickpea flour, milk powder, soybean oil, ghee, and coconut oil were selected due to their commercial availability, cost, and acceptability. The obtained lipid blend is high in saturated and medium chain fatty acids, which increases oxidative stability. Ascorbyl palmitate was the antioxidant of choice based on the recommendations from Nguyen & Bouzambou (2014). Finally, cocoa powder was used to improve flavor and aesthetic appeal.

Enteric infection, a trigger for inflammatory response and intestinal inflammation, is very common among malnourished children in India, resulting in nutrient malabsorption (Gupta *et al.*, 2011). The omega-6/omega-3 ratio is an indicator for the balanced synthesis of eicosanoids in the body and a lower ratio is recommended for undernourished children not only for normal growth, cell functioning, and immune function but also for an adequate anti-inflammatory response (Gogus & Smith 2010; Brenna *et al.*, 2015; Calder, 2006). In a recent study of children with SAM, a two-month supplementation with RUTF high in oleic acid and with a 1:1 LA:ALA ratio increased circulating EPA and DHA and reduced arachidonic acid compared to standard RUTF with LA/ALA ratio of 53:1 (Hsieh *et al.,* 2015). In another study, a peanut-based RUTF fortified with FO significantly increased plasma EPA and docosapentaenoic acid, but not DHA among children

suffering from SAM (Jones *et al.,* 2015). A four-week treatment with FO (14 g/day) decreased the levels of pro-inflammatory cytokines among adult subjects (Caughey *et al.,* 1996). In contrast, a lower dose (i.e. 2 or 3.5 g/day) did not have this effect (Thies *et al.,* 2001; Wallace *et al.,* 2003).

Most companies struggle with the addition of PUFAs into product formulation. Any encapsulation of PUFAs will impact product cost. In addition, plant sources of PUFAs are more suitable for the Indian culture. Although an optimal ratio is important in inflammation, it cannot be at the expense of product's flavor and acceptance. The most difficult problems in formulation with PUFAS are oxidation within storage and the resultant flavors. In this study, we found that reducing the FO content in LNS to 5% will balance the essential fatty acid ratio, while enhancing its stability during storage and consumer acceptability.

RSM studies pinpointed adequate levels of FO, antioxidant, and lecithin that resulted in lower oxidation during storage. Studies also helped identify factor effects and their interactions and to minimize experimental runs and time. A similar optimization technique was used to evaluate physicochemical and sensory properties of peanut pastes (Muego-Gnanasekharan & Resurreccion, 1992). The main changes associated with accelerated storage were increased oxidation (i.e. peroxide value), decreased water activity, decreased product hardness, increased separation of fat and oil from the matrix, and increased vitamin C degradation.

Higher peroxide values in LNS were associated with increased addition of FO (at a level of 10%). Essential fatty acids in FO are less stable and more susceptible to electrophilic attack and metal-catalysed autoxidation (Gunstone, 1996). Briggs *et al.,* (2007) found a similar trend in LNS-A20 samples containing a higher proportion of unsaturated oils. Higher peroxide values were reported in FO-containing cookies (>30%) compared to controls stored at 45 °C for 28 days

(Rangrej *et al.,* 2015). Both storage time and temperature influenced sample oxidation similar to the observations of Riveros *et al.,* (2010) from studies with peanut pastes and Sumainah *et al.,* (2000) from studies with peanut-sesame soy blends. Although the peroxide values of LNS were higher after 3 months, these values remained well below the permitted level of 10 mEq/kg oil, a threshold for perception of rancid flavors in oils (FAO/WHO, 1999).

Water activity (Aw) of LNS products is often low, which enhances their ability to last long in harsh storage conditions. A decrease in Aw from 0 to 3 months could be a result of moisture equilibration in the samples, in which free water migrated from ingredients with high to low Aw, such as from toasted flours and sugar, thus reducing the vapor pressure (Guillard *et al.,* 2003). The Aw remained below 0.6 after 3 months, which prevented the growth of microorganisms, as confirmed by microbial analysis, and resulted in a product safe for consumption at all time points. Despite the changes in Aw, the moisture content remained unchanged. This could be attributed to the packaging material, which prevented migration of moisture from the external environment. Briggs *et al.,* (2007) reported similar moisture results in LNS-A20 samples during a six-month accelerated storage.

Often the oil ingredients in LNS tend to separate after several months in storage. Decreased hardness during high-temperature storage could be attributed to melting of fat crystals and oil penetration in the matrix during the quiescent conditions. Similar results were observed by Muego-Gnanasekharan & Resurreccion (1992), in which hardness of peanut paste samples stored at 40 °C decreased by 43% after 12 months. These authors also reported a higher separation of fat and oil from the product matrix; an indication of lower product quality and that could result in higher oxidation rates. Lecithin content, storage time and their interaction had an effect on oil separation. Oil separation increased with time because melted lipid crystals and lecithin rose to the surface

after the collapse of the lipid crystal network at a high temperature. Furthermore, differences in lipids' specific gravity resulted in flocculation, then partial or complete coalescence with time (Radocaj *et al.*, 2011; Walstra, 2002). UNICEF recommends minimal oil separation (Caron, 2012); however it does not provide any indication as to how to measure separation or quality specifications.

Vitamin C reduction during storage might be attributed to autoxidation at high temperature and in the presence of metal catalysts such as iron and copper. Similar reductions were observed in peanut paste stored at 40 °C for 3 months (Yeh *et al.*, 2002). After 3 months, vitamin C levels remained unchanged probably due to the lower Aw as reported previously (Yeh *et al.*, 2002). In another study, vitamin C losses (~20%) were reported in LNS-A20 after 6 months of storage at 38°C (Briggs *et al.*, 2007). Therefore, overage amounts will be needed to ensure vitamin C is present by the end of the shelf life of products.

Although most times overlooked in emergency relief products for supplementation in low-income settings, consumer acceptability tests are important to evaluate the likelihood of product adoption by end users and to address potential organoleptic issues based on subject's feedback. Women were chosen instead of infants for this study as they often taste the food before feeding it to their children, and their acceptability of a product is positively associated with both their children acceptability and the likelihood to use the product for such purposes (Skinner *et al.*, 2002). Students were selected to determine acceptability among general consumers. Although most products evaluated scored above average on several attributes (i.e. values ≥ 5), the LNS w/o FO was the most acceptable among women and students. FO's taste was the primary reason for low acceptance. Low consumer acceptability was reported by a semi-trained sensory panel who evaluated cookies containing FO at a level of 40% or higher as a direct replacement for shortening

(Rangrej *et al.*, 2015). Similarly, a lower acceptability in terms of crust color, flavor, mouthfeel, and overall acceptability was reported in bagels with increased FO content (Alpaslan & Hayta, 2006). FO's PUFAs oxidize readily, which leads to the accumulation of undesirable flavors (Goh *et al.*, 2006). The brown color of the LNS was appealing among subjects. Chocolate-based products are available in India and thus participants could relate to the LNS products in terms of color, taste, and flavor. In Bangladesh, a study found that 60% of the women did not accept the taste of peanut-based LNS (Ali *et al.*, 2013), which led to reduced acceptance. Therefore, appropriate use of flavors in LNS products is critical for their successful adoption among end users.

Some of the potential limitations of this study may need to be addressed prior to the full adoption of this and similar LNS products. First, the activation energy to predict shelf life was not determined due to lack of at least one additional temperature point (Sewald & DeVries, 2013). Second, the sample size for acceptability tests was small and did not include children or adult men. This reduces the extrapolation of results. Due to significant ethnic diversity in India, it is important that sensory tests be conducted with similar formulations before production and delivery of products to other states and regions.

3.6 Conclusions

An LNS with reduced omega-6/omega-3 PUFA ratio was successfully formulated and optimized for ingredients and stability using RSM; this resulted in recommendations for specific amounts of antioxidant, emulsifier, and FO to obtain an increased storage time with lower oxidation. Flaxseed oil addition was the most significant factor in reducing storage stability. Due to vitamin C reduction during the first three months, adjustments in the micronutrient mix are

needed (i.e. overage). LNS with higher FO content (10%) had lower acceptability than both LNS w/o FO and LNS-A20, which may be attributed to its characteristic off-flavour. Based on the importance of essential fatty acids in growth and development and their role in inflammation, the next generation of LNS products should aim at maximizing the amount of omega-3 fatty acids and their stability in storage and ultimate consumption compliance, while minimizing their oxidation.

3.7 Tables and Figures

Table 6. Recipe formulation for LNS

Ingredient	Quantity (g)
Soybean Oil	10
Coconut Oil	8
Ghee	8
Soy lecithin Emulsifier	1
Shortening (Palm oil)	4
Chickpea flour (Toasted)	17
Soy flour, full fat (Toasted)	10
Non-Fat/ Skimmed Milk Powder	15.25
Sugar	10
Flaxseed oil*	5
Vitamin-Mineral Mix	3.75
Cocoa Powder	2
Maltodextrin M100	6
Total	100

Vitamin mix (for 100 g LNS)- Sodium (sodium chloride) – 100 mg, Potassium (dipotassium phosphate) – 800 mg, Phosphorus (dicalcium phosphate, dipotassium phosphate) – 350 mg, Calcium (dicalcium phosphate, calcium carbonate) – 420 mg, Magnesium (magnesium oxide) – 30mg, Iron (ferrous fumarate) – 10 mg, Zinc (zinc oxide) – 10 mg, Copper (cupric oxide) - 1.2 mg, Iodine (potassium iodide) – 100 µg, Vitamin A (vitamin a palmitate) – 3163.5 IU, Vitamin D3 (cholecalciferol) – 800 IU, Vitamin E (d,l-alpha tocopheryl acetate) – 22.350 IU, Vitamin K (phytonadione) – 20.00 µg, Vitamin C (ascorbic acid) – 45 mg, Thiamin (Thiamin Mononitrate) – 0.2 mg, Riboflavin – 2 mg, Niacin (niacinamide) – 0.5 mg, Pyridoxine (pyridoxine hydrochloride) – 1 mg, Folic acid – 0.2 mg, Vitamin B12 (cyanocobalamin) – 2 µg, Biotin – 0.08 mg, Pantothenic acid – 3 mg, Selenium (sodium selenite) – 30ug.

*Formula to prepare LNS with 5% flaxseed oil. This oil replaced soybean oil in formulation.

Table 7. RSM factor variables and levels

Variables	Coded X_i	Coded levels			ΔX
		-1	0	1	
Antioxidant: L-ascorbyl palmitate (% of sample)	X_1	0.00	0.01	0.03	0.02
Emulsifier: Soy lecithin (% of sample)	X_2	0.5	1	1.5	0.5
Storage time (months)	X_3	0	3	6	3
Flaxseed oil (g)	X_4	0	5	10	5

Table 8. Fatty acid profiles (Area %) of various LNS products

Fatty acid		LNS			A-20
		0% FO	5% FO	10% FO	
Octanoic acid	8:0	3.2	3.3	3.4	0.4
Decanoic acid	10:0	2.6	2.8	2.8	0.8
Lauric acid	12:0	14.1	15.8	16.0	0.8
Myristic acid	14:0	6.7	7.1	7.2	2.1
Myristoleic acid	14:1	0.1	0.1	0.1	0.2
Palmitic	16:0	17.3	16.3	16.0	17.4
Margaric (IS)	17:0	5.4	4.2	3.9	4.5
Stearic	18:0	0.0	0.0	0.0	5.5
Oleic trans	18:1t	6.0	5.7	5.4	0.0
Oleic acid	18:1	16.0	16.0	16.1	18.9
Linoleic acid	18:2	24.0	20.5	17.0	42.9
GLA	18:3n6	0.3	0.2	0.2	0.4
ALA	18:3n3	4.1	8.0	11.8	5.7
LA: ALA ratio		5.8	2.6	1.4	7.5

Table 9. Results of face-centered central composite design for optimization.

Run	Variables				Responses					
	X_1	**X_2**	**X_3**	**X_4**	**Y_1**	**Y_2**	**Y_3**	**Y_4**	**Y_5**	**Y_6**
1	0	0	1	0	0.187	2.090	0.000	10.993	0.087	43.240
2	1	-1	1	1	0.212	2.190	6.869	15.749	0.085	37.560
3	-1	-1	-1	-1	0.226	0.000	0.000	31.803	0.119	49.420
4	1	1	1	1	0.232	1.140	5.941	26.125	0.136	40.120
5	-1	0	0	0	0.179	0.860	0.000	28.145	0.103	43.210
6	1	-1	-1	-1	0.223	0.000	0.000	38.893	0.100	47.630
7	1	-1	1	-1	0.221	2.150	0.000	15.259	0.088	42.150
8	0	0	0	0	0.198	1.220	0.000	29.537	0.052	41.600
9	-1	1	1	-1	0.195	1.910	0.000	22.869	0.147	43.770
10	-1	-1	-1	1	0.227	0.000	0.000	40.129	0.108	49.440
11	0	0	0	0	0.198	1.530	0.000	28.370	0.106	42.250
12	0	0	0	0	0.199	1.230	0.000	30.410	0.102	39.020
13	0	0	-1	0	0.235	0.000	0.000	34.872	0.089	47.210
14	-1	-1	1	-1	0.184	2.270	0.000	20.996	0.166	40.780
15	-1	1	-1	1	0.232	0.000	0.000	45.316	0.102	46.340
16	-1	-1	1	1	0.206	2.040	7.750	23.065	0.048	39.280
17	0	1	0	0	0.194	0.970	0.000	24.644	0.061	37.670
18	1	1	1	-1	0.189	1.110	0.000	24.193	0.099	38.400
19	0	0	0	0	0.230	1.200	0.000	29.430	0.109	41.030
20	0	0	0	1	0.212	1.520	0.000	31.656	0.100	42.880
21	0	0	0	0	0.189	2.170	0.000	26.468	0.097	39.430
22	0	0	0	0	0.194	0.990	0.000	30.606	0.090	43.600
23	1	1	-1	1	0.226	0.000	0.000	31.332	0.110	46.310
24	1	-1	-1	1	0.224	0.000	0.000	38.530	0.120	47.210
25	-1	1	-1	-1	0.238	0.000	0.000	31.705	0.160	41.980
26	1	1	-1	-1	0.225	0.000	0.000	30.901	0.119	47.750
27	0	-1	0	0	0.192	1.710	0.000	31.146	0.161	40.560
28	1	0	0	0	0.185	1.960	0.000	25.938	0.095	42.270
29	0	0	0	-1	0.231	1.430	0.000	28.576	0.102	40.670
30	-1	1	1	1	0.230	1.880	6.440	28.282	0.080	41.100

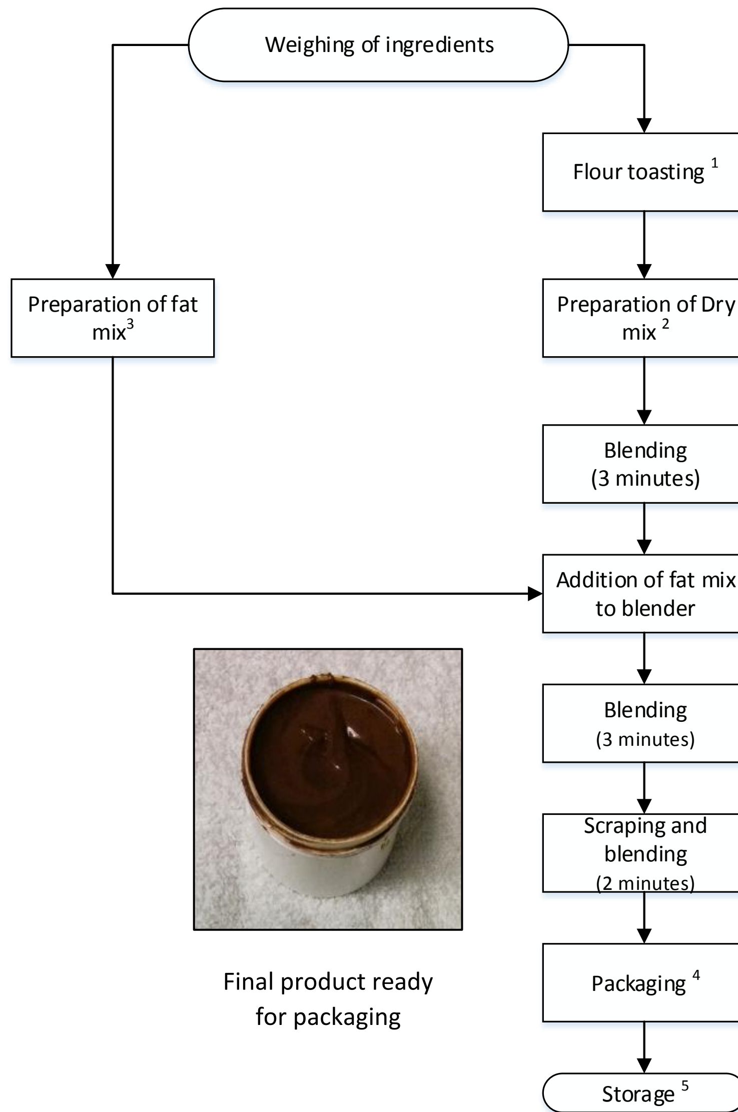
Where X_1, X_2, X_3, X_4 are the independent variables antioxidant (ascorbyl palmitate), emulsifier (soy lecithin), storage time (months) and flaxseed oil (% ingredients), respectively. Y_1, Y2, Y_3, Y_4, Y5 and Y_6 are responses water activity (aw), lipid separation (mm), peroxide value (mEq/ kg fat), hardness (N), moisture content (%wb), and Vitamin C content (mg), respectively.

Table 10. Estimated coefficients of the fitted quadratic equation for different responses based on t-statistic.

Coefficients*	**Y_1**	**Y_2**	**Y_3**	**Y_4**	**Y_5**	**Y_6**
Intercept	0.215	-0.498	1.408	34.462	0.146	42.948
X_1	2.616	22.095	-64.235	-100.753	-1.91	-156.180
X_2	0.039	0.935	-3.527	1.088	0.000	15.538
X_3	-0.016	0.719	-0.256	-2.517	0.000	-3.699
X_4	-0.011	-0.021	-0.154	0.388	0.000	-0.114
X_{12}	-0.546	-14.018	3.412	NS	-0.215	33.571
X_{13}	0.096	-2.247	-2.054	NS	0.014	-12.530
X_{14}	-0.015	0.295	-1.233	NS	0.267	-4.411
X_{23}	0.000	-0.109	-0.093	NS	0.000	0.623
X_{24}	0.001	0.005	-0.056	NS	0.000	0.212
X_{34}	0.000	-0.001	0.113	NS	0.000	-0.040
X_1^2	-78.316	-158.432	2416.837	NS	NS	5669.979
X_2^2	-0.017	-0.404	1.895	NS	NS	-10.055
X_3^2	0.002	-0.044	0.053	NS	NS	0.400
X_4^2	0.001	0.001	0.019	NS	NS	0.006
R^2	0.754	0.907	0.928	0.737	0.404	0.843

*Where X_1, X_2, X_3, X_4 are the independent variables antioxidant (ascorbyl palmitate), emulsifier (soy lecithin), storage time (months) and flaxseed oil (% ingredients), respectively. Y_1, Y2, Y_3, Y_4, Y_5 and Y_6 are water activity (aw), lipid separation (mm), peroxide value (mEq/ kg fat), hardness (N), moisture content (%wb), and vitamin C content (mg), respectively.

Figure 16. Process flowchart for preparation of Indian LNS.



[1] Hot air forced convection toasting of flours (chickpea and soy flour) at 110 °C for 120 min
[2] Dry mix contains toasted chickpea flour, toasted soy flour, non-fat/ skimmed milk powder, sugar, cocoa powder, maltodextrin and vitamin mineral mix
[3] Lipid mix contains soybean oil, coconut oil, ghee, soy lecithin emulsifier, shortening (palm oil) and flaxseed oil
[4] Packaged in three layer minipouches (PAKVF4), heat sealed under nitrogen (99.99% purity) using equipment MVS 31 – XP at conditions vacuum 50%, gas ratio 20% and time 2.5 seconds
[5] Pouches were stored in hot air incubators (Precision by Thermo Scientific) held at 40±2 °C for six months

Figure 17. Response surface plots for peroxide value and lipid separation. A) Peroxide value (mEq/kg) of LNS showing interaction of storage time (months) and flaxseed oil (% ingredients), in which the following values were kept constant: antioxidant (0.01%) and emulsifier (1%). B) Lipid separation value (mm) of LNS showing interaction of storage time (months) and soy lecithin content (% ingredients), in which the following values were kept constant: antioxidant (0.01%) and flaxseed oil (5%).

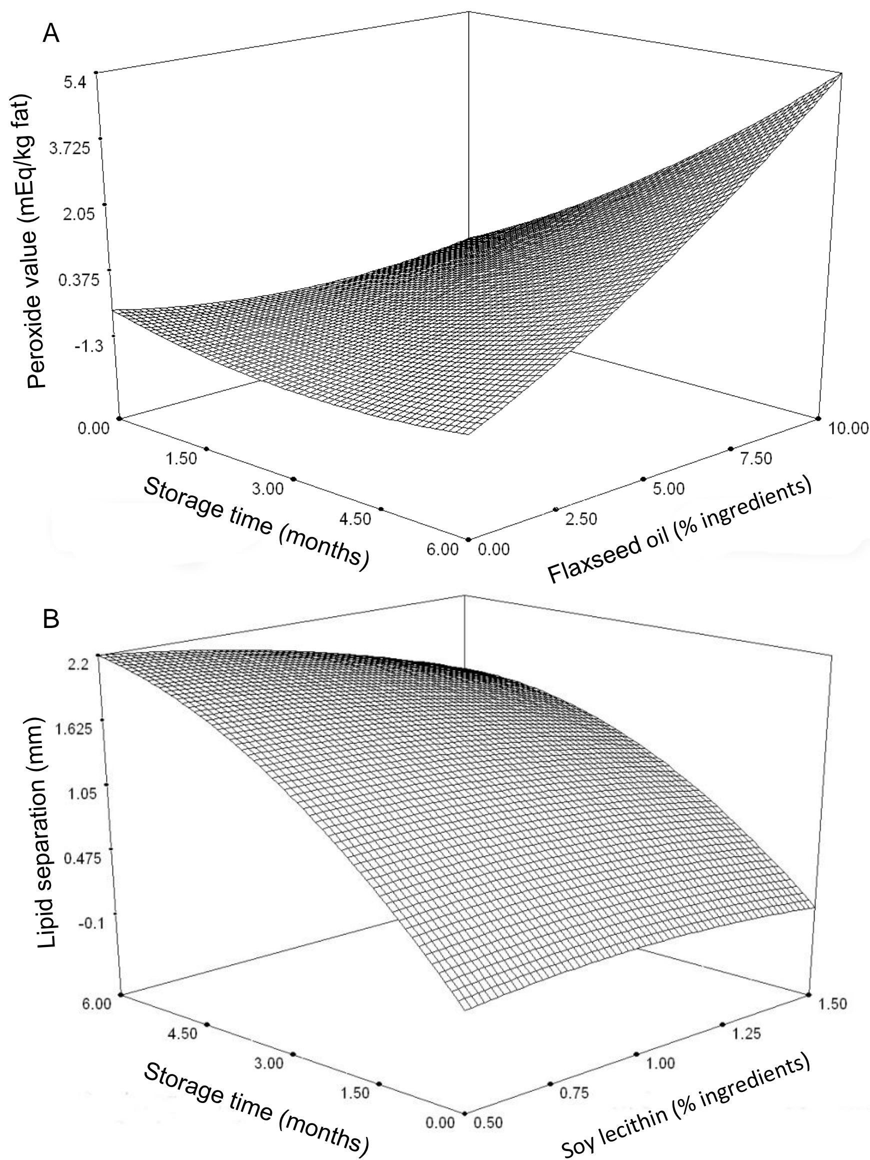


Figure 18. Effect of accelerated storage (40 °C) on (A) water activity (A_w), (B) moisture content (%db), (C) hardness (N), and (D) vitamin C (mg/100g LNS) of LNS. Data points with different letters indicate significant differences ($p \leq 0.05$).

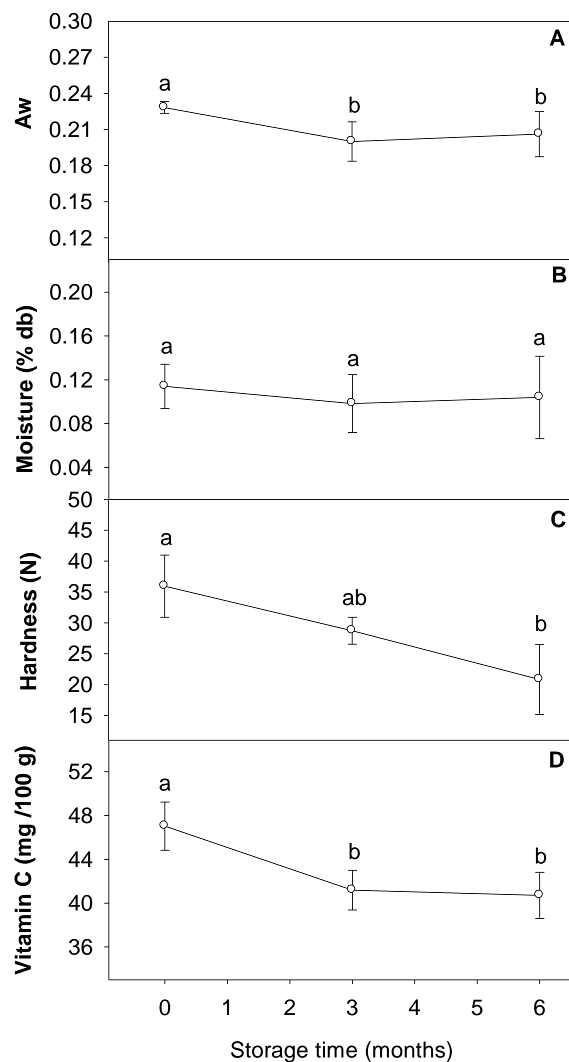
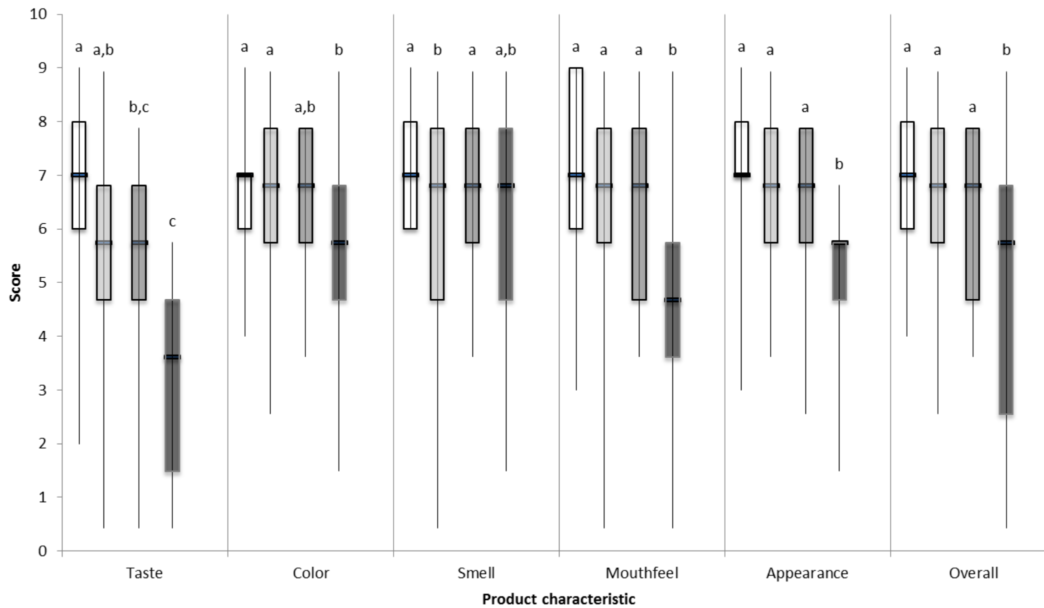
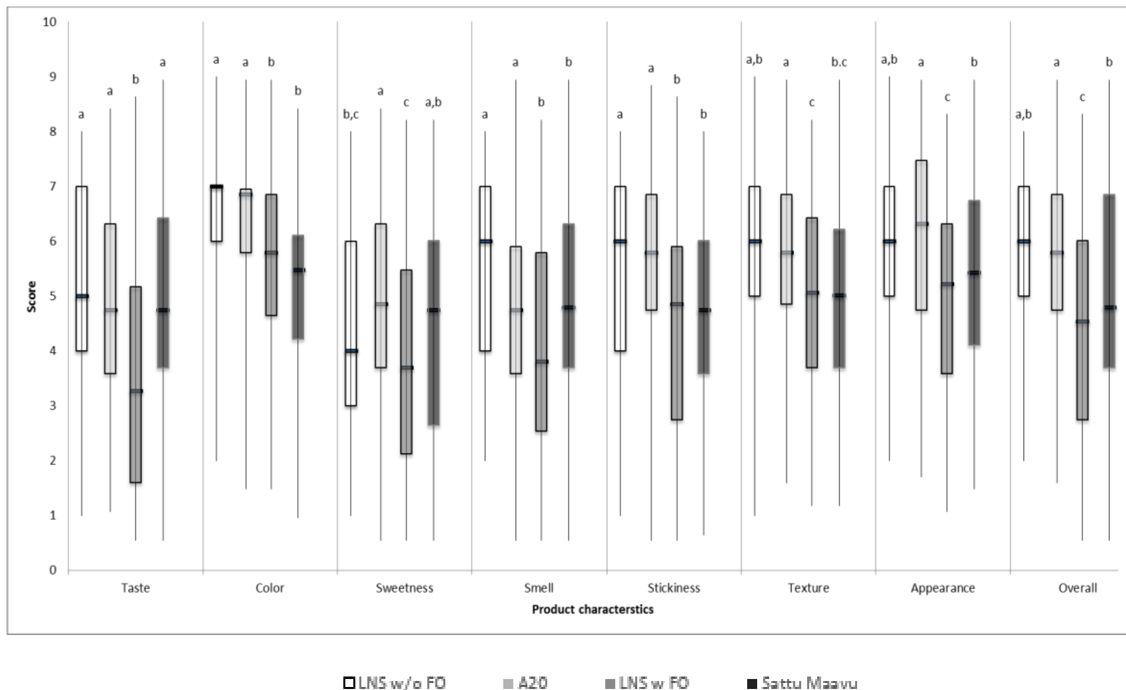


Figure 19. Consumer acceptability test (Hedonic scale 0-9) of several nutrition supplement products among (A) women (n=25) and (B) students (n=86). Box plots represent percentiles and medians. Floating bars with different letters indicate statistical differences after ANOVA and post hoc mean (not median) analyzes ($p \leq 0.05$).

A



B



3.8 References

Ahmed, E. M., & Ali, T. (1986). Textural Quality of Peanut Butter as Influenced by Peanut Seed and Oil Contents 1. Peanut Science, **13(1)**, 18–20

Ali, E., Zachariah, R., Shams, Z., Manzi, M., Akter, T., Alders, P., Allaouna, M., Delchevalerie, P., & Harries, A. D. (2013). Peanut-based ready-to-use therapeutic food: how acceptable and tolerated is it among malnourished pregnant and lactating women in Bangladesh? Maternal & Child Nutrition, **11**, 1028–1035.

Alpaslan, M., & Hayta, M. (2006). The effects of flaxseed, soy and corn flours on the textural and sensory properties of a bakery product. Journal of Food Quality, **29**, 617–627.

Annan, R. A., Webb, P., & Brown, R. (2014). Management of Moderate Acute Malnutrition (MAM): Current Knowledge and Practice Collaborating to improve the management of acute malnutrition worldwide. Retrieved from www.cmamforum.org

Bhagwat, S., Sankar, R., Sachdeva, R., Joseph, L., & Sivaranjani. (2014). Improving the nutrition quality of the school feeding program (Mid-day meal) in India through fortification: a case study. Asia Pacific Journal of Clinical Nutrition, **23**, S12–9.

Bradley Jr, R. L., Bradley, R. L., Nielsen, S. S. (2010). Moisture and Total Solids Analysis. In Food Analysis. (pp. 85-104). Boston, MA: Springer US.

Brenna, J. T., Akomo, P., Bahwere, P., Berkley, J., Calder P. C., Jones, K. D., Liu, L., Manary, M., Trehan, I., & Briend, A. (2015). Balancing omega-6 and omega-3 fatty acids in ready-to-use therapeutic foods (RUTF). BMC Medicine, **13**, 1–4.

Briggs, J., Maguire, P., Sherman, P., Davis, B., Barrett, A., Mahon, J., Doucette, J. (2007). Final report on development of an emergency food product. Retrieved from http://pdf.usaid.gov/pdf_docs/Pnads423.pdf.

Calder, P. C. (2006). n-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. American Journal of Clinical Nutrition, **83**, S1505–1519.

Caron, O. (2012). RUTF product specifications. Retrieved from http://www.unicef.org/supply/files/5._Common_RUTF_Specifications_and_Labelling.pdf.

Caughey, G. E., Mantzioris, E., Gibson, R. a, Cleland, L. G., & James, M. J. (1996). The effect on human tumor necrosis factor alpha and interleukin 1 beta production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. The American Journal of Clinical Nutrition, **63**, 116–122.

Chaparro, C. M., & Dewey, K. G. (2010). Use of lipid-based nutrient supplements (LNS) to improve the nutrient adequacy of general food distribution rations for vulnerable sub-groups in emergency settings. Maternal & Child Nutrition, **6**, 1–69.

Commercial Item Description (CID). (2012). Ready to use therapeutic food (RUTF). Retrieved from

http://www.ams.usda.gov/sites/default/files/media/CID%20Therapeutic%20Food,%20Ready-To-Use.pdf.

Crowe, T. D., & White, P. J. (2001). Adaptation of the AOCS official method for measuring hydroperoxides from small-scale oil samples. Journal of the American Oil Chemists' Society, **78**, 1267–1269.

Dewey, K. G., & Arimond, M. (2012). Lipid-based nutrient supplements: how can they combat child malnutrition? PLoS Medicine, 9(9), e1001314. http://doi.org/10.1371/journal.pmed.1001314El-Waseif, M. A., Hashem, H. A., & Abd EL-Dayem, H. H. (2013). Using flaxseed oil to prepare therapeutical fat spreads. Annals of Agricultural Sciences, **58**, 5–11.

FAO/WHO. (1999). Fats, oils and related product. Food standard program in Codex Alimentarius Commission, (CODEX STAN 19-1981), Food and Agriculture Organization of the United Nations, World Health Organization, Rome, Italy. Retrieved from http://www.fao.org/docrep/004/y2774e/y2774e03.htm#TopOfPage.Ganesan, B., Brothersen, C., & McMahon, D. J. (2014). Fortification of foods with omega-3 polyunsaturated fatty acids. Critical Reviews in Food Science and Nutrition, **54(1)**, 98–114.

Gogus, U. & Smith, C., 2010. n-3 Omega fatty acids: a review of current knowledge. International Journal of Food Science & Technology, **45**, 417–436.

Goh, K. K. T., Ye, A., & Dale, N. (2006). Characterisation of ice cream containing flaxseed oil. International Journal of Food Science and Technology, **41**, 946–953.

Gopalan, C., Sastri, B. V. R., & Balasubramanian, S. C. (1989). Nutritive Value of Indian Foods. National Institute of Nutrition, Indian Council of Medical Research. Retrieved from https://books.google.com/books/about/Nutritive_Value_of_Indian_Foods.html?id=biFBAAAAYAAJ&pgis=1.

Guillard, V., Broyart, B., Bonazzi, C., Guilbert, S., & Gontard, N. (2003). Evolution of moisture distribution during storage in a composite food modelling and simulation. Journal of Food Science, **68**, 958–966.

Gunstone, F. D. (1996). Fatty Acid and Lipid Chemistry. Boston, MA: Springer US.

Gupta, S. Sen, Mohammed, M. H., Ghosh, T. S., Kanungo, S., Nair, G. B., & Mande, S. S. (2011). Metagenome of the gut of a malnourished child. Gut Pathogens, **3:7**.

Hinds, M. J., Chinnan, M. S., & Beuchat, L. R. (1994). Unhydrogenated Palm Oil as a Stabilizer for Peanut Butter. Journal of Food Science, **59(4)**, 816–820.

Hsieh, J.-C., Liu, L., Zeilani, M., Ickes, S., Trehan, I., Maleta, K., Craig, C., Thakwalakwa, C., Singh, L., Brenna, J. T., & Manary, M. J. (2015). High Oleic Ready-to-Use Therapeutic Food Maintains Docosahexaenoic Acid Status in Severe Malnutrition. Journal of Pediatric Gastroenterology and Nutrition, **61**, 138-143.

Jones, K. D., Ali, R., Khasira, M. a, Odera, D., West, A. L., Koster, G., Akomo, P., Talbert, A. W., Goss, V. M., Ngari, M., Thitiri, J., Ndoro, S., Knight, M. A., Omollo, K., Ndungu, A., Mulongo, M. M., Bahwere, P., Fegan, G., Warner, J. O., Postle, A. D., Collins, S., Calder, P. C., & Berkley, J. A. (2015). Ready-to-use therapeutic food with elevated n-3 polyunsaturated fatty acid content, with or without fish oil, to treat severe acute malnutrition: a randomized controlled trial. BMC Medicine, **13:93**.

Muego-Gnanasekharan, K. F., & Resurreccion, A. V. A. (1992). Physicochemical and Sensory Characteristics of Peanut Paste Stored at Different Temperatures. Journal of Food Science, **57**, 1385–1389.

Nwabueze, T. U. (2010). Review article: Basic steps in adapting response surface methodology as mathematical modelling for bioprocess optimisation in the food systems. International Journal of Food Science & Technology, **45**, 1768–1776.

Nguyen, V. H., Bouzambou, H. (2014). Technical Specifications for Lipid based Nutrient Supplement - Large Quantity. Retrieved from http://documents.wfp.org/stellent/groups/public/documents/manual_guide_proced/wfp262353.pdf.O'Mahony, M. (1986). Sensory Evaluation of Food-Statistical Methods and Procedures. New York: Marcel Dekker, Inc.Perlman, D. (1999). Method and composition for preventing oil separation in vegetable kernel butters by combining with microparticulate silicon dioxide. Google Patents. Retrieved from http://www.google.com/patents/US5962064.

Radocaj, O., Dimic, E., Diosady, L. L., & Vujasinovic, V. (2011). Optimizing the texture attributes of a fat-based spread using instrumental measurements. Journal of Texture Studies, **42**, 394–403.

Rangrej, V., Shah, V., Patel, J., & Ganorkar, P. M. (2015). Effect of shortening replacement with flaxseed oil on physical, sensory, fatty acid and storage characteristics of cookies. Journal of Food Science and Technology, **52**, 3694–3700.

Riveros, C. G., Mestrallet, M. G., Gayol, M. F., Quiroga, P. R., Nepote, V., & Grosso, N. R. (2010). Effect of storage on chemical and sensory profiles of peanut pastes prepared with high-oleic and normal peanuts. Journal of the Science of Food and Agriculture, **90**, 2694–2699.

Sewald, M., & DeVries, J. (2013). Food product shelf life. Medallion Laboratories Analytical Progress. Retrieved from http://www.medallionlabs.com/Downloads/food_product_shelf_life_web.pdf.

Skinner, J. D., Carruth, B. R., Bounds, W., & Ziegler, P. J. (2002). Children's Food Preferences: a longitudinal analysis. Journal of the American Dietetic Association, **102**, 1638–1647.

Sumainah, G. M., Sims, C. A., Bates, R. P., & O'Keefe, S. F. (2000). Flavor and Oxidative Stability of Peanut-Sesame-Soy Blends. Journal of Food Science, **65**, 901–905.

Takahata, K., Monobe, K., Tada, M., & Weber, P. C. (1998). The benefits and risks of n-3 polyunsaturated fatty acids. Bioscience, Biotechnology, and Biochemistry, 62(**11**), 2079–85.

Tarrago-Trani, M. T., Phillips, K. M., & Cotty, M. (2012). Matrix-specific method validation for quantitative analysis of vitamin C in diverse foods. Journal of Food Composition and Analysis, **26**, 12–25.

Teitelbaum, J. E., & Allan Walker, W. (2001). Review: the role of omega 3 fatty acids in intestinal inflammation. The Journal of Nutritional Biochemistry, **12(1)**, 21–32.

Thies, F., Miles, E. A., Nebe-von-Caron, G., Powell, J. R., Hurst, T. L., Newsholme, E. A., & Calder, P. C. (2001). Influence of dietary supplementation with long-chain n-3 or n-6 polyunsaturated fatty acids on blood inflammatory cell populations and functions and on plasma soluble adhesion molecules in healthy adults. Lipids, **36**, 1183–1193.

Varma, J. L., Das, S., Sankar, R., Mannar, M. G. V., Levinson, F. J., & Hamer, D. H. (2007). Community-level micronutrient fortification of a food supplement in India: a controlled trial in preschool children aged 36-66 mo. The American Journal of Clinical Nutrition, **85**, 1127–1133.

Wallace, F. A., Miles, E. A., & Calder, P. C. (2003). Comparison of the effects of linseed oil and different doses of fish oil on mononuclear cell function in healthy human subjects. The British Journal of Nutrition, **89**, 679–689.

Walstra, P. (2002). Physical Chemistry of Foods. CRC Press. Retrieved from https://books.google.com/books?id=d4AemlvjJAsC&pgis=1.WHO (1999). Management of severe acute malnutrition: a manual for physicians and other senior health workers. Geneva, Switzerland: WHO.

WHO. (2012). Supplementary Foods for the Management of Moderate Acute Malnutrition in Infants and Children 6–59 Months of Age. Technical Note, WHO, Geneva.

WHO. (2013). World Health Statistics 2013. Geneva: WHO.

Yeh, J.-Y., Phillips, R. D., Resurreccion, A. V. A., & Hung, Y.-C. (2002). Physicochemical and Sensory Characteristic Changes in Fortified Peanut Spreads after 3 Months of Storage at Different Temperatures. Journal of Agricultural and Food Chemistry, **50**, 2377–2384.

Chapter 4: Effect of Oregano Essential Oil and Carvacrol on *Cryptosporidium parvum* Infectivity in HCT-8 Cells

4.1 Abstract

Background: *Cryptosporidium parvum* is the second leading cause of persistent diarrhea among children in low-income settings.

Objective: This study examined the effect of oregano essential oil (OEO) and carvacrol (CV), on prevention of *C. parvum* infectivity *in vitro*.

Methods: HCT-8 cells were seeded (1×10^6) in 96-well microtiter plates until confluency. Cell viability and infectivity were assessed by seeding HCT-8 cell monolayers with *C. parvum* oocysts (1×10^4) in two modalities: 1) 4 h co-culture with bioactive (0–250 µg/mL) followed by washing and incubation (48 h, 37°C, 5% CO_2) in bioactive-free media; and 2) 4 h co-culture of *C. parvum* oocysts followed by washing and treatment with bioactive (0–250 µg/mL) during 48-h incubation. Cell viability was tested using Live/Dead™ assay whereas infectivity was measured using *C. parvum*-specific antibody staining via immunofluorescence detection.

Results: Loss of cell viability (~90%) was found at 125 µg/mL and 60 µg/mL for OEO and CV, respectively. Neither OEO nor CV modulated the invasion of *C. parvum* sporozoites in HCT-8 cells. Treatment with bioactive after invasion reduced relative *C. parvum* infectivity in a dose-dependent manner to 55.6 ± 10.4% and 45.8 ± 4.1% at 60 and 30 µg/mL of OEO and CV, respectively.

Conclusions: OEO and CV are potential bioactives to counteract *C. parvum* infection in children.

4.2 Introduction

Intestinal parasitic infection is considered a global public health problem by the World Health Organization (WHO), afflicting 3.5 billion people and causing clinical morbidity to approximately 450 million people globally (WHO, 1998). Although it affects all ages and socio-economic status, parasitic infections are more prevalent in children (<5 years) living in low-income settings, owing to inadequate sanitation, hygiene, and healthcare system and their less developed immune systems (Scrimshaw, 1994). These infections alter the epithelial integrity and weaken the immune system in children resulting in reduced nutrient digestion and absorption, chronic gut inflammation, iron deficiency anemia, protein-energy malnutrition, and reduced growth and cognitive development (Katona and Katona-Apte, 2008).

Common parasites responsible for morbidity and mortality in children are helminths such as *Ascaris lumbricoides* (roundworm), *Trichiuris trichiuria* (whipworm), *Ancylostoma duodenale*, and *Necator americanus* (hookworms) and protozoans such as *Giardia intestinalis*, *Entamoeba histolytica*, *Cyclospora cayetanensis*, and *Cryptosporidium* spp. After rotavirus, *Cryptosporidium spp.,* mainly *C. parvum* (zoonotic) and *C. hominis* (anthroponotic), are the second largest cause of diarrheal disease and death in infants, posing 2 to 3 times higher risk of mortality (Kotloff *et al.*, 2013). These parasites follow a fecal-oral route of transmission. *C. parvum* oocysts are excreted in the feces of an infected host and are protected by a thick wall resistant to environmental factors and to most disinfection processes, making them highly resilient (Smith *et al.*, 2005). The infection starts with the release of sporozoites from oocysts in the intestinal tract where they attach to and invade mucosal epithelial cells. In these cells, the entire endogenous development including asexual reproduction, gametogony and oocyst formation occurs (Tzipori, 1988). *C. parvum* does

not multiply outside of the host and completes all stages of its development (asexual and sexual) within a single living host. Thus far, *in vitro* models can only replicate the asexual cycle. Although cryptosporidiosis is self-limiting in healthy individuals, the infection can be life threatening among undernourished children with compromised immune systems.

There are a few compounds with some, albeit limited, anticryptosporidial activity including paromomycin, nitazoxanide, azithromycin in combination with paromomycin, roxithromycin, and protease inhibitors often used in highly active antiretroviral therapy (Gargala, 2008). Currently, only one FDA-approved, moderately effective drug, nitazoxanide (Alinia®) (Miyamoto and Eckmann, 2015), is available for treatment of *Cryptosporidium* infection. Due to increasing resistance and severity of side effects, new alternative bioactives are currently under examination (Anthony *et al.*, 2005).

Oregano Essential oil (OEO), also known as the Mediterranean miracle, *Sathra* in Ayurveda or Himalayan Marjoram in India, is known to possess antibacterial, antiviral, antifungal, antiparasitic and antioxidant activities (Burt, 2004). The principal components responsible for the bioactivity of OEO are the small phenolic compounds carvacrol (CV) and thymol (Burt, 2004). Although there is vast literature available on the activity of OEO and its constituents against bacteria, limited evidence exists on its efficacy and effectiveness against gut parasites, specifically *Cryptosporidium spp.* (Burt, 2004; Sivropoulou *et al.*, 1996).

The objective of this study was to systemically examine the effect of OEO and its main monoterpene, CV, on the prevention of *Cryptosporidium parvum* invasion and infectivity using the HCT-8 cell model.

4.3 Materials and methods

Materials. OEO (*Origanum vulgare*) was obtained from Oregano World, Hollywood, FL. Carvacrol (98% pure, 2-methyl-5-[1-methylethyl] phenol, liquid, M≈ 150.2 g/mol, log extinction coefficient 3.262), Pyrazolopyrimidine 1 (PP1; positive control), RPMI 1640 medium, normal goat serum and sodium pyruvate were procured from Sigma-Aldrich (St. Louis, MO). Antibiotic-antimycotic and trypsin/EDTA were purchased from Gibco (Thermo Fisher Sci., Waltham, MA). Fetal Bovine Serum (FBS) and Sporo-Glo™ were obtained from Bio-West (Riverside, MO) and Waterborne Inc. (New Orleans, LA), respectively.

Preparation of C. parvum oocysts. Propagation, collection, and purification of *C. parvum* oocysts were conducted as previously described (Johnson *et al.*, 2004; Schmidt and Kuhlenschmidt, 2008). Briefly, oocysts were purified from homogenized, sieved feces by Sheather's sugar flotation and continuous sucrose density gradient centrifugation. Oocysts were washed and stored at 4°C in 50 mM Tris buffer, pH 7.2 with 10 mM EDTA. Oocysts were used within 1 to 2 weeks of initial isolation from stored feces when viability remained above 80% as judged by excystation. All procedures involving animals were part of protocols approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC).

HCT-8 cell culture. Human colon adenocarcinoma (HCT-8) cells obtained from ATCC (CCL244) were cultured in complete medium (RPMI 1640), supplemented with 2 g of sodium bicarbonate per liter, 2.5 g of glucose per liter, a final concentration of 10% FBS, 5 mL antibiotic-antimycotic solution, and 5 mL sodium pyruvate. Dissociating solution (2 mL; 0.25% Trypsin/EDTA) diluted with sterile PBS was used to subculture the cells in T-75 flasks, following incubation at 37°C and 5% CO_2 for 15 min. Cells were used to seed 96-well plates for invasion and infectivity assays after grown to confluence within 2-3 days.

HCT-8 cell viability. Two-color dual parameter Live/Dead® cell viability assay (Thermo-Fisher Sci.) was used to assess the viability of cells after treatment with OEO and CV at different concentrations (0, 7, 15, 30, 60, 125, 250, 500 and 1000 µg/mL). Live cells were distinguished through the intracellular esterase activity. The green and red fluorescence were detected under phase contrast microscope using common green and red imaging filters used with FITC and Texas Red. HCT-8 cell viability assay was also conducted for both modalities to observe the viability after 4-h cell invasion assay and after 48-h growth inhibition assay. Secondary confirmation of cell viability was made through microscopic observation of cell deformations and damage at different concentrations.

C. parvum invasion and growth inhibition assay. The experimental design was based on previous studies for *in vitro* inhibition of parasitic invasion (modality 1) and infection (modality 2) of human cells using bioactives (Johnson *et al.*, 2004). Two modalities were utilized to conduct the experiment as described below and shown in Figure 20. In either modality, plates with confluent monolayers of HCT-8 cells ($1{\times}10^6$ cells/well) were used. Oocysts (0.5 mL, $5{\times}10^4$) were separately bleached with 40% NaOCl to facilitate oocysts excystation *in situ* on the cell monolayer. A positive control consisting of samples treated with PP1 was used at a level of 1.5 µM (0.179 µg/mL) to inhibit ~98% *C. parvum* infection with no effect on cells. Two empty wells were maintained between each concentration treatment horizontally and vertically to avoid cross contamination due to the volatile nature of the bioactives.

Invasion inhibition assay. In modality 1, oocysts (0.1 mL, $1{\times}10^4$) and cells were co-cultured in complete medium (0.2 mL, triplicate) containing several doses (0, 7, 15, 30, 60, 125, 250 µg/mL) of OEO or CV and incubated for 4 h at 37°C (Figure 20). This was followed by rinsing the cell

monolayers three times with PBS and reconstitution with bioactive-free media and 48 h incubation at 37°C and 5% CO_2.

Growth inhibition/infection assay. In modality 2, oocysts (0.1 mL, 1×10^4) and cells were co-cultured in complete medium (0.2 mL, triplicate) and incubated for 4 h at 37°C and 5% CO_2. Then, cells were washed with PBS three times followed by addition of 0.2 mL of several doses (same as before) of freshly prepared OEO or CV diluted in complete medium (Figure 20). Incubation continued for 48 h with or without complete medium replacement containing a fresh preparation of bioactives after 24 h.

Immunodetection. At the end of the growth inhibition assay, the medium was removed and the cells were fixed with methanol:acetic acid solution (9:1 v/v) for 2 min. Cells were rehydrated, permeabilized by two consecutive rinses with PBS containing 0.1% Triton X-100, blocked with 5% normal goat serum, and stained with Sporo-Glo™ overnight at 4°C. Infectivity was assessed via immunofluorescence detection using phase contrast/fluorescent microscopy after rinsing twice with PBS, followed by imaging in water with a 20X objective as described previously (Kuhlenschmidt *et al.*, 2016). Infected cells containing replicating parasites appeared as fluorescent clusters of infectivity or foci. The numbers of fluorescent particles within each focus per 20X field were determined by an automatic microscopic collection of nine fields per well of a 96-well plate. These nine fields were assembled into a single montage image for each well, representing 75% of the surface area of each well. The number of fluorescent particles in each montage was determined with Image J software, NIH, Bethesda, MD and expressed in fluorescent focus units (FFU). In both modalities, relative *C. parvum* growth was calculated at the doses described for each treatment dose using the control wells as 100% growth.

Carvacrol quantification. Quantification of CV in OEO was conducted via reverse phase HPLC with UV detection, which consisted of a Water 600 delivery system, 717plus autosampler, and 996 PDA detector (Milford, MA). Separation was conducted with a C18 Phenomenex Gemini column (5 µm, 150x4.6 mm; Torrance, CA) and an isocratic mobile phase (100% MeOH). Concentrations were estimated with an external standard curve for pure CV.

Statistics. All the experiments were conducted in triplicates, and the results were reported as mean ± SD. Data analysis was performed using SAS® software (SAS Institute Inc., Cary, NC, USA). Differences between variables were tested for significance by one-way ANOVA. If the model was significant, *post hoc* mean analysis test (Tukey's Honest Significant Difference) was performed. Statistical significance was established at $P<0.05$.

4.4 Results

Cell viability. In modality 1, after the 4 h exposure of HCT-8 cells to various concentrations of OEO and CV, no cell death or alteration in cell texture was observed as assessed by Live/Dead® cell viability assay and phase contrast microscopic observations, respectively. Whereas, in modality 2, small but significant deformation of cell monolayer and loss of cell viability was observed starting at 60 µg/mL CV (5.55 ± 0.72% HCT cell death; $p<0.05$), and no changes in cell viability were observed in cells treated with OEO at doses as high as 125 µg/mL (Figure 21). Treatment with both OEO and CV at doses greater than 250 µg/mL resulted in complete loss of cell viability. Thus, the working concentrations to conduct invasion and infectivity studies were 7, 15, 30, 60, 125 µg/mL for OEO and 7, 15, 30, 60 µg/mL for CV.

Invasion inhibition assay. Relative *C. parvum* growth in cell culture was quantified using immunofluorescence assays. None of the bioactives at any of the tested doses affected the invasion or oocyst excystation of *C. parvum* on HCT-8 cells (Figure 22) during the 4h exposure ($p>0.05$).

Growth inhibition assay. Both, OEO and CV inhibited ($p<0.05$) *C. parvum* growth in a dose-dependent manner (Figure 22). *C. parvum* growth relative to the untreated control was reduced to 55.6 ± 10.4% at 60 µg/mL OEO and 45.8 ± 4.1% at 30 µg/mL CV, without any apparent toxicity to the HCT-8 cells (>97% live). Complete growth inhibition was observed with the positive control (PP1) at a concentration of 1.5 µM (0.179 µg/mL) with no observable cell death (Figure 21). Both bioactives were more effective ($p<0.05$) in inhibiting *C. parvum* infectivity after replacement with fresh treated media 24 hours after incubation. Figure 23 shows the relative *C. parvum* growth at the representative doses of 60 and 30 µg/mL for OEO and CV, respectively.

HPLC Analysis. Carvacrol concentration in OEO was 594.6 ± 10.0 µg/mL.

4.5 Discussion

C. parvum, a monoxenous apicomplexan mucosal parasite, has gained wide recognition due to its association with a severe diarrheal disease that affects mostly infants and children who are immunocompromised and undernourished. Of the large scale of compounds tested for antiparasitic activity against *C. parvum*, only a few have been found to reduce its infectivity (Gargala, 2008). Plant bioactive compounds have a long history of use for prevention and treatment of various human and animal parasitic infections (Kayser *et al.*, 2003). Natural bioactives such as ferulic acid and curcumin from turmeric (Shahiduzzaman *et al.*, 2009), a series of aurones and 4-methoxy-alpha-pyrones (Kayser *et al.*, 2001), and garlic oil (Sreter *et al.*, 1999;

Gaafar, 2012), are reported to have activity against *Cryptosporidium* spp. *in vitro* and *in vivo*. OEO and CV are increasingly acknowledged as having antimicrobial and antiparasitic activities (Burt, 2004). In the Ayurvedic and other ethnopharmacological literature, OEO has been used as a *Krumighna karma or* antiparasitic agent (Lad and Frawley, 1986); however, the evidence on the potential role of OEO or CV against parasitic infection is limited (Table 11).

In this study, we show a dose-dependent inhibition effect of OEO and CV on *C. parvum* infectivity (modality 2), but not invasion (modality 1) of HCT-8 cells. These effective concentrations of 30 µg/mL CV and 60 µg/mL OEO with relative *C. parvum* % growth of 45.8±4.1% and 55.6±10.4%, respectively, were similar to those reported for other parasites as listed in Table 11. Application of fresh treatments after 24 hours, similarly as reported by Shahiduzzaman *et al.* (2009), was found to improve the efficacy of OEO and CV against *C. parvum in vitro*. The high variability in the data could be explained by interexperimental variation of host cell monolayer susceptibility to infection (Rochelle *et al.*, 2002).

The increase in HCT-8 cell death (dead cell % area >2%) with increased dose (CV>30 µg/mL and OEO>60 µg/mL) in modality 2, might be due to a profound increase in the production of reactive oxygen species (ROS) and caspase-3 inducing apoptosis (Liang *et al.*, 2013). Essential oils are known to permeabilize and depolarize the mitochondrial membranes in eukaryotic cells by reducing the membrane potential, affecting the ionic Ca^{+2} cycling, and reducing the pH gradient to impact the proton pump and ATP pool (Suntres *et al.*, 2015). These processes ultimately result in leakage of oxygen radicals, cytochrome *C*, calcium ions and proteins leading to a pro-oxidant status and inducing apoptosis and necrosis of cells (Bakkali *et al.*, 2008). This is consistent with our microscopic observations on cell deformation and damage observed at concentrations >125 and 60 µg/mL for OEO and CV, respectively.

Limited literature exists on the mechanism of antiprotozoal effect of OEO or CV. The drug-resistant properties of *C. parvum* are very different from other apicomplexan parasites due to its unique biology, distinct structure, and biochemical composition. This resilient characteristic is due to its intracellular but extracytoplasmic shelter that creates a parasitophorous vacuole separating it from the cytosol by a feeding organelle (Griffiths *et al.*, 1998). Monoterpenes such as CV can modify the calcium-dependent protein kinase 1 (CDPK1) and affect the Ca^{2+} mediated signaling of the parasite, which is required for invasion, differentiation and regulation of other vital functions (Nagamune *et al.*, 2008; Murphy *et al.*, 2010). In addition, the hydrophobicity (Burt, 2004) and presence of hydroxyl groups in CV and thymol (Ultee, Bennik, & Moezelaar, 2002), may allow the phenols to penetrate the cell membrane and reduce parasitic infection by modulating cytoplasmic metabolic pathways such as ATP synthesis (Turina *et al.*, 2006; Santoro *et al.*, 2007). *C. parvum* relies solely on the host for nutrient acquisition (cholesterol) and for isoprenoids (intermediate isopentenyl pyrophosphate) precursors, which are required for protein prenylation, geranylgeranylation, N-glycosylation, and ubiquinone biosynthesis for parasite growth (Imlay and Odom, 2014; Ehrenman et al., 2013). Essential oil monoterpenes such as linalool, similar in size and structure to CV, are reported to inhibit 3-hydroxy-3-methyl-glutaryl-Co-enzymeA (HMG-CoA) reductase, which is the key regulatory enzyme in isoprenoid synthesis via the Mevalonate pathway in the host cells. This, in turn, limits the acquisition of isoprenoid precursors from the host and blocks the growth and development of *Cryptosporidium spp.* (Bessoff et al., 2013).

This study is part of our long-term goal to explore the role of bioactives in health, especially by incorporating them into foods to improve their functionality. Although, the results of this *in vitro* study are not conclusive as to the effective dose (i.e., EC_{50}) due to the narrow safety window and obvious HCT-8 cell cytotoxicity at high concentrations, the authors contend this work provides

evidence of the bioactivity of small phenolic molecules and instigates the need for further research. Extrapolating these toxicity and efficacy effects to *in vivo* conditions is difficult due to the continuous flow of fluids through the gastrointestinal tract and the rapid metabolism of CV in living organisms (Atanasov *et al.*, 2015). Other parameters such as absorption, distribution, metabolism and excretion (ADME), as well as permeability and solubility of OEO and CV also influence their bioefficacy *in vivo*. Previous ADME studies in rats showed rapid metabolism and urinary excretion of CV. Plasma C_{max} (maximum plasma concentration) for CV was reached at 0.5 h, in which a small amount of metabolites, such as glucuronide and sulfate conjugates and oxidation derivatives of benzyl alcohol and 2-phenylpropanol were excreted at 24 h. This was followed by complete disappearance of metabolites at 48 h after oral administration (Beck *et al.*, 2010; Austgulen *et al.*, 1987). Furthermore, dose exposure time *in vivo* might be much lower than that used in cell *in vitro* models. In the HCT-8 cell model, for instance, the exposure time was 48 h, with or without medium replacement after 24 h. Toxicological information for OEO or CV is limited; however, OEO was found to be non-toxic in humans at a dose of 600 mg in emulsified oil (Force *et al.*, 2000). In addition, the Natural Health Products Directorate of Canada established the safe limit for CV as an oral medicinal ingredient at 2.7 mg/kg body weight per day for adults. This dose is a 100-fold higher than that found to be cytotoxic in this study.

Although the results from this study are promising, there are some limitations. The immunodetection of infectivity using phase contrast/fluorescent microscopy is not as comprehensive and efficient as polymerase chain reaction techniques for quantification purposes. The *in vitro* model used was limited to those concentrations tolerable by the HCT-8 cells. Moreover, the extent to which this model has clinical relevance has to be evaluated. Nonetheless, the HCT-8 cell line model for cultivation of *C. parvum* and determination of infectivity is reported

to be an effective and adequate alternative to animal models (Rochelle *et al.*, 2002; Upton *et al.*, 1994). The *in vitro* model has been critical in the identification of other bioactive compounds currently used in the treatment of protozoans-related diseases (Gargala *et al.*, 2000). We recommend further *in vivo* testing in suitable animal models to provide basic pharmacological and toxicological data followed by controlled human clinical trials.

4.6 Conclusion

The present study supports the role of OEO as an anticryptosporidial agent. To our knowledge, this is the first study to demonstrate the bioactivity of both OEO and CV as inhibitors of *C. parvum* infection in HCT-8 cells. Studies should continue with the characterization of these and similar compounds in terms of their mechanisms of action *in vitro* and in pre-clinical and clinical models. Although our work shows the therapeutic potential for OEO and CV as antiparasitic agents, more research is needed to determine the effective dose of bioactives, establishing mechanisms of action, and elucidating their possible use in food-based applications. Such studies will contribute to our limited knowledge of the use of OEO and CV in foods to treat parasitic infections and will expand our ability to address one of the immediate causes of undernutrition in low-income countries.

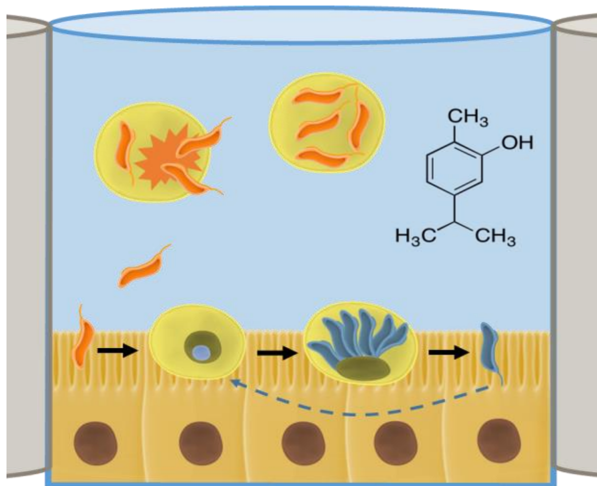
4.7 Table and Figures

Table 11. Literature on activity of oregano essential oil (OEO) and carvacrol (CV) against parasites**.**

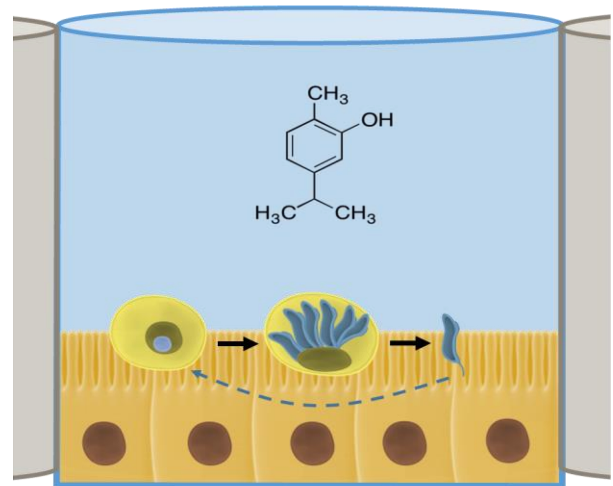
Bioactive	Parasite	Dose and Effect	Model
OEO[1]	*Blastocytis hominis, Entamoeba hartmanni,* and *Endolimax nana*	600 mg of emulsified oil for 6 weeks resulted in disappearance of these parasites in stool specimens	Human adults (n=14)
OEO[2]	*Trypanosoma cruzi*	Essential oil IC_{50}/24 h (µg/mL) = 175 for inhibition of epimastigote growth; 115 to induce trypomastigote lysis	*In vitro* incubation in LIT (for bloodstream tryptomastigote) and RPMI-1640 (for epimastigote) medium
OEO[3]	*Echinococcus granulous* and proloscoleces cysts	Essential oil (10 µg/mL of thymol) resulted in complete loss of protoscolex viability after 72 days post incubation	*In vitro* incubation in medium 199
OEO[4]	*Eimeria tennella*	300 mg/kg body weight Resulted in body weight gains and reduced bloody diarrhea	Broiler chickens infected with 5×10^4 sporulated oocysts
CV and Thymol[5]	Nematode *Haemonchus cotortus*	LC_{50} fiducial limits (mg/mL)= Thymol: 0.13; CV: 0.11; both: 0.037	*In vitro* egg hatch test
CV and Thymol[6]	*Trypanosoma cruzi epimastigotes, Trypanosoma cruzi amastigotes Leishmania chagasi* promastigotes	IC_{50} (µg/mL) for Epimastigotes = CV: 3.0, Thymol: 0.33 Amastigotes = CV: 27.3, Thymol: 3.2 Promastigotes = CV: 0.33, Thymol: 65.2	Vero and THP-1 cells

[1.] Force *et al.*, 2000, [2.]Santoro *et al.*, 2007, [3]Pensel *et al.*, 2014, [4]Giannenas *et al.*, 2003, [5]Katiki *et al.*, 2014, [6]Escobar *et al.*, 2010

Figure 20. Schematic of *in vitro* assessment of *C. parvum* growth and invasion. In modality 1, oocysts and excysting sporozoites are exposed to different dosages of OEO or CV. In modality 2, sporozoites (trophozoites) attached or permeated in HCT-8 cell monolayers, undergoing asexual division into merozoites, are exposed to different dosages of bioactive.



Modality 1. 4-h co-culture with OEO or CV during invasion.



Modality 2. After 4-h invasion, treatment with OEO or CV during infection.

Figure 21. Images of Live (green)/ dead (red) cell assay (A) at various concentrations of oregano essential oil (OEO) and carvacrol (CV) and Immunofluorescence staining (B) of control (no treatment), positive control (Pyrazolopyrimidine (PP1), 100% effective dose), and representative dose of 60 µg/mL OEO and 30 µg/mL CV with 56% and 46% relative *C. parvum* growth, respectively, compared to the control wells.

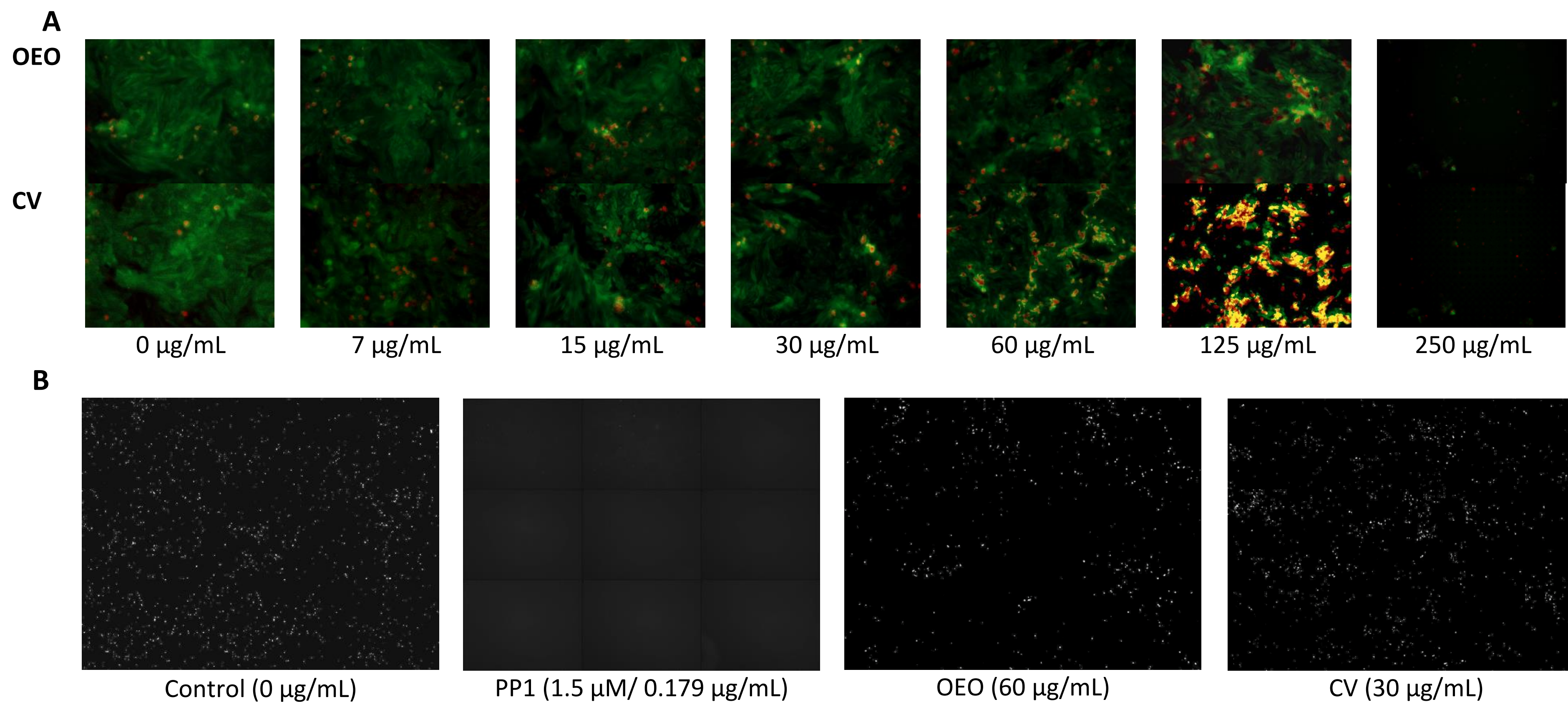


Figure 22. Effect of OEO (A) and CV (B) on relative *C. parvum* % growth at various doses (0, 7, 15, 30, 60, 125 µg/mL of OEO and 0, 7, 15, 30, 60 µg/mL of CV) determined by immunofluorescent assays. Values are means ± standard deviation of two replicates from three repeated trials. *Cell death different from other points. [a<b<c] modality 2 only, difference among treatments, P<0.05

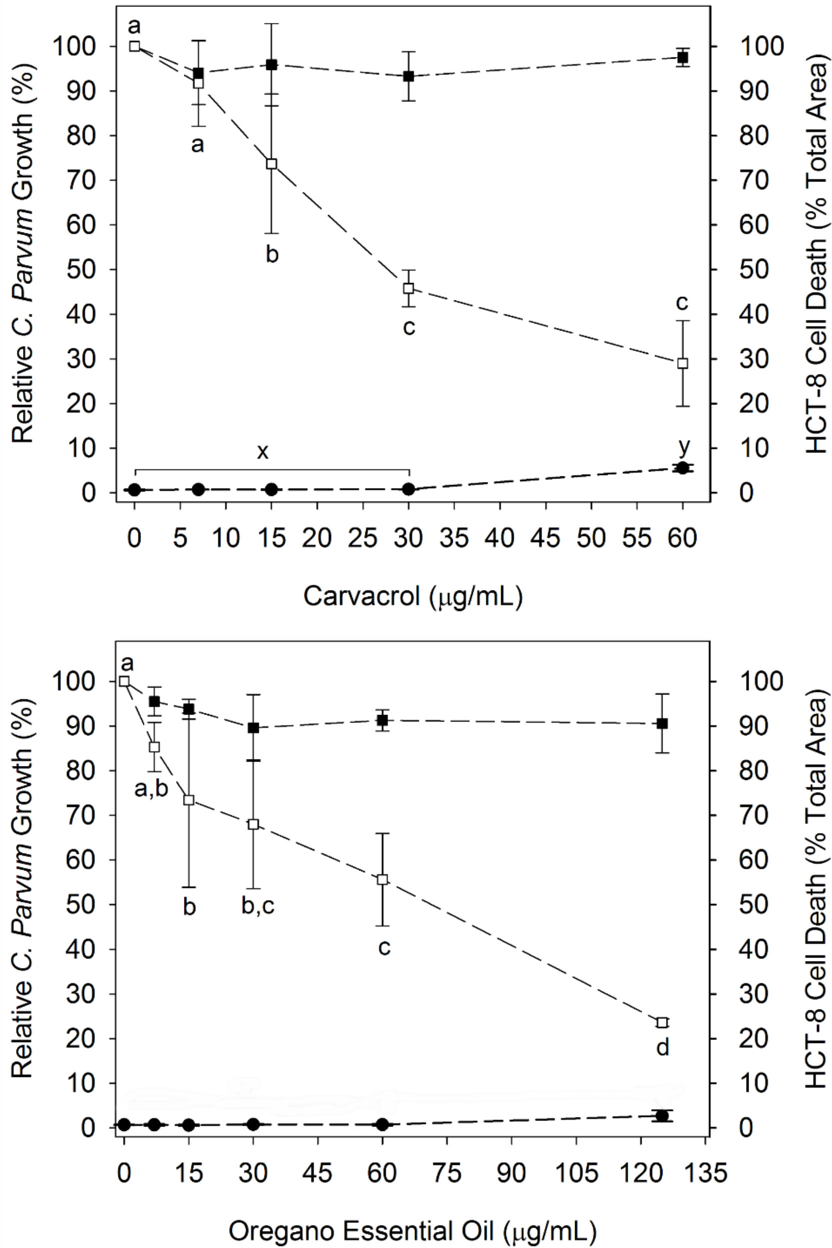
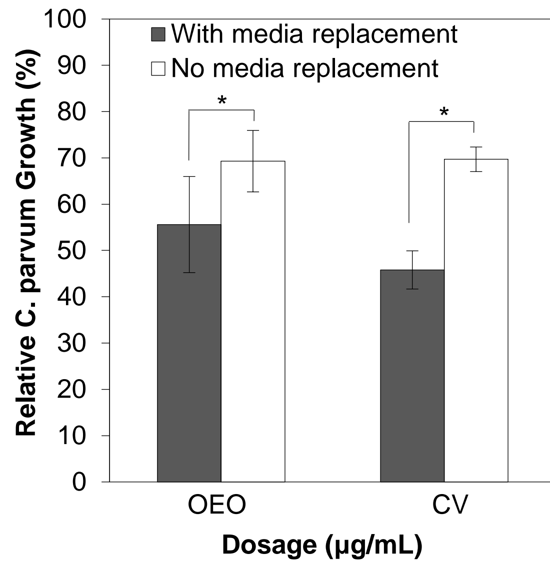


Figure 23. Effect of OEO and CV on relative *C. parvum* % growth at representative dose of 60 µg/mL OEO and 30 µg/mL CV with or without complete medium replacement containing a fresh preparation of bioactives after 24 h, during the 48 h incubation. Values are means ± standard deviation of two replicates from three repeated trials. *difference between treatments, $p<0.05$.



4.8 References

Anthony J-P, Fyfe L, Smith H. 2005. Plant active components - a resource for antiparasitic agents? Trends Parasitol. 21: 462–8.

Atanasov AG, Waltenberger B, Pferschy-Wenzig E-M, Linder T, Wawrosch C, Uhrin P, et al. 2015. Discovery and resupply of pharmacologically active plant-derived natural products: A review. Biotechnol. Adv. 33: 1582–1614.

Austgulen L-T, Solheim E, Scheline RR. 1987. Metabolism in Rats of p -Cymene Derivatives: Carvacrol and Thymol. Pharmacol. Toxicol. 61: 98–102.

Bakkali F, Averbeck S, Averbeck D, Idaomar M. 2008. Biological effects of essential oils – A review. Food Chem. Toxicol. 46: 446–475.

Beck M, Martine B, Elste V, Mair P, Rümbeli R. 2010. Studying Absorption, Distribution, Metabolism, and Excretion of a Complex Extract. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA.

Bessoff K, Sateriale A, Lee KK, Huston CD. 2013. Drug repurposing screen reveals FDA-approved inhibitors of human HMG-CoA reductase and isoprenoid synthesis that block Cryptosporidium parvum growth. Antimicrob. Agents Chemother. 57: 1804–14.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods--a review. Int. J. Food Microbiol. 94: 223–53.

Can Baser K. 2008. Biological and Pharmacological Activities of Carvacrol and Carvacrol Bearing Essential Oils. Curr. Pharm. Des. 14: 3106–3119.

Ehrenman K, Wanyiri JW, Bhat N, Ward HD, Coppens I. 2013. Cryptosporidium parvum scavenges LDL-derived cholesterol and micellar cholesterol internalized into enterocytes. Cell. Microbiol. 15: 1182–1197.

Escobar P, Milena Leal S, Herrera LV, Martinez JR, Stashenko E. 2010. Chemical composition and antiprotozoal activities of Colombian Lippia spp essential oils and their major components. Mem. Inst. Oswaldo Cruz 105: 184–90.

Force M, Sparks WS, Ronzio R a. 2000. Inhibition of enteric parasites by emulsified oil of oregano in vivo. Phyther. Res. 14: 213–214.

Gaafar MR. 2012. Efficacy of Allium sativum (garlic) against experimental cryptosporidiosis. Alexandria J. Med. 48: 59–66.

Gargala G, Delaunay A, Li X, Brasseur P, Favennec L, Ballet JJ. 2000. Efficacy of nitazoxanide, tizoxanide and tizoxanide glucuronide against Cryptosporidium parvum development in sporozoite-infected HCT-8 enterocytic cells. J. Antimicrob. Chemother. 46: 57–60.

Gargala G. 2008. Drug treatment and novel drug target against Cryptosporidium. Parasite 15: 275–81.

Giannenas I, Florou-Paneri P, Papazahariadou M, Christaki E, Botsoglou N a., Spais a. B. 2003. Effect of dietary supplementation with oregano essential oil on performance of broilers after experimental infection with eimeria tenella. Arch. Anim. Nutr. 57: 99–106.

Griffiths JK, Balakrishnan R, Widmer G, Tzipori S. 1998. Paromomycin and geneticin inhibit intracellular Cryptosporidium parvum without trafficking through the host cell cytoplasm: implications for drug delivery. Infect. Immun. 66: 3874–83.

Imlay L, Odom AR. 2014. Isoprenoid metabolism in apicomplexan parasites. Curr. Clin. Microbiol. reports 1: 37–50.

Johnson JK, Schmidt J, Gelberg HB, Kuhlenschmidt MS. 2004. Microbial adhesion of

Cryptosporidium parvum sporozoites: purification of an inhibitory lipid from bovine mucosa. J. Parasitol. 90: 980–90.
Katiki L, Evangelista A, Canova E, Piza A, Fornazari B, Araujo R, et al. 2014. Synergic interaction of aromatic compounds thymol and carvacrol against nematode Haemonchus contortus resistant strain. Planta Med. 80: P1L28.
Katona P, Katona-Apte J. 2008. The interaction between nutrition and infection. Clin. Infect. Dis. 46: 1582–8.
Kayser O, Kiderlen AF, Croft SL. 2003. Natural products as antiparasitic drugs. Parasitol. Res. 90: S55–S62.
Kayser O, Waters WR, Woods KM, Upton SJ, Keithly JS, Kiderlen AF. 2001. Evaluation of in vitro activity of aurones and related compounds against Cryptosporidium parvum. Planta Med. 67: 722–5.
Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382: 209–222.
Kuhlenschmidt TB, Rutaganira FU, Long S, Tang K, Shokat KM, Kuhlenschmidt MS, et al. 2016. Inhibition of Calcium-Dependent Protein Kinase 1 (CDPK1) In Vitro by Pyrazolopyrimidine Derivatives Does Not Correlate with Sensitivity of Cryptosporidium parvum Growth in Cell Culture. Antimicrob. Agents Chemother. 60: 570–579.
Lad V, Frawley D. 1986. The yoga of herbs : an Ayurvedic guide to herbal medicine. Lotus Press.
Liang W-Z, Chou C-T, Lu T, Chi C-C, Tseng L-L, Pan C-C, et al. 2013. The mechanism of carvacrol-evoked [Ca2+]i rises and non-Ca2+-triggered cell death in OC2 human oral cancer cells. Toxicology 303: 152–161.
Miyamoto Y, Eckmann L. 2015. Drug Development Against the Major Diarrhea-Causing Parasites of the Small Intestine, Cryptosporidium and Giardia. Front. Microbiol. 6: 1208.
Murphy RC, Ojo KK, Larson ET, Castellanos-Gonzalez A, Perera BGK, Keyloun KR, et al. 2010. Discovery of Potent and Selective Inhibitors of CDPK1 from C. parvum and T. gondii. ACS Med. Chem. Lett. 1: 331–335.
Nagamune K, Moreno SN, Chini EN, Sibley LD. 2008. Calcium regulation and signaling in apicomplexan parasites. Subcell. Biochem. 47: 70–81.
Pensel PE, Maggiore MA, Gende LB, Eguaras MJ, Denegri MG, Elissondo MC. 2014. Efficacy of Essential Oils of Thymus vulgaris and Origanum vulgare on Echinococcus granulosus. 2014.
Rochelle PA, Marshall MM, Mead JR, Johnson AM, Korich DG, Rosen JS, et al. 2002. Comparison of in vitro cell culture and a mouse assay for measuring infectivity of Cryptosporidium parvum. Appl. Environ. Microbiol. 68: 3809–17.
Santoro GF, das Graças Cardoso M, Guimarães LGL, Salgado APSP, Menna-Barreto RFS, Soares MJ. 2007. Effect of oregano (Origanum vulgare L.) and thyme (Thymus vulgaris L.) essential oils on Trypanosoma cruzi (Protozoa: Kinetoplastida) growth and ultrastructure. Parasitol. Res. 100: 783–90.
Schmidt J, Kuhlenschmidt MS. 2008. Microbial adhesion of Cryptosporidium parvum: identification of a colostrum-derived inhibitory lipid. Mol. Biochem. Parasitol. 162: 32–9.
Scrimshaw N. 1994. The consequences of hidden hunger for individuals and societies. Food

Nutr. Bull. 15: 3–24.
Shahiduzzaman M, Dyachenko V, Khalafalla RE, Desouky AY, Daugschies A. 2009. Effects of curcumin on Cryptosporidium parvum in vitro. Parasitol. Res. 105: 1155–61.
Sivropoulou A, Papanikolaou E, Nikolaou C, Kokkini S, Lanaras T, Arsenakis M. 1996. Antimicrobial and Cytotoxic Activities of Origanum Essential Oils.
Smith H V, Nichols RAB, Grimason AM. 2005. Cryptosporidium excystation and invasion: getting to the guts of the matter. Trends Parasitol. 21: 133–42.
Sreter T, Szell Z, Varga I. 1999. Attempted Chemoprophylaxis of Cryptosporidiosis in Chickens, Using Diclazuril, Toltrazuril, or Garlic Extract. J. Parasitol. 85: 989.
Suntres ZE, Coccimiglio J, Alipour M. 2015. The Bioactivity and Toxicological Actions of Carvacrol. Crit. Rev. Food Sci. Nutr. 55: 304–318.
Turina A de. V., Nolan MV, Zygadlo JA, Perillo MA. 2006. Natural terpenes: Self-assembly and membrane partitioning. Biophys. Chem. 122: 101–113.
Tzipori S. 1988. Cryptosporidiosis in Perspective. Adv. Parasitol. 27: 63–129.
Upton SJ, Tilley M, Brillhart DB. 1994. Comparative development of Cryptosporidium parvum (Apicomplexa) in 11 continuous host cell lines. FEMS Microbiol. Lett. 118: 233–236.
World Health Organization. Partners for parasite control: geographical distribution and useful facts and stats. Geneva: WHO, 2007. Available at: http://www.who.int/wormcontrol/statistics/geographical/en/index.html (accessed December 2 2016)

Chapter 5: Functionalization of Lipid-based Nutrient Supplement with β-Cyclodextrin Inclusions of Oregano Essential Oil: Preparation, Characterization and Sensory Evaluation

5.1 Abstract

Background: Intestinal parasitic infection is one of the main causes of acute undernutrition in children. It alters the intestinal membrane integrity, induces inflammation, undermines nutrient absorption, and causes diarrheal nutrient losses. Oregano essential oil (OEO) is known to reduce intestinal parasitic infections when consumed orally, however, no study exists on their use as an ingredient in lipid-based nutrient supplement (LNS) formulations. OEO is certified GRAS by FDA for food use; however, its therapeutic use as a supplement has been limited due to its unpleasant flavor, high volatility, and instability to heat and during digestion, all which reduce its effectiveness against intestinal parasites.

Objective: The objective of this study was to develop a functional LNS containing OEO, which is stable, acceptable and provides targeted intestinal delivery of bioactive.

Methods: β-cyclodextrin (β-CyD) inclusion complexes of OEO (β-CyD-OEO) and carvacrol (β-CyD-CV) (1:1 molar) were prepared using slurry complexation (-20°C) method and characterized based on encapsulation efficiency, moisture content, morphology, and 2-phase *in vitro* digestion stability. Carvacrol (CV) content was measured using reverse phase HPLC-UV. LNS containing β-CyD-OEO (27.2 mg encapsulate/ 20 g LNS) was formulated using Indian staples and ingredients. Discriminatory sensory tests (triangle) were performed with college students (n=58)

and low-income women (n=25), with young children at home (1-6 years), living in Mehsana, India to evaluate differences between LNS with and without bioactive ingredient (β-CyD-OEO only).

Results: Moisture content of the dried complexes ranged from 9.1 to 9.7% d.b. and water activity ranged from 0.35 to 0.412. The complex sizes of β-CyD-OEO and β-CyD-CV were 1.5-7 μm and 4-20 μm, respectively. The encapsulation efficiency was 86.04±4.48% and 81.39±3.34 for β-CyD-OEO and β-CyD-CV, respectively, and the CV content of OEO was 0.6 g/mL. The β-CyD-OEO and β-CyD-CV complexes were stable through the gastric and intestinal phases and CV bioaccessibility ranged from 7.2-7.7% and 6.0-6.5%, respectively. Sensory tests with student and women revealed no differences ($p>0.05$) in color, aroma, and taste between LNS with and without β-CyD-OEO complexes.

Conclusion: Functionalization of LNS with β-CyD-OEO is feasible based on *in vitro* stability and sensory studies. Future studies will address effects of functional LNS on parasite outcomes using pre-clinical models.

5.2 Introduction

Undernutrition is a major cause of morbidity and mortality in children under five and is associated with long-term consequences on the physical growth, cognitive development, susceptibility to infection and diseases, and economic performance (Black et al., 2008). Almost half of all deaths in children under 5 is attributable to undernutrition, translating into an unnecessary loss of about 3 million children a year (UNICEF/WHO/World Bank Group, 2016). According to the UNICEF, 1990 conceptual framework, the two immediate causes of undernutrition are inadequate dietary intake and repeated infectious diseases, such as parasitic infections, both of which are closely related and are found responsible for impairing the immune system interdependently (Hughes and Kelly, 2006; Cunnigham-Rundles, 2008). Inadequate dietary intake can cause sudden weight loss, growth faltering and immunity suppression, furthering the children susceptibility to infections and diseases. Whereas infection with enteric parasites can result in reduced nutrient digestion and absorption (Northrop-Clewes et al., 2001), frequent persistent diarrhea (Gendrel et al., 2003) and chronic gut inflammation (Wang, 2008) resulting in nutrient deficiency related conditions such as iron deficiency anemia (Farid et al., 1969) and protein-energy malnutrition (Muniz-Junqueira and Queiróz, 2002). Intestinal parasitic infections are endemic worldwide, and the infections are more prevalent in children (<5 years) living in low-income settings, owing to inadequate sanitation, hygiene and healthcare system and their less developed immune systems (Scrimshaw, 1994).

Lipid-based nutrient supplements (LNS) are fortified food products, artificially high in energy and nutrients (protein, essential fatty acids, and micronutrients). They are widely used to address undernutrition under different settings such as in emergency relief efforts, food aid, school feeding, or day care centers (Arimond et al., 2013). LNS are reported to be effective in addressing

undernutrition through improvement of dietary protein quality and micronutrients. Nonetheless, there is a paucity in interventions that use LNS to tackle other immediate causes of undernutrition such as diseases from bacterial and parasitic infection, enteropathy and its characteristic chronic inflammation (Hughes and Kelly, 2006; Cunnigham-Rundles, 2008). Undernutrition is a multi-etiological problem and interventions focused only on providing nutritious foods are not sufficient (Egger et al., 1990).

Oregano Essential oil (OEO), also known as the Mediterranean miracle, Sathra in Ayurveda or Himalayan Marjoram in India, is known to reduce intestinal parasitic infections when consumed orally. No study exists, however, on its use as an ingredient in LNS (Force et al., 2000). The principal compound in OEO is the phenolic monoterpene carvacrol (CV) (2-Methyl-5-(1-methylethyl) phenol), which is also responsible for the bioactive properties (Can Baser, 2008). The major phenolics present in OEO are shown in Figure 24. Both, OEO and CV are classified as generally recognized as safe (GRAS) and are approved for food use (European Parliament and Council., 1996; EAFUS, 2006).

Incorporation of OEO or its constituents in food products, such as LNS is a challenging task due to its high volatility, instability, interaction with food ingredients, and potent flavor. These compounds have extremely low flavor threshold (odor threshold 2.29 µg/mL and taste threshold 5 µg/mL). Furthermore, they evaporate easily, and decompose and oxidize during formulation, processing and storage due to exposure to heat, pressure, light, or oxygen (Beirão-da-Costa et al., 2013; Hosseini et al., 2013). Moreover, their therapeutic effect against parasites might be limited by their rapid metabolism and instability during digestion. Thus, a small fraction of the oral dose, if any, reaches the lower gut, where often parasites reside (Kohlert et al., 2002; Kohlert et al., 2000). CV was found to almost completely absorb in the stomach and proximal small intestine

before reaching the action site, i.e., lower intestine (Kohlert et al., 2000; Michiels et al., 2008). Encapsulation is a widely used technique for improving stability, controlled delivery and reducing the unpleasant odor and taste (Wang et al., 2009; McClements et al., 2009). The inclusion of CV and OEO in cyclodextrin (CyD) complexes is a unique method to overcome such problems (Szente and Szejtli, 2004).

β-Cyclodextrins (β-CyD) are cyclic oligosaccharides with seven glucose units joined by α-1,4 glycosidic bonds and have a singular hydrophobic cavity, due to the absence of hydroxyl group, which allows inclusion of hydrophobic molecules such as CV to mask the potent flavor and generate controlled and targeted delivery systems (Szente and Szejtli, 2004). β-CyD are certified GRAS, non-toxic, and have an adequate size to forming 1:1 inclusion complexes with CV at a high CyD/CV formation constant of 2620 M^{-1} (USFDA, 2001; Hirayama and Uekama, 1999; Kfoury et al., 2016b). β-CyD have been previously used as food ingredients in chocolate and mayonnaise for emulsification, grape drinks, coffee, ginseng extract, soybean lecithin for taste masking, and in instant tea drink for preserving flavor (Szente and Szejtli, 2004).

β-CyD complexes are not absorbed across the gastrointestinal tract due to their chemical structure (large number of hydrogen donors and acceptors), their molecular weight (>972 Da), and their low octanol/water partition coefficient (approx. log $P_{o/w}$ between less than -3 and 0) (Irie and Uekama, 1997; Matsuda, 1999). The release of the active compound in the lower gut is triggered through the fermentation and breakdown of CyD into small saccharides by the gut microflora (approx. 1011 – 1012 CFU/mL), especially *Bacteroides*, which use CyD as sole carbon source and stimulate the cyclodextrinase (Antenucci and Palmer, 1984). *In vitro* simulated digestion systems are widely used to assess the stability and bioaccessibility of active compounds in foods and microencapsulated materials (Hur et al., 2011; Flores and Kong, 2017).

The aim of this research was to develop an LNS 2.0 via preparation and characterization of β-CyD complexes of CV and OEO, and discriminatory sensory evaluation of LNS containing OEO-β-CyD complex.

5.3 Materials and Methods

Materials – OEO (Origanum vulgare) was supplied by Oregano World, FL. Carvacrol (98% pure, 2-methyl-5-[1-methylethyl] phenol, liquid, M≈ 150.2 g/mol, log extinction coefficient 3.262) and carvacrol food grade (≥98%) were procured from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Methanol and Acetonitrile were HPLC grade and purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Materials used for the *in vitro* digestion study were all purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). The filters used were 0.2 μm PTFE filter (VWR, Radnor, Pa., U.S.A.). Methanol was procured from Fisher Scientific, Pittsburgh, PA. β-CyD (Cavamax® W7) was procured from Wacker Chemie AG, Adrian, MI. LNS Ingredients – Chickpea flour (Spicy World, Houston, TX), soy flour (Bob's Red Mill, Milwaukie, OR), maltodextrin (Honeyville, Inc, Brigham City, UT), FO (Spectrum Naturals, Inc, Melville, New York), clarified butter (ghee; AMUL, Anand, India), skim milk powder (Barry Farm, Cridersville, OH), soybean oil (Crisco, Orville, OH), coconut oil (Spectrum Naturals, Inc, Melville, New York), palm oil shortening (Crisco, Orville, OH), vitamin mineral mix (Watson Inc, West Haven, CT), soy lecithin (Now, Bloomingdale, IL), antioxidant ascorbyl palmitate (Spectrum Chemical, Gardena, CA), and cocoa powder (Hershey's, Hershey, PA).

Methods.

Preparation of β-CyD inclusion complexes. A slurry complexation method modified from Karathanos et al., 2007 and Donovan et al., 2015 was used for the preparation of inclusion complexes (Figure 25). β-CyD (Cavamax® W7 Wacker Chemie AG, München, Germany)(MW 1,134.98 g/mol) was mixed in distilled water at a 50% solids content. OEO or CV were added at a 1:1 molecular ratio in a 500 mL glass bottle with a lid and mixed using a magnetic stirrer for 48 h at room temperature (25±5°C) to allow the complex formation and prevent loss of the active compound. The solution was centrifuged in 50 mL tubes at 3,000 RCF for 10 min at 4 °C and the supernatant water was decanted using a Pasteur pipette. The solid sediment was spread thinly in a 500 mL mortar (16 cm diameter, Fisher Scientific) and placed in a vacuum desiccator (Scienceware® vacuum desiccator, Sigma-Aldrich) at -20°C for drying for 2-4 d. The moisture content of the ground powder was measured using a moisture content analyzer (HR83 Halogen Moisture Analyzer, Mettler-Toledo, LLC, Columbus, OH). Drying was completed when the powder reached a moisture content of 8-10%. Light grinding with pestle was used to break the particles into powder. Dried samples were stored in tightly sealed (parafilm), aluminum wrapped, 50 mL tubes in dark cabinet at -20°C.

HPLC quantification of carvacrol– Separation and quantification of CV were carried out by reverse phase HPLC and UV detection (PDA at 275 nm) using a Waters 600 delivery system, 717plus autosampler and 996 PDA detector (Milford, Mass., U.S.A.). Separation was conducted with a Phenomenex Gemini 5 μm, C18, 110A, 150x4.6 mm column (Phenomenex, Torrance, Calif., U.S.A.) and an isocratic mobile phase of 100% methanol. Samples were extracted and diluted in DI water, and the sample size was adjusted to not exceed 1 mg/mL CV (CV solubility in H_2O). An external standard curve of pure CV was constructed with varying

concentrations (5-100 µg/mL in water, under the same conditions. All samples were filtered with 0.2 µM filters (VWR, Radnor, PA) before HPLC analysis.

Encapsulation efficiency. The entrapment efficiency (EE) of inclusion complexes was determined using a method modified from (Santos et al., 2015). 204 mg of β-CyD-CV (27 mg CV) or β-CyD-CV (23 mg CV) sample was dissolved in 27 mL acetonitrile and 27 mL of water for determination of total CV and surface adsorbed CV, respectively (Equation 2 and Equation 3). The samples were capped, vortexed for 5 minutes, and left for 48 h at room temperature. Next, the tubes were centrifuged at 3,000 RCF for 15 min to remove any CyD from the solution, leaving only the active compound. CV in the supernatant was estimated using HPLC as described above after filtering using 0.2 µM filters. The entrapment efficiency was calculated using equation 4.

$$\text{Total CV } (\%)=\frac{\text{Total CV recovered}(\frac{\mu g}{mL})}{\text{CV added }(\frac{\mu g}{mL})}\times 100 \qquad \text{Equation 2}$$

$$\text{Surface CV } (\%)=\frac{\text{Surface adsorbed CV }(\frac{\mu g}{mL})}{\text{CV added }(\frac{\mu g}{mL})}\times 100 \qquad \text{Equation 3}$$

$$\text{EE}=\text{Total CV } (\%) - \text{Surface CV } (\%) \qquad \text{Equation 4}$$

Moisture content and water activity. Moisture content was analyzed using a Moisture Analyzer (HR83 Halogen Moisture Analyzer, Mettler-Toledo, LLC, Columbus, OH) and water activity was assessed using an Aqualab water activity meter (Aqualab 4TE, Decagon Devices Inc.).

LNS preparation. LNS formulations were prepared using staple ingredients from India as described by Gaur et al., 2017. Briefly, weighed flours were toasted at 110°C for 120 min in a forced convection hot air oven (Precision Scientific Mechanical Oven, Fisher Sci.). Flours were

added to other dry ingredients and mixed further to prepare a dry mix (2 min). The dry mix was added to a wet grinder (PG503, Premier Wonder), and contents were mixed (3 min) to ensure homogenous mixing and size reduction. The fats and oils were brought to room temperature (23±2°C) and mixed in a glass bowl for 2 min. The lipid mixture was added to dry contents in a wet grinder and blended for 5 min. The samples were stored in air-tight glass jars at room temperature (23±2°C) until used.

***In vitro* digestion test.** Digestive stability and bioaccessibility of CV was evaluated using a 2-phase digestion model simulating the gastric and intestinal phase, using a modified method from Dean A. Garrett et al., (1999) and Miller et al., (1981). Briefly, two enzyme solutions were used where Enzyme A mix consisted of pepsin (4 g/L) solution in 0.1 mol/L HCl and Enzyme B mix consisted of 250 mL of pancreatin (2 g/L) and bile extract (12 g/L) in 0.1 M $NaHCO_3$. The enzyme mixes were prepared to simulate the gastric and intestinal phases, respectively. For digestive stability assessment, 27 mg CV and OEO were dissolved in 27 mL saline solution (0.9% NaCl) in a 50 ml tube (CV solubility ~1 mg/mL), vortexed, sonicated and stored for 3 h under refrigeration (5±2 °C). Next, for the gastric phase, 2 mL enzyme A was added to the mix and the pH was adjusted to 2.0 using a few drops of 1 M HCl. The samples were then incubated at 37°C in orbital shaking at 95 rpm for 1 h. For the intestinal phase, the pH was adjusted to 5.3 using a few drops of 0.9 M bicarbonate solution, and 9 mL of enzyme B was added with a few drops of 2 M NaOH to bring the pH to 7.5. The tubes were then incubated for 2 more hours. Sample triplicates were extracted at each phase, diluted and filtered with 0.2 μM filters for analysis of CV content of digesta via reverse phase HPLC. The digestive stability was calculated using equation 5.

Bioaccessibility provides an estimate of active compound available for intestinal absorption by virtue of being solubilized in the continuous aqueous micellar fraction of the digesta.

For the bioaccessibility assessment, 204 mg (~27 mg CV) of β-CyD-CV and β-CyD-OEO, with or without LNS (4 g) were dissolved in 27 mL saline solution (0.9% NaCl), vortexed, and stored for 3h under refrigeration (5±2 °C). Next, the gastric and intestinal digestion were carried out similar to the digestive stability assessment method as described above. For isolation of the micellar fraction, the tubes at each phase were centrifuged at 3,000 RCF for 15 min, and the aqueous micellar fraction was carefully isolated using a Pasteur pipette. Next, the isolated micellar fraction was vortexed, diluted and filtered using 0.2 µM filters for CV content analysis. The bioaccessibility was calculated using equation 6.

Weights of the tubes were measured after each phase for correction by weight. DI water, β-CyD empty complex served as blank controls. Three measurements were taken.

$$\text{Digestive stability (\%)} = \frac{\text{CV in digesta}}{\text{Initial CV}} \text{X } 100 \qquad \text{Equation 5}$$

$$\text{Bioaccessibility (\%)} = \frac{\text{CV in micellar fraction}}{\text{Initial CV}} \text{X } 100 \qquad \text{Equation 6}$$

Morphology and particle. Morphology of the microcapsules was observed using a scanning electron microscope (SEM; FEI Quanta FEG 450 ESEM, Hillsboro, OR, U.S.A.) at Beckmann Institute for Advanced Science and Technology (Urbana, IL, U.S.A.). The Samples were dispersed on a double-stick conductive carbon substrate and were imaged in low vacuum with a large field detector at 15 kV at spot 3. Particle size was estimated from the SEM images using ImageJ software.

Sensory Difference Tests. Research protocols using humans were approved by the Institutional Review Board at the University of Illinois, Urbana-Champaign. The study was conducted in collaboration with Mansinhbhai Institute of Dairy and Food Technology (MIDFT),

Mehsana, India. 58 college students (18-24 years) and 25 women (18-45 years), with young children at home (1-6 years), otherwise healthy (no diarrhea, cold, or fever), were randomly recruited from MIDFT and within a 20 km radius from MIDFT, respectively. Written consents were obtained from the participants. Triangle tests were performed by the participants to evaluate differences between LNS with and without β-CyD-OEO, using the formulations as shown in Table 12 (Meilgaard et al., 2007). The panelists were presented with three coded samples (~2 ± 0.5 g) in clear disposable sensory cups at a time and were asked to mark the odd (different) sample based on three criteria i.e., color, smell, and taste. The samples were maintained at 25 ± 2°C and all tests were performed in well-lit and ventilated rooms. Water (~25 ± 2°C), and crackers were provided for rinsing between the samples. All testing sessions were scheduled between 11:00 AM and 1:00 PM. The subjects who were able to identify the different sample (correct) were given a score of 1 whereas the others (incorrect) were given a score of 0. The total number of correct responses were then compared with the critical number at α=0.05, calculated using the equation 7 (Meilgaard et al., 2007).

$$Z_{critical} = \frac{k-\left(\frac{1}{3}\right)\times n}{\sqrt{\frac{2}{9}\times n}} \qquad \text{Equation 7}$$

Where Z critical is 1.64 for α=0.05, k is the minimum number of correct responses to reject the assumption of "no difference," n is the total number of participants.

Statistics. All the experiments were conducted in triplicates, and the results are reported as mean ± SD. For all analyses, determinations were made in triplicate as independent experiments. Data analyses were performed using SAS (SAS Institute, Cary, NC, USA). Differences between

variables were tested for significance using one-way ANOVA. Significantly different means ($P \leq 0.05$) were separated by the Tukey's mean separation test.

5.4 Results

CV content of OEO - The CV content of OEO was 604.45 ± 10.03 μg/mL.

Moisture content and water activity – Moisture content of the β-CyD-CV and β-CyD-OEO ranged from 9.1 to 9.7 % d.b. and the water activity ranged from 0.35 to 0.412.

Encapsulation efficiency – The total CV, surface CV and encapsulation efficiencies β-CyD-CV and β-CyD-OEO are listed in Table 13. The encapsulation efficiency of β-CyD-CV and β-CyD-OEO were 85.9 ± 2.06% and 80.9 ± 4.55%, respectively.

Morphology and particle size – The β-CyD complexes were non-spherical and rectangular in shape and their size ranged from 25-90 µm. Figure 26 shows the β-CyD empty, β-CyD-CV and β-CyD-OEO complexes at magnification 1000X. The shape of β-CyD-CV and β-CyD-OEO were similar to the β-CyD empty complexes; however, their size were significantly smaller in size of 4-20 µm and 1.5-7 µm, respectively, compared with the β-CyD empty samples. The smaller particles were found to adhere to the surfaces of the crystals. The complexes had cracks, invaginations and breaks (Figure 26).

***In vitro* digestive stability –** Stability of OEO was measured in terms of CV content. Both CV and OEO were stable throughout the *in vitro* digestion process and no difference was observed in the percent digestive stability of CV in samples from initial, gastric, and intestinal phase (Figure 27). Digestive enzymes had no effect on the stability of CV or OEO (Figure 28).

Bioaccessibility - Bioaccessibility of CV from LNS containing CV alone or OEO, ranged from 70-80% of the initial amount loaded. Bioaccessibility was not different between the digestion phases for LNS with CV, however, in the sample LNS with OEO the bioaccessibility reduced significantly ($p<0.05$) in the intestinal phase (Figure 29). Bioaccessibility of CV from samples β-CyD-CV or β-CyD-OEO was significantly lower ($p<0.0001$) compared to the samples containing free CV or OEO. Bioaccessibility of CV slightly increased ($p<0.05$) in the presence of LNS (Figure 30).

Discriminatory sensory studies – Both student and women participants were not able to discriminate ($p<0.05$) between the LNS samples with or without β-CyD-OEO (Figure 31).

5.5 Discussion

To our knowledge, this is the first attempt to functionalize LNS with bioactives, such as CV and OEO, encapsulated in CyD to address intestinal parasitic infections in children. CyDs are widely used in food and pharmaceutical industry to encapsulate and improve the biological, chemical and physical properties of biologically active compounds, especially those derived from plants (Pinho et al., 2014). The 1:1 molar ratio of host (β-CyD MW= 1,135 g/mol) to guest (CV MW= 150.21 g/mol) molecules was chosen because β-CyD-CV inclusions are reported to form stable complexes with the high formation constant of 2,620 M^{-1} at this stoichiometric ratio. CV is a hydrophobic ($\log P_{0/w} = 3.64$), non-polar monoterpenic phenol isomer and only one CV molecule interacts with the apolar cavity of CyD through van der Waals and hydrophobic interactions (Kfoury et al., 2016a; Szente and Szejtli, 2004).

The encapsulation efficiency of β-CyD-CV complex was in agreement with that previously reported for β-CyD-CV complexes (Santos et al., 2015) and was similar to that for other phenolic complexes such as β-CyD-eugenol (Choi et al., 2009; Seo et al., 2010). Both CV and OEO were encapsulated effectively in β-CyD complexes as indicated by the high encapsulation efficiency, suggesting excellent suitability of CV for inclusion in β-CyD. This could be attributed to the high hydrophobicity (log $P_{o/w} = 3.64$ (Ultee et al., 2002)), low solubility (1 mg/mL (Chen et al., 2014)), high formation constant with β-CyD (2,620 M-1 (Kfoury et al., 2016a) and small molecular size of CV molecule with excellent complementarity of the benzene ring.

The morphology and particle size of β-CyD complexes were in agreement with those reported for CV, linalool and citronella oil (Guimarães, Oliveira, Alves, Menezes, Serafini, de Souza Araújo, et al., 2015; Songkro et al., 2012; Menezes et al., 2014). The relatively larger size of β-CyD empty complexes could be attributed to agglomeration of β-CyD particles via hydrogen bonding. In the absence of a guest molecule, β-CyD tend to cluster and agglomerate due to lack of significant net charge on the inclusion complex particles i.e. no repulsive forces to prevent agglomeration (Hill et al., 2013). This is also consistent with observation of smaller particles attraction and adherence to the larger particles (Santos et al., 2015). The reduction in the particle size in the β-CyD-CV and β-CyD-OEO complexes indicated a conformational change of β-CyD that obstructed the agglomeration β-CyD similar to those observations reported by Guimarães et al., (2015) and Seo et al., (2010).

Encapsulated CV and OEO were stable under the simulated gastro-intestinal digestion conditions. This was in agreement with the observations from *in vitro* studies reported by Michiels et al., 2008, in which no degradation of CV was found in the proximal segments (gastric and jejunal simulations) but about 29% degradation was observed in the caecal simulation. The caecal

degradation reported in their study was due to the higher biocatalytic capacity of caecal bacteria (Michiels et al., 2008). The heat stability of OEO and CV could be explained by the high boiling point of OEO and its constituent CV, which is 238°C, thus losses due to volatilization were minimum under the *in vitro* temperature conditions at 37-39°C (Michiels et al., 2008). Yang et al. (2007) reported that CV was very stable (<10% degradation) at temperatures as high as 150°C, when heated in water for 30 minutes. (Yang et al., 2007).

Bioaccessibility of unencapsulated bioactives in the LNS matrix was relatively high (>60%). Although most of the CV was recovered and stable under these conditions, the lower bioaccessibility in the samples containing LNS was probably due to the interaction of CV with LNS ingredients. The ingredients included the emulsifier, fats and oils, proteins and micronutrients that might have promoted the migration of CV to the top lipid layer or bottom pellet of the centrifuged digesta resulting in incomplete micellarization (McClements and Li, 2010). This was in agreement with the results reported by Roman et al., (2012), in which β-Carotene recovery from fortified almond butter was low at all simulated stages of digestion. The authors suggested that the protein or carbohydrate compounds in the almond butter might emulsify or encapsulate β-carotene, thereby modifying its release from the food matrix. Our findings and those of Roman et al., (2012) require further verification using *in vivo* models.

Encapsulation of CV in β-CyD reduced its bioaccessibility (<10%) regardless of the presence of LNS. This indicates a limited degradation of the β-CyD by gastric or pancreatic enzymes under digestion conditions. This enables desired lower-gut targeted release of the CV or OEO through the fermentation and breakdown of CyD by gut microflora (Minami et al., 1998; Hirayama and Uekama, 1999). This controlled and targeted release property of β-CyD was utilized by Shyale et al, 2005 to develop an antiparasitic drug delivery system to target helminths residing

in the lower gut and to prevent the absorption of the drug in the upper gut. In that study, tablets of β-CyD-albendazole were developed using guar gum as a matrix carrier and were subjected to a 3-step *in-vitro* drug release study, involving gastric, intestinal and a colonic phase using rat caecal content medium, under anaerobic conditions. The encapsulated drug formulations, especially those with higher guar gum content (>30%), had a lower bioaccessibility or drug release during the gastrointestinal phase and a considerably higher release in the colonic phase, indicating the microbial fermentation and breakdown of β-CyD in the presence of rat caecal contents. Similarly, García-Rodriguez et al., 2001 reported β-CyD-albendazole to have higher antihelmintic activity compared to carboxymethylcellulose-albendazole suspension. In their study, mice were infected with *Trichinella spiralis* and both antihelmintic suspensions were delivered orally at 50 mg/kg. Only β-CyD-albendazole was shown to be effective due to lower gut release of the drug.

The increased release and thus higher bioaccessibility of CV with addition of LNS could be explained by the high affinity of hydrophobic lipids in LNS towards CyD complexes, which when dissolved in the aqueous matrix were probably able to extract and/or replace the CV from the hydrophobic cavity in CyD complexes as described by Saenger, (1980).

Encapsulation strategy also resulted in the effective reduction in the perception of essential oil when presented to subjects. Both groups, students and women participants were not able to identify ($p<0.05$) the odd sample. This is similar to the results reported by Kant et al, (2004), in which the panelist were not able to differentiate between the high fat and fat-free lemon flavored yogurt, when β-CyD encapsulates of lemon flavor were used for fat-free formulations. In another study, incorporation of β-CyD in ginseng solutions, at a level of 1 g β-CyD in 100 mL solution, was found to reduce the bitterness intensity, as evaluated using a descriptive sensory analysis (Tamamoto et al., 2010). Similarly, addition of 0.4% (w/v) β-CyD to goat milk was found to reduce

the goaty flavor (short chain fatty acids) as determined using a triangle sensory test (Meier et al., 2001).

Although the results of this study are promising, there are some limitations. *In vitro* digestion model used in the study was limited to the gastric and intestinal digestion stages and we did not simulate the lower gut phase. Nonetheless, other groups have evaluated the release of albendazole, (Shyale et al., 2005) and biphenyl acid (Hirayama et al., 1996) incubation under simulated colon conditions using rat caecal contents and have found preferential drug release in the colonic phase due to the presence of microflora. The *in vitro* model used in this study was successful in accurately predicting the release characteristics of CV in terms of bioaccessibility in a static environment, however, future *in vivo* studies are necessary to assess such characteristics in the dynamic environment of the intestine. Triangle sensory tests with inexperienced panelists are known to be more accurate when conducted in duplicates (i.e., each participant performs the test twice) (Dacremont and Sauvageot, 1997). We did not conduct the triangle test in duplicates or compared it with other sensory tests to increase the power of the test.

5.6 Conclusions

Functionalization of LNS with β-CyD-OEO was technically feasible based on physicochemical characterization, *in vitro* stability and bioaccessibility, and sensory studies. Future studies will address the effects of functional LNS on parasite outcomes using pre-clinical models.

5.7 Tables and Figures

Table 12. Recipe formulation for 100 g LNS with or without β-CyD-OEO

Ingredient	**LNS with β-CyD-OEO (g)**	**LNS without β-CyD-OEO (g)**
Soybean oil	10	10
Coconut oil	8	8
Ghee	8	8
Soy lecithin emulsifier	1	1
Shortening (palm oil)	4	4
Chickpea flour (toasted)	17	17
Soy flour, full fat (toasted)	10	10
Nonfat/skimmed milk powder	15.25	15.25
Sugar	10	10
Flaxseed oil	5	5
Vitamin–mineral mix	3.75	3.75
Cocoa powder	2	2
Maltodextrin M100	5.6	6
β-CyD-OEO	0.4	0
Total	100	100

Table 13. CV recovery, surface CV and encapsulation efficiency of β-CyD inclusions of CV and OEO.

Sample	Total CV recovered (%)	Surface CV (%)	Encapsulation efficiency (%)
β-CyD-CV	92.0 ± 2.2	6.11 ± 0.29	85.9 ± 2.1%
β-CyD-OEO	88.1 ± 4.8	7.20 ± 0.59	80.9 ± 4.6%

Figure 24. Major phenolics in oregano essential oil.

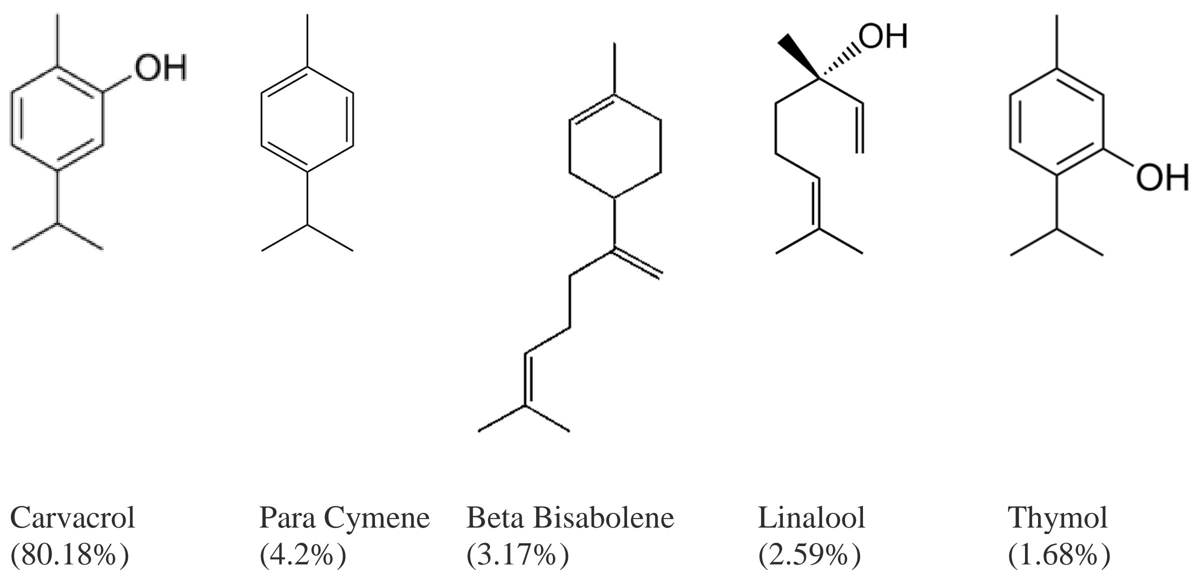


Figure 25. Procedure to prepare β-CyD inclusions of CV and OEO.

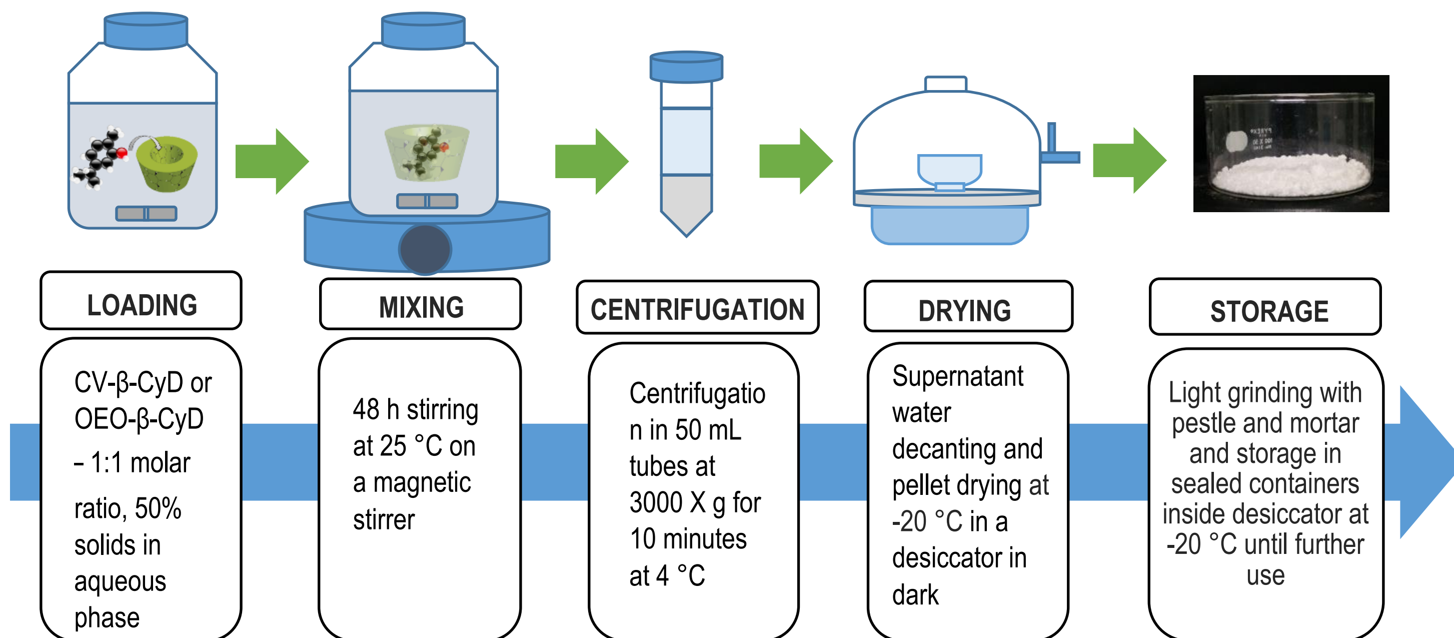
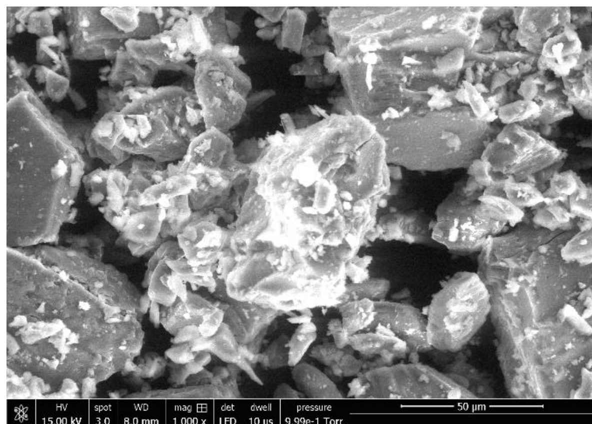
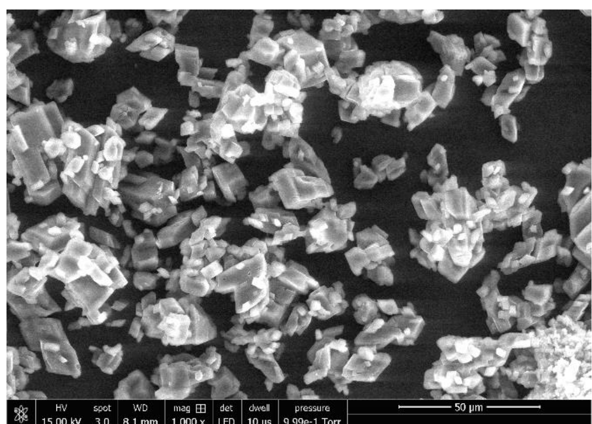


Figure 26. Scanning electron microscope images of (A) β-CyD empty complex, (B) β-CyD-CV and (C) β-CyD-OEO at 1000X magnification.





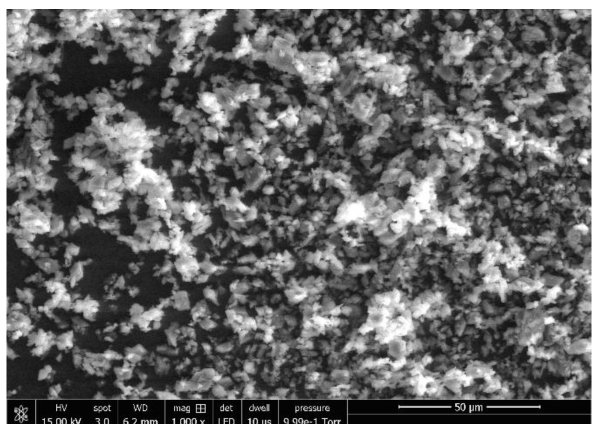


Figure 27. Stability of CV and OEO during the simulated digestion conditions. Data points represent means ± SD

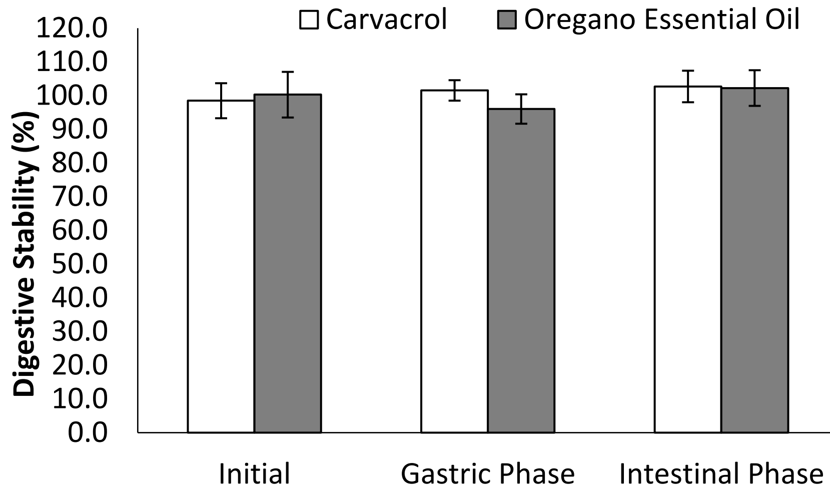


Figure 28. Effect of digestive enzymes on digestive stability of CV and OEO. Data points represent means ± SD.

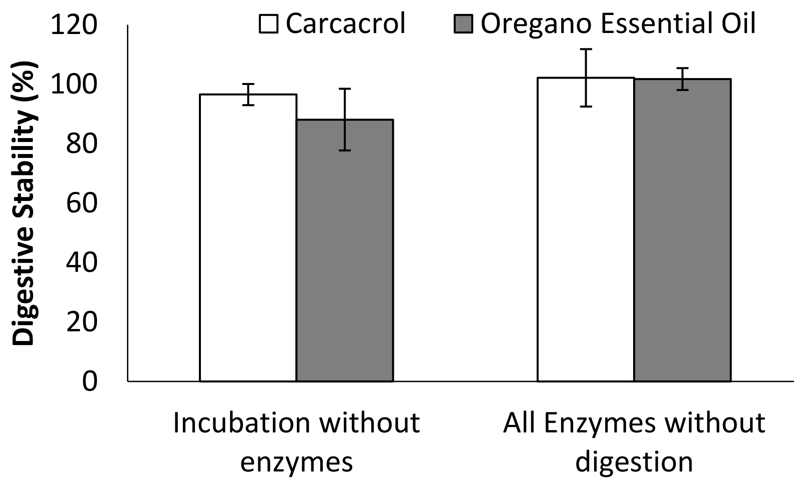


Figure 29. Bioaccessibility of OEO and CV from LNS samples. Data points represent means ± SD. *statistical differences in between treatments within each digestive phase, $p<0.05$.

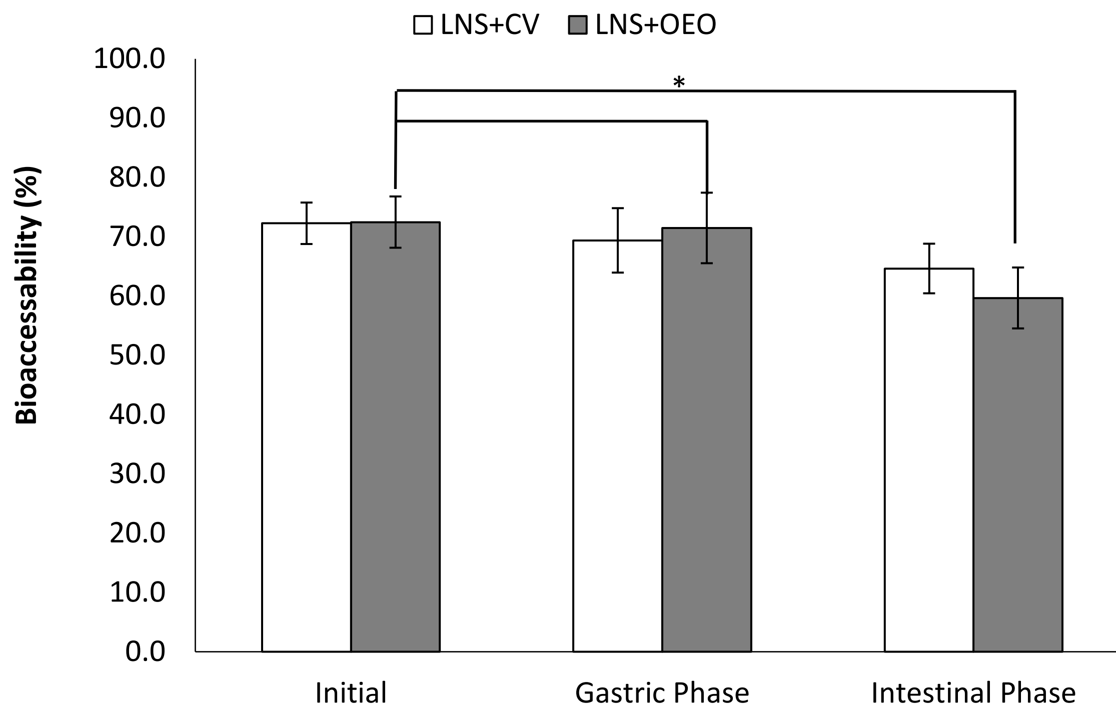


Figure 30. Bioaccessibility of β-CyD-CV and β-CyD-OEO in LNS under *in-vitro* gastro-intestinal digestion conditions. Data point represent mean ± SD. *statistical differences in bioaccessibility between LNS samples containing CV and OEO, $p<0.05$.

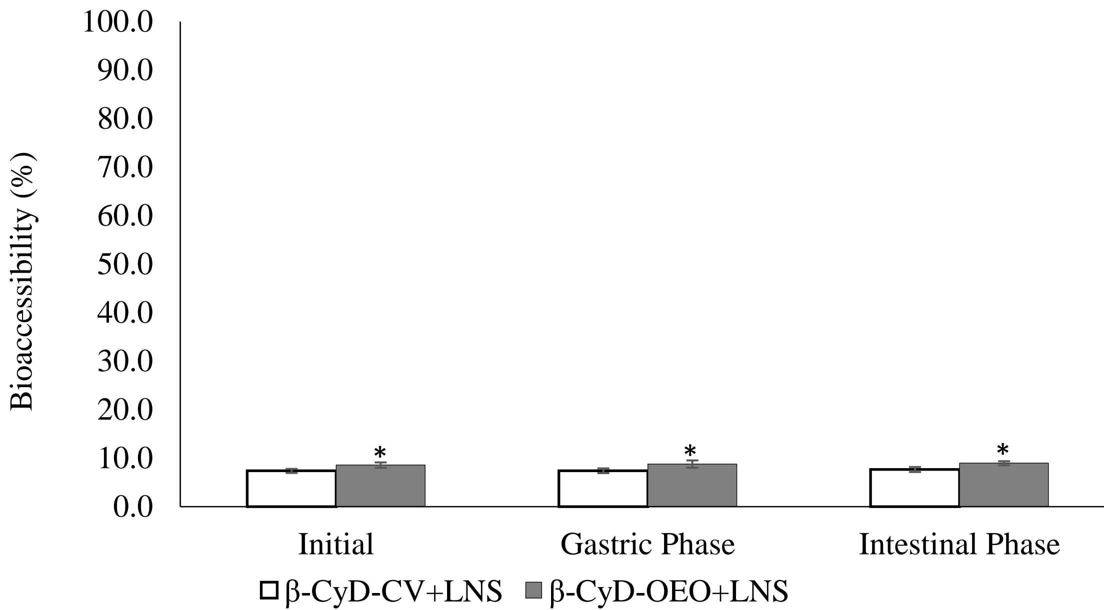


Figure 31. Bioaccessibility of β-CyD-CV and β-CyD-OEO under *in-vitro* gastro-intestinal digestion conditions. Data point represent mean ± SD. *statistical differences in bioaccessibility between β-CyD complexes containing CV and OEO, $p<0.05$.

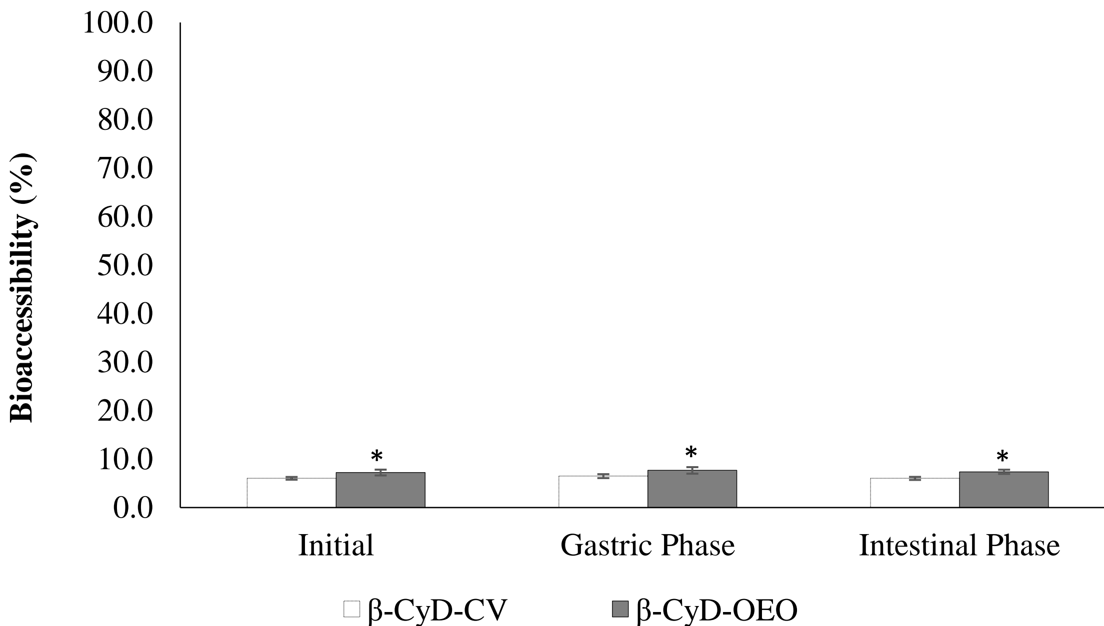
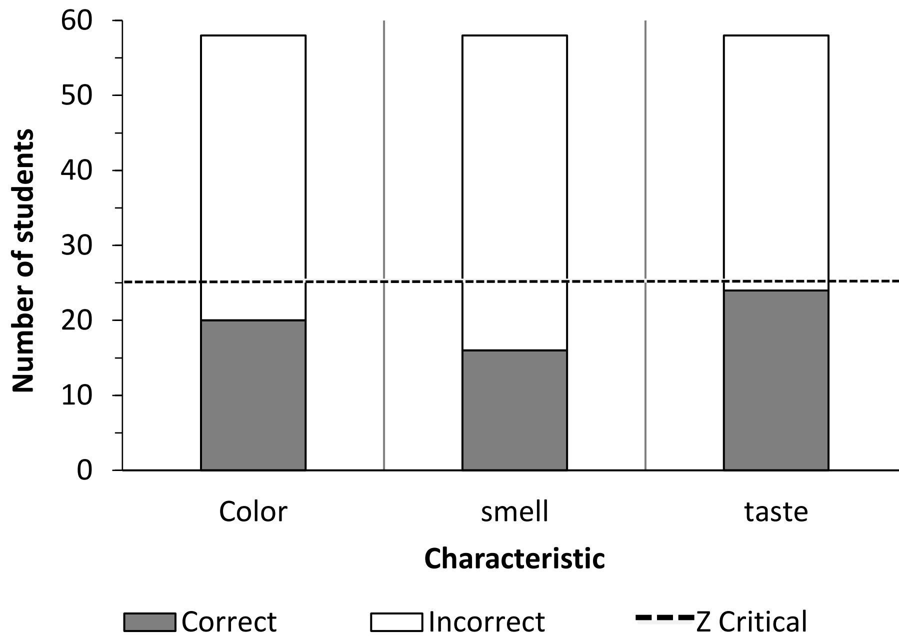
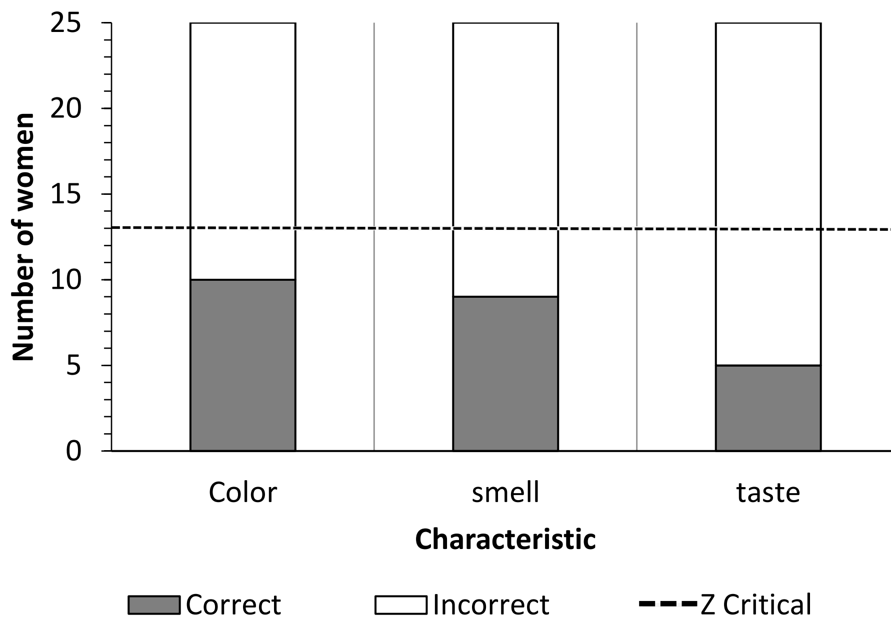


Figure 32. Triangle test results showing no significant differences ($p<0.05$) between LNS with or without β-CyD-OEO in students (n=58) and women (n=25) with young children at home (1-6 years).





5.8 References

Antenucci RN, Palmer JK. 1984. Enzymatic degradation of α- and β-cyclodextrins by Bacteroides of the human colon. 32: 1316–1321.

Arimond M, Zeilani M, Jungjohann S, Brown KH, Ashorn P, Allen LH, et al. 2013. Considerations in developing lipid-based nutrient supplements for prevention of undernutrition: experience from the International Lipid-Based Nutrient Supplements (iLiNS) Project. Matern. Child Nutr. 11: 31–61.

Beirão-da-Costa S, Duarte C, Bourbon AI, Pinheiro AC, Januário MIN, Vicente A a., et al. 2013. Inulin potential for encapsulation and controlled delivery of Oregano essential oil. Food Hydrocoll. 33: 199–206.

Black RE, Allen LH, Bhutta ZA, Caulfield LE, de Onis M, Ezzati M, et al. 2008. Maternal and child undernutrition: global and regional exposures and health consequences. Lancet 371: 243–60.

Can Baser K. 2008. Biological and Pharmacological Activities of Carvacrol and Carvacrol Bearing Essential Oils. Curr. Pharm. Des. 14: 3106–3119.

Chen H, Michael Davidson P, Zhong Q. 2014. Impacts of sample preparation methods on solubility and antilisterial characteristics of essential oil components in milk. Appl. Environ. Microbiol. 80: 907–916.

Choi M-J, Soottitantawat A, Nuchuchua O, Min S-G, Ruktanonchai U. 2009. Physical and light oxidative properties of eugenol encapsulated by molecular inclusion and emulsion–diffusion method. Food Res. Int. 42: 148–156.

Dacremont C, Sauvageot F. 1997. Are replicate evaluations of triangle tests during a session good practice? Food Qual. Prefer. 8: 367–372.

Garrett D, Failla ML, and, Sarama RJ. 1999. Development of an in Vitro Digestion Method To Assess Carotenoid Bioavailability from Meals. J Agric Food Chem. 47(10): 4301-9

EAFUS. 2006. A Food Additive Database. Centre for Food Safety, and Applied Nutrition. Retrieved from http://www.usc.es/caa/EdulcWeb/EAFUS.pdf

Egger RJ, Hofhuis EH, Bloem MW, Chusilp K, Wedel M, Intarakhao C, et al. 1990. Association between intestinal parasitoses and nutritional status in 3-8-year-old children in northeast Thailand. Trop. Geogr. Med. 42: 312–23.

European Parliament and Council. 1996. Regulation (EC) No 2232/96 the European Parliament, and of the Council on 28 October 1996, Commission Decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs.

Farid Z, Patwardhan VN, Darby WJ. 1969. Parasitism and Anemia. Am J Clin Nutr 22: 498–503.

Flores FP, Kong F. 2017. In Vitro Release Kinetics of Microencapsulated Materials and the Effect of the Food Matrix. Annu. Rev. Food Sci. Technol. 8: 237–259.

Force M, Sparks WS, Ronzio R a. 2000. Inhibition of enteric parasites by emulsified oil of oregano in vivo. Phyther. Res. 14: 213–214.

García-Rodriguez JJ, Torrado J, Bolás F. 2001. Improving bioavailability and anthelmintic activity of albendazole by preparing albendazole-cyclodextrin complexes. Parasite 8: S188–S190.

Gaur S, Sloffer EM, Ojha A, Patra F, Shukla D, Engeseth NJ, et al. 2017. Omega-3-Fortified Lipid-Based Nutrient Supplement. Food Nutr. Bull.: 037957211770123.

Gendrel D, Treluyer JM, Richard-Lenoble D. 2003. Parasitic diarrhea in normal and malnourished children. Fundam. Clin. Pharmacol. 17: 189–197.

Guimarães AG, Oliveira MA, Alves R dos S, Menezes P dos P, Serafini MR, Araújo AA de S, et al. 2015. Encapsulation of carvacrol, a monoterpene present in the essential oil of oregano, with β-cyclodextrin, improves the pharmacological response on cancer pain experimental protocols. Chem. Biol. Interact. 227: 69–76.

Guimarães AG, Oliveira MA, Alves R dos S, Menezes P dos P, Serafini MR, de Souza Araújo AA, et al. 2015. Encapsulation of carvacrol, a monoterpene present in the essential oil of oregano, with β-cyclodextrin, improves the pharmacological response on cancer pain experimental protocols. Chem. Biol. Interact. 227: 69–76.

Hill LE, Gomes C, Taylor TM. 2013. Characterization of beta-cyclodextrin inclusion complexes containing essential oils (trans-cinnamaldehyde, eugenol, cinnamon bark, and clove bud extracts) for antimicrobial delivery applications. LWT - Food Sci. Technol. 51: 86–93.

Hirayama F, Minami K, Uekama K. 1996. In-vitro Evaluation of Biphenylyl Acetic Acid-β-Cyclodextrin Conjugates as Colon-targeting Prodrugs: Drug Release Behaviour in Rat Biological Media. J. Pharm. Pharmacol. 48: 27–31.

Hirayama F, Uekama K. 1999. Cyclodextrin-based controlled drug release system. Adv. Drug Deliv. Rev. 36: 125–141.

Hosseini SF, Zandi M, Rezaei M, Farahmandghavi F. 2013. Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: preparation, characterization and in vitro release study. Carbohydr. Polym. 95: 50–6.

Hughes S, Kelly P. 2006. Interactions of malnutrition and immune impairment, with specific reference to immunity against parasites. Parasite Immunol. 28: 577–88.

Hur SJ, Lim BO, Decker EA, McClements DJ. 2011. In vitro human digestion models for food applications. Food Chem. 125: 1–12.

Irie T, Uekama K. 1997. Pharmaceutical Applications of Cyclodextrins. III. Toxicological Issues and Safety Evaluation. J. Pharm. Sci. 86: 147–162.

Kant A, Linforth RST, Hort J, Taylor AJ. 2004. Effect of β-Cyclodextrin on Aroma Release and Flavor Perception. J. Agric. Food Chem. 52: 2028–2035.

Karathanos VT, Mourtzinos I, Yannakopoulou K, Andrikopoulos NK. 2007. Study of the solubility, antioxidant activity and structure of inclusion complex of vanillin with β-cyclodextrin. Food Chem. 101: 652–658.

Kfoury M, Landy D, Ruellan S, Auezova L, Greige-Gerges H, Fourmentin S. 2016a. Determination of formation constants and structural characterization of cyclodextrin inclusion complexes with two phenolic isomers: carvacrol and thymol. Beilstein J. Org. Chem. 12: 29–42.

Kfoury M, Landy D, Ruellan S, Auezova L, Greige-Gerges H, Fourmentin S. 2016b. Determination of formation constants and structural characterization of cyclodextrin inclusion complexes with two phenolic isomers: carvacrol and thymol. Beilstein J. Org. Chem. 12: 29–42.

Kohlert C, van Rensen I, März R, Schindler G, Graefe EU, Veit M. 2000. Bioavailability and pharmacokinetics of natural volatile terpenes in animals and humans. Planta Med. 66: 495–505.

Kohlert C, Schindler G, März RW, Abel G, Brinkhaus B, Derendorf H, et al. 2002. Systemic availability and pharmacokinetics of thymol in humans. J. Clin. Pharmacol. 42: 731–7.

Matsuda H. 1999. Cyclodextrins in transdermal and rectal delivery. Adv. Drug Deliv. Rev. 36: 81–99.

McClements DJ, Decker EA, Park Y. 2009. Controlling lipid bioavailability through physicochemical and structural approaches. Crit. Rev. Food Sci. Nutr. 49: 48–67.

McClements DJ, Li Y. 2010. Review of in vitro digestion models for rapid screening of emulsion-based systems. Food Funct. 1: 32.

Cunnigham-Rundles S, Moon A, Mcneeley DF. 2008. Malnutrition and Host Defense. In: Duggan C, Watkins JB, walker WA eds. Nutrition in Pediatrics: Basic Science and Clinical Application, 4th ed. London: BC: Decker, Hamilton. 261-271.

Meier MM, Drunkler DA, Bordignon Luiz MT, Fett R, Szpoganicz B. 2001. The influence of β-cyclodextrin on goaty flavour - Characterization of synthetic inclusion complexes with capric acid and caprylicacid. Br. Food J. 103: 281–290.

Meilgaard M, Civille GV, Carr BT. 2007. Sensory evaluation techniques. Taylor & Francis.

Menezes PP, Serafini MR, Quintans-Júnior LJ, Silva GF, Oliveira JF, Carvalho FMS, et al. 2014. Inclusion complex of -linalool and β-cyclodextrin. J. Therm. Anal. Calorim. 115: 2429–2437.

Michiels J, Missotten J, Dierick N, Fremaut D, Maene P, De Smet S. 2008. In vitro degradation and in vivo passage kinetics of carvacrol, thymol, eugenol and trans -cinnamaldehyde along the gastrointestinal tract of piglets. J. Sci. Food Agric. 88: 2371–2381.

Miller DD, Schricker BR, Rasmussen RR, Van Campen D. 1981. An in vitro method for estimation of iron availability from meals. Am. J. Clin. Nutr. 34: 2248–56.

Minami K, Hirayama F, Uekama K. 1998. Colon-Specific Drug Delivery Based on a Cyclodextrin Prodrug: Release Behavior of Biphenylylacetic Acid from Its Cyclodextrin Conjugates in Rat Intestinal Tracts after Oral Administration. J. Pharm. Sci. 87: 715–720.

Muniz-Junqueira M, Queiróz E. 2002. Relationship between protein-energy malnutrition, vitamin A, and parasitoses in children living in Brasília. Rev. da Soc. Bras. ….

Northrop-Clewes CA, Rousham EK, Mascie-Taylor CN, Lunn PG. 2001. Anthelmintic treatment of rural Bangladeshi children: effect on host physiology, growth, and biochemical status. Am J Clin Nutr 73: 53–60.

Pinho E, Grootveld M, Soares G, Henriques M. 2014. Cyclodextrins as encapsulation agents for

plant bioactive compounds. Carbohydr. Polym. 101: 121–135.

Roman MJ, Burri BJ, Singh RP. 2012. Release and Bioaccessibility of β-Carotene from Fortified Almond Butter during in Vitro Digestion. J. Agric. Food Chem. 60: 9659–9666.

Saenger W. 1980. Cyclodextrin Inclusion Compounds in Research and Industry. Angew. Chemie Int. Ed. English 19: 344–362.

Santos EH, Kamimura JA, Hill LE, Gomes CL. 2015. Characterization of carvacrol beta-cyclodextrin inclusion complexes as delivery systems for antibacterial and antioxidant applications. LWT - Food Sci. Technol. 60: 583–592.

Seo E-J, Min S-G, Choi M-J. 2010. Release characteristics of freeze-dried eugenol encapsulated with β -cyclodextrin by molecular inclusion method. J. Microencapsul. 27: 496–505.

Shyale S, Chowdhary KPR, Krishnaiah YSR. 2005. Development of colon-targeted albendazole-β-cyclodextrin-complex drug delivery systems. Drug Dev. Res. 65: 76–83.

Songkro S, Hayook N, Jaisawang J, Maneenuan D, Chuchome T, Kaewnopparat N. 2012. Investigation of inclusion complexes of citronella oil, citronellal and citronellol with β-cyclodextrin for mosquito repellent. J. Incl. Phenom. Macrocycl. Chem. 72: 339–355.

Szente L, Szejtli J. 2004. Cyclodextrins as food ingredients. Trends Food Sci. Technol. 15: 137–142.

Tamamoto LC, Schmidt SJ, Lee S-Y. 2010. Sensory Properties of Ginseng Solutions Modified by Masking Agents. J. Food Sci. 75: S341–S347.

Ultee A, Bennik MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. Appl. Environ. Microbiol. 68: 1561–8.

USFDA. 2001. U.S. Food and drug administration. Agency response letter GRAS notice No. GRN 000074. CFSAN/Office Food Addit. Saf.

Wang L. 2008. Helminth infections and intestinal inflammation. World J. Gastroenterol. 14: 5125.

Wang Q, Gong J, Huang X, Yu H, Xue F. 2009. In vitro evaluation of the activity of microencapsulated carvacrol against Escherichia coli with K88 pili. J. Appl. Microbiol. 107: 1781–8.

Yang Y, Kayan B, Bozer N, Pate B, Baker C, Gizir AM. 2007. Terpene degradation and extraction from basil and oregano leaves using subcritical water. J. Chromatogr. A 1152: 262–267.

Chapter 6: Summary and Future Directions

In this dissertation study, considerable progress was made towards developing an LNS 2.0 that could potentially address undernutrition by combining a nutrient dense supplementary food (i.e., LNS) with a functional ingredient that can reduce enteric parasitic infections in Indian children (i.e., carvacrol from OEO).

In the first study, LNS with staple ingredients from India fortified with FO was successfully formulated and optimized using RSM. A four-factor-three-level: omega-3 fatty acid source (flaxseed oil) (0-10%), antioxidant (ascorbyl palmitate: 0.0-0.03%), emulsifier (soy lecithin: 0.5-1.5%), and storage time (0-6 months) model was employed to optimize LNS's functionality and storage stability. The designed LNS formulations yielded approximately 452 kcal, 13.2 g of protein, 30 g of fat, and 53 g of carbohydrates and the optimal levels to maximize storage time and FO, and minimize oxidation were identified as 0.02% antioxidant, 1.5% emulsifier, and 4.9% FO. In addition, the newly developed LNS was found highly acceptable among Indian mothers and students with acceptability scores similar to those of other commercial supplements tested. To our knowledge, this is the first study that used RSM for LNS ingredient and storage stability optimization. A similar methodology could be utilized in future to optimize the existing or new LNS formulations, fortified with other omega-3 fatty acid sources such as fish oil, camelina oil, and algal oil. Also, future LNS formulations could target other at-risk populations such as pregnant and lactating women and elderly groups living in the low- and middle-income countries. Further research on the effect of omega-3 fortified LNS on growth and development of children is warranted.

The second study of this dissertation aimed at identifying the antiparasitic, more specifically, the anticryptosporidial effect of OEO and CV on the invasion and infection of HCT-8 cells using the *in vitro* cell culture model. Neither OEO nor CV modulated the invasion of *C. parvum* sporozoites. Treatment after invasion, however, was found to reduce relative *C. parvum* infectivity in a dose-dependent manner to 55.6 ± 10.4% and 45.8 ± 4.1% at 60 and 30 μg/mL of OEO and CV, respectively. To our knowledge, this was the first study to demonstrate the therapeutic potential of OEO and CV as anticryptosporidial agents (Gaur et al., 2016). Future studies on characterization of these antiparasitic agents in terms of their mechanisms of action *in vitro* and in pre-clinical and clinical models are recommended. Such studies will contribute to our limited knowledge of the use of OEO and CV in foods to treat parasitic infections and will expand our ability to address one of the immediate causes of undernutrition in low-income countries.

In the final study of this dissertation, the author functionalized LNS to contain CV and OEO, and thus, to develop an improved LNS (i.e., LNS 2.0), which could potentially address intestinal parasitic infections in children. This is the first example of a product aimed at addressing both immediate causes of undernutrition in poor settings. Functionalization of LNS with β-CyD-OEO was found feasible based on physicochemical characterization, *in vitro* stability and bioaccessibility, and sensory studies. The inclusion of OEO in β-CyD was found successful in masking the potent flavor of OEO. This was indicated by the discriminatory sensory test results in which students and mothers of children under the age of 5 were not able to discriminate ($p>0.05$) between LNS with and without β-CyD-OEO complexes in terms of color, aroma, and taste. β-CyD-OEO and β-CyD-CV complexes were found stable throughout the oral, gastric and intestinal phases with a limited release of CV in the micellar phase, which was represented by low CV bioaccessibility ranging from 7.2-7.7% and 6.0-6.5%, respectively. This stability and controlled

release characteristics are critical for the targeted delivery of OEO from LNS in the lower gut, where CyD are fermented and broken down by the gut microflora. Future studies are required to establish the *in vivo* controlled release characteristics of OEO from β-CyD-OEO with or without LNS, and to evaluate the effects of functional LNS on parasite and clinical outcomes. Other studies could examine the inclusion of broad spectrum antiparasitic drugs into CyD and their effects using pre-clinical and clinical models.

Appendix A: Institutional Review Board Approval Notice

UNIVERSITY OF ILLINOIS
AT URBANA-CHAMPAIGN

Office of the Vice Chancellor for Research

Office for the Protection of Research Subjects
528 East Green Street
Suite 203
Champaign, IL 61820

May 4, 2015

Juan Andrade
Food Science & Human Nutrition
457 Bevier Hall
905 S Goodwin Ave
Urbana, IL 61801

RE: *Consumer acceptability testing of lipid-based nutrient supplements in India*
IRB Protocol Number: 15778

Dear Dr. Andrade:

Your response to stipulations for the project entitled *Consumer acceptability testing of lipid-based nutrient supplements in India* has satisfactorily addressed the concerns of the UIUC Institutional Review Board (IRB) and you are now free to proceed with the human subjects protocol. The UIUC IRB approved, by expedited review, the protocol as described in your IRB-1 application with stipulated changes. The expiration date for this protocol, IRB number 15778, is 05/03/2016. The risk designation applied to your project is *no more than minimal risk*. Certification of approval is available upon request.

Copies of the attached date-stamped consent form(s) must be used in obtaining informed consent. If there is a need to revise or alter the consent form(s), please submit the revised form(s) for IRB review, approval, and date-stamping prior to use.

Under applicable regulations, no changes to procedures involving human subjects may be made without prior IRB review and approval. The regulations also require that you promptly notify the IRB of any problems involving human subjects, including unanticipated side effects, adverse reactions, and any injuries or complications that arise during the project.

If you have any questions about the IRB process, or if you need assistance at any time, please feel free to contact me at the OPRS office, or visit our Web site at http://www.irb.illinois.edu.

Sincerely,

Anita Balgopal, PhD
Director, Office for the Protection of Research Subjects

Attachment(s)

c: Shashank Gaur

U of Illinois at Urbana-Champaign • IORG0000014 • FWA #00008584
telephone (217) 333-2670 • *fax* (217) 333-0405 • *email* IRB@illinois.edu

Appendix B: Institutional Review Board Approved Consent Form

Appendix 2a.

UNIVERSITY OF ILLINOIS

AT URBANA-CHAMPAIGN

Juan Andrade

Assistant Professor

Dept of Food Sci & Human Nutrition
457 Bevier Hall, MC-187
905 S. Goodwin Avenue
Urbana, IL 61801

Office Telephone No. .217 333-9653
Fax No. .217 244-2455
E-mail jandrade@illinois.edu

Consent form: Women (mothers), Milk Collection Center, Mehsana, Gujarat

Greetings!

My name is Shashank Gaur. I am a doctoral student from the University of Illinois in the United States working in collaboration with MIDFT, Mehsana. I want to help reducing malnutrition in India. We have made new food products for you and your family and our intention is to know how acceptable these products are. For this, you will have to taste the food products and tell us what you think of them based on different characteristics. Before this taste test, you will receive instructions. Next, here are eight important details that you need to know before considering your participation in this research study.

1. **Acceptability testing.** You will taste several food products. This activity can take between 30 to 45 minutes to complete including the initial training.
2. **Confidentiality.** The information you provide will be kept strictly confidential, it will be used for academic research, and it will not identify you directly. Nobody will know your answers to the test.
3. **Voluntary participation.** Participation in this activity is voluntary. You can decline to participate at any time. You can leave this study and place at any time and for any reasons without any penalties. However, we will ask you to let us know if this is the case.
4. **Benefits and Payments.** There are no direct benefits associated with your participation in this study. There will be no payment for your participation in this study. However, we will provide you a gift (stainless steel utensil worth $4) as a token of our thanks.
5. **Risks.** There are no risks associated with this study beyond those that you face daily. Your relationship with any of the institutions involved will not change if you decide to drop out of this study before, during or after.
6. **Costs.** Your participation in this study will have no cost to you.
7. **Photographs:** During the study we will take some individual photographs and group photographs, upon your permission. The individual photos will not show your face. See the examples below:

8. **Consent**. Your participation in this study is very important for us and it will help us understand consumer preferences of our products. If you desire to participate, please write your name at the bottom of the form. You will be provided with a copy of this form for your records.

If you have any questions about this project, please contact:

Contact Persons

Dr. Juan E. Andrade
Assistant Professor and Principal Investigator
Department of Food Science and Human Nutrition
University of Illinois, Urbana-Champaign
Telephone: +01-(217) 333-9653
E-mail: jandrade@illinois.edu

Dr. Ankur Ojha
Assistant Professor
Mansinhbhai Institute of Dairy and Food Technology,
Dudhsagar Dairy Campus, Highway,
Mehsana - 384002
Gujarat
+91 8238430214
E-mail: ankur@midft.com

Mr. Shashank Gaur
Doctoral Student
Food Science and Human Nutrition
University of Illinois Urbana-Champaign
Telephone: +1-(217) 419-8286
E-mail: gaur2@illinois.edu

If you have any questions about your rights as a participant in research involving human subjects, please feel free to contact the University of Illinois Institutional Review Board (IRB) Office at +01-(217) 333-2670 or irb@uiuc.edu. You are welcome to call this number collect if you identify yourself as a research participant.

CONSENT
Please check (X) the box.

I understood the information provided to me and voluntarily consent to participate in this study.	☐
I consent that photographs can be taken during the consumer acceptability study.	☐
I have been given a copy of this consent form.	☐

Print Name

Signature

Date

UNIVERSITY OF ILLINOIS
APPROVED CONSENT
VALID UNTIL
MAY 0 3 2016

Appendix C: Institutional Review Board Approved Consent Form in Gujarati

Appendix 2b.

UNIVERSITY OF ILLINOIS

AT URBANA-CHAMPAIGN

Juan Andrade

Assistant Professor

Dept of Food Sci & Human Nutrition
457 Bevier Hall, MC-187
905 S. Goodwin Avenue
Urbana, IL 61801

Office Telephone No. .217 333-9653
Fax No. .217 244-2455
E-mail jandrade@illinois.edu

સંમતી પ્રત્રક : મહિલાઓ (માતાઓ),દૂધ મંડળીઓ

મહેસાણા – ગુજરાત

નમસ્કાર:-

માર઼ુ નામ શશાંક ગૌર છે. હું અમેરિકા ની યુનિવર્સીટી ઓફ ઈલીનોઈ નો વિદ્યાર્થી છું અને મહેસાણામાં દુધસાગર ડેરીની MIDFT સંસ્થાની સાથે રહીને કામ કરૂં છું. ખાદ્યપદાર્થના ઉપયોગ થી ભારત માંથી કુપોષણ દુર કરવામાં મને રસ છે. આ સંશોધનનો હેતુ કુપોષણથી સંવેદનશીલ લોકોને જરૂરપુંરતું પોષણ મળી રહે તે માટે અમોએ એક પોષણ યુક્ત ખાધપદાર્થ બનાવેલ છે તેની સ્વીકાર્યતા કેટલી છે તે નક્કી કરવાનું છે. આ અભ્યાસમાં તમારે ખાધપદાર્થ ને ચાખવો પડશે અને તેની વિવિધ લાક્ષણીકતાઓને ક્રમ તથા મૂલ્યાંકન કરવું પડશે. ખાદ્યપદાર્થ ની આ સંવેદનાત્મક તપાસ કરવા પહેલા ક્રમાંક તથા મૂલ્યાંકન કસોટી કેવી રીતે કરવાની તે માટેની જરૂરી સૂચનાઓ તમને આપવામાં આવશે. આ સંસોધન અભ્યાસમાં આપની ભાગીદારી નોધાવતા પહેલા નીચે જણાવેલ કેટલીક મહત્વની બાબતો-માહિતીઓ જાણી લેવી જરૂરી છે.

૧.બોર્ડ સ્વીકાર્યતા કસોટી:- તમે કેટલાક ખાધપદાર્થો ને ચાખશો તમારી જડપના આધારે, આ કસોટીને પૂરી કરતા આશરે ૩૦-૪૫ મીનીટ લાગશે. આ સમય માં શરૂઆતમાં ની તાલીમ પણ આપી દેવામાં આવશે.

૨.ગુપ્તતા:- તમે આપેલી માહિતીઓ ચોકસ પણે ગુપ્ત રાખવામાં આવશે. આ માહિતીનો ઉપયોગ શૈક્ષણીક સંશોધન માટે કરવામાં આવશે અને તેના ધ્વારા પ્રત્યક્ષ રીતે આપની ઓળખ છતી થશે નહિ.

૩.સ્વેચ્છિક ભાગીદારી:- આ અભ્યાસમાં સ્વેચ્છિક રીતે ભાગીદારી લઇ શકાય છે. તમે આમાં ભાગીદારી નહિ લેવા માટે ગમેત્યારે મનાઈ કરી શકો છો. તમે કોઈપણ કારણસર આ અભ્યાસ માંથી ખસી સકો છો. ના પડી શકો છો. જેના માટે તમને કોઈપણ પ્રકારનો દંડ થશે નહિ.તેમ છતાં જો આપ આવું કરોતો અમે તમને આમ કેમ કરો છો તે અમને જણાવવા કહીશું.

૪.ફાયદાઓ અને આર્થિક ચુકવણી:- આ અભ્યાસમાં આપની ભાગીદારીથી આપને કોઈ પ્રત્યક્ષ ફાયદાઓ નથી. આ અભ્યાસમાં આપની ભાગીદારી ક જોડાવાથી આપને કોઈપણ જાતનું આર્થિક ચૂકવણું કરવામાં આવશે નહિ.

૫.જોખમો:- આ અભ્યાસના સાથે જોડાવાથી આપને કોઈ જોખમો ક ભય નથી.શિવાય કે આપ જે રોજ બરોજના જીંદગીમાં અનુભવતા હોય. આ અભ્યાસ પહેલા કે તે દરમિયાન કે અભ્યાસ પછી જો આપ એમાંથી પણ જવા માંગતા હોયતો તેમ કરવાથી તમારા કોઈપણ સંસ્થા સાથેના સંબંધોને કોઈપણરીતે અસર કરતુ નથી.

૬.ફોટોગ્રાફ:- આ અભ્યાસ દરમ્યાન તમારી સંમતી બાદ કેટલાક ફોટોગ્રાફ લઈશું. જે વ્યક્તિગત હશે અને અમુક સામુહિક પ્રકાર ના હશે. વ્યક્તિગત ફોટાઓ એવા ખાસ એંગલ થી લેવામાં આવશે જે શુનિચિત કરશે કે તમારો ચહેરો ફોટોમાં દેખાય નહિ. આ માટે નીચેના ઉદાહરણ જુઓ.

૭.ખર્ચ:- આ અભ્યાસમાં જોડાવાથી ભાગીદારી થવાથી તમોને કોઈપણ જાતનો ખર્ચ થવાનો નથી.

૮.સંમતી:- આ અભ્યાસમાં આપની ભાગીદારી ખુબજ અગત્યની/કટોકટીની છે.અમને ઉપભોક્તાના સ્વાદની પસંદગી વિશે ઊંડાણપૂર્વકણી સમાજ અપાશે. આ બાબત અમને અમારી ખાદ્યપદાર્થની બનાવટમાં સુધારા તથા માન્યતા કરવામાં મદદ કરશે. આ ફોર્મના અંતે સહી કરીને આ અભ્યાસમાં આપ ભાગીદાર/જોડાવવા આપની સંમતી આપો છો કે કેમ તે જણાવજો.

આ ફોર્મ ની એક નકલ આપને આપના રેકર્ડ માટે આપવામાં આવશે.

જો આપને કોઈ પ્રશ્ન હોયતો મહેરબાની કરી નીચે જણાવેલ વ્યક્તિઓનો સંપર્ક કરો.

<u>Contact Persons</u>

ડૉ આન્દ્રડે.જુઆન ઈ.
આસીપ્રોફેસેર અને મુખ્ય .
સંશોધક
ડીપાર્ટમેન્ટ ઓફ ફૂડ અને હુમન
નુંટ્રીજન
યુની– ઓફ એલીનોઈ. અરબના
ચેમ્પૈન
ટેલીફોન -૩૩૩.(૨૧૭)-૦૧+-:
૯૬૫૩
ઈ-:મૈલ- jandrade@illinois.edu

ડૉ.અંકુર ઓજા
આસી.પ્રોફેસર
માનસિંહભાઈ ઇન્સ્ટીટ્યુટ ઓફ
ડેરી અને ફૂડ ટેક્નોલોજી,
દૂધસાગર ડેરી કેમ્પસ,હાઈવે,
મેહસાણા-૩૮૪૦૦૨.ગુજરાત
+૯૧ ૮૨૩૮૪૩૦૨૧૪
ઈ-મેલ:- **ankur@midft.com**

મી. શશાંક ગૌર
ડોક્ટરલ વિદ્યાર્થી
ફૂડ સાયન્સ અને હુમન
નુટરીસન યુની.ઓફ
ઈલિનોઈસ અર્બના-ચેમ્પૈન.
ટેલીફોન: +1-(217) 419-8286
ઈ-મેલ: gaur2@illinois.edu

જો આપને મનુષ્યો ને સેલ કરતા સંશોધનો માં આપ સહભાગી થતા આપના હક્કો વિશે કોઈપણ પ્રશ્ન હોય તો આપ યુનિવર્સિટી ઓફ ઈલીનોઈ રીસર્ચ રીવ્યુ બોર્ડ (IRR) ની ઓફીસ ઉપર +01 – (214) 333 – 2670 અથવા IRB@UIUC.EDU. ઉપર સંપર્ક કરી શકો છો. જો આપ આપની જાતને સંશોધનના સહભાગી હો તો આ નંબર ઉપર સંપર્ક કરવા આવકારીએ છીએ

સંમતી

હું ઉપર જણાવેલ બધી વિગતો સમજુ છું. અને સ્વેચ્છાએ થી હું આમાં ભાગ લેવા સંમતી આપું છું.	☐
હું અભ્યાસ દરમિયાન ફોટા પડાવવાની સંમતી આપુ છું.	☐
મને આ સંમતી ફોર્મ ની એક નકલ આપવામાં આવી છે.	☐

નામ:

સહી:-

તારીખ:-

UNIVERSITY OF ILLINOIS
APPROVED CONSENT
VALID UNTIL
MAY 03 2016

Appendix D: Demographic Information Questionnaire

Please read the following questions and mark the option that represent your situation.

1. What is your age in years?

   a) Less than 18 ☐
   b) 18 – 24 ☐
   c) 24 – 30 ☐
   d) 30 – 45 ☐
   e) Above 45 ☐

2. What is your occupation (check all that apply)?

   a) Farming ☐
   b) Cattle rearing ☐
   c) House wife ☐
   d) Office worker (factory, Anganwadi, hospital, post office, etc.) ☐
   e) Other ☐

3. How many children do you have?

   a) 1 ☐
   b) 2 ☐
   c) 3 ☐
   d) 4 ☐
   e) More than 4 ☐

4. What is the age of your youngest children?

   a) 0 to 3 years ☐
   b) 4 to 8 years ☐
   c) 9 to 13 years ☐
   d) More than 13 years ☐
   e) More than 4 ☐

5. For the most part of the day, who feeds the younger children at home?

   a) You ☐
   b) Your husband ☐
   c) Your older children ☐
   d) Your parents in-law ☐

e) Other relatives ☐

6. Does your family feed any supplementary food to the children?

a) Yes ☐ If YES, complete the questions below
b) No ☐ If NO, finish this questionnaire

6. 1. If yes, what kind of supplementary food is given to children at home?

a) Homemade supplementary food ☐
b) Commercial supplementary foods (Amulspray, Cerelac, Lactogen, Nestogen etc.) ☐

6.2. What ingredients do you use to make supplementary food for children at home? Check all that apply.

a) Grains (e.g. rice, wheat, millet etc.) ☐
b) Pulses (e.g. chana dal, urad dal, moong dal etc.) ☐
c) Fat (e.g. ghee, oil, dalda etc.) ☐
d) Fruits (e.g. banana, mango, apple etc.) ☐
e) Sugar (e.g. white, brown etc.) ☐
f) Salt ☐
g) Spices (curry, oregano, etc.) ☐

--x-------------------END-------------------x---

Thank you for filling the questionnaire. Please submit the form and collect the receipt.

Note: The receipt contains important information on date, time and venue for test. Please bring it with you on the test day.

Appendix E: Template for Participant Receipt

CONSUMER ACCEPTABILITY TEST

(To be provided to the subject after signing the consent form)

Unique Identification Code	[insert three digit code]
Date of Test	[insert date]
Time of Test	[insert time]
Venue	[insert venue]

Questions? Contact Mr. Shashank Gaur at ___________________________ [local phone number]

____

Authorized Signature

Thank you for your participation!

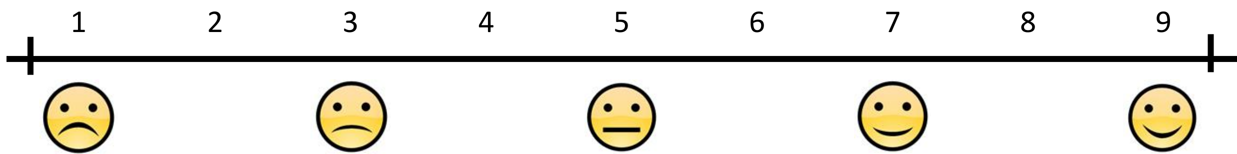
Appendix F: Ballot Template for Consumer Acceptability Test

Directions: Place the given cup in front of you and evaluate it based on the criteria below using the scale provided (1 to 9)

Sample number: _____________

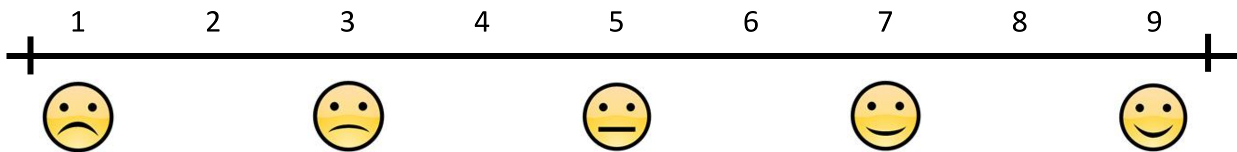
To start, look at the cup XXX and rate how much you like its COLOUR.

COLOUR



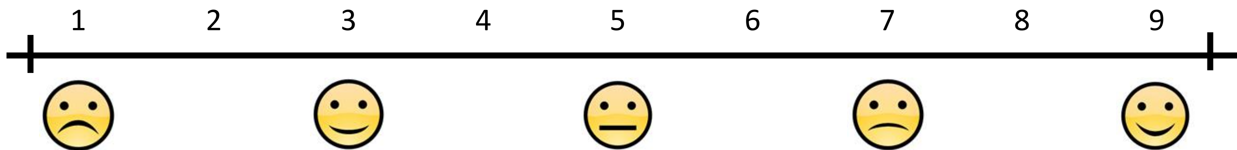
Now, bring the sample to your nose and rate how much you like its SMELL.

SMELL



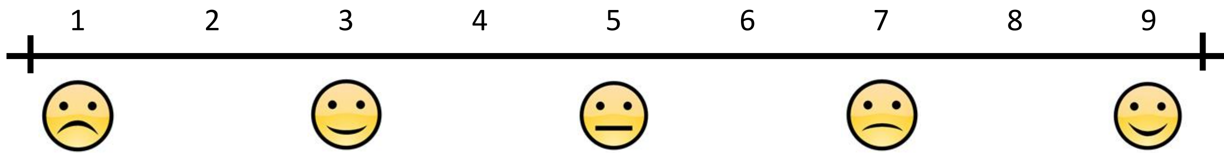
Now, place a spoonful of product in your mouth and rate how much you like mouthfeel.

MOUTHFEEL



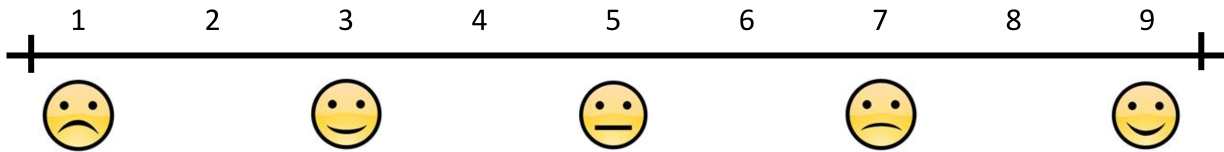
Please rate how much you like its SWEETNESS.

SWEETNESS



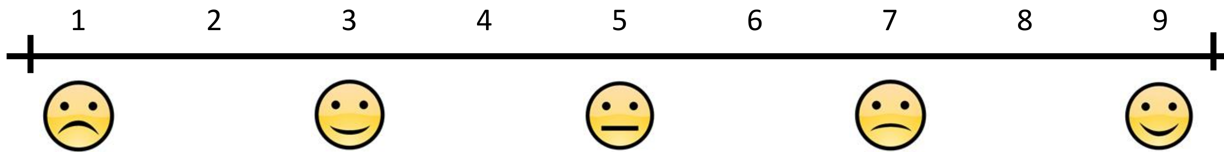
Please rate how much you like its APPEARANCE

APPEARENCE



Rate the product in terms of overall ACCEPTABILITY.

OVERALL ACCEPTABILITY



Now, please eat the cracker provided to you. Rinse your mouth and spit in the disposable cup.

Appendix G: Ballot Template for Ranking Test

Directions: You are presented with four cups. Taste product from each cup and rank them excellent, good, fair or bad based on your overall acceptability. Please use tick marks (✓)

Sample number	Excellent	Good	Fair	Bad
453				
334				
221				
515				

Thank you!

Please submit the forms.

Appendix H: Ballot Template for Discriminatory Sensory Tests (Triangle Tests)

Please select the sample which is different based on color

Participant no. ___________

---------------------✂--------------------cut here----------------✂---------------------------

Participant no. ___________

Please select the sample which is different based on smell

---------------------✂--------------------cut here----------------✂---------------------------

Participant no. ________

Please select the sample which is different based on taste