UNIVERSITY OF COPENHAGEN

Master's thesis in Human Nutrition

Acceptability of cricket-based biscuits and assessment of gut microbiota composition in schoolchildren. A study in Bondo, Kenya

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Preface

This report represents my master thesis, with which I complete my degree as M.Sc. in Human Nutrition at the Department of Nutrition, Exercise and Sports at the University of Copenhagen. The thesis accounts for 45 ECTS and is carried out in the period from 01.01.2015 to 30.09.2015.

All primary data collection and analysis was performed by myself. Exceptions are DNA sequencing, performed by Department of Environmental Science, Aarhus University, Roskilde, and primary trimming and analysis of the sequenced data, performed by postdoc Lukasz Krych from Department of Food Science, University of Copenhagen.

GREEiNSECT is an international project that includes different private and public partners and organizations around the world ("GREEiNSECT," 2014). This particular study involves the department of Nutrition, Exercise and Sports (NEXS) at University of Copenhagen and Jaramogi Oginga Odinga University of Science and Technology (JOOUST) in Bondo, Kenya. Nutritional guidance was also provided by GREEiNSECT partners in Nairobi: Dr. Victor Owino from Technical University of Nairobi and Dr. John Kinyuru from Jomo Kenyatta University of Agriculture & Technology. GREEiNSECT is sponsored by Danida, the development cooperation under the Danish Ministry of Foreign Affairs. Sponsorships for the present study were awarded by Danida Fellowship Center travel grant, PLAN Danmark, the Danish Nutrition Society, and the Augustinus foundation.

The study was carried out in collaboration with Nyakasumbi primary school adjacent to JOOUST.

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Last but definitely not least, I want to thank my supervisor, Dr. Nanna Roos. Good discussions, great advice, smiles and encouragement in person and via email made the whole process possible.

Abstract

BACKGROUND: Children in Kenya have a high risk of undernutrition. House crickets (*Acheta Domesticus)* are rich in many nutrients important for growth and development. Including crickets in products for school feeding programs could prevent undernutrition.

OBJECTIVES: The objectives of this study were to develop a biscuit based on cricket powder nutritionally suited for growing children in Kenya and to assess acceptability of the proposed biscuit as well as the effect on the microbiota composition.

METHODS: The study was a randomized, parallel study. Fifty-four children aged 5-10 years were served 98-102 g biscuits containing either 10 % cricket powder (intervention) or 10 % milk powder (control) during school days for four weeks. At baseline, anthropometry (weight, height, and mid upper arm circumference) was performed and information on prior insect consumption collected. Daily, measures of consumption (weight of biscuits eaten), consumption of breakfast, measures of hesitation and refusal to eat, and morbidity (diarrhea, nausea, and vomiting) were taken. Weekly, hedonic ratings were performed. At the end of the trial, bodyweight was measured and one stool sample was collected per child. Microbiota composition was determined by high throughput sequencing in Denmark.

RESULTS: The cricket biscuit contains linoleic acid, complete protein, vitamin A and B12, iron, and zinc, deficiencies of which are important public health concerns in Kenya. Consumption was 96.9 % and 94.2 % for cricket and milk biscuits (p=0.14), respectively. Hedonic ratings were significantly lower in cricket biscuits for looks (p=0.006), smell (p=0.04), texture (p=0.02), and overall (p=0.01) compared to milk biscuits. No change in microbiota composition was seen between the groups.

CONCLUSION: The biscuits contribute with many macro- and micronutrients important for a growing child in a developing country. The acceptability of the cricket biscuits is high and long-term based on set criteria (>75 % eaten >75 % of the days). Organoleptic properties are rated above average, but further development of the biscuits could possibly increase ratings. The microbiota composition did not change between intake of crickets or milk.

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Abbreviations

AI – adequate intake

AMDR - acceptable macronutrient distribution range

ANOSIM - analysis of similarities

ANOVA – analysis of variance

ASF – animal-source food

BAZ – BMI-for-age z-score

DALY – disability-adjusted life year

DNA – deoxyribonucleic acid

FDR – false discovery rate

HAZ – height-for-age z-score

HIV - human immunodeficiency virus

JOOUST – Jaramogi Oginga Odinga University of Science and Technology

KEBS – Kenyan Bureau of Standards

MAM – moderate acute malnutrition

MUAC – mid upper arm circumference

OTU – operational taxonomic unit

PCoA - principal coordinate analysis

PCR – polymerase chain reaction

QIIME – Quantitative Insight Into Microbial Ecology

RNI – recommended nutrient intake

rRNA – ribosomal ribonucleic acid

RUTF – ready-to-use therapeutic food

SAM – severe acute malnutrition

SCFA – short-chain fatty acid

SD – standard deviation

SE – standard error

WFP – World Food Programme

WHO – World Health Organization

WHZ – weight-for-height z-score

Introduction and background

Undernutrition is a major public health concern in Kenya, especially in children. Poor dietary quality in childhood lead to physical and psychological retardation. Society is affected when ability to work is compromised and demand for health care is increased. Prevention and treatment of undernutrition include school feeding programs and ready-to-use therapeutic foods (RUTF) with high-energy foods rich in important nutrients not present in the daily diet of most children. Insects can contribute to the daily diet with several important nutrients. House crickets (*Acheta Domesticus)* can be reared locally providing income for local farmers. Microbiota composition is important for health and metabolism, and can possibly change with dietary alterations.

Undernutrition

Definition

Undernutrition is a vicious cycle of poor dietary intake and infection, which affects each other in a negative feedback loop. Underlying causes are many and complex. Anthropometric indices are used to define cutoffs for undernutrition. Z-scores are created from the World Health Organization (WHO) growth standards (2006) for 0-5 year olds and WHO growth references (2007) for 5-19 year olds. The growth standards are based on the growth of children receiving recommended feeding and care, whereas the references are based on the description of growth of a certain population at a given time and place. Z-score cutoffs for moderate and severe undernutrition are <-2 standard deviation (SD) and <-3 SD, respectively. Additionally, mid upper arm circumference (MUAC) is a good indicator of undernutrition. MUAC is correlated to muscle mass, which could explain why MUAC is a good predictor of death (Briend, Garenne, Maire, Fontaine, & Dieng, 1989).

Undernutrition can be either chronic or acute. The prominent feature of chronic undernutrition is stunting. In order to survive, the body compromises linear growth. The specific mechanisms are unknown. Height-for-age z-scores (HAZ) determine stunting. Acute undernutrition is divided into moderate (MAM) and severe (SAM) and is referred to as wasting. In children under 5 years, weight-for-height z-scores (WHZ) and a MUAC below 115 mm determine wasting. In children above 5 years BMI-for-age z-scores (BAZ) determine wasting, and MUAC cutoffs have been proposed to be 129 mm for children 5-9 years and 160 mm for 10-13 years (WHO, 2009b). Bilateral edema indicates SAM regardless of other measures.

Micronutrient deficiencies are referred to as ‘the hidden hunger’. The four most prevalent deficiencies worldwide are vitamin A, iron, iodine and zinc. Deficiencies in iron and zinc lead to conditions of anemia, growth failure, and death if severe. Vitamin A deficiency can lead to eye disorders and blindness, and

defects in growth and immune function. Iodine deficiency during infancy and childhood can result in goiter, mental retardation, and cretinism. Kenyan children have previously been shown to suffer from high rates of vitamin B12 deficiency (Neumann et al., 2003). Animal-source food (ASF) (e.g. meat, fish, dairy products, and eggs) is the only source of vitamin B12. The vitamin is important for deoxyribonucleic acid (DNA) synthesis among other things. Deficiency therefore causes symptoms affecting the whole body including fatigue, anemia, disturbed vision, and mental disorders.

Using 24-hour recall interviews, it has been shown that Luo[1] adults in Bondo district (Nyanza province) have a very low intake of ASF and a very high intake of cereals and grain products (Hansen et al., 2011). In other words, dietary diversity is low in the region around Lake Victoria indicating that undernutrition is prevalent.

Prevalence

Table 1 shows the prevalence of undernutrition worldwide, in Kenya, and in Nyanza province. Data is from 2012 worldwide and 2009 Kenya (Kenya National Bureau of statistics and ICF Macro, 2009; UNICEF, WHO, & World Bank, 2012).

Table 1 Prevalence of undernutrition recorded as stunting, wasting and underweight in children under 5 years worldwide (2012), in Kenya, and in Nyanza province (2009).

	Worldwide	Kenya	Nyanza province
Stunting	25 %	35 %	31 %
Wasting	8 %	7 %	4 %
Underweight	15 %	16 %	11 %

The prevalence of human immunodeficiency virus (HIV) in Nyanza province is 13.9 %, which by far is the highest in Kenya, with a country mean of 6.3 %.

The prevalence of micronutrient deficiency can be estimated in terms of the impact on human health. WHO has estimated disability-adjusted life years (DALYs), i.e. years lost to disability and disease, in children under 5 years in low-income settings. DALYs are 1.6 % for iron deficiency, 2.4 % for vitamin A deficiency, 1.7 % for zinc deficiency, and 0.2 % for iodine deficiency out of total global DALYs (WHO, 2009a). Childhood undernutrition is together with maternal and fetal undernutrition a great burden worldwide and is estimated to be the cause of 3.1 million child deaths annually (Black et al., 2013).

Prevention and treatment of undernutrition

Nutritional prevention of undernutrition in children under 5 years focuses on the promotion of complete breastfeeding until 6 months and continued breastfeeding until 2 years or later (Bhutta et al., 2013).

[1] The tribe in the region around lake Victoria

Another area of interest is to optimize weaning foods to include all nutrients the child needs. For children above 5 years, school feeding programs are a widespread type of nutritional prevention of undernutrition (WFP, 2013).

Nutritional treatment of undernutrition is performed with therapeutic foods in either an in- or outpatient manner. Prevention and treatment of illnesses concurrently is necessary to combat undernutrition.

School feeding programs

The incentive for school feeding programs is to increase both school attendance and the nutritional status of primary school children by making it economically attractive for parents to send their children to school. It is hypothesized that growth and cognition is optimized in the population, and society as a whole will benefit. School feeding programs have been implemented in Kenya for 3 decades and are in transition from World Food Programme (WFP)-assisted to government-run programs (Bundy et al., 2009).

School feeding programs are divided into two groups: On-location meals or take home rations. Take home rations normally consist of country specific staples such as rice and oil, often fortified. The ration is meant as a supplement for the whole family and the school acts as a distributor. On-location meals are usually hot meals or fortified biscuits served throughout the school day. It is estimated that fortified biscuits are the most cost-effective school meal and increase micronutrient status best (Gelli, Al-Shaiba, & Espejo, 2009).

School feeding programs have shown to increase energy and nutrient intake, measures of anthropometry and micronutrient status (Adelman, Gilligan, & Lehrer, 2008). Besides increasing the nutritional status of the children, it is proposed that school attendance and enrollment increase, especially for the girls and hereby contribute to gender equity. School attendance has been shown to increase with school feeding in Kenya (Omwami, Neumann, & Bwibo, 2011). Adelman et al. (2008) finally conclude that impact of school feeding programs is greatest in societies where school enrollment and attendance and/or nutritional status is low, which is the case in Kenya.

A large school feeding study in Kenya has shown that ASFs have a great impact on growth, physical activity and cognition (Grillenberger et al., 2003, 2006; McLean et al., 2007; Neumann, Murphy, Gewa, Grillenberger, & Bwibo, 2007; Siekmann et al., 2003). The trial compared meals that included milk or meat compared to an isocaloric control without ASF and a control group that received no meal. Although biochemical analyses only revealed an improvement in vitamin B12 status, the children receiving ASF improved significantly compared to both control groups in most aspects, thereby indicating an important role for ASF in growth and development.

A transition from imported to local produce is recommended by WFP. Establishment of 'home-grown school feeding' is described in depth elsewhere (Espejo, Burbano, & Galliano, 2009). WFP states that sustainable nutritious foods are needed from a local source and should be based on local taste.

Therapeutic foods

Therapeutic foods are used in the treatment of SAM and recently of MAM (Lenters, Wazny, Webb, Ahmed, & Bhutta, 2013). The golden standard for treatment of SAM is F-75 and F-100. Both products are milk-based and the names refer to their energy content (75 and 100 kcal per 100 ml, respectively). F-75 is used as initial treatment to avoid refeeding syndrome in SAM patients. Refeeding syndrome is metabolic disturbances that occur with reintroduction of nutrients in starved individuals, but the mechanism behind the syndrome is unclear. When metabolically stable, the patient is switched to F-100. Both products are powders to be mixed with clean water.

RUTF is a special group of high-energy dense foods developed for treatment of uncomplicated SAM, i.e. SAM patients with good appetite and no complicated disease. RUTFs have also been used in the treatment of MAM (Lenters et al., 2013). It is recommended that the food is soft or crushable, since RUTF is mainly fed to small children and babies. Examples of RUTF products are Plumpy'Nut® and BP-100™. The recommended nutrional profile of the products is similar to F-100 with the addition of iron (WHO, WFP, UNSCN, & UNICEF, 2007) (appendix 1). Most therapeutic foods have the same four core ingredients: Oil, sugar, a micronutrient supplement, and skimmed milk powder. They have a low water content (<2.5%) in order to increase shelf life by preventing bacterial growth. Instead, lipids bind the products together and increase the energy density significantly. Because no preparation is necessary, they are distributed to SAM patients in an outpatient manner. Community treatment has several advantages. The patients have a lower risk of infection than in a hospital setting and the patient and family can continue their normal life without risking their livelihood due to long distances to hospitals in low-income settings. In addition, the lower cost per child also allow treatment of more patients.

Substitution for milk powder in prevention and treatment of undernutrition

Milk powder is highly nutritious, shelf-stable, and is an easy way of providing ASF to children in areas with regular low consumption of meat. It is stated in RUTF guidelines that at least half the protein in a RUTF should come from milk (WHO et al., 2007). Milk powder is commonly produced in industrialized countries and transported to low-income countries. However, in recent years, it has been tested whether local ingredients can displace milk powder to increase local revenue and minimize overall costs including transportation.

Legumes have been proposed as a good local nutrient source. The originally developed RUTF product Plumpy'Nut® is produced in France by the inventing company Nutriset, but can easily be produced on a local basis elsewhere (Manary, 2006). In this product, part of the milk powder ration is substituted with peanuts. However, peanut-based products are at risk of aflatoxin contamination. In general, introducing legumes decreases energy density due to the high fiber content, antinutrients are introduced, and they have a low content of absorbable phosphorus.

Insects have been proposed to substitute milk. Insects are in a dietary context ASF. ASFs are not only rich in both macro- and micronutrients, but the nutrients are also highly bioavailable compared to a plant-based diet. This means that the content of antinutrients such as phytic acid is low and the nutrients are more easily absorbed. A good example is heme iron from meat which has a much larger absorption rate (15-35 %) than non-heme iron (2-20 %) (Monsen, 1988). Other components in meat can further enhance absorption, such as the meat factor. Bioavailability of nutrients and presence of meat factor in insects are currently unknown. In the WinFood project, termites were utilized in a weaning food product for prevention of undernutrition with promising results (Konyole et al., 2012). The WinFood Classic product contained 10 % termites (*Macrotermes Subhylanus*) and 3 % Dagaa fish (*Rastrineobola Argentea*). Mothers assessed organoleptic qualities and their children's reactions towards the food and the product was evaluated to be as acceptable as the conventional milk-containing product CSB+.

Why eat insects?

In general, numerous insects have been recorded as edible throughout the world. Fig. 1 shows the most recently updated list (01.06.2015) from Wageningen University in the Netherlands with more than 2,000 species documented.

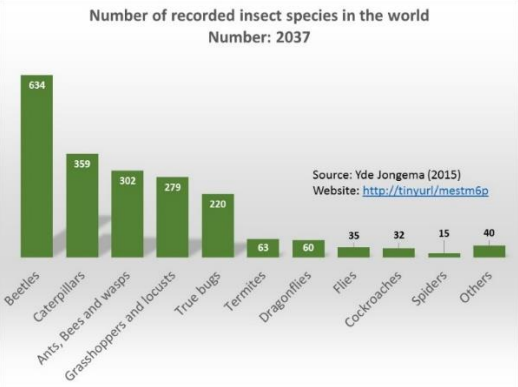


Figure 1 Recorded edible insects species ("List of edible insects of the world (June 1, 2015)," 2015)

Palatability

The palatability of a food is one of the main reasons why we want to eat it (Yeomans, 1996). Furthermore, the detection of deliciousness appears to control food intake through both the satiety and reward system in the brain thereby enhancing overall food consumption (Sørensen, Møller, Flint, Martens, & Raben, 2003).

In general, insects are variable in organoleptic properties and can vary from soft to crunchy, acidic to fatty. The knowledge on palatability of insects has so far been informal. Nordic Food Lab is a non-profit organization that runs the research project "Deliciousness as an Argument for Entomophagy" ("Nordic food Lab," 2013). Through their research, they discover different molecules in insects that are palatable and subsequently optimize their flavor by different cooking methods. Glutamate and formic acid are among the components they have highlighted (Evans, 2014).

Food security

Access to food is not a given in many parts of the world. Drought, floods, political instability and more can have large impacts on the availability of food. Food supply variates throughout the year and infrastructure greatly influences the population's access. Insects can contribute to food security since they thrive in circumstances where other nutrient-dense foods do not. The GREEiNSECT project proposes domestication of crickets, which has the possibility of improving the availability of edible insects compared to traditional measures of capturing insects in the wild seasonally. Incorporating insects into regularly consumed food products can increase intake compared to the traditional way of eating most insects as a snack. In situations of food insecurity, incorporation of insects into food supplements for prevention and treatment of undernutrition can possibly reduce the prevalence of undernutrition in vulnerable groups.

Environment

A growing global population and an increasing demand for livestock production generate a burden for the environment. Crickets are highly preferable compared to the major livestock animals such as poultry, pigs and cattle for two main reasons. First, feed conversion rates[2] are much lower. According to Van Huis (2013), it only takes 1.7 kg of feed to produce 1 kg of cricket, whereas the same amount for chicken, pork and beef is approximately 2.5, 5, and 10 kg, respectively. In addition, the amount that is edible of the cricket is much larger than any of the three other types of livestock, amounting to a 12-fold difference in feed conversion rates between cricket and cattle (van Huis, 2013). However, there is still doubt about how efficient crickets are at converting feed to biomass (Lundy & Parrella, 2015).

[2] How much feed to give in order to obtain a kg of livestock.

Secondly, the emission of greenhouse gasses from insect rearing has been assessed to be much lower than from conventional livestock production under experimental conditions. In general, livestock is calculated to be responsible for 18 % of greenhouse gas emissions globally (Steinfeld et al., 2006). As crickets do not emit CH_4, a highly potent greenhouse gas, and only emit low levels of CO_2 and N_2O, rearing of crickets and possibly other insects could be a part of a sustainable solution to the climate crisis (Oonincx et al., 2010). Furthermore, Oonincx provided evidence that crickets are favorable to other forms of livestock in emission of the indirect greenhouse gas ammonia.

The use of water in cricket rearing can possibly be much lower than traditional livestock, which can greatly increase food security in dry regions of the world. No research has been published up until now on this matter.

Entomophagy in Lake Victoria region of Kenya

The Luo people of the Lake Victoria region have a long history of entomophagy, i.e. consuming insects. However, Western dietary culture has expanded to the region and has had a great impact on especially younger generations. They do not consider insects as a source of food anymore, adopting the 'disgust factor' of the West (Ayieko & Oriaro, 2008).

Species that are consumed seasonally in the region include the lakefly, longhorn grasshopper, ants, crickets and termites (Ayieko & Oriaro, 2008; Christensen et al., 2006; Kinyuru, Kenji, Muhoho, & Ayieko, 2010). Among these, the termite is by far the most common. Termites are harvested from the wild during the long rains (February – April) when they come out of their nests for mating. Harvesting also occurs to a lesser degree in the dry season by beating the ground around the nest to simulate heavy rain (Christensen et al., 2006; van Huis, 2003). Termites have not yet been successfully reared in captivity, which inhibits the use of termites as a sustainable food source in food security. During the WinFood project researchers experienced production issues due to low availability of termites (Owino et al., 2015).

Food safety policies in Kenya currently state that indigenous foods (including insects) are allowed for consumption, but not for trade unless approved by Kenya Bureau of Standards (KEBS) (Halloran, Vantomme, Hanboonsong, & Ekesi, 2015).

Previous research in insect-based food products in Lake Victoria region

Previous experiments in the Lake Victoria region with incorporation of insects into processed food products have mainly focused on termites and lakeflies. Kinyuru et al. (2009) incorporated termite flour into wheat buns at different percentages with promising results. Ayieko et al. (2010) incorporated termites and lakeflies into various products: crackers, muffins, sausages and meat loaf. Although sausage and meat loaf

were not considered good food vectors of these insects, crackers and muffins were acceptable to the test subjects. Based on these results, the biscuit was chosen as food vector for the present study. Furthermore, the low water content and activity of biscuits would ensure longer shelf life for the product.

Rearing of crickets in Kenya – GREEiNSECT

Orthoptera (grasshoppers, locusts and crickets) is one of the orders of insects most commonly eaten by people worldwide (van Huis et al., 2013). The house cricket is the insect of choice to be investigated for human consumption in the GREEiNSECT project. The project aims to develop farming and business strategies for small, medium, and large-scale production of insects for food and feed ("GREEiNSECT," 2014). The main reason for the choice is that the female cricket lays eggs continuously throughout the year in high amounts after mating once (Murtaugh & Denlinger, 1985).

Nutritional profile of crickets in comparison to whole milk powder

Several publications of the nutrional composition of crickets are available (Mark D. Finke, 2002, 2007; Rumpold & Schlüter, 2013). The values presented are close to the values declared for the cricket powder from Thailand used in this study. It is worth noting, that the nutritional value of crickets changes throughout life stages. The information stated here and used in further calculations is derived from adult crickets. In table 2 below, nutritional information is shown for cricket powder and whole milk powder along with the recommended nutrient intake (RNI) and acceptable macronutrient distribution range (AMDR) for children 5-10 years (FAO, WHO, & UNU, 2001; Institute of Medicine of the National Academies, 2005; WHO & FAO, 2004). All information about the cricket powder is from the supplier unless indicated. Information about whole milk powder is from the online Danish food composition databank FoodComp ("FoodComp," 2009). Whole milk powder was used instead of skimmed milk powder in the trial, as it was available locally.

A public health concern in Kenya is low intake of essential amino acids, polyunsaturated fats, vitamins, and minerals. Crickets have a high content of complete protein (including all nine essential amino acids in adequate amount) and the quality is superior to soy when fed to rats (M D Finke, DeFoliart, & Benevenga, 1989). Crickets are high in unsaturated fatty acids. 60 % of the fatty acid content is unsaturated of which most is linoleic acid (18:2, n-6) (Grapes, Whiting, & Dinan, 1989). The dietary fiber in cricket is mainly derived from chitin. Chitin is a polymer of *N*-acetylglucosamine and is the second most abundant biopolymer after cellulose. The exoskeleton of insects consists mainly of chitin bound to minerals and proteins. Chitin is proposed to be indigestible by humans and therefore function as a dietary fiber. Nonetheless, human chitinases have been found (Paoletti, Norberto, Damini, & Musumeci, 2007). Milk

powder contains complete protein, albeit only half the amount as cricket. Milk powder has very low amounts of polyunsaturated fatty acids and no dietary fiber.

In terms of micronutrients, crickets have high contents of iron and zinc. Vitamin A is not present in crickets. Milk, on the other hand is high in vitamin A, but low in both iron and zinc. Iodine content is low in both milk and cricket. Crickets contain 40 % more vitamin B12 than milk, but content in both is high.

Table 2 Nutritional information for cricket powder, whole milk powder and RNI/AMDR

Nutrient	**Cricket** **Amount per 100 g**	**Milk** **Amount per 100 g**	**RNI/AMDR[1]**
Total energy (kJ)	1808	2095	5565-8996
Fat (g)	20	27	25-35 %
Saturated fat (g)	6	17	-
Protein (g)	56	28	10-30 %
Carbohydrate (g)	7	37	45-65 %
Dietary fiber (g)	5	0	25-31 g/day (AI[2])
Vitamin A (RE[4], µg)	Not detected	238	450-600
Vitamin B12 (µg)	5.4[4]	3.3	1.2-2.4
Iron (mg)	5.2	0.6	6.1-8.9[5]
Zinc (mg)	20[6]	3.9	4.8-8.6[7]
Iodine (µg)	21[4]	7	90-120

[1] RNI – recommended nutrient intake. AMDR – acceptable macronutrient distribution range
[2] AI – adequate intake
[3] RE – retinol equivalents
[4] From Finke 2002, measured on an as is basis
[5] based on a bioavailability of 10 % (because the ingredients will be incorporated into a biscuit)
[6] From Rumpold & Schlüter 2013, measured in dry matter
[7] based on moderate bioavailability (because the ingredients will be incorporated into a biscuit)

Microbiota and health

The significance of microbiota in human health

Humans and microbes live in symbiosis with each other. The human body consists of 10 times more microbes than human cells (Guarner & Malagelada, 2003). Not only do different microbes live in different environments in the human body, the composition of microbes also varies from person to person. The importance of the interaction between bacteria and humans is best shown in mice raised in a germ-free environment. Fecal transplants from mice and humans suffering from obesity, metabolic syndrome, and colitis transferred into germ-free mice can induce the same phenotype in the recipient as in the donor.

Observation studies in humans have also linked several diseases with an altered gut microbiota (Spor, Koren, & Ley, 2011).

Microbiota is the assemblage of microorganisms present in a defined environment. The human gut microbiota develops over time. Bacteria in the womb are the first to colonize the fetus' gut (Aagaard et al., 2014). The mode of delivery has an impact on early colonization. Vaginal birth causes colonization of vaginal and to some extent fecal bacteria through interaction with the birth canal and caesarean section causes colonization with a skin-like gut microbiota from the mother (Huurre et al., 2008). Research has indicated that the gut microbiota is stable at age 2-3 (Yatsunenko et al., 2012).

Besides age, internal factors that are likely to affect the gut microbiota composition include genetics, use of antibiotics, physiology and diet. Externally, the type of bacteria that you are in contact with frequently are dependent on geography and the mode of subsistence. Together, the multiple factors mean that inter-individual differences in microbiota composition are great (Wu et al., 2011).

Historically, microbes from different environments was studied using microscopy and cultivation in different growth media. However, many microbes are morphologically similar, but perform very different functions. Furthermore, most microbes (around 99 %) are not able to be cultivated (known as the great plate count anomaly) (Hugenholtz, 2002). High throughput sequencing has revolutionized microbial research. The 16S ribosomal ribonucleic acid (rRNA) gene is a highly conserved gene throughout evolution of bacteria and archaea with hypervariable regions, making it an optimal target for sequencing and taxonomic classification of the microbes in a given sample. Other techniques known as the 'omics' explore the functionality of the gut microbiome. An example is metatranscriptomics.

Gut microbiota and diet

Recently, research has focused on the association between gut microbiota and diet. The interplay between ingested food and the composition and function of the microbiota is complex. The microbiota is involved in the metabolism of several nutrients. Correlations have been noted between different diets and the microbiota composition. Lastly, microbiota composition can shift in response to dietary changes.

The fact that energy metabolism and microbiota is closely associated is best studied in the cases of obesity and undernutrition. Turnbaugh et al. (2009) presented results that people with a certain microbiome had a higher risk of becoming obese. The hypothesis is that certain bacteria are more efficient at breaking down food for the body to absorb. At the other end of the spectrum, microbial dysfunction is part of the cause of undernutrition in young children (<3 years). Several studies have seen a correlation between changes in microbiota composition and presence of undernutrition in India, Bangladesh, and Malawi (Ghosh et al.,

2014; Monira et al., 2011; Smith et al., 2013; Subramanian et al., 2014). Importantly, Smith et al. (2013) showed that mice feeding on a typical Malawian diet who had a fecal transplant from a human SAM patient showed marked weight loss and metabolic disturbances implying a causal effect of the microbiota. Immaturity of the microbiota according to the child's chronological age appears to be the culprit. Subramanian et al. (2014) have created a model of microbiota-for-age z-score and relative microbiota maturity based on the results from Malawi, which seem to be valid for cohorts in both Bangladesh and Malawi.

Correlations between long-term diet and the microbiota composition are evident. Microbiota from people eating a diet of plant-based foods high in dietary fiber is different from a typical Western diet of animal-protein and high-fat products (Wu et al., 2011). A typical Kenyan diet is rich in dietary fiber, which is expected to produce high rates of short-chain fatty acids (SCFAs). SCFAs are produced when dietary fiber is fermented in the colon by some types of bacteria. Butyrate specifically is important for colonic health. Butyrate is the primary source of energy for colonocytes and possess anti-carcinogenic and anti-inflammatory properties (Canani et al., 2011). Such correlations are also evident on a country level (De Filippo et al., 2010; Schnorr et al., 2014; Yatsunenko et al., 2012).

Changing the diet can lead to a change in microbiota composition as Wu et al. (2012) have presented. A change can lead to overall better health and improved homeostasis in the colon.

Based on the current knowledge on prevalence of undernutrition in Kenya, improvement of nutritional interventions for both prevention and treatment is desirable. ASF intake in children has a positive impact on growth and development. Milk powder is currently the golden standard for ASF in prevention and treatment products, but substituting the imported milk powder for locally reared crickets can improve local revenue, create jobs, and is more environment friendly than other alternatives. Crickets are rich in essential nutrients, which are consumed in low amounts in the Lake Victoria region, making them an ideal option for incorporation into prevention and treatment food products. Inclusion of crickets could also change microbiota composition.

Objectives

The overall objective of the study was to develop a biscuit based on cricket powder nutritionally suited for growing children and to assess acceptability of the proposed biscuit as well as the effect on the microbiota composition. In order to pursue this objective several research questions were recognized:

1) What is the nutritional composition of the cricket biscuits?
2) How is the consumption of cricket biscuits compared to a test biscuit made with milk powder?
3) How are the hedonic ratings of cricket biscuits compared to the milk biscuit?
4) Does ingestion of insects alter the microbiota composition of human faeces compared to ingestion of milk?

Methods and materials

Formulation and production of biscuits

Ingredients and recipe formulation

The recipe for both biscuits were developed from an existing recipe previously created by GREEiNSECT partner Prof. Dr. Monica Ayieko. The final composition of the biscuits for the intervention was developed in a stepwise procedure.

Macronutrient distribution in the original recipe was calculated and adjustments considered for suitability for the nutrient needs of children age 5-10 years. FoodComp was used for this purpose, as no specific up-to-date Kenyan tables could be located ("FoodComp," 2009). Any ingredients not in the database either had nutritional information printed on the label or it was sought from the producer.

Next, a series of pilot biscuits were produced with variation in cricket and amaranth contents. Pre-tasting was performed in two stages. First, four colleagues from the GREEiNSECT project tasted and reviewed biscuits with either GREEiNSECT crickets or imported cricket powder. All were experienced entomophagists. All biscuits produced contained 20 % crickets by ingredient. Furthermore, they tasted biscuits with and without popped amaranth. Popped amaranth was a part of the original recipe but was considered to be left out due to availability and nutritional value. They rated the biscuits either 'like', 'neutral', or 'dislike' on the following aspects: Appearance, smell, texture, and flavor. Based on the results, an overall rating was calculated for each biscuit. The biscuit without amaranth and with imported cricket powder had the highest rating. Second, based on the result from the first pre-tasting, three biscuits were produced and presented for a panel of four children from the target group, who did not attend the school chosen for the study. The biscuits were without amaranth and with either 10, 15, or 20 % imported cricket powder (per ingredient). The children were presented with the biscuits individually in a random order and asked to fill out the hedonic rating questionnaire produced for the trial. The highest rating was given to the biscuit with 10 % cricket powder and was therefore chosen as the test biscuit. In addition, the forms and questionnaires to present to the parents were looked at and discussed with the two local adults present at the pre-tasting. One was a colleague from GREEiNSECT. Both the hedonic rating questionnaire as well as the consent and screening form was then adapted for better understanding in the local community.

Cricket biscuits

Dried crickets grinded into powder was imported from a company in Thailand through collaborating partners. Sufficient amounts of locally produced cricket powder was not available at the time of the study.

In the pre-tasting, biscuits containing imported cricket powder were compared with biscuits with local crickets. The imported crickets were rated higher in the pre-tasting.

Milk biscuits

For the production, whole milk powder was used in the same amount as cricket powder. However, due to professional opinion from the collaborating bakers, baking soda was left out of the milk biscuit recipe at the beginning of production since milk powder is a lighter substance than cricket powder. This did not change the milk powder percentage greatly (from 10 % to 10.06 % by ingredient).

Methods of preparation and production

Two production facilities were used: First, a local bakery. Second, the food laboratory at Jaramogi Oginga Odinga University of Science and Technology (JOOUST). A trained baker was hired to produce the full amount of biscuits. Ingredients were supplied by the head researcher. The biscuits were packaged after cooling in plastic freezer bags of three (98-102 g) and kept on -20°C for storage until serving.

Safety control

Biscuits produced by the original bakery was sent to KEBS for safety testing for the following microbes: *Escherichia coli*, *Salmonella* (species not specified), *Staphylococcus aureas*, and yeasts and molds (not specified). After the production was moved to the university food lab, no further testing was deemed necessary since the baker was staff from the bakery and all hygienic standards were followed.

Acceptability study

Sample size and subjects

Sample size was calculated according to the main outcome, which was consumption. The study by Nga et al (2005) was used as a template since general guidelines were not available. A clinically relevant difference in consumption was set at 20 %, which yields a sample size of 50 children assuming an SD of 0.8 with a power of 0.8 and a significance of 0.05. Assuming an attrition rate of 20 %, 60 children was the target of recruitment.

Inclusion criteria were apparently healthy children aged 5 to 10 years attending Nyakasumbi Primary School in Bondo. Children started primary school at age 5. Children >10 years were excluded due to the many bodily changes associated with puberty.

Exclusion criteria were:

- Children suffering from Irritable Bowel Syndrome, Crohn's Disease or similar bowel syndromes (self-reported by caretaker)

- Children with signs of bilateral pitting edema
- In children age 5-9 years a MUAC <129 mm (corresponding to a -3 z-score according to growth standards for 5 year old boys). In children age 10 years a MUAC <160 mm
- Children with a BAZ <-3 or >2
- Children suffering from diarrhea, fever or any other condition that could interfere with food intake
- Children suffering from allergies towards any of the ingredients in the biscuits or shellfish (self-reported by caretaker)

As this was an acceptability study, any condition that could affect food intake and hunger lead to exclusion. Anthropometric indices (BAZ, MUAC and clinical signs of bilateral pitting edema) were used as a measure of under- or overnutrition. Furthermore, any children with bowel syndromes were excluded since it has previously been shown that the microbiota composition is altered, which could affect the results in the present study (Guinane & Cotter, 2013). The reason for excluding children with shellfish allergy is that allergy for insects has previously been linked to shellfish allergy (Belluco et al., 2013).

Recruitment took place at the school on four different days by convenience sampling. All parents with children in the right age bracket were invited on several different days to hear about the study and give consent and general information for screening. Furthermore, one parent appeared at the university to give permission on a separate day. The screening questionnaire can be seen in appendix 2.

Study setting

The study was performed at JOOUST as well as the adjacent Nyakasumbi Primary School. Collection of consent forms and stool samples as well as anthropometric measurements at baseline and end of study was performed at the primary school. Due to space limitations and ethical considerations for the children at the school not included in the study, the meals and hedonic rating questionnaires were carried out at the food laboratory at JOOUST.

Before the study, all data enumerators had received thorough training in daily procedures such as anthropometric measurements, dealing with children, and questionnaires.

Anthropometry

The following anthropometric measurements were performed at baseline: height, weight, and MUAC. All measurements were performed twice and the mean was used for statistical analysis. If the two measurements differed more than a set value (0.5 cm, 0.5 kg and 0.3 cm for height, weight and MUAC, respectively) a third measurement was performed. Height and MUAC was measured using Schorrboard

length measurer and measuring tape, respectively. Weight was measured using a CAMRY glass electronic personal scale model EB9318.

Weight was likewise performed on the last day of the study to measure impact of the intervention. Anthropometry forms can be seen in appendix 3.

Study design

The study design was parallel with two groups. The participants were randomized individually in blocks of four using randomization.com. The participants were not informed which biscuit they were consuming. However, blinding was not possible due to differences between cricket and milk biscuits in appearance and taste. The study period was 4 weeks and included 19 school days (Monday in week 4 was a public holiday).

Daily procedure

Each school day the children arrived at the university food lab in their mid-morning break from 11.00-11.30. Due to the distance from the school, the mealtime was set to 15 minutes to minimize disruption of their education. The two groups were seated at two tables in the food lab with a table in the middle to separate them. Three pre-weighed biscuits were served daily with an overall weight of 98-102 g. Communication was allowed between the children, but swapping food, leaving the room, giving food to someone else or taking the food with them was not. While children were eating, observations on morbidity and morning food intake were noted down. The data enumerators asked the children about their diet in the morning and the morbidity pattern the last 24 hours in terms of nausea, vomiting and diarrhea. In case the child gave information on other morbidity aspects, this was noted down as well. The consumption and morbidity form can be seen in appendix 4. After 15 minutes, the children were sent back to school and leftovers were weighed and noted down.

Acceptability criteria and tools

Two criteria was set up to measure acceptability. First, intake was measured. Average acceptance was set at a consumption >50 % and good acceptance at >75 %. Long-term good acceptance was set at a consumption > 75 % for at least 75 % of the days. A scale (PAPYRUS AGS-3000) was used to measure the amount of biscuits on a pre-weighed plate before and after consumption to the nearest gram. This was done when the children were not present in order not to influence their eating behavior as well as due to time constraints.

Second, subjective scoring of organoleptic properties (looks, color, smell, taste, texture, and overall liking) was performed each week using a 5-point Likert Scale with smileys suitable for children (Mellor & Moore, 2014; Reynolds-Keefer & Johnson, 2011) (appendix 5). The measures were performed on Fridays, except in week 2, where it was performed Thursday due to changed school activities Friday. The questionnaire was

written in English, Swahili and Luo to accommodate the large span in languages used in the community. The children were instructed on how to fill out the questionnaire and to answer truthfully and individually without communicating with the other children.

Faeces sampling and analysis

Sampling procedure

During the two study days prior to sample collection, the data enumerators explained to the children how to take the sample. Explaining the procedure twice was done in order to ensure that the children had correctly understood the technique. The children were asked to bring a fresh morning stool sample to school during a given time period and note down the time of defecation on the container. Furthermore, they were provided with collection containers as well as an informal letter for their parents in English and Luo. The parents had prior been informed about the collection when consent forms were signed.

The collection period was the last three days of the study. The collection period was set to accommodate irregularities in bowel movements. Collection took place from 06.45 to 08.00 am at the primary school. Samples were checked for correct time of defecation. If the child or parents had not filled out the form on the container, an estimated time was given by the child if possible. The time of delivery was noted down and the samples subsequently put in a cooler box and transported to the chemistry lab at JOOUST. One sample was handed in after the collection period. The sample was delivered at 11.00 am when eating began.

At JOOUST, the condition of the samples were estimated using the Bristol stool scale (appendix 6). A fecal matter sample weighing 0.8 g was then transferred to a pathology vial and mixed well with 4 ml RNAlater. Vials were securely packaged individually in zip-lock bags containing absorbable paper and collectively in four heat-sealed bags. The samples were stored at 5°C and transported to Denmark within a week with a cooling element. In Denmark the samples were immediately stored at - 80°C until analysis.

Microbiota analysis – 16S Metagenomic Sequencing

Illumina next generation sequencing (MiSeq system) was used to determine microbiota composition of the fecal samples using sequencing of the 16S rRNA gene. DNA extraction, amplification and purification of product, and library formation was performed at the Department of Food Science, University of Copenhagen. All procedures followed standard protocols established in the laboratory (Pyndt Jørgensen et al., 2014). Sequencing was performed at Department of Environmental Science, Aarhus University, Roskilde.

DNA extraction

DNA extraction was performed at the Department of Food Science, University of Copenhagen.

The PowerSoil® DNA Isolation Kit (MO BIO laboratories, Inc., California, USA) was used to extract DNA from the samples as instructed by the manufacturer (MO BIO Laboratories, 2013). Additional steps were added to suit the fecal samples following standard procedures in the laboratory. First, approximately 2 ml of the thawed sample was transferred each to two microtubes. The samples were centrifuged at 13,000 rpm for 10 min and the supernatant (RNAlater) discarded. Minimum 200 mg (min 201.7mg, max 325.0 mg) of the pellet was transferred to the PowerBead tube. Two rounds of heating (10 min. at 65°C and 90°C, respectively) were performed in order to disrupt more cells. The beat-beading of the PowerBead tubes was performed in three cycles of 15 seconds at speed 6.5 M/s at a FastPrep-24 5G instrument (MP Biomedicals, LLC., California, USA). Samples were stored at -20°C before each polymerase chain reaction (PCR) cycle and sequencing.

Library preparation – amplification, purification and library formation of DNA

Library preparation was performed at the Department of Food Science, University of Copenhagen according to standard protocols (Pyndt Jørgensen et al., 2014).

The extracted DNA underwent two PCRs in order to amplify the desired sequences (fig. 2). All samples were loaded randomly unto the 96-wells plate. The first PCR amplified the variable regions V3 and V4 of the 16S rRNA gene using primers compatible with the Nextera Index Kit (Illumina) (fig. 3).

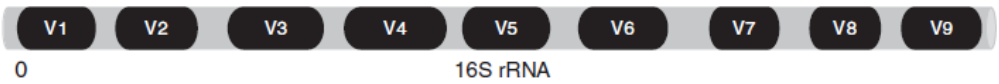


Figure 2 model of the 16S rRNA gene. Hypervariable regions are dark and conserved regions light grey (from Tyler et al. 2014)

The second PCR added specific primertags (Illumina Nextera XT Index Kit, Illumina Inc., California, USA) to each sample in order for the results to be analyzed per child after sequencing. Electrophorese gels were run after each PCR to verify the reaction succeeded using a 1.5% agarose gel (1.5g UltraPure™ Agarose per 100 mL 1x TAE buffer, dyed with 4 µl Midori Green Advance DNA Strain), which was run at 120V for about 30 minutes.

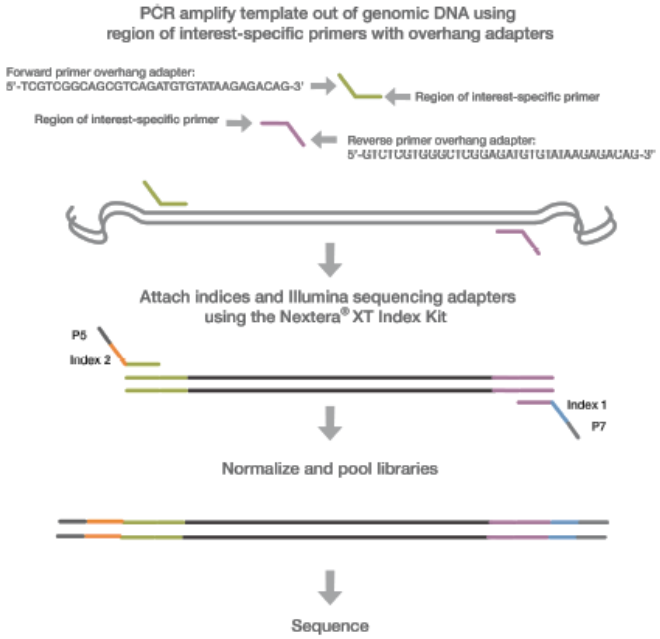


Figure 3 PCR reactions in library preparation (image from Illumina 2013)

The samples were purified using AMPure XP beads (Beckman Coulter Genomic, CA, USA). The instructions from the manufacturer were followed (Beckman Coulter, 2013). The concentration of DNA in the samples was accurately measured with a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) and the samples were pooled to a library according to their concentration. Each sample provided 30 ng DNA to the library.

High throughput sequencing of the gut microbiota and primary analysis

Sequencing was performed by the Department of Environmental Science, Aarhus University, Roskilde. The fecal microbiota samples were determined using tag-encoded 16S rRNA gene MiSeq-based (Illumina, CA, USA) high throughput sequencing according to instructions from the manufacturer (Illumina, 2014).

Primary analysis of the raw dataset was performed by Lukasz Krych at Department of Food Science, University of Copenhagen. Details can be found in appendix 7. Operational taxonomic units (OTUs) were constructed for sequences with 97 % similarity. The Greengenes (13.8) 16S rRNA gene collection was used as a reference database to taxonomically classify OTUs (McDonald et al., 2012). Quantitative Insight Into Microbial Ecology (QIIME) open source software package (1.7.0 and 1.8.0) was used for subsequent analysis steps (Caporaso et al., 2010). Analyses included observed species and principal coordinate analysis (PCoA) plots using the UniFrac method, both of which describes diversity of the microbial samples. Analysis of similarities (ANOSIM) was applied to evaluate group differences. Correlations between trial parameters (Bristol grading, BAZ, weight, height, MUAC, missing days from trial, age) and genera relative abundance were investigated using Pearson's product-moment correlation coefficient.

Ethical considerations

The protocol was approved by the ethical committee at Jaramogi Oginga Odinga Teaching & Referral Hospital, Kisumu. Permission to do the study at the school was afterwards obtained from the Ministry of Education and the head teacher. Furthermore, the Ministry of Health was informed about the study.

There were no suspected side effects with the biscuits or any major discomfort or risks with the procedures. During faecal sampling, the children were instructed in proper hygiene to minimize contamination and illness. The limited risks were estimated to be outweighed by the perspectives of the findings. No monetary compensation was given to either children or caretakers. A small gift (a pen) was given to the children if they showed up for the last weighing on the last study day.

All children and their caretakers involved in the study were thoroughly informed about the study orally as well as in writing for the caretakers. Written consent was gathered from caretakers. It was furthermore emphasized that subjects and/or caretakers could withdraw the consent at any given time without giving a reason. No measurements were done without consent.

Statistical analysis

All data besides fecal analysis was collected and entered into Microsoft Excel 2013. The statistical software R (Version 3.2.1) was used to perform statistical tests on the transferred data. Statistical significance was defined as $p<0.05$. Z-scores were calculated using WHO AnthroPlus software based on WHO growth reference for 5-19 year old children 2007.

All data was inspected for assumptions. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality.

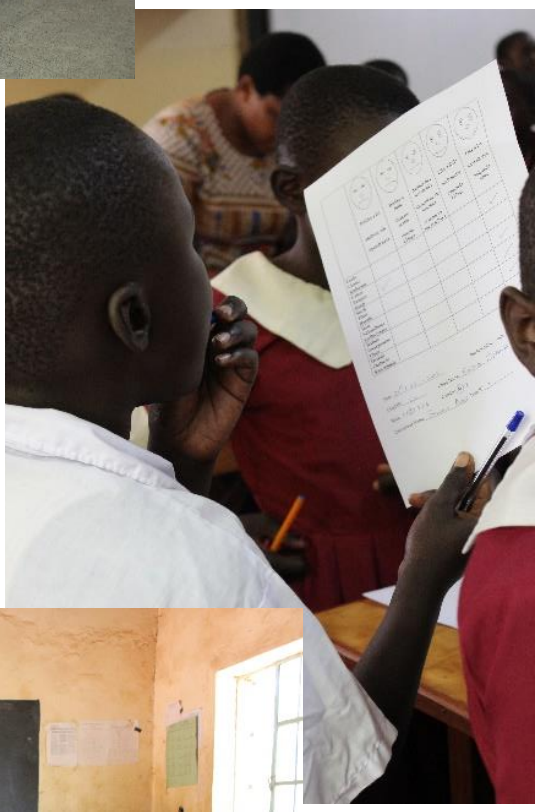
Description of baseline data was shown as mean (SD) for numeric variables and % (n) for binomial variables. Post-hoc chi-square tests were performed to inspect missing data distribution in the two groups for consumption and hedonic rating data.

Confidence intervals (95 %) for the mean of consumption was used to determine whether consumption was acceptable, good, and long-term and good according to set standards.

Linear mixed models (*lme4* package in R) was applied to hedonic ratings and intake according to group. The model can accommodate missing values and can control for non-independence among repeated observations, which is why it is preferred over other statistical methods. In both cases subject was set as random effect for intercept, and gender, age and intervention day was controlled for as fixed effects.

Furthermore, the interaction between time and group was tested. *P*-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question.

Unadjusted analysis of variance (ANOVA) analyses were used to detect differences in bacteria genera between groups. False discovery rate (FDR) corrections were subsequently applied to significant *p*-values to adjust for multiple comparisons, i.e. to reduce the rate of type I errors.



Results

Pre-tasting

In the first pre-tasting, the biscuit containing 20 % imported cricket powder and no amaranth was rated higher than GREEiNSECT crickets and with amaranth in different combinations. The biscuit had an overall rating of 2.50 out of 3. The other biscuits rated between 2.00-2.44.

In the second pre-tasting, the biscuit rated highest in the first pre-tasting was created with 10, 15, and 20 % imported cricket powder. The four children rated the biscuit with 10 % highest at 4.38 out of 5. The biscuits containing 15 and 20 % cricket powder rated 3.75 and 3.83, respectively. Based on the pre-tastings, the biscuit containing 10 % imported cricket powder and no amaranth was chosen as the trial biscuit.

Trial biscuit description and composition

Based on the two pilot tests the final intervention and control biscuit contained 10 % cricket and milk powder, respectively. The final biscuits can be seen in fig. 4. The cricket biscuits were darker in color and slightly bigger due to the higher content of leavening agents. The biscuits were presented as seen on the picture in order to keep uniformity and to keep flies away from the food between preparation and arrival of the children.

Figure 4 Cricket biscuit (left) and milk biscuit (right) as presented to the children.

The nutritional composition of both cricket and milk biscuits is shown in table 3. Likewise, the contribution of one daily portion (100 g) to RNI is presented.

Table 3 Nutritional composition of cricket biscuit and milk biscuit including contributions to RNI of a daily portion (100 g).

Per 100 g[1]	10 % cricket biscuit	10 % milk biscuit	RNI/AMDR[2]	% of RNI from daily portion (cricket)	% of RNI from daily portion (milk)
Energy (kJ)	1935	1942	5565-8996	21-35	21-35
Fat (E%[3])	37	38	25-35		
Saturated fat (g)	6.9	8.1	-		
Protein (E%[3])	12	9	10-30		
Carbohydrates (E%[3])	51	53	45-65		
Dietary fiber (g)	2.4	1.7	25-31 (AI[4])	8-10	6-7
Moisture (%[5])	4.5	4.5	-	-	-
Ash (G)	1.9	2.2	-	-	-
Vitamin A (RE[6], µg)	184	209	450-600	31-41	35-47
Vitamin B12 (µg)	1.0[7]	0.8	1.2-2.4	43-85	32-63
Iron (mg)	1.6	1.0	6.1-8.9[8]	18-26	11-17
Zinc (mg)	3.1[9]	1.1	4.8-8.6[10]	36-65	13-23
Iodine (µg)	7.7[7]	5.9	90-120	4-6[10]	5-7

[1] Based on nutritional composition tables of ingredients (http://www.foodcomp.dk/v7/fvdb_search.asp), except for cricket powder (manufacturer information except otherwise stated)
[2] RNI – recommended nutrient intake (FAO et al., 2001; WHO & FAO, 2004). AMDR – acceptable macronutrient distribution range (Institute of Medicine of the National Academies, 2005).
[3] E % for carbohydrates, fat, and protein is calculated from total kJ based on food energy per mass (37 kJ/g fat, 17 kJ/g protein)
[4] AI – adequate intake (Institute of Medicine of the National Academies, 2005)
[5] Moisture estimated via nutritional composition table search for biscuits (http://www.foodcomp.dk/v7/fvdb_search.asp).
[6] RE - retinol equivalents
[7] Information on cricket from Finke (2002)
[8] Based on a bioavailability of 10 %
[9] Information on cricket from Rumpold & Schlüter (2013)
[10] Based on moderate bioavailability

Subject recruitment and characterization

An overview of the study enrollment is presented in fig. 5. One subject had to be excluded after baseline anthropometric measurements as he had a MUAC of 15.8 cm, which was an exclusion criterion. After randomization, three subjects failed to attend the meal sessions. As the children were not reporting for class throughout the study period, it was assumed that they had changed schools. In total 54 children were included for analysis, based on the available case principle.

Table 4 shows characteristics of the intervention and control group. The random differences between the groups included a slightly higher age and proportion of boys in the intervention group. Consequently, the anthropometric indices were likewise slightly higher. In contrast, the proportion of moderately undernourished children was higher in the intervention group (BAZ and HAZ <-2).

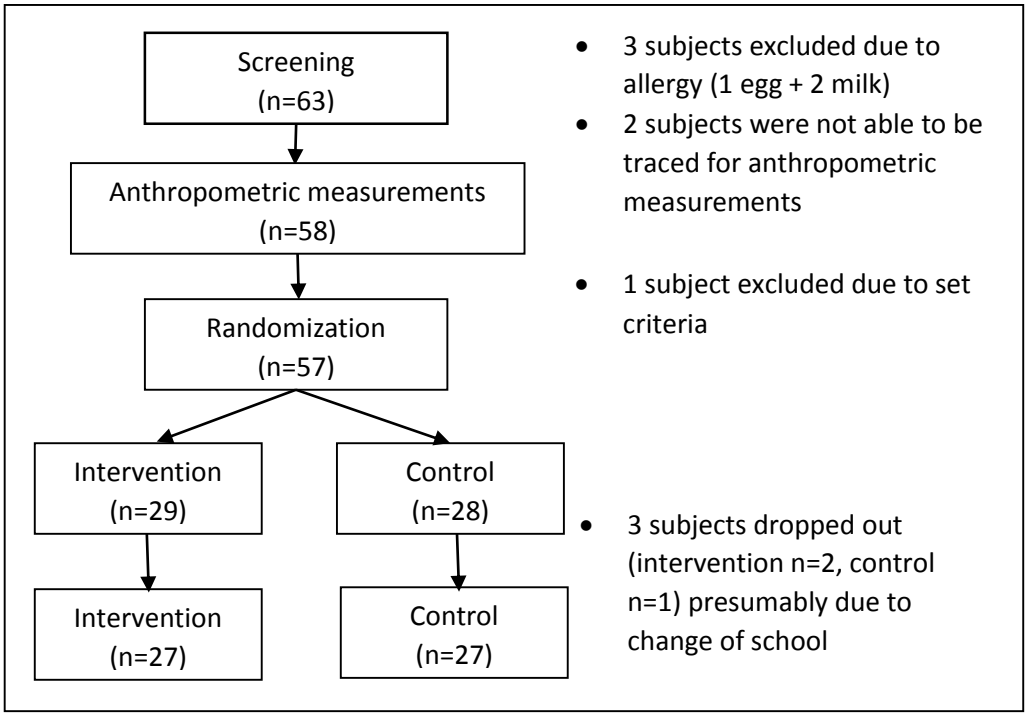


Figure 5 Flowchart of enrollment of the children into the intervention

Table 4 Baseline characteristics for intervention and control group. All variables are shown as mean (SD) unless otherwise indicated.

	Intervention group (n=27)	**Control group (n=27)**	**Total (n=54)**
Age (years)	8.7 (1.5)	8.2 (1.6)	8.5 (1.5)
Male, % (n)	41 (11)	37 (10)	39 (19)
Height (cm)	130.6 (8.3)	126.9 (11.6)	128.7 (10.2)
Weight (kg)	26.8 (5.1)	24.5 (6.0)	25.6 (5.6)
BAZ[1]	-0.4 (0.8)	-0.7 (1.0)	-0.5 (0.9)
BAZ < -2, % (n)	7 (2)	0 (0)	3.70 (2)
MUAC (cm)	18.2 (1.9)	17.4 (1.9)	17.8 (1.9)
HAZ[2]	-0.1 (1.3)	-0.2 (1.1)	-0.2 (1.2)
HAZ < -2, % (n)	4 (1)	4 (1)	4 (2)
Have tried eating insects, % (n)[3]	93 (25)	67 (18)	80 (43)
Eats insects now (in season), % (n)	81 (22)	70 (19)	76 (41)
Self-reported allergy of red meat[4]	22 (6)	19 (5)	20 (11)

[1] BAZ – BMI-for-age z-score
[2] HAZ – Height-for-age z-score
[3] Termites were the only type of insect indicated for consumption
[4] Reported by caretaker

Not all children showed up on all study days. Missing data was evenly distributed (chi-square test, p=0.49) with 8.4 % of possible daily measures missing in the intervention group (43 out of 513 possible recordings) and 7.4 % in the control group (38 out of 513).

Consumption and acceptability

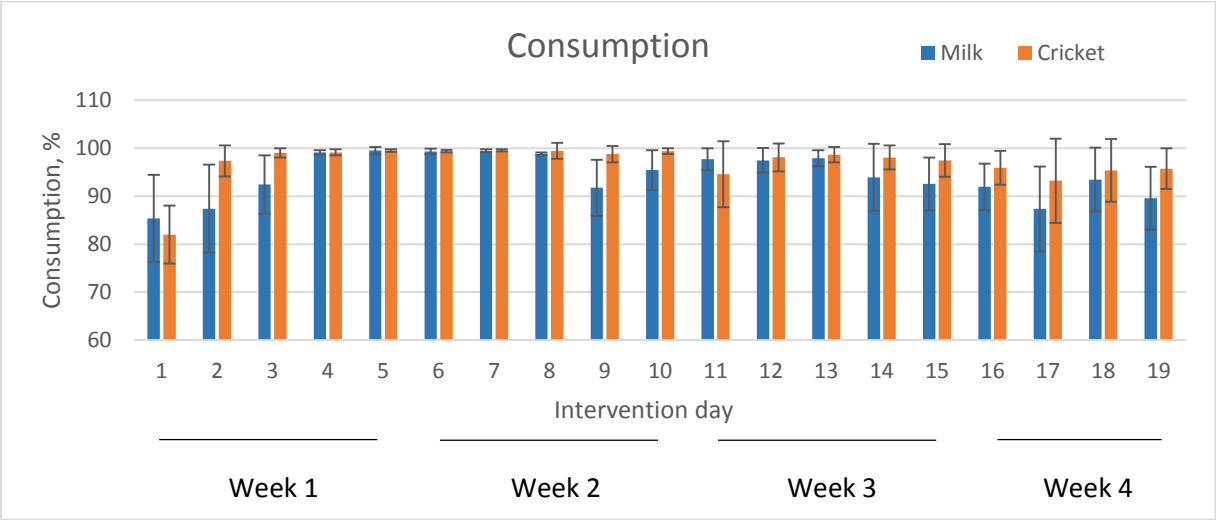


Figure 6 Bar chart of mean consumption per group per day. Error bars indicate 95 % confidence interval. N ranges from 20-27 per group.

The consumption pattern per day of cricket and milk biscuits can be seen in fig. 6. The 95 % confidence interval for the overall mean consumption was 95.3-97.5 % for cricket and 93.0-95.5 % for milk. This means that both groups reached good acceptance (>75 %). The daily consumption was above 75 % on all days meaning that long-term acceptance was likewise achieved. On day 11, one recording was removed, because the child had spilled water and soaked the biscuits.

Total mean consumption of the cricket and milk biscuits over the intervention period was analyzed using linear mixed models adjusted for days, age, and gender (fixed effects), and subject (random intercept). The children eating cricket biscuits consumed 2.5 percentage points more than the control group (96.9 % and 94.2 % for cricket and milk biscuits, respectively), though statistically non-significant (p=0.14). The percentage points is equivalent to about 2.5 grams. No interaction was found between days and group (p=0.38[3]), which means that the groups do not have different consumption patterns over time.

Hesitation and refusal

In addition to the high consumption pattern across both groups, very few incidents occurred where the data enumerators had to encourage the children to start eating. Out of 945 recordings, only five recordings of hesitation to eat were made. Twice, a child refused to eat, which was defined as two encouragements by data enumerators followed by refusal. However, both children had consumed some of the biscuits by the end of the session.

[3] model adjusted for age and gender as fixed effects and subject as random intercept

Breakfast

Table 5 shows how many children ate breakfast per trial day in percentage of children present. Most children ate breakfast that consisted of one or two types of starchy items (rice, potatoes, ugali, porridge) and tea.

Table 5 Percentage of children consuming breakfast per trial day (self-reported).

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
n	46	49	52	52	52	52	46	49	51	53	46	48	49	50	45	50	52	50	53
% had breakfast	72	80	85	79	79	81	72	84	80	70	72	88	82	80	93	86	83	86	75

Hedonic ratings

In table 6 and fig. 7 the overall means and graphic representation of the hedonic ratings per week are presented, respectively. Hedonic ratings were generally high. Two aspects (cricket biscuit texture and smell) graded under 4 ("like a little"). Number of respondents was not significantly different throughout the weeks or by group for any organoleptic properties (p>0.05).

Table 6 Overall means (SD) of hedonic ratings according to biscuit type.

	Cricket biscuits	Milk biscuits
Looks	4.34 (1.36)	4.83 (0.65)
Color	4.17 (1.25)	4.31 (1.07)
Smell	3.96 (1.44)	4.56 (1.04)
Taste	4.02 (1.37)	4.43 (1.09)
Texture	3.96 (1.52)	4.53 (0.98)
Overall	4.19 (1.36)	4.69 (0.77)

Linear mixed models was used for hedonic ratings in the same way as for consumption with same adjustments. Ratings for looks, smell, texture and overall liking were significantly higher for milk biscuits than cricket biscuits. All ratings were around 0.5 Likert points higher in the control group (table 7).

Table 7 Linear mixed model analysis for difference in hedonic ratings per biscuit type. The coefficient indicates difference in rating from cricket to milk biscuits. Standard error (SE) is shown in brackets. Adjusted for days, age and gender (fixed effects) and subject (random intercept). Significant *p*-values are highlighted.

	Looks	Color	Smell	Taste	Texture	Overall
Coefficient (SE)	0.57 (0.20)	0.07 (0.20)	0.54 (0.26)	0.39 (0.23)	0.55 (0.24)	0.49 (0.19)
p-value	**0.01**	0.74	**0.04**	0.09	**0.02**	**0.01**

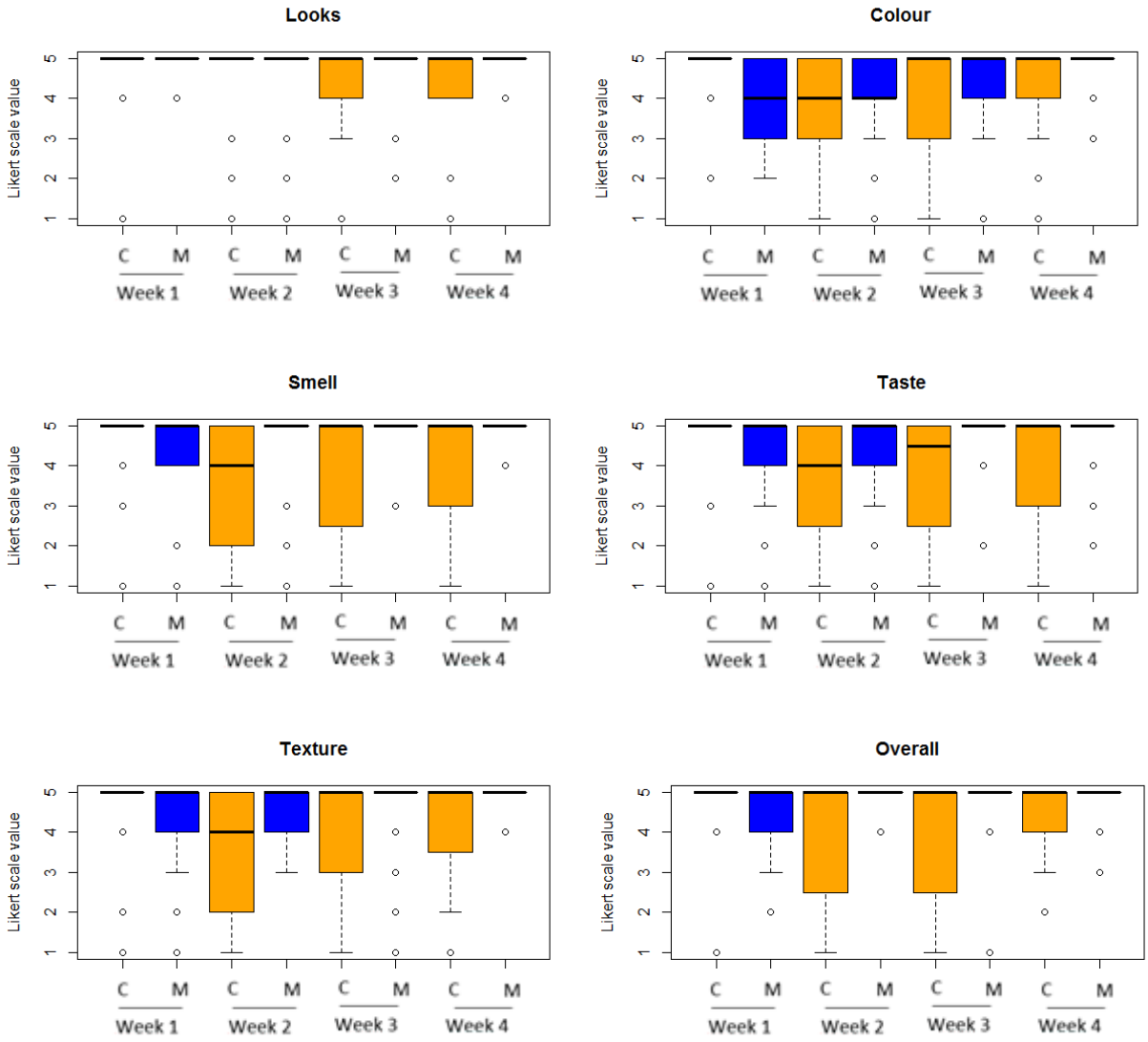


Figure 7 Box plots for hedonic ratings (looks, color, smell, taste, texture, overall) with medians (line), inter-quantile range (box) and 95 % confidence interval (error bars) presented. N ranges from 19-27 per group. C=Cricket, M=Milk.

An interaction between days and group was shown for color (p=0.004) and smell (p=0.005). Analyses for looks, taste, texture, and overall did not show an interaction between days and group.

Morbidity

Table 8 Recordings of morbidity for all children. Other indicates stomachache, headache, common cold, cough, flue, chest pain, malaria.

	Number of cases (out of 950 recordings)
Nausea	7
Vomiting	2
Diarrhea	1
Other	54

Morbidity was recorded 56 times as seen in table 8. Five of those recordings were of children not present, but reported by friends or siblings. Stomachache (14 recordings) and headache (24 recordings) were by far the most common noted morbidity.

The stool samples collected were rated with the Bristol stool scale (appendix 6). The results for the 54 samples are shown in table 9.

Table 9 Bristol grading for stool samples

Bristol grading	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Number of stool samples	6	6	10	7	4	14	7

Impact on anthropometry

Due to challenges in the study, children were measured at baseline in two different groups. Forty-two children were measured a month before the trial started whereas 12 were measured in the week leading up to the study. At the end of the study, 52 children were measured. The following analysis was conducted on the 40 children that had data from both baseline (one month before study) and end of study. The analyses was likewise run including all 52 children with two measurement sets, which did not show a different result.

Table 10 Impact on anthropometry in 40 children that were measured within the same period. Weight difference is in percentage increase from first measurement. Mean (SE) is shown.

	Intervention group (n=20)	**Control group (n=20)**	***p*-value**
Weight difference, %	4.8 (0.5)	7.0 (1.9)	0.27
BAZ difference	0.4 (0.1)	0.6 (0.2)	0.18

Unpaired t-tests were conducted on weight difference in percentage from the original measure and difference in BAZ. It is noteworthy that height was not measured at the end of study due to time constraints. As seen in table 10, the impact on anthropometric measurements were not significantly different.

Microbiota composition

Sequences purified for chimeric reads[4] yielded 2,296,278 sequences giving an average of 43,967 sequences per sample (min = 20,034; max = 98,905; SD = 20,703). Mean sequence length was 398 bp (± 98 bp). Eight

[4] Chimeric reads are two or more joined biological sequences and are formed during PCR.

of 54 samples were removed from the analysis due to the low reads number (below 1000) (Lukasz Krych, personal communication).

As seen in fig. 8, the number of observed species did not differ between the intervention (blue) and control (red) groups. Furthermore, as both groups appear to be reaching a plateau for coverage, it indicates that the majority of the present bacteria types was sequenced and deeper sequencing was only expected to add information on the very low abundant bacteria.

Species level identity (i.e. 97 % similarity in OTUs) showed no significant effect of biscuit group in either unweighted (qualitative) (fig. 9A), weighted (quantitative) (fig. 9C), or generalized (unified) (fig. 9E) UniFrac PCoA plots. An unknown systematic effect was found. In order to check if the unknown effect was obstructing an effect of biscuit intake, data was reanalyzed as described by Østergaard et al. (2015). OTUs determining the unknown systematic effect were excluded in the new analysis. No significant effect of biscuit group was detected in unweighted (fig. 9B), weighted (9D), or generalized (9F) UniFrac PCoA plots. The subsequent ANOSIM analyses showed no significant differences in unweighted nor weighted UniFrac distance matrices (all $p > 0.05$) (Lukasz Krych, personal communication).

There were no correlations found between trial parameters (Bristol grading, BAZ, weight, height, MUAC, missing days from trial, age) and genera relative abundance (data not shown).

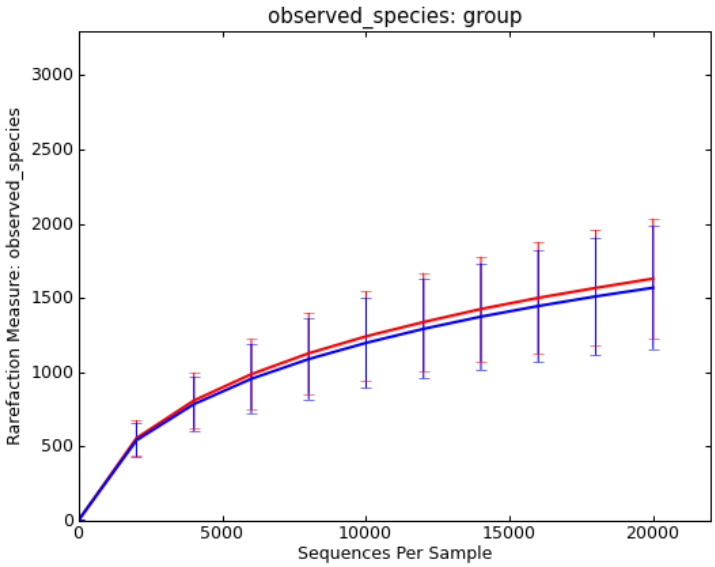


Figure 8 Observed species per group (blue = intervention group, red = control group).

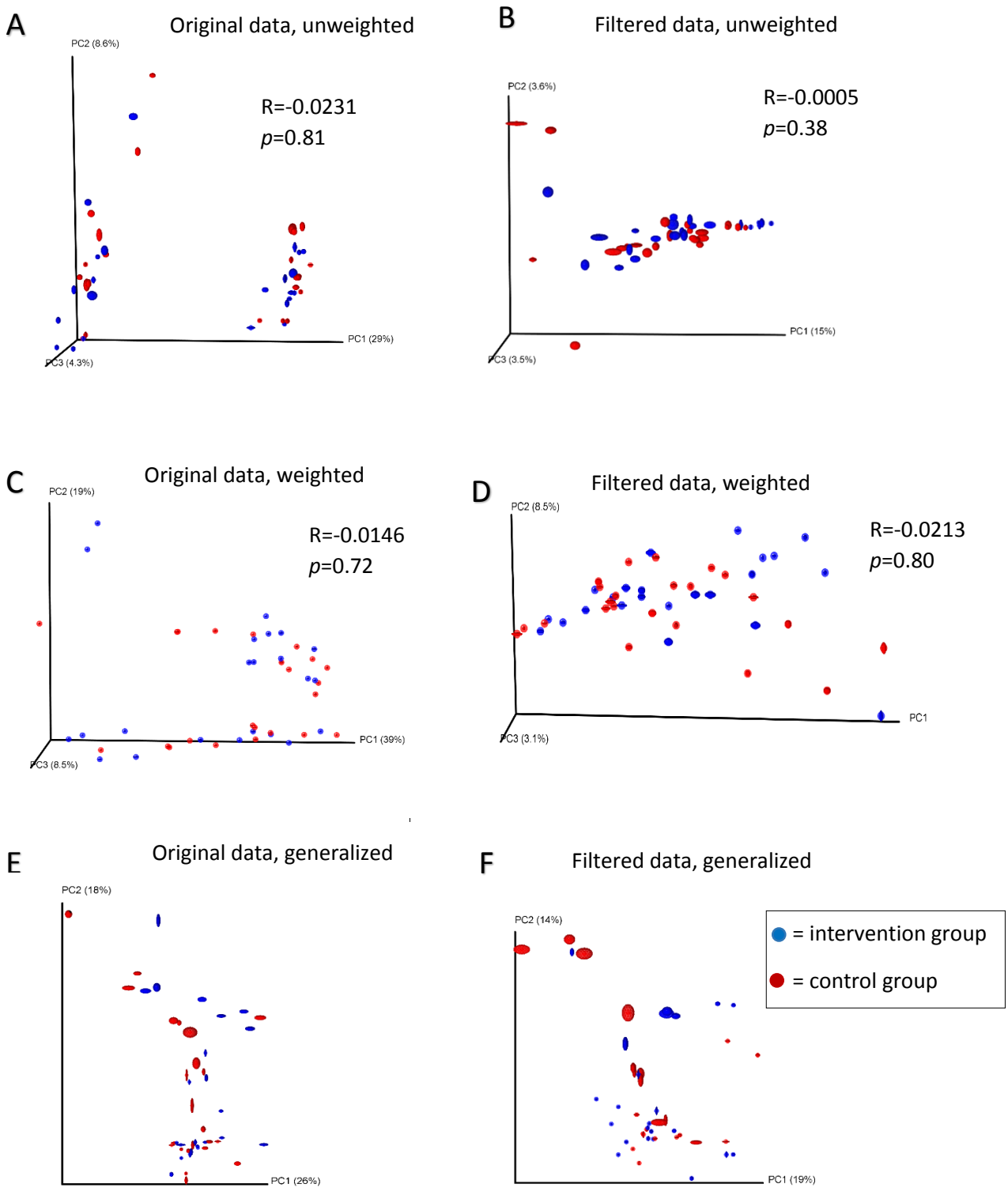


Figure 9 Unweighted, weighted and generalized UniFrac PCoA plots of original data (A, C, E) and filtered data (B, D, F) based on identified OTUs and constructed out from the three primary PCs. Each point represents a sample from 46 subjects (eight samples were taken out due to low reads number) and is colored according to group (blue = intervention group, red = control group). The sphere around the point specifies the 95 % confidence interval. ANOSIM R statistics and p-value is shown for unweighted and weighted plots. Data analyzed at the Department of Food Science, University of Copenhagen following established procedures.

In order to test if there were any differences between groups among individual bacteria genera, unadjusted ANOVAs were run. The filtered data was used for the analyses because it was not clarified what systematic effect caused the difference between the samples. Four of 106 ANOVAs were significant. The Cyanobacterium was more prevalent in children consuming milk biscuits, whereas the Firmicutes were more prevalent in children consuming cricket biscuits. All abundances were below 0.1 %. However, when corrected for multiple testing using FDR, none of the *p*-values reached significance as seen in table 11.

Table 11 unadjusted ANOVAs on genera level according to group. Only significant *p*-values are represented alongside FDR-corrected *p*-values

Phylum	Class	Order	Family	Genus	*p*-value	FDR-*p*
Cyanobacteria	4C0d	YS2	unassigned	unassigned	0.01	0.68
Firmicutes	Clostridia	Clostridiales	other	other	0.05	0.68
Firmicutes	Clostridia	Clostridiales	Christensenellaceae	unassigned	0.03	0.68
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillospira*	0.04	0.68

The general microbiota composition in Kenyan children age 5-10 years is described in fig. 10. All data was pooled for the children in the study, since no significant difference was seen with respect to diet or any other measured variable (intervention, age, gender, and methodological variables in faeces preparation). In fig. 10, it is shown that 95.1 % of the bacteria belonged to three phyla: Bacteroidetes, Firmicutes, and Proteobacteria. Bacteroidetes was the most represented phylum (52.8 %) and *Prevotella* the most commonly found genus (46.0 %). All results are percentages of the relative distribution.

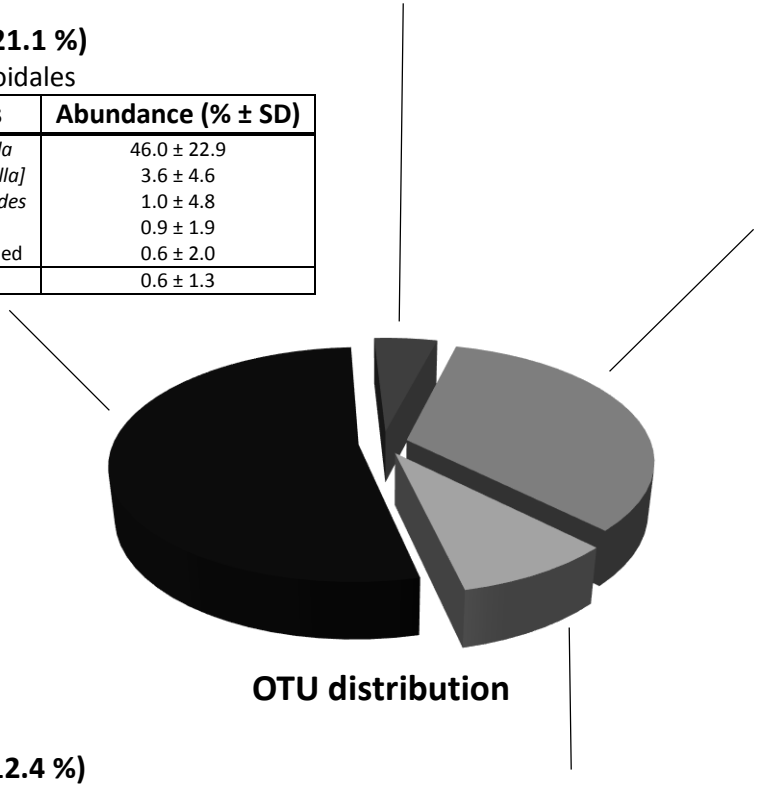
Other (4.9 ± 1.3 %)

Phylum	Class	Order	Family	Genus	Abundance (% ± SD)
Cyanobacteria	4C0d	YS2	unassigned	unassigned	2.5 ± 3.8
other	other	other	other	other	2.1 ± 1.5
remaining					0.3 ± 1.4

Bacteroidetes (52.8 ± 21.1 %)

C: Bacteroidia, O: Bacteroidales

Family	Genus	Abundance (% ± SD)
Prevotellaceae	*Prevotella*	46.0 ± 22.9
[Paraprevotellaceae]	*[Prevotella]*	3.6 ± 4.6
Bacteroidaceae	*Bacteroides*	1.0 ± 4.8
[Paraprevotellaceae]	CF231	0.9 ± 1.9
unassigned	unassigned	0.6 ± 2.0
remaining		0.6 ± 1.3



Firmicutes (33.5 ± 21.3 %)

C: Clostridia, O: Clostridiales

Family	Genus	Abundance (% ± SD)
Ruminococcaceae	unassigned	7.1 ± 7.8
Lachnospiraceae	unassigned	4.3 ± 3.5
Veillonellaceae	*Anaerovibrio*	3.6 ± 5.2
Ruminococcaceae	*Faecalibacterium*	3.0 ±3.5
unassigned	unassigned	2.4 ± 1.8
Veillonellaceae	*Megasphaera*	1.9 ±4.6
*Lactobacillaceae	*Lactobacillus*	1.5 ±3.8
Ruminococcaceae	*Ruminococcus*	1.3 ± 5.4
Lachnospiraceae	other	0.9 ± 0.8
Lachnospiraceae	*Roseburia*	0.9 ± 0.8
Clostridiaceae	unassigned	0.8 ±1.0
other	other	0.8 ± 0.6
Lachnospiraceae	*Blautia*	0.7 ± 0.7
Veillonellaceae	*Phascolarctobacterium*	0.5 ± 1.7
Lachnospiraceae	*Coprococcus*	0.5 ± 0.5
Clostridiaceae	other	0.5 ± 0.8
Veillonellaceae	unassigned	0.4 ± 0.6
Ruminococcaceae	*Oscillospira*	0.4 ± 0.6
Lachnospiraceae	*[Ruminococcus]*	0.3 ± 0.3
remaining		1.6 ± 3.4

* C: Bacilli, O: Lactobacillales

Proteobacteria (8.8 ± 12.4 %)

Class	Order	Family	Genus	Abundance (% ± SD)
Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	*Succinivibrio*	5.0 ± 11.0
Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Campylobacter*	3.2 ± 7.8
remaining				0.6 ± 1.9

Figure 10 Overview of the relative abundance of identified OTUs in all stool samples (n=46). Pie chart shows relative abundance of OTUs at phyla level and tables for each phylum show relative abundance of OTUs at genera level. At genera level, only OTUs with an average abundance ≥0.30 % are listed, and OTUs are listed in descending order regarding their relative abundance. Abundance refers to relative abundance (%) and is reported as mean ± SD. Taxa denoted as "unassigned" means that the reference database does not have an official taxonomy for this cluster. Taxa denoted as "other" (unidentified), indicates ambiguity in the assignment meaning that more than one bacterial taxa could be assigned to this cluster. Taxa mentioned in the square brackets indicate a proposed taxonomy. Abbreviations for bacterial taxonomy used: C: Class, O: Order.

Discussion

The present study showed that it is possible to produce a biscuit with crickets nutritionally suitable for 5-10 year old children. No significant difference in consumption of cricket biscuits was found compared to milk biscuits. However, hedonic ratings of looks, smell, texture and overall liking were significantly lower for cricket biscuits. Microbiota composition did not differ between the groups.

Composition of cricket biscuit in the context of the diet in Bondo district

In general, the nutritional profile of the cricket biscuits is better suited for prevention of undernutrition in children in the Lake Victoria region than milk biscuits in all aspects except vitamin A (table 3).

The nutritional profile of the cricket biscuits add value to the habitual diet for the Luo people in Bondo district, which consists mainly of energy from carbohydrates, in particular starch (Hansen et al., 2011). It should be noted, that Hansen et al. investigated adult food intake and child nutrition can be somewhat different. The higher value of energy from fat in the biscuits is important since habitual intake of this key macronutrient is low (15 and 18 % for men and women, respectively). Only 36 % of the fat in the cricket biscuits is saturated. Linoleic acid (18:2, n-6) content has been shown to be around 40 mol % of the fatty acid content in mature female crickets, whereas little α-linolenic acid (18:3, n-3) is present (Grapes et al., 1989). Due to fish consumption in the region, recommended doses of α-linolenic acid are consumed (Wanjihia, Kiplamai, Waudo, & Boit, 2009).

Maize is one of the most common foods eaten in Nyanza province, often in the form of the thick porridge *ugali*. The protein quality of maize is low. The essential amino acids lysine and tryptophan are present in insufficient amounts. Mixed with other sources of protein, the amino acid profile of the diet can be enhanced, as is the case in the dish *githeri*, where maize is mixed with beans (FAO, 1992). Complementing maize-based diets with crickets adds complete protein and increase total amount of protein intake compared to milk biscuits.

Concerning micronutrients, the cricket biscuits provide zinc, iron, vitamin A and little iodine. Deficiencies of these four micronutrients are important public health concerns in Kenya as well as worldwide (Siekmann et al., 2003; WHO, 2009a). Even though crickets contain very high amounts of zinc and iron, the low amount of cricket powder in the biscuits means that content is on average only 51 % and 22 % of RNI for zinc and iron, respectively. Antinutrients such as phytic acid present in wheat flour can lower bioavailability, and therefore moderate bioavailability was assumed for RNI. High amounts of vitamin A in margarine and egg make up for the absence in crickets. Vitamin B12 from the cricket biscuits cover a high percentage of RNI. Children in Kenya are presumably at risk of vitamin B12 deficiency, indicated by the high prevalence shown

in school children at baseline in the study by Mclean et al. (2007). None of the five micronutrients covers 100 % of RNI. For vitamin A (β-carotene), an intake of 50 % of recommended dietary allowance[5] was shown to maintain serum retinol concentrations from day to day, but not enough to sustain levels during the long school holiday break (van Stuijvenberg et al., 2001). This indicates that high amounts of micronutrients should be present in school feeding programs.

The remaining intake can be hard to reach with the habitual diet in the region. Meat, vegetables, nuts, and fruits, which are foods rich in vitamin A and bioavailable iron and zinc, are consumed in very low amounts (1-4 % of daily energy intake) (Hansen et al., 2011). Hansen et al. (2011) conducted their survey in August-November, where many fruits and vegetables are available after the long rains. Sources of iodine in the region are iodized salt and fish from Lake Victoria (Eckhoff & Maage, 1997). 79 % of respondents in the survey made by Hansen et al. (2011) had eaten fish during at least one of two 24-hour recalls, but the intake only amounted to 6 % of daily energy consumed. According to United Nations Children's Fund statistics, 93 % of the population in Kenya used adequate iodized salt from 2009-2013 (UNICEF, 2013). Intake of iodine appears adequate. Vitamin B12 is only found in ASF. Although only consumed in small amounts, the majority of the Luo population consume ASF (Hansen et al., 2011). It can be assumed that for most children included in the school feeding program, the RNI of vitamin B12 was covered.

Eleven caretakers indicated at screening that their child was allergic or intolerant to red meat. Hence, intake of meat must be assumed very low or non-existent in these children and they could be at higher risk of micronutrient deficiencies. Consumption of insects could to some extent substitute consumption of red meat and decrease symptoms of food allergy or intolerance. Galactose-α-1,3-galactose (α-gal) is an oligosaccharide recently described in meat (except fish and poultry) that can elicit allergic responses in sensitized subjects. Sensitization appears to be in the form of tick bites and have been described in USA, Europe and Australia (Commins & Platts-Mills, 2013). High prevalence of antibodies to the oligosaccharide have been found in a population of rural Kenyan children (76 %) (Commins et al., 2011). It would be worth exploring if the same is true in the present population.

Composition of cricket biscuit in the context of RUTF

BP-100™ is a bar that can be crushed and mixed with water and is the established RUTF product that the cricket biscuits resemble most in appearance and texture. Overall, the composition of the cricket biscuits do not match the recommendation for RUTF (Table 3 and appendix 1). Recommendations for energy content, percentage of fat, and micronutrient content (vitamin A and B12, iron, zinc, and iodine) are higher

[5] Recommended dietary allowance is the daily intake sufficient for 97.5 % of the population (US), whereas RNI covers 95 % of the population (UK).

than the values in the biscuits. However, the recommendations for micronutrients are based on supplementation and the recommended sources of some micronutrients are presumably less bioavailable or active than their counterparts in cricket biscuits (Chaparro & Dewey, 2010). The recommended moisture content is lower than what is presumed for the cricket biscuits. Nonetheless, crickets can contribute to RUTF products with high amounts of complete protein, unsaturated fatty acids, vitamin B12, iron and zinc (table 2).

Consumption pattern

Based on the consumption, it is clear that all children eating cricket biscuits accepted entomophagy on a daily basis. The consumption of both biscuits were continuously high throughout the trial. Children involved in the study asked the data enumerators on several occasions if they could get more biscuits. Since the biscuits were meant as a snack to supplement the daily diet, providing more than ⅓ of the recommended energy intake could possibly affect consumption of food outside of school negatively as indicated in the prior Kenya study (Murphy et al., 2003). On two days, the program had to change time from 11 am to 9 am due to altered school activities (trial day 10 and 11). Consumption did not seem to differ (fig. 6). However, the percentage of children reporting not to eat breakfast was high on the two days (table 5). It could indicate that the time of the school feeding program is important to avoid a negative impact on food intake outside the school.

The aforementioned school feeding study in Kenya showed that including meat in the snack increased the intake of the snack (93 %) compared to a snack with milk (89 %) and isocaloric control (76 %) (Murphy et al., 2003). This result resembles what is seen in the present study. Inclusion of beef or cricket could have a savory effect that increase intake of the snack (Sørensen et al., 2003).

Acceptance was good and long-term. The set criteria for acceptance were used because larger trials studying weaning foods and RUTFs in healthy children had incorporated similar ones (Konyole et al., 2012; Nga et al., 2013). However, such cutoffs are arbitrary and not comparable across studies, since variables such as energy of the food presented and characteristics of the study population vary greatly. Validated cutoffs based on age and nutritional status of the children as well as energy of the food presented is desirable.

Hedonic ratings

Both biscuits were rated above average in all aspects at all times. Overall, the same tendency could be seen in the hedonic ratings with time (fig. 7). Milk biscuits generally rated high from week 1 and continued to rise, whereas cricket biscuits followed the same slope but at lower ratings (table 7). This indicates that

children need longer time to adjust to the organoleptic properties of biscuits containing insects. Furthermore, color and smell had significantly different slopes for cricket and milk biscuit, indicating slower adaption for these properties in cricket compared to milk biscuits. Both biscuits were new to the children, but cricket was the only new ingredient. Prior research has shown that repeated exposure of new foods increases the preference for the food in schoolchildren (Caton et al., 2013; Hausner, Hartvig, Reinbach, Wendin, & Bredie, 2012). Although the two studies looked at intake and not hedonic ratings, the same trend is evident as in the present study.

All results from the intervention group from week 1 were higher than any other week. Data on hedonic ratings were generally inadequate in week 1 due to confusion on how to fill out the questionnaire. For instance, the translation from English to Luo of the term 'neither like nor dislike' (appendix 5) included the word for taste (*tamu*), which led to confusion. Together with the consumption pattern and results from the other weeks, it could indicate that the children answered what they thought the research team wanted. This is a common problem in subjective research as the children most likely were not comfortable in the environment yet.

Further exploration of the reasons for the ratings could be done using a focus group of children to discuss the qualities of the biscuits. Qualitative research has the advantage of understanding quantitative data in depth. Limited time and resources made it impossible in the present study.

Further development of biscuits for school feeding programs

In the present study, the biscuits were stored in freezer bags at -20°C (fig. 4). Proper packaging for shelf-storage is important to consider in further development of the biscuits for school feeding programs. Moisture content and water activity is related and baking time lowers both (Czuchajowska, Pomeranz, & Jeffers, 1989). Water activity is low in biscuits (<0.5) and higher in humid air (0.7), which means that over time the biscuits will absorb water. This will have an impact on both texture and the risk of mold growth. Furthermore, it was not possible within the period of this study to assess shelf life of the product. For future research, it will be necessary to determine safety aspects of storage in tropical areas.

One way to optimize micronutrient content is addition of a micronutrient premix. An issue is organoleptic changes of the food vehicle. Taste changes and rancidity issues have been shown for iron and change of color for β-carotene (Allen, de Benoist, Dary, & Hurrell, 2006). Furthermore, risk of adverse effects with excessive intake include infection risk for iron and liver damage for vitamin A (Allen et al., 2006; Esan et al., 2013). Fortification should be done with caution and in coordination with other public health measures in the area, for example malaria prevention. Another option would be to test biscuits with a higher amount of

cricket. Although pilot-tests concluded that biscuits with 10 % cricket were highest rated, biscuits with 15 or 20 % were still accepted (average ratings >3).

Anthropometry and morbidity

Anthropometric measurements were limited by time constraints at baseline and end of study. Children were measured at baseline either a month or the week before the study and only weight was possible to assess after the trial. All children except for one had gained weight by the end of the trial, indicating a positive impact of the trial biscuits, although accurate depictions of anthropometric impact is not possible with the data. Comparisons between the groups are valid. No difference was detected between the groups, indicating that cricket can substitute milk in the biscuits without negative implications for short-term growth (table 10).

In general, the study population was apparently healthy. However, two children were moderate acute undernourished (BAZ <-2) and two were moderately stunted, suggesting chronic undernutrition (HAZ <-2) (table 4). Unfortunately, it was not possible to gain knowledge on HIV status due to ethical considerations, but the general prevalence in Nyanza province was 13.9 % in 2009 (Kenya National Bureau of statistics and ICF Macro, 2009). HIV and nutrition is highly interrelated with negative feedback loops that worsen the patient's condition (Ivers et al., 2009). Knowledge on the nutritional requirement of children used in this study is based on healthy children, which might skew the analysis for the children affected by HIV infection.

Headache and stomachache were the two predominant morbidities noted throughout the trial (table 8). However, these symptoms were not systematically investigated as nausea, vomiting and diarrhea were, so the real number could be higher. Although only one case of diarrhea was reported, 21 stool samples scored 6 or 7 on the Bristol stool scale (table 9 and appendix 6). This indicates a concern in the interpretation of diarrhea. Data enumerators were instructed in the interpretation from WHO (three or more loose or liquid stools per day). However, local interpretation of diarrhea is often regarded as sickness concurrent with fever, stomach cramps etc. (Monica Ayieko, personal communication). Increased focus should be given to correct definitions of conditions according to WHO in the future. Furthermore, use of antibiotics was not recorded, which is valuable information in accordance to morbidity as well as microbiota composition.

There was no reason to suspect that morbidity was caused by ingestion of the test foods in any of the children, since no pattern in morbidity was found. Complaints were not received by children or parents. On the contrary, intake did not seem affected due to morbidity.

Microbiota composition

Methodology

The unknown systematic effect that was detected in the samples is assumed a systematic error during library preparation. All samples that were clustered together in unweighted analysis of the original data (fig. 9A) were from the same columns on the 96-wells plate. Since all known parameters that could affect results (collection day of specimen, day of DNA extraction and group) were randomly distributed on the plate, an error appears a likely explanation. Time constraints made it impossible to re-run samples in time for publication of the thesis.

Several of the studies mentioned below have used primers for other regions of the 16S rRNA gene (fig. 2). The relative distribution is dependent on primer selection and number of 16S rRNA gene copies in the different bacteria. Hence, caution should be taken when comparing the results to other studies. Similarly, it is good to keep in mind that many bacteria found in the bacterial communities in the present study have not been characterized in depth. Taxonomy is dependent on database, and rare or uncultivable bacteria are often missing.

No difference in microbiota composition due to intervention was detected in either weighted, generalized, or unweighted analyses. This indicates that neither the rare, intermediate, nor commonly occurring intestinal bacteria differed according to intervention. Prior research has shown that variation in microbiota composition is primarily explained by subject specific differences, especially in children (Wu et al., 2011; Yatsunenko et al., 2012). Entomophagy could as such have an important impact on microbiota, but not large enough to overcome inter-individual differences. In future studies it would be valuable to test the effect of entomophagy using a crossover design with baseline measurements in order to detect diet difference when eliminating subject variability. Furthermore, day-to-day variation is common and several samples could eliminate temporal variability. The fact that no difference was detected could mean that the gut microbiota of the children had reached stability prior to the intervention, which is in line with previous observations (Yatsunenko et al., 2012).

Chitin

As to my knowledge, no research has focused on the effect of chitin on microbiota composition. However, it has previously been shown that chitin-glucan[6] can restore butyrate-producing Clostridia bacteria in mice fed a high fat diet (Neyrinck et al., 2012). Butyrate is important as an energy source for colonocytes and in

[6] A complex of chitin and beta-(1,3)-D-glucan (poly-D-glucose).

modulation of the immune system (Canani et al., 2011). Furthermore, chitosan[7] was shown to decrease the frequency of occurrence of lecithinase-negative Clostridia bacteria in humans as well as inhibit growth of several Clostridia bacteria in vitro (Simůnek, Tishchenko, Hodrová, & Bartonová, 2006; Terada et al., 1995). It is possible, that not only chitin derivates, but also chitin itself influences the regulation of the Clostridia class of bacteria. The fact that three of four genera found in different quantities between the intervention and control groups (although not significant when corrected with FDR) were Clostridia bacteria supports this hypothesis (table 11). Cell and animal studies could help elucidate the hypothesis that chitin is an important prebiotic.

Prior research has shown that although no change is seen in the microbiota composition with diet change, the metatranscriptome (i.e. the genes transcribed in bacteria for protein synthesis) can still change (David et al., 2014; Eloe-Fadrosh et al., 2015). For instance, ingestion of chitin could promote a change in transcription of genes involved in carbohydrate metabolism in order for the bacteria to be able to break the polymer down with chitinases. Prior results with human chitinases support this hypothesis (Paoletti et al., 2007).

Diet and microbiota composition

The OTU relative distribution of the combined data (fig. 10) showed interesting similarities compared to other dietary studies. Wu et al. (2012) found that a long-term diet predominant in carbohydrate consumption increases relative amount of the *Prevotella* genus compared to *Bacteroides*. This is also the case in the present study where the relative distribution of *Prevotella* is 46.0 % and *Bacteroides* 1.0 %. Populations from Venezuela, Mali and Burkina Faso similarly had a high prevalence in Prevotella, whereas Americans and Italians predominated in Bacteroides (De Filippo et al., 2010; Yatsunenko et al., 2012). The populations from Venezuela, Mali and Burkina Faso were noted to consume mainly vegetarian diets rich in maize, cassava and other starchy components.

Interestingly, in the study by De Filippo et al. (2010) it was recorded that children in Burkina Faso eat termites when in season. It is not stated if fecal sampling was done during this time. Since fecal sampling in the present study took place during the season where consumption of termites is common, the possibility exists that children from the control group had consumed insects, which could affect the results.

Overall, the studies that have shown a significant difference in microbiota composition with diet, have researched major diet differences such as a vegetarian, starch-based diet versus a typical Western diet of refined grains, high fat and animal protein. Changing one ingredient in a snack could induce a small change,

[7] A deacetylated derivate of chitin.

but a much larger cohort would be necessary to decipher it. A longer trial period could likewise be necessary for a change in microbiota composition to be detected. Studies in mice under very controlled circumstances have shown that longer periods are necessary to detect a change (Jacob Bak Holm, personal communication). On the contrary, Wu et al. (2012) detected a change in microbiota composition within 24 hours of initiating controlled feeding in humans with either a high fat/low fiber diet or vice versa.

Effect of geography and livelihood on microbiota composition

Geography and mode of subsistence have been associated with microbiota differences in several studies. The microbiota composition of the Kenyan children (fig. 10) resemble rural children in Burkina Faso and Hadza hunter-gatherers from Tanzania more than urban children and adults in Italy (De Filippo et al., 2010; Schnorr et al., 2014). The higher distribution of the phylum Bacteroidetes relative to Firmicutes is evident in all three African populations where the reverse is true for the Italian populations. However, some bacterial genera in the present study are similar to the Italian children and not present in children from Burkina Faso (De Filippo et al., 2010). The presence of Proteobacteria as a subdominant component is similar between the Kenyan children and Hadza hunter-gatherers (Schnorr et al., 2014). The presence of the genus *Succinivibrio* seems to be linked to the African continent as seen in the present study, Burkina Faso, Cameroon, Tanzania and Malawi (De Filippo et al., 2010; Morton et al., 2015; Schnorr et al., 2014; Yatsunenko et al., 2012). When comparing the Kenyan children to different populations in Cameroon in terms of livelihood, the children are more similar to Bantu farmers and fishing populations than Pygmy hunter-gatherers (Morton et al., 2015). Together, the sample from Kenya resembles samples from the African continent more than Europe, but the variance within Africa is great according to subsistence showing a much more diverse picture than previously thought.

Limitations of study

The age and date of birth provided by the caretakers often did not match. Date of birth was considered most reliable and was used in subsequent analyses. However, birth certificates were not provided by the majority of caretakers. For six children date of birth was not provided. In those cases, a random date of birth was assigned according to the provided age. Age in general is therefore a measure with a high risk of error.

Most children did not show up at JOOUST on at least one occasion. Numerous missing days were not accounted for. Only two episodes of sickness were accounted for by the children, one of which was malaria. For future studies, it would be of great interest to investigate whether children chose to stay at school and play, were sick, or stayed away for other reasons such as work in the field with the family. This would give a better picture on acceptance of biscuits and overall health.

One of the important limiting factors for interpreting the data of the present study is the lack of knowledge on the habitual diet of the children, including detailed questions on insect consumption. In order to understand in more detail how entomophagy could enhance the dietary intake of certain nutrients and energy, it is necessary to know which part of the diet is unbalanced and whether an intervention such as school feeding has an impact on the diet outside of school as noted by Murphy et al. (2003).

There is a risk that convenience sampling of the children led to systematic bias. As the recruitment phase was in the beginning of the long rainy season, most caretakers were busy in the fields sowing. Since an estimated 85 % of the community are farmers, it could be an underrepresented group of children compared to reality (Hansen et al., 2011). Lack of socioeconomic data from the families make it difficult to assess if bias occurred. Furthermore, it was very difficult to access caretakers and the recruitment phase had to be extended several times. In the end, only 57 out of 60 planned children were included in the study and 54 included in analysis (fig. 5). Fifty children were enough to have power to show a difference of consumption of 20 % (Nga et al., 2013). Due to missing days, the analysis did not live up to the power calculations. However, since the consumption of cricket biscuits was very high throughout the whole trial it is of little importance.

Conclusion

This study proves that it is possible to create a cricket-based (10 % by ingredient) biscuit for school feeding programs that are nutritionally suitable for children in primary school.

The biscuits were very well accepted in a long-term manner for children age 5-10 years with an overall consumption of 96.9 % corresponding to about 97 g daily, which was not significantly different from milk biscuit consumption. Consumption of this amount covers RNI partly for several macro- and micronutrients of public health concern in Kenya.

All hedonic ratings for the cricket biscuits were above average. A pattern for all hedonic ratings was that cricket biscuits followed the same ascending trend over time as milk biscuits, but at a lower rating. Four organoleptic properties were overall significantly lower than milk biscuits: Looks, smell, texture and overall liking.

The microbiota composition did not change for the children eating cricket biscuits compared to the children eating milk biscuits. However, a systematic effect during library preparation could have caused an error, and the sequencing will need to be performed again to verify the result. It is possible that a functional change had occurred even though no taxonomic change did.

Perspectives

The trial showed that a school feeding program with cricket biscuits is feasible within the present setting. For the future, it should be established whether crickets would be accepted in communities where entomophagy is not practiced to the degree that the Luo people around Lake Victoria do. Furthermore, cost-effectiveness needs to be determined. Preferably, both nutritional status as well as education should be endpoints. Social aspects of school feeding programs ought to be taken into consideration.

Besides optimizing the cricket biscuits as discussed in this report, other products can be evolved. Based on the high consumption pattern and the nutritional profile of crickets, foods for weaning and RUTF products are possibilities worth exploring. An example would be to base a weaning product on the WinFood formula, including crickets instead of termites. The product would be more sustainable, since it is possible to rear crickets throughout the year in Kenya. RUTF using local ingredients instead of imported milk powder is a vast area of research. Due to the high amount of fatty acids in crickets, a high-energy spread formula is realistic. In alignment with RUTF product development, impact of entomophagy on the microbiota in the undernourished child should be explored for any negative or positive effects.

The socioeconomic perspectives of home-grown school feeding are important. Not only will the children benefit, but also farmers, supermarkets and bakeries. Before implementing school feeding programs with crickets, the government in Kenya needs to implement safety regulations for edible insects.

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Appendix 1 – Recommended nutritional composition of RUTF

The recommended nutritional composition of RUTF as established by WHO et al. (2007). Highlighted items are discussed in the text. Recommended chemical forms of micronutrients are shown in brackets (Chaparro & Dewey, 2010).

	Recommended value per 100 g
Moisture content	**2.5 % maximum**
Energy	**2176-2301 kJ**
Protein	**10-12 % total energy**
Lipids	**45-60 % total energy**
Sodium	290 mg maximum
Potassium	1,110–1,400 mg
Calcium	300–600 mg
Phosphorus (excluding phytate)	300–600 mg
Magnesium	80–140 mg
Iron (encapsulated ferrous sulfate)	**10-14 mg**
Zinc (zinc sulfate)	**11-14 mg**
Copper	1.4-1.8 mg
Selenium	20–40 µg
Iodine (potassium iodate)	**70-140 µg**
Vitamin A (retinyl acetate)	**800-1100 µg**
Vitamin D	15–20 µg
Vitamin E	20 mg minimum
Vitamin K	15–30 µg
Vitamin B1	0.5 mg minimum
Vitamin B2	1.6 mg minimum
Vitamin C	50 mg minimum
Vitamin B6	0.6 mg minimum
Vitamin B12 (cyanocobalamin (0.1%))	**1.6 µg minimum**
Folic acid	200 µg minimum
Niacin	5 mg minimum
Pantothenic acid	3 mg minimum
Biotin	60 µg minimum
n-6 fatty acids	3%–10% of total energy
n-3 fatty acids	0.3%–2.5% of total energy

Appendix 2 – Screening form

Child's name:_____ Child ID:_____ date:_____

Interviewer name:_____ Interviewer ID: _____

I will be asking you a few questions to make sure all requirements are met. Some of the questions can be personal. We will also have to weigh and measure the height and mid-upper-arm circumference of your child before inclusion in the study. All of the information you provide will remain confidential, but when there is a question you are not comfortable answering, please tell us.

	Questions	**Response categories**	**code**
1	Is your child a boy or a girl?	Boy ☐	1
		Girl ☐	2
2	How old is your child?	Age in years ……	
3	What is the date of birth? (Ask for certificate. If not known write 00/00-0000, Researcher: check 'unconfirmed if no certificate)	Date (day/month-year) ……/……..-…….. ☐ unconfirmed	
4	Will your child be available for the duration of the 2 months acceptability and impact study? (March 25th-May 22nd)	Yes ☐	1
		No ☐	2
5	Does the child have any acute or chronic illnesses such as ARI (Acute Respiratory Infections), fever, diarrhea (>3 loose stools per day, WHO), IBS (Irritable Bowel Syndrome) etc.? Or other illnesses which interfere with food intake?	Yes ☐	1
		No ☐	2
6	Does your child have any known food allergies/intolerances?	Yes ☐	1
		No ☐	2
6a	If yes, which?	……..	
7	Is the child eating at least one meal a day (not considering the biscuit trial)?	Yes ☐	1
		No ☐	2

8	Is the child currently participating in any other study?	Yes ☐	1
		No ☐	2
9	Has the child ever tasted insects?	Yes ☐	1
		No ☐	2
10	If yes, which insects has the child eaten?	……..	
11	Does the child eat insects currently, when available?	Yes ☐	1
		No ☐	2
12	Has the child received deworming treatment within the last 7 days?	Yes ☐	1
		No ☐	2
13	If yes, specify the date of treatment	Date (day/month-year) ……/……..-……..	
14	Researcher: Has the child's caregiver given an informed verbal or signed consent on behalf of the child?	Yes ☐	1
		No ☐	2

Appendix 3 – Anthropometry forms

Baseline

measurer name:_____ measurer ID: _____

Child's name:_____ Child ID:_____

Gender: M / F Age:_____ Date_____

	Anthropometry screening	**Response categories**	**code**
1a	Weight, 1st measurement	Kg ☐☐.☐	
1b	Weight, 2nd measurement	Kg ☐☐.☐	
1c	Weight, 3rd measurement (if 1 and 2 is more than 0.5 kg apart)	Kg ☐☐.☐	
2a	Height, 1st measurement	Cm ☐☐☐.☐	
2b	Height, 2nd measurement	Cm ☐☐☐.☐	
2c	Height, 3rd measurement (if 1 and 2 is more than 0.5 cm apart)	Cm ☐☐☐.☐	
3a	MUAC (mid-upper-arm circumference), 1st measurement	Cm ☐☐.☐☐	
3b	MUAC (mid-upper-arm circumference), 2nd measurement	Cm ☐☐.☐☐	
3c	MUAC (mid-upper-arm circumference), 3rd measurement (if 1 and 2 is more than 0.3 cm apart)	Cm ☐☐.☐☐	

4	Does the child have bilateral pitting oedema?	Yes ☐	1
		No ☐	2
5	Anja: Does the child have a BMI-for age <-3?	Yes ☐	1
		No ☐	2
6	Anja: Does the child have a BMI-for age >2?	Yes ☐	1
		No ☐	2
7	Anja: Does the child have a MUAC under 12.9 cm (5-9 y)/16.0 cm(10 y)?	Yes ☐	1
		No ☐	2
8	Anja: Is the child eligible for the study?	Yes ☐	1
		No ☐	2

End of trial

Measurer name:_____ measurer ID: _____

Child's name:_____ Child ID:_____

Gender: M / F Date_____

Weight, 1st measurement	Kg ☐☐.☐
Weight, 2nd measurement	Kg ☐☐.☐
Weight, 3rd measurement (if 1 and 2 is more than 0.5 kg apart)	Kg ☐☐.☐

Appendix 4 – Daily intake and morbidity form

Dates: _____/_____-_____ to _____/_____-_____ Week: 1 / 2 / 3 / 4

Product color: white / orange Gender: M / F

Child ID:_____ Child Name:_____

team ID:_____ Interviewer Names:_____

Definitions:

Hesitation to eat: Hesitation to eat the product is defined as refusing to eat the product when the research assistant presents it, but after encouragement by the assistant, the child tries the offered food.

Refusal to eat: Refusal to eat the product means that the child completely refuses the offered product even after being encouraged twice by the assistant.

Nausea: a feeling of sickness with an inclination to vomit

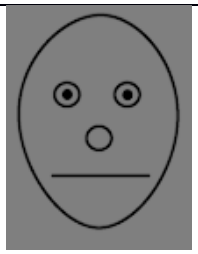
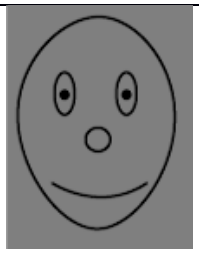
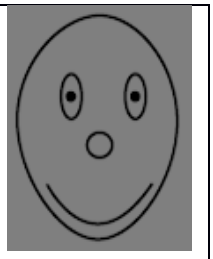
Vomiting: Throwing up stomach content. If the child has thrown up once or more, write in brackets how many times on the form.

Diarrhea: 3 or more loose or liquid bowel movements per day (WHO)

What was eaten: a short, precise description of the food (preferably in English, otherwise state (S) for swahili or (L) for Luo, and I will translate later)

	monday	tuesday	wednesday	thursday	Friday
Intake (amount measured and noted down to nearest 1.0 g)					
Plate					
Product number (see on package)					
Plate w. food – BEFORE					
Plate w. food – AFTER					
Previous food intake (on the day of feeding)					
eaten today (1=YES, 2=NO)					
What was eaten?					
Eating pattern (write 1 for YES or 2 for NO)					
Hesitation to eat					
Refusal to eat					
Morbidity (write 1 for YES or 2 for NO)					
Nausea					
Vomiting (if yes write times/day in brackets)					
Diarrhea					
Comments or other medical complications:					

Appendix 5 – Hedonic ratings

	 **Dislike a lot** **okahere ndi** **sipendi hata**	 **Dislike a little** **okahere matin** **sipendi kidogo**	 **Neither like nor dislike** **ok omit be ok orachna** **si tamu na pia si mbaya**	 **Like a little** **omit matin** **naipenda kidogo**	 **Like a lot** **omit ahinya** **naipenda sana**
Looks **Chalne** **Kufanana**					
Color **Rangine** **Rangi**					
Smell **Tikne** **Harufu**					
Taste **Ndhandhone** **Ladha (onja)**					
Texture **Gwargwarne** **Ulaini**					
Overall **Chalne te** **Kwa ujumla**					

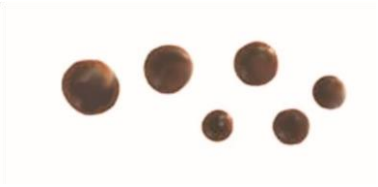
Date: _____/_____-_____ Product color: white / orange

Child ID:_____ Child Name:_____

Week: 1 / 2 / 3 / 4 Gender: M / F

Interviewer Name_____ Team ID:_____

Appendix 6 – Bristol Stool Scale

Bristol Stool Form Scale		
Type	**Description**	**Image**
Type 1	Separate hard lumps, like nuts	
Type 2	Sausage-shaped but lumpy	
Type 3	Like a sausage or snake but with cracks on its surface	
Type 4	Like a sausage or snake, smooth and soft	
Type 5	Soft blobs with clear-cut edges	
Type 6	Fluffy pieces with ragged edges, a mushy stool	

Type 7	Watery, no solid pieces	

Appendix 7 – Details of bioinformatics and primary analysis

Written by postdoc Lukasz Krych, Department of Food Science, University of Copenhagen

The raw dataset containing pair-ended reads with corresponding quality scores were merged and trimmed using fastq_mergepairs and fastq_filter scripts implemented in the UPARSE pipeline. The minimum overlap length was set to 10bp. The minimum length of merged reads was 250bp. The max expected error E = 2.0, and first truncating position with quality score N ≤ 4. Purging the dataset from chimeric reads and constructing de novo Operational Taxonomic Units (OTU) were conducted using the UPARSE pipeline (uchime_ref) (Edgar, 2013). The green genes (13.8) 16S rRNA gene collection was used as a reference database (McDonald et al., 2012). Quantitative Insight Into Microbial Ecology (QIIME) open source software package (1.7.0 and 1.8.0) was used for subsequent analysis steps (Caporaso et al., 2010).

Principal coordinate analysis (PCoA) plots were generated with the Jackknifed Beta Diversity workflow based on 10 distance metrics calculated using 10 subsampled OTU tables. The number of sequences taken for each jackknifed subset was set to 85% of the sequence number within the second most indigent sample (20,000 reads/sample). Analysis of similarities (ANOSIM) was used to evaluate group differences using weighted and unweighted UniFrac distance metrics that were generated based on rarefied (20,000 reads/sample) OTU tables (Lozupone & Knight, 2005). The relative distribution of the genera registered was calculated for unified and summarized in the genus level OTU tables.

Alpha diversity measures expressed with an observed species (sequence similarity 97% OTUs) value were computed for rarefied OTU tables (20,000 reads/sample) using the alpha rarefaction workflow. Differences in alpha diversity were determined using a t-test-based approach employing the non-parametric (Monte Carlo) method (999 permutations) implemented in the compare alpha diversity workflow.

The analysis of variance (ANOVA) was used to determine quantitative (relative abundance) association of OTUs with given diet. These were calculated based on 1000 subsampled OTU-tables rarefied to an equal number of reads (20,000 per sample) and summarized to the genus level.

Correlations between trial parameters (Bristol grading, BAZ, weight, height, MUAC, missing days from trial, age) and genera relative abundance were verified with the Pearson's product-moment correlation coefficient implemented in the observation_metadata_correlation.py script (QIIME 1.9.0).